

Dissecting the binding behaviour of the multi-RRM protein Rrm4 during endosomal mRNA transport

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Nina Kim Stoffel
aus Hagen

Düsseldorf, August 2025

aus dem Institut für Mikrobiologie
der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf
Berichterstatte:

1. Prof. Dr. M. Feldbrügge
2. Prof. Dr. H. Schaal

Tag der mündlichen Prüfung:

For

Heiner Schaal

Anne Ephrussi

Dorothee Staiger

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1 Summary

Spatiotemporal gene regulation relies on RNA-binding proteins (RBPs) that coordinate mRNA transport and translation. Many RBPs feature modular architectures with multiple RNA-binding domains (RBDs), yet the domain-specific contributions to RNA and protein interactomes *in vivo* remain poorly understood.

This thesis presents a systematic dissection of the binding behavior of Rrm4, the key RBP that mediates translation-active endosomal mRNA transport during polarized growth in the pathogen *Ustilago maydis*. To resolve the functions of its three RNA recognition motifs (RRMs), domain-specific mutants were analyzed through a multi-omics strategy combining iCLIP2 (individual-nucleotide resolution UV crosslinking and immunoprecipitation), differential RNA sequencing, endosome- and co-immunoprecipitation mass spectrometry (endo-IP MS, co-IP MS), and cell biological validation.

RNA interactome profiling revealed that the tandem domains RRM1/2 recognize functionally important binding sites (RRM12-BS), which are required for mRNA transport and abundance. RRM12-BS are enriched in transcripts linked to polarity, mitochondrial activity, and cell wall plasticity—linking RNA binding to unipolar growth. In contrast, RRM3 enhances RNA affinity via sequence-specific UAUG binding but is largely dispensable for polarity establishment. Moreover, the endosomal proteome depends on Rrm4’s RNA-binding capacity. Mutation of RRM1/2 or deletion of Rrm4 selectively reduced endosome-associated proteins encoded by Rrm4-target mRNAs (e.g., polarity factors) and co-localized ribosomes, whereas neither RRM3 mutation nor proteins encoded by non-target mRNAs showed detectable changes. These findings point to a functional hierarchy among the RRMs, with RRM1/2 mediating endosomal mRNA hitchhiking and local translation, thereby shaping the endosomal proteome.

Since RBPs rarely act in isolation, the RRM-dependent Rrm4 protein interactome was dissected. RRM1 mediates RNA-independent interactions with the mRNP scaffold protein Upa2 and the translational repressor Ssd1, positioning RRM1 as a multivalent hub that integrates RNA binding, mRNP assembly, and translational control. The Rrm4–Ssd1 interaction likely facilitates the repressed transport of cell wall-related mRNAs, highlighting a new regulon-specific model of translation-regulated endosomal mRNA trafficking.

Beyond mRNAs, Rrm4 binds pre-tRNAs, expanding its interactome to non-coding RNAs. Pre-tRNAs co-localize with endosomes and are delivered to mitochondria, where intron removal occurs. Deletion of Rrm4 impairs splicing efficiency and global translation, revealing a novel link between endosomal transport and tRNA maturation. Together, this work defines how the multi-RRM RBP Rrm4 coordinates RNA and protein networks and expands the concept of endosomal RNA transport in space and time—from fungi to neurons.

2 Zusammenfassung

Die raumzeitliche Genregulation beruht auf RNA-bindenden Proteinen (RBPs), die den Transport und die Translation von mRNAs koordinieren. RBPs besitzen häufig eine modulare Architektur mit mehreren RNA-Bindedomänen (RBDs), wobei die individuellen Beiträge dieser Domänen zu RNA- und Proteininteraktionen *in vivo* bislang nur unzureichend verstanden sind. Diese Arbeit untersucht systematisch das Bindevverhalten von Rrm4, einem zentralen RBP, das den translational aktiven endosomalen mRNA-Transport während des polaren Wachstums in dem pathogen Pilz *Ustilago maydis* vermittelt. Um die spezifischen Funktionen der drei RNA-Erkennungsmotive (RRMs) aufzuklären, wurden Domänen-spezifische Mutanten mithilfe einer Multi-Omics-Strategie analysiert – einschließlich iCLIP2 (individual-nucleotide resolution UV crosslinking and immunoprecipitation 2), RNA-Sequenzierung, endosomaler und ko-immunpräzipitativer Massenspektrometrie (endo-IP MS, co-IP MS) sowie zellbiologischer Validierung.

Die RNA-Interaktomanalyse zeigte, dass die tandemartig angeordneten Domänen RRM1/2 funktionell relevante Bindestellen (RRM12-BS) erkennen, die essenziell für mRNA-Transport und -Stabilität sind. Diese Sequenzen sind angereichert in Transkripten, die mit Polarität, mitochondrialer Funktion und Zellwandplastizität assoziiert sind – und verknüpfen somit RNA-Bindung mit gerichteter Zellpolarität. RRM3 hingegen bindet sequenzspezifisch UAUG-Motive und erhöht die RNA-Affinität, ist jedoch weitgehend entbehrlich für die Etablierung der Polarität.

Darüber hinaus hängt das endosomale Proteom von der RNA-Bindekapazität von Rrm4 ab. Mutation der RRM1/2 oder Deletion von Rrm4 führte zu einer selektiven Reduktion endosomaler Proteine, die von Rrm4-Ziel-mRNAs kodiert werden (z. B. Polarisationsfaktoren), sowie der kolokalisierten Ribosomen. Weder die RRM3-Mutation noch Proteine kodiert von nicht-Ziel-mRNAs zeigten vergleichbare Veränderungen. Diese Befunde deuten auf eine funktionelle Hierarchie der RRMs hin, bei der RRM1/2 den endosomalen mRNA Transport und die lokale Translation vermitteln und so das endosomale Proteom prägen.

Da RBPs selten isoliert agieren, wurde zudem das proteinbasierte Interaktom von Rrm4 in Abhängigkeit der RRMs analysiert. RRM1 vermittelt RNA-unabhängige Interaktionen mit dem Gerüstprotein Upa2 und dem Translationsrepressor Ssd1 und fungiert so als multivalenter Knotenpunkt, der RNA-Bindung, mRNP-Stabilität und Translationskontrolle integriert. Die Rrm4–Ssd1-Interaktion ermöglicht wahrscheinlich den translational repremierten Transport zellwandbezogener mRNAs und offenbart damit ein regulonspezifisches, translationsreguliertes Transportsystem.

Darüber hinaus wurde gezeigt, dass Rrm4 auch prä-tRNAs bindet und sein Interaktom somit auf nicht-kodierende RNAs erweitert. Diese prä-tRNAs kolokalisieren mit

Endosomen und werden zu Mitochondrien transportiert, wo das prä-tRNA-Spleißen erfolgt. Der Verlust von Rrm4 beeinträchtigt die Spleißeffizienz und stört die globale Translation, was eine unerwartete Verbindung zwischen endosomalem RNA-Transport und tRNA-Prozessierung offenbart. Insgesamt liefert diese Arbeit neue Einblicke in die koordinierte Funktion des multi-RRM-haltigen RNA-bindenden Proteins Rrm4 und erweitert das Modell des endosomalen RNA-Transports in Raum und Zeit – von Pilzen bis zu Neuronen.

3 Introduction

3.1 Post-transcriptional regulation

RNA-binding proteins (RBPs) are ubiquitous across all kingdoms of life, playing fundamental roles in post-transcriptional regulation (Fig. 1A) [1, 2]. The fate of an mRNA is dictated by its associated RBPs, which collectively form messenger ribonucleoprotein particles (mRNPs) [3, 4, 5]. These complexes orchestrate key processes in the mRNA life cycle, including synthesis, processing, modification, export, transport, localization, translation, and decay (Fig. 1A) [6, 7]. In humans, hundreds of canonical RBPs have been identified, distinguished by the presence of one or more classical RNA-binding domains (RBDs) [2, 8]. Additionally, non-canonical RBPs, including moonlighting proteins, that lack conventional RBDs, yet still interact with RNA, have been identified [3, 9]. This highlights the complexity of the RBPworld [4, 8]. Given their central role in gene expression, RBPs are tightly regulated, and their dysfunction is implicated in a broad spectrum of diseases, from developmental disorders to aging-related conditions [6, 10, 11]. To understand how an RBP contributes to cellular physiology, it is essential to define its RNA interactome and elucidate the mechanisms underlying its binding specificity [7, 12, 13]. Moreover, RBPs rarely function in isolation, instead, they operate within complex mRNP networks (Fig. 1A), interacting with RBPs and other protein partners to fine-tune gene expression [5, 14, 15]. Deciphering these RNA–protein and protein–protein interactions *in vivo* is essential for understanding the functional impact of an RBP within the RNA lifecycle (Fig. 1A) [2, 16, 17].

3.2 RNA-binding domains

Canonical RBPs are defined by the presence of well-characterized RNA-binding domains (RBDs) that recognize specific RNA sequences or structures [20, 21]. The most extensively studied RBDs include the RNA recognition motif (RRM), K-homology (KH) domain, DEAD-box helicase domain, double-stranded RNA-binding domain (dsRBD), La motif (LA/LaM), Pumilio (Puf) domain, arginine–glycine–glycine (RGG) motifs, and zinc finger (ZnF) domains (Fig. 1B) [5, 8, 18, 21].

A hallmark of RNA-binding proteins (RBPs) is their modular domain architecture, typically comprising multiple RBDs arranged in tandem or combined with other RBD types (Fig. 1B) [19, 22]. This organization enhances RNA-binding specificity and affinity while enabling dynamic regulation of RNA targets [19, 23]. Notable examples of multi-RBD RBPs include the RNA transporter TDP-43 (Transactive Response DNA-Binding Protein 43 kDa), which contains tandem RRM [24], and Staufén, characterized by five dsRBDs [25]. Splicing regulators such as FUBP1 (Far Upstream Element-Binding Protein 1), with four KH domains [26], and hnRNP A1 (Heterogeneous Nuclear

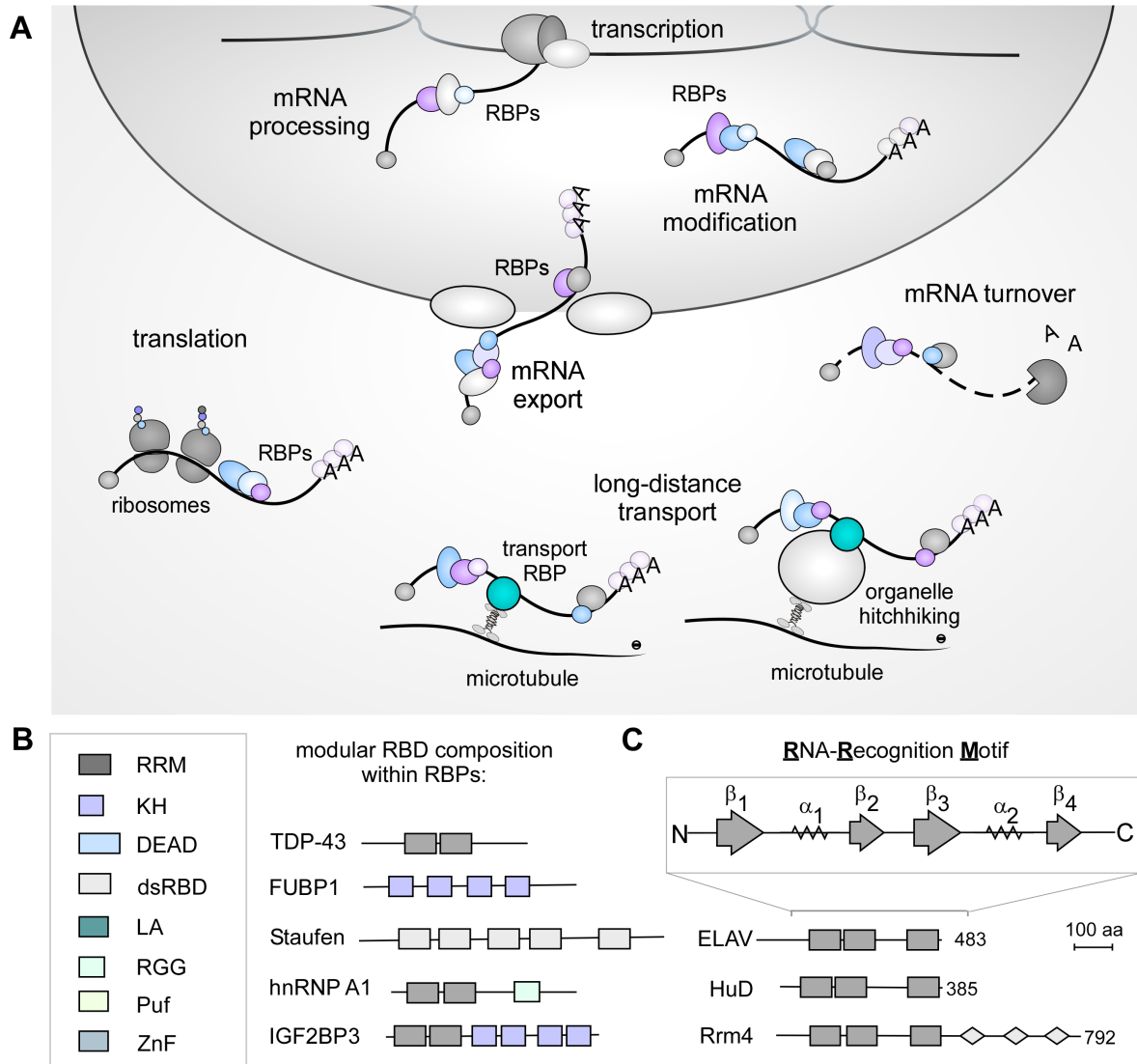


Figure 1: Post-transcriptional gene regulation mediated by RNA-binding proteins

(A) Schematic representation of the mRNA lifecycle—from transcription to decay—highlighting the diverse regulatory roles of RNA-binding proteins (RBPs). (B) Overview of common RNA-binding domains (RBDs, box), alongside exemplary RNA-binding proteins that illustrate the modular organization. (C) Conserved structural fold of an RNA recognition motif (RRM), characterized by a four-stranded antiparallel β -sheet flanked by two α -helices [18, 19]. Representative RBPs from the ELAV-like family illustrate the conserved arrangement of multiple RRM within individual proteins.

Ribonucleoprotein A1), which harbors both tandem-RRMs and an RGG motif [18]. Another well-characterized example is IGF2BP3/IMP3 (Insulin-like Growth Factor 2 mRNA-Binding Protein 3), a key mRNP granule component containing two RRM and four KH domains [27, 28]. Together, these examples underscore that modular RBD architecture is a conserved and versatile feature of RBPs across diverse stages of the mRNA lifecycle (Fig. 1A–B) [22, 23, 28].

Among all known RNA-binding domains, the RNA recognition motif (RRM) is the

Among all known RNA-binding domains, the RNA recognition motif (RRM) is the most prevalent (Fig. 1C) [8]. Typically comprising 80-90 amino acids, RRM s adopt a conserved fold formed by four antiparallel β -strands that create a β -sheet surface, flanked by two α -helices [18, 19]. Multi-RRM proteins such as *Drosophila melanogaster* ELAV (Embryonic Lethal Abnormal Vision), the mammalian HuD (Hu Antigen D), and the fungal Rrm4 (RNA Recognition Motif 4) exemplify a conserved domain architecture [29, 30, 31]. These ELAV-(like) proteins typically contain tandem-RRMs as well as a third, more distantly separated RRM located more N-terminally (Fig. 1C) [31, 32]. Although it is well established that multiple RBDs enhance RNA-binding affinity and target specificity, the precise functional contributions of individual domains remain elusive [1, 17, 22, 23].

3.3 RNA-protein interaction

To unravel RNA-protein interactions *in vivo*, either RNA-centric or protein-centric approaches can be applied, depending on the research question [12, 13, 33]. RNA-centric methods aim to identify unknown RBPs by capturing RNA followed by mass spectrometry analysis [9, 34]. In contrast, protein-centric methods identify unknown RNA targets for a given RBP and can be categorized into two major groups: those that chemically modify associated RNAs, such as Hyper-TRIBE [35, 36], and those that rely on protein purification, including RNA immunoprecipitation (RIP) [37], UV crosslinking and immunoprecipitation (CLIP) [38], or UV crosslinking and analysis of cDNA (CRAC) [39]. These techniques vary significantly in experimental complexity and resolution [13, 40]. Among these techniques, iCLIP(2) (individual-nucleotide resolution CLIP 2) [41, 42] and other comparable high-resolution CLIP methods [13, 43, 44] stand out for their ability to not only identify associated RNAs but also precisely map binding sites at single-nucleotide resolution [42, 45]. For example, eCLIP (enhanced CLIP) has been used to define the RNA interactomes of hundreds of human RBPs in a large-scale study, identifying thousands of binding sites for individual RBPs [13, 43]. However, the underlying mechanisms that define binding specificity, the processes of RNA recognition, and the functional relevance of specific binding sites remain poorly understood [17, 21].

3.4 mRNP composition and RBP interactomes

While iCLIP(2) and related high-resolution approaches offer valuable insights into RNA-binding profiles [13, 40], they do not fully capture the complexity of mRNP protein composition or dynamics [16, 17, 46, 47]. RBPs frequently engage in diverse protein-protein interactions that modulate their RNA-binding affinity, specificity, and functional output [15, 16, 48]. Therefore, a comprehensive understanding of an RBP requires not only the identification of its RNA targets but also the delineation of the

broader mRNP network it forms with other proteins [47, 49].

Traditional approaches for investigating mRNP composition include modification-based methods such as Bio-ID, or protein purification strategies like co-immuno-precipitation (co-IP) followed by mass spectrometry [16, 50, 51]. Notably, combining co-IP with RNase treatment allows for distinguishing between RNA-dependent and RNA-independent interactions [46, 52], thereby providing deeper insights into interaction modes. Recently, large-scale co-IP MS studies in yeast and mammals have been applied to decipher mRNP networks in greater detail, unveiling new insights into various stages of the mRNA lifecycle [46, 52, 53]. Despite these advances, the regulatory principles underlying these complex networks and the specific contributions of individual RBDs to the protein interactome remain incompletely understood [23, 48].

3.5 Subcellular mRNA localization and transport systems

A critical layer of spatiotemporal gene expression is the precise transport and localization of mRNAs [54, 55], enabling translation at subcellular sites where proteins are functionally required (Fig. 1A) [56, 57]. RNA trafficking is tightly regulated by RBPs, which selectively bind to target transcripts and coordinate their subcellular delivery with high specificity and fidelity [55, 58, 59]. Across diverse systems, cells employ multiple mRNA transport strategies, ranging from passive diffusion to directed, motorprotein-driven translocation along cytoskeletal tracks [55, 60, 61, 62]. In highly polarized cells such as neurons, active transport is indispensable for localizing transcripts to regions far from the nucleus, which is crucial for neuronal plasticity (Fig. 1A) [56, 58, 60, 63]. A well-characterized example is the RBP FMRP (Fragile X Mental Retardation Protein), which interacts with kinesin-I to mediate the transport and localized translation of *CaMKII α* mRNA—an essential mechanism for synaptic function [64, 65]. Disruptions in this process are linked to the neurological disorder fragile X syndrome (FXS) [66]. Beyond direct interactions with motor proteins, growing evidence points to an alternative, evolutionarily conserved transport mechanism in which mRNPs hitchhike on motile organelles—such as endosomes or lysosomes—along microtubule tracks [55, 60, 61]. Initially described in fungi, this vesicle-coupled mRNA transport mechanism has since been identified in plants and mammals (Fig. 1A) [63, 67, 68]. Disruption of vesicle-mediated mRNA trafficking has been linked to neurological disorders such as amyotrophic lateral sclerosis (ALS) and Charcot-Marie-Tooth disease, as well as impaired morphogenesis in fungi [58, 60, 63].

A prime example is the endosome-mediated delivery of nuclear-encoded mitochondrial mRNAs, which are locally translated on endosomes adjacent to mitochondria—an essential process for neuronal integrity and function [63, 67, 69, 70]. Beyond mitochondrial targets, cytoskeletal mRNAs are also transported on early endosomes in a

translation-dependent manner in HEK cells [71]. Notably, endosomes also mediate the subcellular distribution of non-coding RNAs, including pre-miRNAs in neurons [72]. Overall, this underscores the multifunctional role of vesicle-mediated RNA hitchhiking in maintaining cellular homeostasis and plasticity [63, 73].

3.6 Endosomal mRNA transport in *U. maydis*

Endosomal mRNA transport was first discovered in hyphal cells of the plant pathogenic fungal microorganism *Ustilago maydis* (Fig. 2A) [31, 74, 75]. Here, early endosomes shuttle bidirectionally along microtubule bundles, driven by kinesin-3 and dynein motor proteins (Fig. 2A) [76, 77]. The key regulator of mRNA cargo recruitment onto moving endosomes is the RBP Rrm4 (Fig. 2A) [62, 74, 78, 79]. Loss of Rrm4 or defective transport disrupts hyphal polarity, leading to a characteristic bipolar growth phenotype [31, 80, 81]. Rrm4 contains three N-terminal RNA recognition motifs (RRMs) and three C-terminal Mademoiselle (MLLE) domains essential for endosomal attachment (Fig. 2A) [31, 82, 83]. Notably, the third MLLE domain is both necessary and sufficient for interaction with the anchoring protein Upa1, which directly associates with the early endosomal membrane [81, 83]. In contrast, the RRM domains of Rrm4 define its RNA-binding capacity [31, 82]. Initial analyses reveal distinct contributions of these domains: mutations in RRM1 and RRM2 impair polar growth, whereas the RRM3 mutant shows a wild-type growth phenotype (Fig. 2A) [31]. Interestingly, preliminary UV-crosslinking experiments suggest that the RNA-binding capacity of Rrm4 is more strongly affected by mutations in RRM3 than in RRM1 [31]. However, the precise mechanisms by which individual RRMs regulate target recognition, binding site specificity, and their functional impact remain elusive [23, 82]. Rrm4-mediated transport of septin mRNAs—including *cdc3*, *cdc10*, *cdc11*, and *cdc12*—is coupled to active translation during transit, a process critical for septin complex assembly and for establishing a gradient extending from the hyphal tip onwards [78, 79]. Consistently, ribosomal proteins co-localize with motile early endosomes in a translational- and Rrm4-dependent manner (Fig. 2A) [63, 78, 84]. Beyond septin transcripts, Rrm4 also binds to the stop codons of nuclear-encoded mRNAs for ATPase subunits of mitochondrial respiratory chain complex V [63, 82]. This highlights the conserved link between endosomal mRNA transport and mitochondrial supply across species—from fungi to neurons [63, 67, 69, 70, 85, 86].

Like other RBPs, Rrm4 functions within a dynamic protein network (Fig. 2A) [87], which includes the small RBP Grp1 (glycine-rich protein 1) and the mRNP scaffolding protein Upa2 [63, 82, 88]. The RNA-binding protein Grp1 shares a broad set of targets with Rrm4 but plays only an accessory role in endosomal mRNA transport and polarized growth [82]. In contrast, loss of Upa2 reduces mRNA shuttling and leads to pronounced

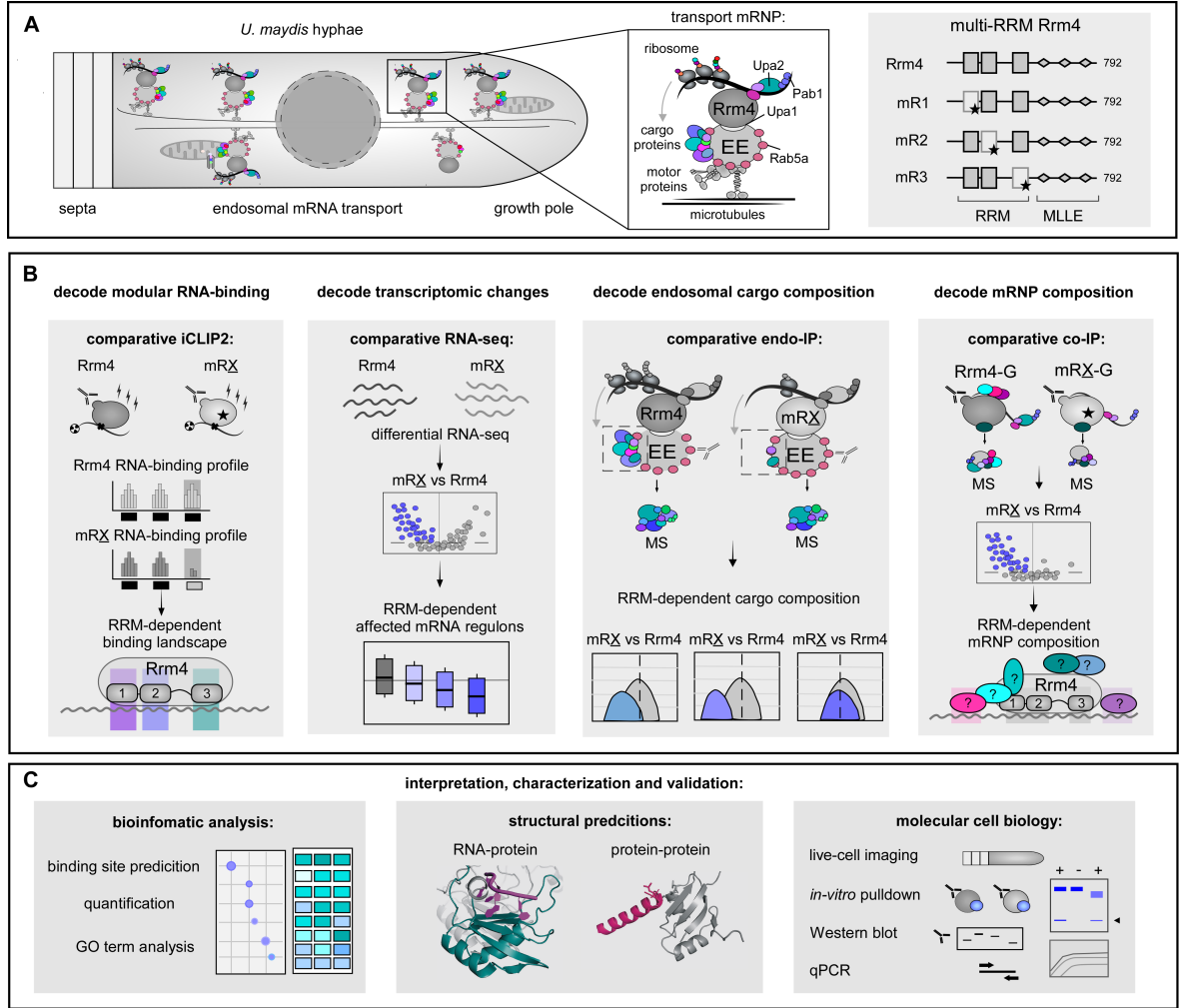


Figure 2: Multi-omics dissection of the RRM-mediated interactome of the RBP Rrm4

(A) Schematic overview of endosomal mRNA transport in polarized *Ustilago maydis* hyphae. The magnified view highlights translationally active endosomal mRNP complexes organized by the RNA-binding protein Rrm4. The domain architecture of Rrm4 comprises three RNA recognition motifs (RRMs) and three C-terminal MLLE domains. RRM mutants (mR1, mR2, mR3) harbor alanine substitutions in the conserved RNP1 motifs (asterisks). (B) Experimental strategy to dissect domain-specific functions of the RRM through an integrated multi-omics workflow. RNA-binding profiles are analyzed using iCLIP2 (first panel), transcriptome-wide expression changes are assessed by RNA-seq (second panel), and protein composition are resolved via endosomal immunoprecipitation followed by mass spectrometry (endo-IP MS, third panel) and co-immunoprecipitation mass spectrometry (co-IP MS, fourth panel). (C) Integration of bioinformatic analysis (first panel), structural predictions (second panel), and biochemical validation (third panel) enables functional annotation of RRM-dependent regulatory networks at both RNA and protein levels.

transport mRNP complex [88]. Upa2 interacts with the poly(A)-binding protein Pab1, which associates with the mRNA poly(A) tail and thereby stabilizes the mRNP complex during transport [53, 63, 88]. Initial findings indicate that the C-terminal GWW motif of Upa2 is essential for its co-localization with motile endosomes [88]. However, the

of Upa2 is essential for its co-localization with motile endosomes [88]. However, the exact mechanism by which it anchors to the endosomal transport mRNP remains unresolved [63, 88, 89].

Furthermore, preliminary co-IP MS data, supported by live-cell imaging, suggest that the translational repressor Ssd1 (Suppressor of SIT4 Deletion) associates with the Rrm4-mediated transport mRNP [89, 90]. These findings raise the question of whether additional RBPs are components of the endosomal transport mRNPs and may play regulatory roles during transport (Fig. 2A). To date, a high-resolution compositional dissection of the transport mRNP and its interaction networks remains conspicuously absent [16, 49]. Despite the evolutionary conservation of endosomal RNA hitchhiking from fungi to neurons, the molecular mechanisms governing RNA cargo selection and RBP cooperation during transit remain poorly understood [67, 68, 71, 82, 91]. Deciphering these principles will be essential to understanding how spatiotemporal mRNA transport and localization orchestrate cellular identity and functional specialization, as well as their roles in disease pathogenesis—such as ALS—and impaired fungal growth [57, 58, 63, 67, 70, 86].

3.7 Aim of the thesis

In *U. maydis*, endosomal mRNA hitchhiking is orchestrated by the multi-RRM RBP Rrm4, which is crucial for precise polarized growth [63, 75, 87]. Although the essential role of Rrm4 in mRNA trafficking is well established [62, 78], the molecular mechanisms underlying mRNA cargo recognition and its functional consequences remain poorly understood [82]. In particular, how the individual RRMs of Rrm4 contribute to RNA-binding specificity and mRNP assembly has not yet been mechanistically resolved [23, 82].

Thus, this thesis aims to systematically decode the modular interaction modes of the three RRMs of Rrm4, elucidating their distinct and cooperative functions (Fig. 2A). In details, it seeks to uncover mechanistic insights into the principles of cargo mRNA selection, RNA binding site definition, and the distinction between functional and accessory binding sites essential for precise endosomal mRNA transport (Fig. 2A–C). To resolve RNA-binding specificity at transcriptome-wide scale with nucleotide resolution, a high-sensitivity iCLIP2 protocol [41] will be adapted and improved for Rrm4, ensuring the preservation of both RNA and protein integrity (Fig. 2B). Comparative iCLIP2 experiments using RRM-specific mutants will delineate the individual and cooperative contributions of each RRM to RNA target site selection [31, 82]. In parallel, comparative RNA-seq of RRM-mutant strains will assess the transcriptome-wide consequences (Fig. 2A–B). This integrated strategy—combining high-resolution RNA-binding maps with transcriptomic profiling—will define how the RRMs govern target selection and their functional consequences, thereby providing mechanistic insights into mRNA cargo regulation.

While the Rrm4-mediated RNA-binding of septin mRNAs and their translation on shuttling endosomes is well characterized (Fig. 2A) [58, 79, 87], additional translational regulons associated with endosomes remain largely unexplored. This study will investigate how Rrm4 and its individual RRMs influence the composition of endosomal cargo proteome, with a particular focus on Rrm4-dependent mRNA regulons (Fig. 2B). To enable this, an endosome immunoprecipitation (endo-IP) protocol will be developed, allowing quantitative profiling of endosomal-associated RNA and protein cargo (Fig. 2B). A comparative mutant-based approach will further elucidate the functional relevance of individual RRMs in modulating the cargo proteome composition, enabling the discrimination of transport regulons (Fig. 2B).

Moreover, given that RBPs typically function within higher-order mRNP assemblies [5, 48, 49], this study also investigates the proteome-wide interactome of Rrm4. Thus, an improved comparative co-IP MS strategy will be developed and applied to Rrm4, its RRM mutants, and RNase-treated samples to define the RRM- and RNA-dependent protein interactome of Rrm4. Integrated with live-cell imaging and biochemical valida-

tion, this approach will elucidate how individual RRMs shape the mRNP profile and how RBPs cooperate to facilitate selective mRNA transport (Fig. 2B).

Finally, iCLIP2, transcriptomics, endo-IP MS, and co-IP MS will be integrated using bioinformatic analyses, structural modeling, live-cell imaging, and targeted molecular validation (Fig. 2C). This high-resolution, multi-omics framework will elucidate how the modular RRM architecture of Rrm4 governs both the RNA and protein composition of transport mRNPs, and how this influences endosomal cargo selection. By resolving the mechanistic roles of individual RRMs, this thesis will advance the understanding of mRNA transport and localized translation [55, 56, 60, 63, 92]. Moreover, it will establish a generalizable framework for decoding multi-RBD RNA-binding proteins [15, 22, 23], such as the mammalian RBP HuD (Fig. 1C) [30, 32]. These mechanistic insights will be pivotal for understanding how RNA-binding specificity and cooperative RBP networks are regulated—processes essential for defining mRNA fate (Fig. 2A–C) [3, 5, 6, 15, 21, 23, 48, 49, 58].

4 Microbial iCLIP2: Enhanced mapping of RNA-Protein interaction by promoting protein and RNA stability

The manuscript is published in *RNA*:

Microbial iCLIP2: Enhanced mapping of RNA–protein interaction by promoting protein and RNA stability

Nina Kim Stoffel, Srimeenakshi Sankaranarayanan, Kira Müntjes, Nadine Körtel, Anke Busch, Kathi Zarnack, Julian König, and Michael Feldbrügge

<http://www.rnajournal.org/cgi/doi/10.1261/rna.080193.124>

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Microbial iCLIP2: enhanced mapping of RNA–protein interaction by promoting protein and RNA stability

NINA KIM STOFFEL,¹ SRIMEENAKSHI SANKARANARAYANAN,¹ KIRA MÜNTJES,¹ NADINE KÖRTEL,² ANKE BUSCH,² KATHI ZARNACK,^{3,4} JULIAN KÖNIG,^{2,4} and MICHAEL FELDBRÜGGE¹

¹Institute of Microbiology, Heinrich Heine University Düsseldorf, Cluster of Excellence on Plant Sciences, 40204 Düsseldorf, Germany

²Institute of Molecular Biology GmbH, 55128 Mainz, Germany

³Buchmann Institute for Molecular Life Sciences (BMLS), Goethe University Frankfurt, 60438 Frankfurt am Main, Germany

⁴Theodor Boveri Institute, Biocenter, University of Würzburg, 97074 Würzburg, Germany

ABSTRACT

The entire RNA life cycle, spanning from transcription to decay, is intricately regulated by RNA-binding proteins (RBPs). To understand their precise functions, it is crucial to identify direct targets, pinpoint their exact binding sites, and unravel the underlying specificity *in vivo*. Individual-nucleotide resolution UV cross-linking and immunoprecipitation 2 (iCLIP2) is a state-of-the-art technique that enables the identification of RBP-binding sites at single-nucleotide resolution. However, in the field of microbiology, optimized iCLIP protocols compared to mammalian systems are lacking. Here, we present the first microbial iCLIP2 approach using the multi-RRM domain protein Rrm4 from the fungus *Ustilago maydis* as an example. Key challenges, such as inherently high RNase and protease activity in fungi, were addressed by improving mechanical cell disruption and lysis buffer composition. Our modifications increased the yield of cross-link events and improved the identification of Rrm4-binding sites. Thereby, we were able to pinpoint that Rrm4 binds the stop codons of nuclear-encoded mRNAs of mitochondrial respiratory complexes I, III, and V—revealing an intimate link between endosomal mRNA transport and mitochondrial physiology. Thus, our study using *U. maydis* as an example might serve as a blueprint for optimizing iCLIP2 procedures in other microorganisms with high RNase/protease conditions.

Keywords: RNA–protein interaction; iCLIP2; RNase activity; protease activity

INTRODUCTION

RNA-binding proteins (RBPs) are ubiquitous and play a pivotal role in determining the physiological state of the cell. Dysfunctional RBPs have been implicated in various human diseases (Gebauer et al. 2021). Hence, it is crucial to investigate RNA–protein interactions *in vivo* with a focus on understanding the interactome, its specificity, and regulation.

Depending on the research question, RNA–protein interactions can be studied from either an RNA- or protein-centric perspective (Hentze et al. 2018; Ramanathan et al. 2019; Hafner et al. 2021). The protein-centric approach facilitates the identification of unknown RNA targets for the RBP of interest and can be further categorized into two major groups. Methods involving chemical modifications of bound target RNAs (e.g., RNA tagging, TRIBE; Qin et al. 2021) and techniques that include protein purifi-

cation like RIP (RNA immunoprecipitation, Tenenbaum et al. 2000), CLIP (UV cross-linking and immunoprecipitation, Ule et al. 2003), and CRAC (UV cross-linking and analysis of cDNA, Granneman et al. 2009). In particular, iCLIP (individual-nucleotide resolution CLIP, König et al. 2010) stands out due to its high resolution, allowing the identification of precise binding motifs. The advantages of the method inspired the establishment of numerous optimized variants mainly in mammalian cells, such as irCLIP (infrared CLIP, Zarnegar et al. 2016), eCLIP (enhanced CLIP, Van Nostrand et al. 2016), and iCLIP2 (Fig. 1A; Buchbender et al. 2020).

The iCLIP2 protocol (Fig. 1A) includes the fundamental steps of iCLIP. However, drastic improvements were made to the procedure after cDNA synthesis (Buchbender et al. 2020). This leads to reduced material loss, thereby

Corresponding author: feldbrue@hhu.de

Handling editor: Javier Caceres

Article is online at <http://www.majournal.org/cgi/doi/10.1261/rna.080193.124>.

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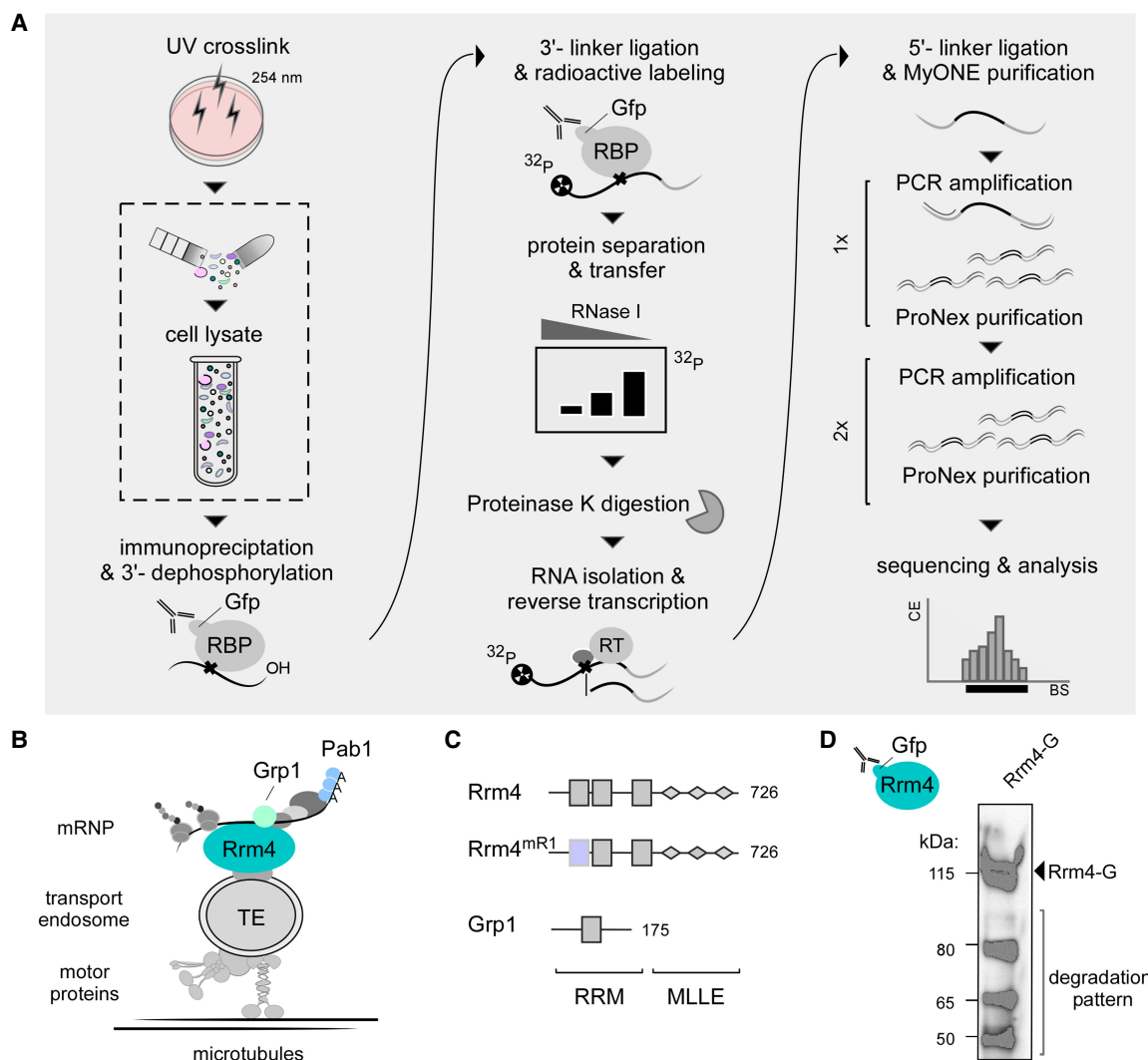


FIGURE 1. Adaptation and improvement of iCLIP2 procedure. (A) Diagrammatic overview of iCLIP2 procedure, with a specific emphasis on cell lysis (marked). (B) Endosomal mRNA transport model in *U. maydis*. The RBP Rrm4 (RNA recognition motif protein 4, petrol) binds directly to cargo mRNAs (black) and cotransports the associated RBPs like Grp1 (glycine-rich protein 1, mint) or the poly(A)-binding protein (Pab1, blue) in an RNA-dependent manner. (C) Schematic overview of the size (in AA) and domain architecture ([RRM] RNA recognition motif; [MLLE] Mademoiselle domains) of Rrm4, the amino acid substitutions mutant Rrm4^{mr1} and the RBP Grp1. (D) Western blot analysis of immunoprecipitated Rrm4-G (Rrm4-G; 112 kDa).

increasing the library depth. In the past, iCLIP has been adapted for use in plants (Meyer et al. 2017; Lewinski et al. 2024a), archaea (Bathke et al. 2022), as well as in fungi (Olgeiser et al. 2019). Since the protocol involves numerous critical and sensitive steps, establishing improved versions such as iCLIP2 in systems with high protease and RNase activities is nontrivial.

We are investigating endosomal mRNA transport, which is best studied in the model microorganism *Ustilago maydis* and highly polarized cells like neurons (Das et al. 2021; Bourke et al. 2023). In *U. maydis*, RNA recognition motif protein 4 (Rrm4) is the key RBP mediating endosomal RNA transport (Fig. 1B; Müntjes et al. 2021). Components of the transport complex are additional RBPs such as the

glycine-rich protein 1 (Grp1; Fig. 1B) and the poly(A)-binding protein 1 (Pab1). Rrm4 has an ELAV-like arrangement of a tandem RNA recognition motif (RRM1,2) and an additionally separated third RRM (RRM3, Fig. 1C). At its C-terminus, it contains a binding platform of three Mademoiselle domains (MLLE, Fig. 1C) important for endosomal attachment (Devan et al. 2022). To analyze the contribution of the different RRM of Rrm4, variants with amino acid block mutations in the RNA contact region RNP1 of the respective domains were generated (Becht et al. 2006). Pilot studies revealed that all mutants exhibit strongly reduced RNA binding (Becht et al. 2006). Consequently, a comparative iCLIP2 approach posed several technical difficulties, necessitating further improvements

of the method. Firstly, the reduced binding efficiency of the variants diminishes the amount of RNA starting material. Secondly, the high molecular weight of Rrm4 makes immunoprecipitation, gel separation, and blotting challenging. Thirdly, the protein Rrm4 is inherently unstable in vitro (Olgeiser et al. 2019). Fourthly, the method needs to be optimized for high RNase and protease activity common in fungi, leading to protein and RNA degradation upon cell lysis (Cortes-Maldonado et al. 2020). Of note, increased RNase and protease activity is also observed in other systems, such as pancreatic and liver tissue, as well as in plants (Augereau et al. 2016; Hafner et al. 2021). Thus, addressing these challenges with a well-defined lysis protocol that ensures high-quality, nondegraded starting material is essential and constitutes a significant advancement.

RESULTS

Advanced lysis techniques for consistent and high-quality iCLIP2 data

A crucial factor for maintaining adequate protein and RNA integrity is the implementation of an efficient lysis protocol. This is particularly important to ensure the quality and depth of an iCLIP2 experiment. As an example, we used Rrm4-G (Fig. 1D), a functional C-terminal fusion with the green fluorescent protein (Gfp) and Rrm4^{mR1}-G, a mutant with substitution of four amino acids in the RNP1 region of the first RRM domain (Fig. 2A, TVEF changed to AAAA; Becht et al. 2006). This mutation resulted in reduced RNA-binding in previous UV cross-linking experiments (Becht et al. 2006). Aliquots of the same batch (see Materials and Methods), i.e., hyphal cells expressing either Rrm4-G or Rrm4^{mR1}-G, were used as starting material to analyze four different cell disruption techniques (Fig. 2A; Supplemental Table S1). The approaches differed in terms of equipment, time efficiency, throughput/volume accessibility, temperature conditions, and the separation of mechanical and chemical lysis (dry or wet milling; see Supplemental Table S1; Materials and Methods). For techniques 1 and 2, “Bead mill jar” and “Bead mill tubes,” we used dry-cryogenic milling in a steel jar with two steel beads (12 mm diameter) or 2 mL tubes with one steel bead (5 mm diameter), respectively. In technique 3, “Homogenizer,” cell disruption relied on applying a Precellys 24 homogenizer, also using cryo conditions. In technique 4, “Vibrax,” cell disruption was achieved in 2 mL tubes containing glass beads (0.5 mm diameter) by rigorous shaking on a Vibrax apparatus. Cell material was initially also frozen using liquid nitrogen.

In direct comparison, dry-cryogenic milling (Bead mill tubes) using a test tube (2 mL) with a steel bead (5 mm diameter) greatly reduced the degradation of Rrm4-G and Rrm4^{mR1}-G (Fig. 2A; Supplemental Table S1). In addition,

it offers the advantage of processing numerous samples simultaneously in a time-efficient manner (Fig. 2A; Supplemental Table S1). The achieved results are technically (Fig. 2B) and biologically (Fig. 2C; Supplemental Fig. S1B) highly reproducible, indicating robust and solid cell disruption. Similar results were obtained for additional high-molecular weight RBPs, including Khd4 and Ssd1, as well as the chitinase interactor Jps1 (Fig. 2D; Khd4-G: Sankaranarayanan et al. 2023, Ssd1-G: Bayne et al. 2022, and Jps1-G: Reindl et al. 2020). In previous experiments in *U. maydis*, several degradation products were detected resulting in reduced amounts of full-length protein.

Next, we analyzed the compatibility of the improved lysis protocol with the immunoprecipitation approach of iCLIP2 (IP; Fig. 2E) using Rrm4-G and Rrm4^{mR1}-G. The input material displayed nondegraded, full-length protein versions (Fig. 2E). As expected, the degradation pattern was slightly increased after immunoprecipitation, attributed to the extended incubation time (see Materials and Methods). Nevertheless, the experimental setup was proven to be compatible. Thus, the new cell lysis protocol represents a significant improvement in degradation pattern, time efficiency, and simplicity of handling compared to previous protocols (Figs. 1D, 2E).

Optimizing iCLIP2 buffer conditions for enhanced protein–RNA complex integrity

Due to the high protease activity in fungal cell extracts, our standard lysis buffer includes 8 M urea to ensure protease denaturation (Devan et al. 2022). Typically, UV cross-linking-based approaches allow stringent highly denaturing conditions like urea and high salt concentrations without affecting the fixed protein–RNA interactions (Rosenberg et al. 2017; Challal et al. 2022). To assess the compatibility of the chaotropic agent urea with our microbial iCLIP2 approach, we verified that the pull-down efficiency remained consistent irrespective of urea concentration for Gfp heterologously expressed in *Escherichia coli* (Supplemental Fig. S1A).

To evaluate the influence of urea, we next analyzed Grp1-G, a small RRM-containing RBP that is highly amenable for iCLIP studies (Meyer et al. 2017; Olgeiser et al. 2019; Lewinski et al. 2024b). Unexpectedly, increasing urea concentrations above 2 M drastically reduced the signal of radiolabeled RNA (Fig. 3A), while the protein abundance remained unchanged (Supplemental Fig. S1C). This could be due to the impact of the urea concentration in either RNA integrity or radioactive labeling. Thus, we selected 2 M urea for iCLIP2 experiments with the more challenging protein variants Rrm4-G and Rrm4^{mR1}-G. Although the overall radioactive signal decreased when 2 M urea was added to the buffer, the radioactive signal was more concentrated in the region of interest, most likely due to an increase in full-length protein–RNA complexes

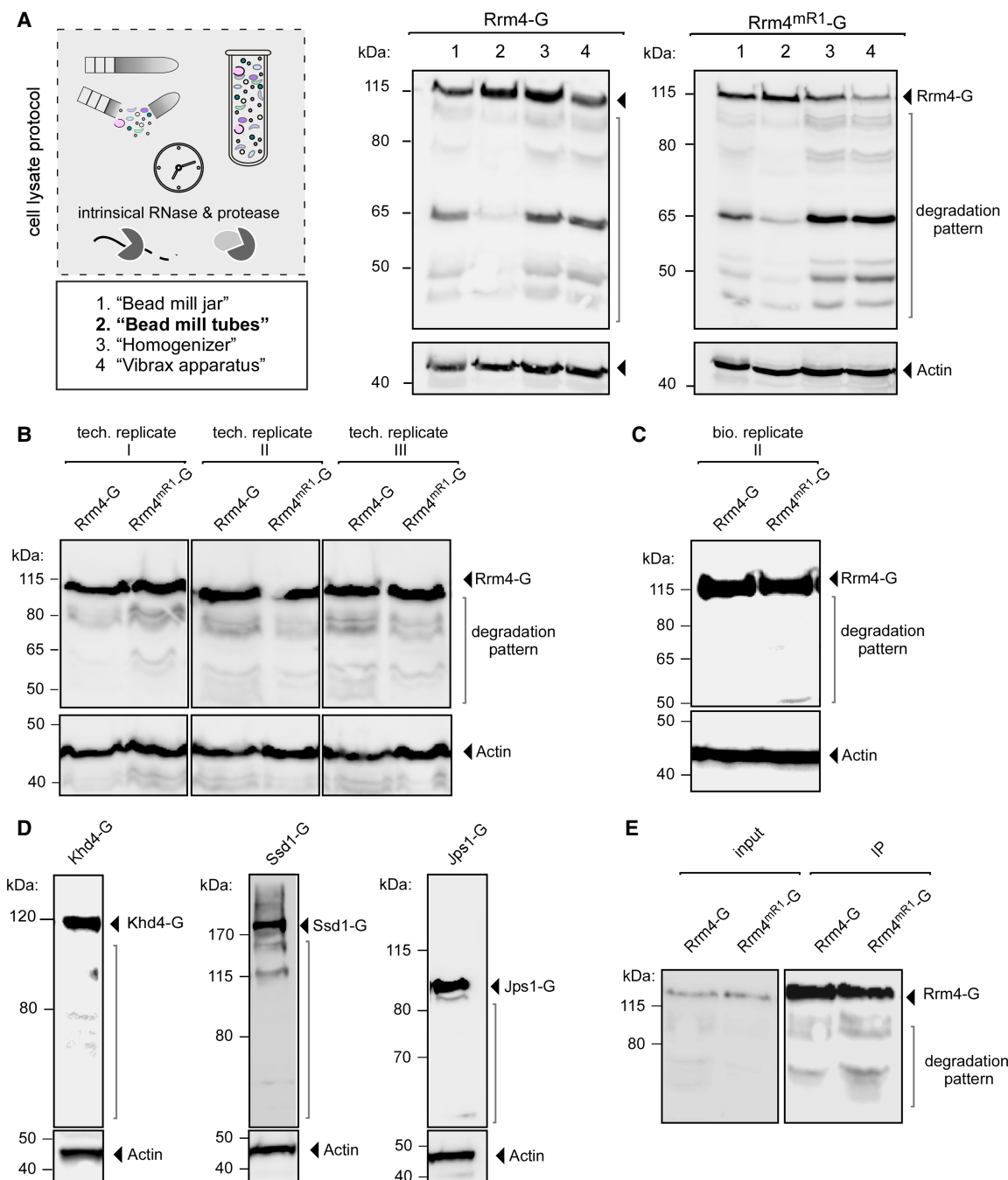


FIGURE 2. Improved mechanical cell distribution. (A) Four techniques to mechanically lyse cells were analyzed (1 = Bead mill jar, 2 = Bead mill tubes, 3 = Homogenizer, 4 = Vibrax apparatus). Start material from the same batches (Rrm4/Rrm4^{mR1}-G) was used. The respective cell lysates were subjected to western blot analysis (Rrm4/Rrm4^{mR1}-G = 112 kDa, indicated by arrow). The area of protein degradation is marked in dark gray (Actin, 42 kDa, served as control). (B) Western blot analysis of three technical replicates of technique 2 (Bead mill tubes) are shown. Technical replicates of Rrm4/Rrm4^{mR1}-G cell lysates were prepared from the same batches of frozen materials. The full-length protein is indicated by an arrow (112 kDa), and the respective degradation patterns are marked (Actin, 42 kDa, served as control). (C) Western blot analysis as in B. The respective biological replicates were performed as described previously. (D) Western blot analysis of the RBPs Khd4-G (178 kDa), Ssd1-G (180 kDa), and the protein Jps1-G (90 kDa; actin, 42 kDa, served as control). (E) Western blot analysis of immunoprecipitation of Rrm4/Rrm4^{mR1}-G (IP, 112 kDa, indicated by arrow).

(above 112 kDa; Fig. 3B,C; Supplemental Fig. S2A–C). Next, we tested the RBP Grp1 as positive control and Gfp as negative control (Supplemental Fig. S2D). As ex-

pected, Grp1 showed a strong radioactive signal, whereas Gfp did not (Supplemental Fig. S2D). Thus, by adding 2 M urea to our lysis buffer, we could reduce unspecific binding

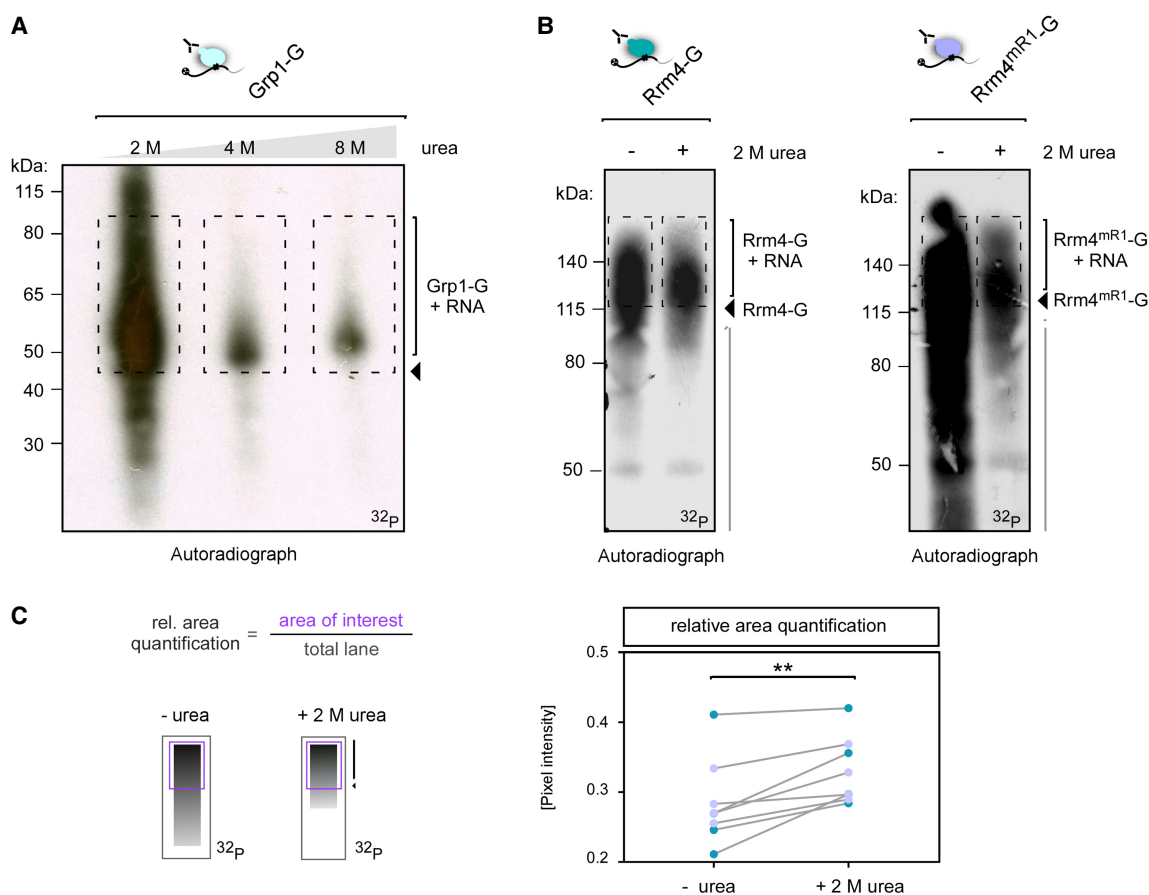


FIGURE 3. Optimized lysis buffer conditions. (A) Autoradiograph of Grp1-G-RNA (area of interest >45 kDa) complexes, purified with lysis buffer containing 2–8 M urea. (B) Autoradiograph of Rrm4/Rrm4^{mR1}-G-RNA complexes, immunoprecipitated with or without 2 M urea-containing lysis buffers. The area of interest is indicated (>112 kDa). The inclusion of 2 M urea in the lysis buffer results in a reduced overall radioactive signal, suggesting higher specificity. (C) The relative area quantification was determined by the signal intensity of the area of interest (purple) divided by the overall signal of the lane (marked black). Four independent biological replicates of Rrm4 and Rrm4^{mR1}-G/RNA-complexes, purified with and without 2 M urea-containing lysis buffer, were quantified (paired t-test; *P*-value: ** <0.01).

to Gfp (Supplemental Fig. S2D,E). In summary, the optimization of the urea concentration led to an increase in full-length protein and thus more starting material was available for the iCLIP2 experiment.

Improved cell lysis protocol maximizes RNA integrity

To prepare the sequencing library and to verify the length distribution of PCR products, it is necessary to determine the required minimum number of PCR cycles using a so-called optimization PCR. In contrast to the original protocol that resulted in very short PCR products, we now observed the opposite, i.e., unusually long PCR products (>250 bp; Fig. 4A) indicative of less RNase activity. In our previous iCLIP protocol, we used high concentrations of RNase inhibitors in our lysis buffer without adding external RNases. The remaining endogenous RNase activity was sufficient to degrade RNA resulting in short PCR fragments (Olgeiser et al. 2019). We were therefore interested in

whether the modified lysis procedure using 2 M urea, which reduced protease activity, also contributed to reduced RNase activity. By separating the corresponding area detected within autoradiography (Fig. 4B) into short (S: 115–140 kDa) and long fractions (L: >140 kDa), we confirmed that the cDNA library primarily consisted of longer RNAs (>250 bp) co-precipitated with Rrm4-G/Rrm4^{mR1}-G (Fig. 4B). Thus, our modification of the lysis procedure increased RNA integrity, highlighting the substantial impact of our improvements. We used 2 M urea throughout the remaining study. Although improved RNA integrity is desirable in principle, too long RNAs can hinder the effective separation of the RNA–protein complexes during gel electrophoresis (Haberman et al. 2017a). Furthermore, libraries prepared from extended RNAs can interfere with standard sequencing read length. Hence, our aim is an optimal range between 150 and 250 nt.

To further improve this step, we titrated the added RNase I within the recommended range of 1:50 to

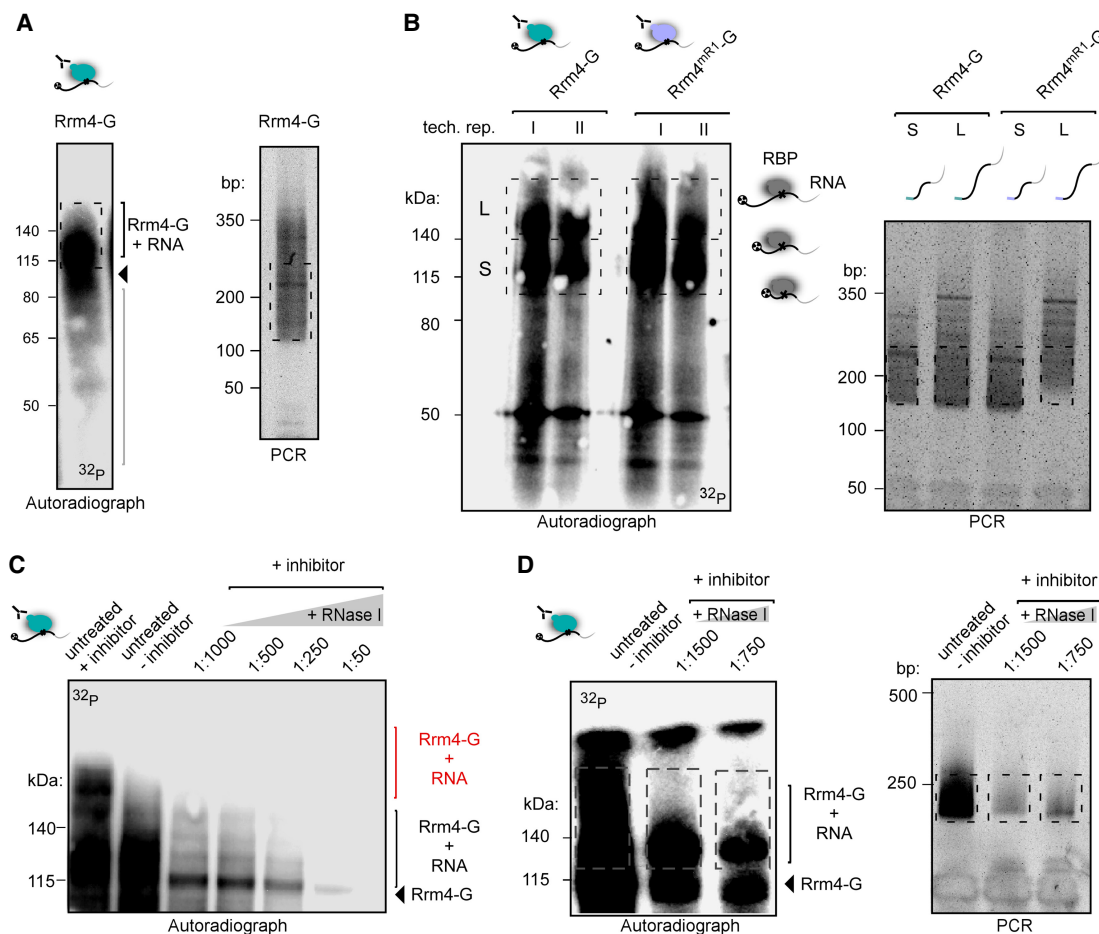


FIGURE 4. Enhanced RNA integrity. (A) Autoradiograph of Rrm4-G-RNA complex purified with 2 M urea. The optimization-PCR showed “unusually long” >250 bp cDNA products (optimal range is indicated). (B) Autoradiograph of two technical replicates of Rrm4/Rrm4^{mR1}-G. The area of interest was segmented into short (marked as S, 112–140 kDa) and long (marked as L, >140 kDa) RNAs. The respective cDNA libraries (S and L) were used for PCRs. The optimal range of cDNA length is indicated. (C) Autoradiograph of the RNase I titration series of Rrm4-G-RNA-complexes (>112 kDa, Rrm4-G full-length band indicated by an arrow, 112 kDa). Indicated in red are Rrm4-G/RNA-complexes exhibiting RNA fragments. The previous protocol procedure (untreated + inhibitor = no RNase I treatment, high amount of RNase inhibitor), internal RNase treatment (untreated, – inhibitor = no RNase inhibitor), and different RNase concentrations (1:1000–1:50 RNase I dilution + inhibitor) were analyzed by autoradiography. The inhibitor was added to the cell lysis buffer to reduce internal RNase activity. The respective RNase I (external) treatment was performed after IP within 1× PNK buffer, which does not include any RNase inhibitors. (D) Autoradiograph and optimization-PCR of Rrm4-G/RNA-complexes under diverse RNase I conditions. The experimental procedure as described in C was repeated with a dilution of 1:750 and 1:1500 RNase I instead, the cDNA library was synthesized, and respective optimization-PCRs were performed. PCR products show cDNA sizes in the expected area (marked).

1:1000 dilutions (Buchbender et al. 2020). Examining the intrinsic RNase activity revealed a strong signal of radioactively labeled RNA even in the absence of RNase inhibitors during lysis and immunoprecipitation steps (untreated, –inhibitor, Fig. 4C). Subsequently, we tested RNase I dilutions of 1:750, 1:1500, and intrinsic RNase activity (untreated, –inhibitor, Fig. 4D). As can be seen in Figure 4D, the intrinsic RNase gave the best results in terms of RNA length. Therefore, we omitted the RNase inhibitor from our lysis buffer for the subsequent steps.

In addition, we also examined the RNase conditions for Grp1-G (Supplemental Fig. S3A,B). Consistent with the previous results for Rrm4-G, Grp1-G co-purified RNAs were rather long in the presence of RNase inhibitors. The

smear of the Grp1-G/RNA complex improved under intrinsic RNases (untreated, –inhibitor) and external RNase I titration conditions. Interestingly, for Grp1, a dilution of 1:750 RNase I yielded the best result. This highlights the uniqueness of each RBP (Fig. 4D; Supplemental Fig. S3A,B), underscoring the importance of optimizing the conditions for each RBP of interest individually.

Reproducibility and enhanced resolution by microbial iCLIP2

To determine the quality and consistency of our microbial iCLIP2 protocol, we further investigated Rrm4-G and Rrm4^{mR1}-G (Fig. 5A–C). In both cases, the RBP-RNA

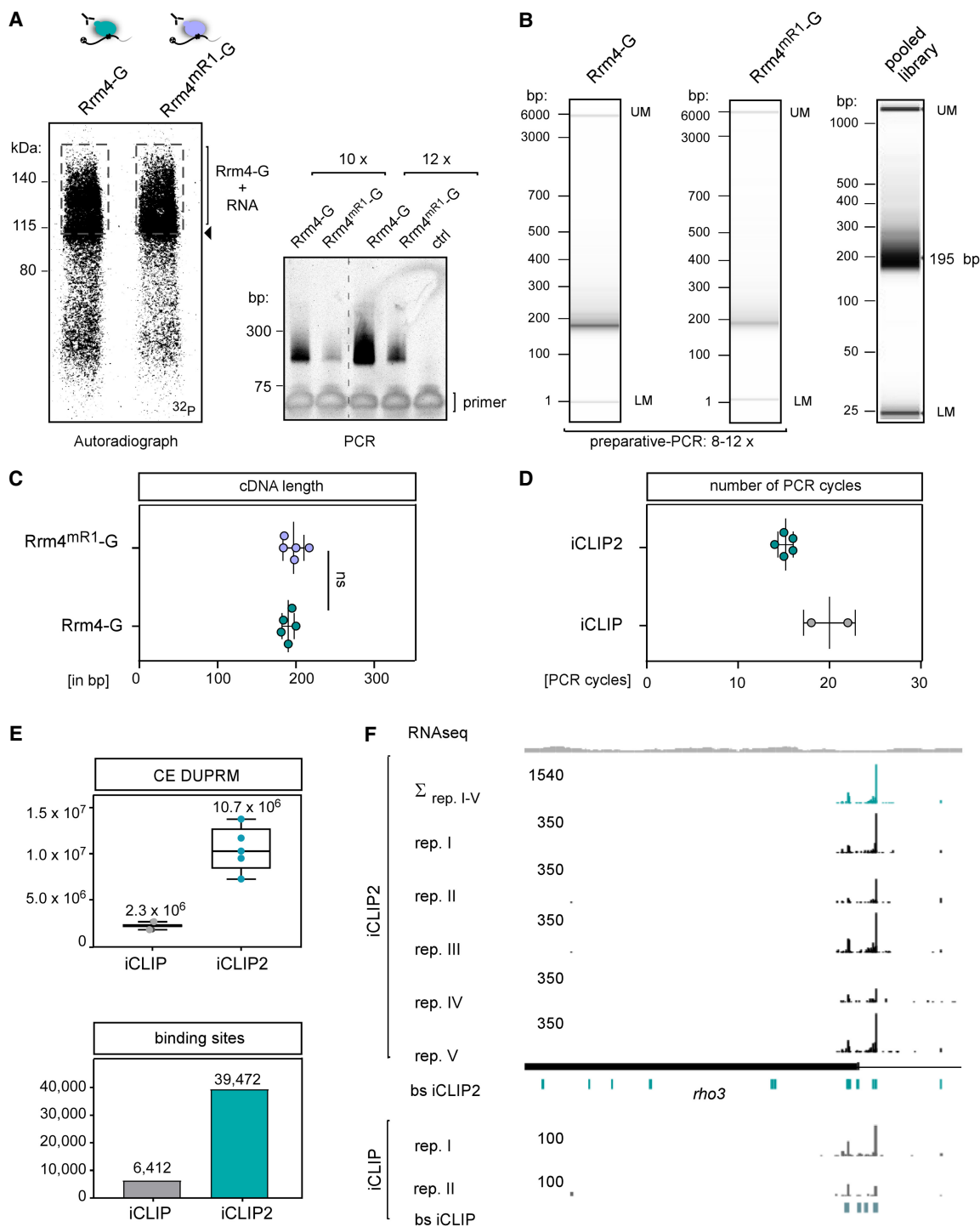


FIGURE 5. Adaptation of improved iCLIP2 protocol. (A) Autoradiograph and optimization-PCR of Rm4/Rm4^{mR1}-G. (B) Capillary gel electrophoresis image of the final PCR of Rm4/Rm4^{mR1}-G libraries before and after pooling. The respective number of PCR cycles depends on the RBP (Rm4 vs. Rm4^{mR1}-G) and the quantity of the respective cDNA library. The pooled library includes additional samples beyond Rm4-G. (C) Reproducible cDNA length based on the mean of $n = 5$ biological replicates of Rm4-G/Rm4^{mR1}-G. (D) Number of PCR cycles for Rm4-G needed for library preparation by iCLIP (18–22x; $n = 2$) or iCLIP2 (1. PCR: 6x; 2. PCR: 8–10x; in total = 14–16x; $n = 5$) procedure. (E) Quantified numbers of cross-link events after duplicate removal (CE after DUPRM) of Rm4-G iCLIP ($n = 2$) and Rm4-G iCLIP2 ($n = 5$). Number of binding sites determined for Rm4-G in iCLIP ($n = 6412$) versus iCLIP2 ($n = 39,472$). For both data sets, a nucleotide window of 9 nt has been chosen. (F) Genome browser view of *rho3* mRNA. The numbers represent the upper limit of stack heights for iCLIP(2) cross-link events (CE) and RNA-seq reads. The RNA-seq coverage of Wt filaments is depicted in gray. The five biological Rm4 replicates of iCLIP2 are depicted in black (mentioned as rep. I–V). The respective merged cross-link events of all five biological replicates and the determined binding sites (bs) are depicted in petrol. The *rho3* ORF is shown as a filled rectangle. The two biological Rm4 iCLIP replicates (gray, rep. I/II) and the determined bs (light petrol) are depicted underneath.

complex (above 112 kDa) and cDNA libraries were in the optimal range (about 150–250 bp; Fig. 5B,C). This validates the reproducibility of our procedure, demonstrating a consistent cDNA length without fluctuations attributed to intrinsic RNases (Fig. 5B,C). In addition, we were able to decrease the number of required cycles for the preparative PCR of Rrm4-G compared to our previously achieved Rrm4-G iCLIP experiments (Fig. 5D, our previous iCLIP: 18–22 cycles [Olgeiser et al. 2019]; modified iCLIP2: 14–16 cycles). In summary, our protocol is suitable for RBPs with lower binding capacity, as confirmed by Rrm4^{mr1}-G.

Next, we conducted the entire iCLIP2 procedure, analyzing five biological replicates of Rrm4-G, to obtain a more comprehensive understanding of the library quality. Data evaluation and quality control steps indicated that these libraries possessed high quality with the majority of the reads uniquely mapped (Supplemental Fig. S4A). For bioinformatics analysis, we used a slightly modified version of the iCLIP2 data processing workflow described in the original publication (see Materials and Methods; Busch et al. 2020). We obtained an average of 10.7 million cross-link events after duplicate removal per replicate ($n = 5$; CE after DUPRM; Fig. 5E, top), a great improvement from the previous data set ($n = 2$; average of 2.3 million CE after DUPRM, Olgeiser et al. 2019). This suggests a substantial enhancement in the signal-to-noise ratio, thereby improving binding site predictions (Fig. 5E). Also, the majority of binding sites were detected in all five biological replicates (Supplemental Fig. S4B), indicating a high level of reproducibility in the procedure. A comparison of the previous iCLIP (Olgeiser et al. 2019) and the improved iCLIP2 approach revealed that 94% of the previously defined target mRNAs were also detected in iCLIP2, with the identification of an additional 1757 targets (Supplemental Fig. S4C). As an example of the quality of iCLIP2, the well-characterized Rrm4-binding target mRNA *rho3* is depicted (Fig. 5F; König et al. 2009). All iCLIP2 replicates (rep I–V) show a similar binding (Fig. 5F; Supplemental Fig. S4B). Comparing iCLIP2 and iCLIP reveals a highly reproducible Rrm4-binding pattern, with both data sets showing predominant cross-link events within the 3' UTR of *rho3* (Fig. 5F). This demonstrates that the redefined protocol and the improved bioinformatic pipeline, including iCLIP data preprocessing (Busch et al. 2020), PureCLIP (Krakau et al. 2017), and BindingSiteFinder (Busch et al. 2020), did not introduce inconsistencies and that, across independent experiments, the RNA interactome remains consistent.

Rrm4 binds at stop codons of mRNAs encoding mitochondrial proteins of the respiration chain complexes

The previous Rrm4 iCLIP data had exhibited binding at the stop codon of a distinct set of target mRNAs (Fig. 6A;

Olgeiser et al. 2019). iCLIP2 showed a similar binding distribution (Fig. 6A). Furthermore, iCLIP2 revealed an expansion in stop codon targets ($n = 968$; Fig. 6B). The overlap between iCLIP and iCLIP2 ($n = 206$) demonstrated significant enrichment of the respiratory complex V, as previously reported (Supplemental Fig. S4D; Olgeiser et al. 2019). This new subset ($n = 968$) significantly enriched for components related to mitochondrial function, particularly within complex I (Fig. 6B). Therefore, we conducted a more in-depth analysis of the *U. maydis* respiratory complex I (Fig. 6C). A comparison of bacterial, fungal, and mammalian complex I components revealed that *U. maydis* comprises 27 nuclear and seven mitochondrial components, including the evolutionarily conserved core complex N, Q, and P, suggesting a fully functional complex I (Supplemental Table S2). Compared to the human version, *U. maydis* lacks 11 components, which are primarily located in the periphery (Supplemental Table S2), positioning *U. maydis* as an evolutionary intermediate between mammals and *Saccharomyces cerevisiae*, the latter of which lacks the multimeric complex I.

Analyzing transcriptome-wide Rrm4 binding revealed an interesting pattern for mRNAs encoding mitochondrial respiratory complexes (Fig. 6C). Binding sites at the stop codon position were evident for complexes I, III, and V of the mitochondrial respiratory chain complex within our iCLIP2 data set (Fig. 6C). Interestingly, for complex I, we found a prominent binding pattern within core subunits N and Q (Fig. 6C). As representative examples of the mitochondrial respiratory complexes, *nuo2* (UMAG_10820, Comp I; Fig. 6D), *qcr7* (UMAG_04237, Comp III; Fig. 6D), and *atp10* (UMAG_12212, Comp V; Fig. 6D) are depicted.

In summary, the enhanced resolution achieved with the improved iCLIP2 protocol has revealed a stronger connection between Rrm4 and nuclear-encoded mRNAs, specifically those encoding components of the respiratory complexes, with an emphasis on stop codon binding (Fig. 6A–D). Rrm4 potentially regulates the transport and translation of these mRNAs, thereby influencing mitochondrial energy production. However, it is important to note that our understanding of this biological mechanism is currently incomplete, and further experiments will be needed.

DISCUSSION

Establishing a tailor-made protocol for the study of microbial RBPs

To gain a deeper understanding of RNA biology in microorganisms, we aimed to adopt iCLIP2 (Fig. 6E; Buchbender et al. 2020). To overcome the challenge of high intrinsic RNase and protease activities in our system, a crucial step was the implementation of a new cell disruption protocol involving dry-cryogenic bead-milling for cell

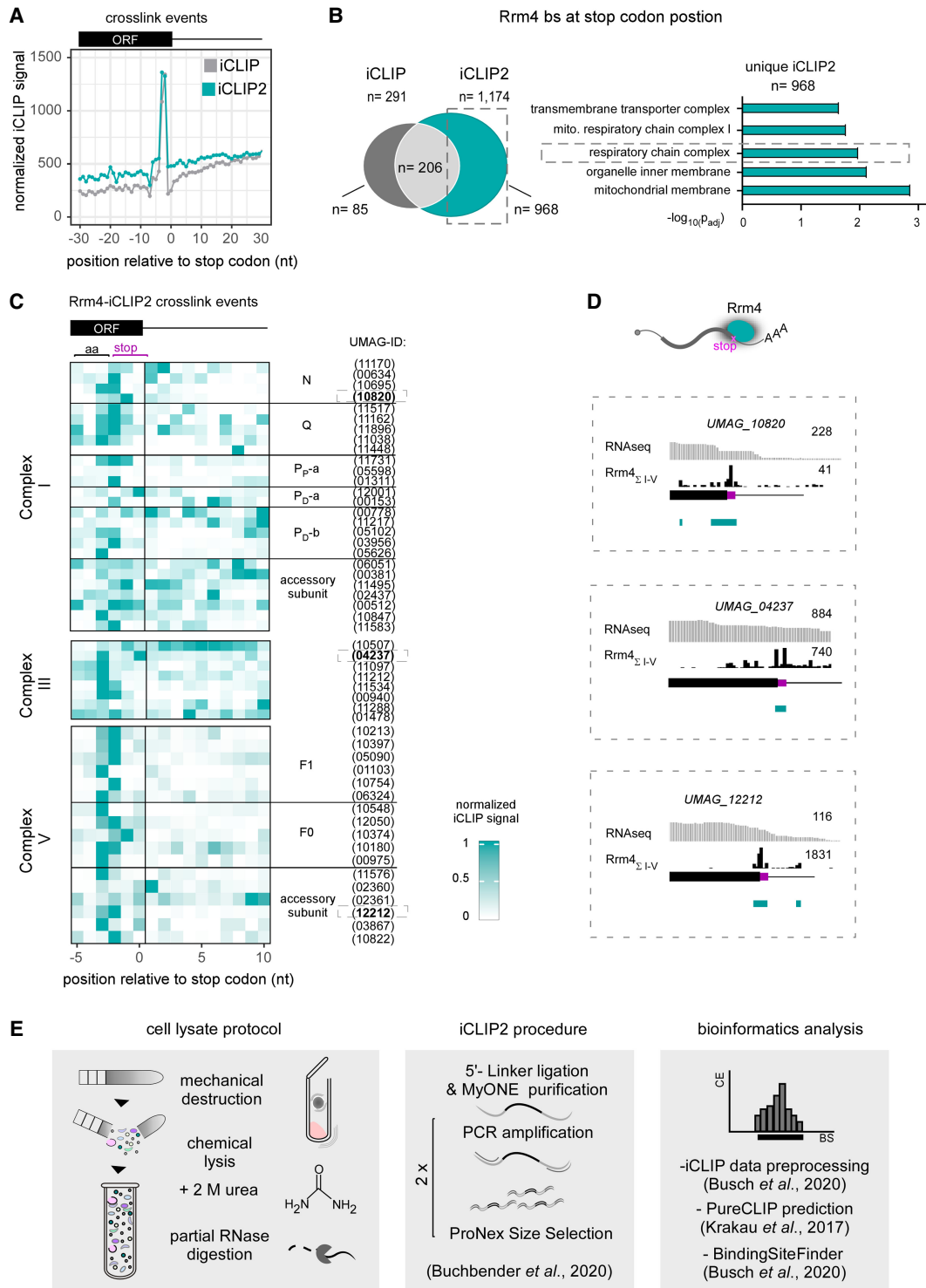


FIGURE 6. Rrm4 binds at stop codons of nuclear-encoded mRNAs of the respiratory chain complex I, III, and V. (A) Metaprofile of Rrm4 iCLIP (gray) and iCLIP2 (petrol) normalized signals relative to stop codon. (B) Venn diagram showing the number of Rrm4-binding sites at the stop codon position as detected by iCLIP and iCLIP2 (dark gray: uniquely found in iCLIP $n = 85$; light gray: shared targets $n = 206$; petrol: uniquely found in iCLIP2 $n = 968$). Selected bar chart from GO-term analysis of stop codon binding targets uniquely determined within the iCLIP2 data set. (C) Heatmap of Rrm4 iCLIP2 cross-link events in a window (-5 to 10 nt) around the stop codon position (0 is defined as the last nucleotide of the stop codon). The amino acid codon preceding the stop codon is indicated (aa). The core complex subunits N, Q, and P, as well as the accessory subunits of respiratory complex I, are indicated. Complex III and the subunits of complex V (F_1 and F_0) and their accessory subunits are also indicated. The respective identifiers (UMAG_ID) of the individual proteins are listed alongside. (D) Genome browser views of *nuo2* (UMAG_10820, Comp I), *qcr7* (UMAG_04237, Comp III), and *atp10* (UMAG_12212, Comp V) mRNAs. The predicted binding sites are depicted in petrol, and the stop codon is highlighted in magenta. (E) Schematic overview of the optimized microbial iCLIP2 procedure. Lysis protocol (left) was improved by the adaption of dry-cryogenic milling, buffer optimization by adding the chaotropic detergents urea (2 M), and internal RNase digestion. The adaptation of the advanced iCLIP2 procedure (center) and defined bioinformatics analysis (right), including data preprocessing, PureCLIP, and BindingSiteFinder is indicated (described in Materials and Methods).

lysis. Besides microbes, the method has proven applicability in plants (Box et al. 2011) and mammalian tissues (LaCava et al. 2016; Dou et al. 2020). Its efficiency in terms of time, suitability for high-throughput, and ability to enhance RNA integrity, particularly under conditions of high RNase activity such as in pancreatic tissues (Li et al. 2009; Chougoni and Grossman 2020; Munro et al. 2023) make it a versatile and valuable method.

Our refined lysis buffer, including 2 M urea, resulted in more precisely detected radiolabeled RBP-RNA complexes by improving protein stability. In addition, urea can reduce unspecific binding (Huppertz et al. 2014; Rosenberg et al. 2017; Lewinski et al. 2024a) and denature RNases (Almarza et al. 2009). By the reduction of RNase activity, the RNA integrity increased. Consequently, RNase titration became crucial, and within our setup, internal RNases have provided the best results for Rrm4. In contrast, for the highly abundant, small RBP Grp1 containing a single RRM, an external RNase I treatment has proven to be the most effective. This highlights the significance of optimizing the protocol based on the RBP used. It is important to note that relying on intrinsic RNase activity is generally not recommended due to the potential bias it can introduce through cleavage (Haberman et al. 2017b). However, we have not identified such a bias so far.

Applying an optimized protocol to study microbial RBPs

Due to the improved protocol (Fig. 6E), we achieved a deeper resolution, resulting in the identification of additional binding sites and targets of Rrm4. Hence, we identified Rrm4-binding sites at the stop codons of nuclear-encoded mRNAs for the respiratory chain complexes I, III, and V. Interestingly, our findings further strengthen the connection between vesicle-mediated mRNA transport and mitochondria (Müntjes et al. 2021), a relationship that has also been observed in neurons of *Xenopus laevis* and human (Cioni et al. 2019; De Pace et al. 2024) as well as HEK293 cells (Schuhmacher et al. 2023). However, whether Rrm4 plays a role in the translational regulation of these targets is not known so far. Overall, we gained a more extensive RNA interactome of Rrm4 by iCLIP2. This observation supports the idea that endosomes act as a universal mRNA transporter, contributing to the overall distribution of mRNAs (Olgeiser et al. 2019).

iCLIP studies are rare in organisms like plants and fungi or tissues like pancreatic, which exhibit high RNase/protease activity (Alvelos et al. 2021; Arribas-Hernandez et al. 2021). Generally, the realm of RNA-protein interactions in the microbial kingdom, particularly in vivo with high resolution, remains relatively underexplored. In *S. cerevisiae*, one study has utilized the iCLIP/miCLIP protocol (Varier et al. 2022). Most other studies have employed the CRAC methodology (Granneman et al. 2009; Challal

et al. 2022; Esteban-Serna et al. 2023), which includes a (tandem) affinity-purification step under denatured conditions and differs in cDNA library preparation. We recommend the optimized iCLIP2 procedure, as it yields highly reproducible cross-link events with excellent library depth.

Conclusions

In essence, the refined protocol has established a solid framework for successfully implementing a comparative microbial iCLIP2 approach of Rrm4 and respective RRM variants in *U. maydis*. We are optimistic that our enhanced procedure, specifically designed for biological samples with elevated RNase/protease activity, such as fungal hyphae, can yield substantial advantages. Finally, we envision that our improved lysis procedure is transferable to other RNA biochemistry methods, including CLIP, CRAC, RIP, and more.

MATERIALS AND METHODS

Cultivation of *Ustilago maydis*

The laboratory strain AB33 from *U. maydis* was used to synchronize filamentous growth by changing the nitrogen source (Brachmann et al. 2001). The cultures ($OD_{600} = 0.5$) were grown in complete medium (CM) supplemented with 1% glucose at 28°C with agitation at 200 rpm (described in detail in Devan et al. 2022). Filamentous growth was induced by shifting the cells to nitrate minimal (NM) medium supplemented with 1% glucose at 28°C, 200 rpm for 6 h postinduction (h.p.i; Brachmann et al. 2001). The filaments were harvested by centrifugation (7150g, 10 min, 4°C) and washed with 5 mL PBS (phosphate-buffered saline pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4).

Cell harvesting and lysis

Four different techniques were compared (Fig. 2A).

Technique 1: “Bead mill jar”

The cell pellet (50 mL cell culture) was resuspended in 2 mL lysis buffer, transferred into “steel jar” flash-frozen using liquid nitrogen (Supplemental Table S1), and stored at –80°C (Olgeiser et al. 2019). Two steel beads (12 mm diameter) were added to each jar. Subsequently, the filled steel jars were subjected to three rounds of shaking at 30 Hz for 10 min each (RetschMill M400), with periodic refreezing steps in liquid nitrogen. The frozen lysate was thawed for ~1 h at 4°C and transferred to a 2 mL reaction tube and centrifuged at 16,200g, 4°C, 10 min. The resulting supernatant was utilized for subsequent steps or flash-frozen as needed. Overall, the experiment is very time-consuming and not well-suited for processing multiple samples simultaneously or handling a large sample volume, primarily because only two steel cups can be run at the same time. To conduct a complete iCLIP(2) approach, each sample typically requires a minimum of 150 mL of culture. For two different samples, this means a total of six jars, 100 min of shaking time, an additional hour for thawing,

and the centrifugation step. The entire procedure typically takes ~3.5 h.

Technique 2: “Bead mill tubes”

The cell pellet (50 mL culture) was resuspended in 2 mL PBS, split, and transferred into 2 mL reaction tubes (each 1 mL). The tubes were subsequently centrifuged at 16,200g, 4°C, for 5 min, and the supernatant was completely removed. The tubes were placed in a floating holder and flash-frozen in liquid nitrogen (Supplemental Table S1). The tubes can be stored at –80°C until use. To each tube, one steel bead (5 mm diameter) was added and placed into a precooled TissueLyser Adapter (Qiagen). The samples underwent two rounds of cell lysis, each lasting for 30 sec at 30 Hz, with a liquid nitrogen cooling step in between. The resulting powder was reconstituted using 1 mL of lysis buffer. Cellular debris was separated by centrifugation at 16,200g for 10 min at 4°C. The supernatant was carefully transferred, and the samples were pooled again into a fresh precooled tube. An additional centrifugation step was performed for 5 min. The resulting cell lysate can be stored at –80°C. Utilizing the TissueLyser Adapter (Qiagen) in the RetschMill (M400), up to 48 of the 2 mL reaction tubes (24 tubes per adapter) can be accommodated simultaneously. For optimal thawing, tubes were placed in a Vibrax (Ika-Vibrax-Vxr, Electronic), and resuspension was achieved through gentle mixing for 5 min, <500 rpm at 4°C. In contrast to strategy 1, this method offers higher throughput and is more time-efficient (1–1.5 h).

Technique 3: “Homogenizer”

An alternative common method for disrupting difficult cell tissues involves the use of a homogenizer (Precellys 24, Peqlab). In this approach, the cell pellet from a 50 mL culture was resuspended in 2 mL of lysis buffer and transferred into 2 mL reaction tubes. A spoon of 0.5 mm glass beads was added, and the homogenization was carried out using a Precellys 24 homogenizer for 1 min at full speed under liquid nitrogen conditions (Supplemental Table S1). The suspension was thawed on ice and centrifuged at 16,200g for 10 min at 4°C to separate the cell debris. The resulting supernatant was carefully transferred into a new tube, and the centrifugation step was repeated for an additional 5 min.

Technique 4: “Vibrax technique”

As an additional option, we tested the “Vibrax technique” (Schmitz et al. 2019). In this method, a 50 mL culture pellet was resuspended in 650 μ L of buffer and transferred to a new 2 mL reaction tube containing 300 mg of 0.5 mm glass beads (Supplemental Table S1). The suspension was flash-frozen and subsequently thawed by shaking on a Vibrax apparatus (Ika-Vibrax-Vxr, Electronic) at 2000 rpm for 30 min at 4°C. The samples were centrifuged at 16,200g, 10 min, and 4°C. The complete supernatant was transferred into a fresh reaction tube and subjected to an additional spin (16,200g, 5 min, at 4°C). The resulting cell lysate can be stored (–80°C) or directly used for further experiments.

Western blot analysis

The protein concentrations of cell lysates were determined by Pierce BCA assay (Thermo Fisher; 23225) or standard Bradford assay (Bio-Rad, 1976). Samples were adjusted to similar concentrations (1 mg/mL) in NuPAGE. LDS sample buffer containing DTT

(Thermo Fisher, NP0007; DTT-Dithiothreitol) was added, according to the manufacturer’s instructions. The samples were loaded onto a 4%–12% NuPAGE Bis-Tris gel (Thermo Fisher, NP0321) and run for 1.5 h, 180 V. The gel was wet-blotted onto a nitrocellulose membrane (0.45 μ m, Amersham Protran) using 1 $\times$ NuPAGE Transfer buffer + containing 15% methanol (Thermo Fisher; NP00061) for 1.5 h at 30 V. For western blot detection, α -Gfp (1:2000 dilution; Roche, 11814460001) and α -Actin (1:2000, MP Biomedicals 0869100) from mouse were used as primary antibodies, while α -mouse HRP conjugate (1: 5000 dilution; Promega; W4021) was used as the secondary antibody. All antibodies were dissolved in 1 $\times$ TBS-T, including 5% milk powder. Detailed descriptions of general western blot performance are described in Devan et al. (2022). For detection, the Image Analyzer LAS400 (GE Healthcare) and ECL Prime (Amersham, RPN2232) were used, according to the manufacturer’s instructions.

Microbial iCLIP2 procedure

A detailed, step-by-step protocol is provided in the Supplemental Material (Supplemental Microbial iCLIP2 protocol).

For the iCLIP2 experiment, 150 mL of filamentous cell culture (OD_{600} = 0.5; 6 h.p.i) was used. The experiment was carried out in a 4°C cold room using material and buffers cooled with ice. The culture was divided into three 50 mL tubes and harvested by centrifugation (7150g, 10 min, 4°C). The pellets were washed with 5 mL of PBS (phosphate-buffered saline pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4) and centrifuged again (7150g, 10 min, 4°C). The final cell pellets were resuspended in 5 mL of PBS, transferred into a Petri dish (100 $\times$ 100 $\times$ 20 mm, Sarstedt: 82.9923.422), and kept on ice. The resuspended cells were UV irradiated (254 nm, 1 $\times$ 200 mJ/cm², Biolink UV-Crosslinker, Vilber-Lourmat) and pooled in a 50 mL tube. Cells were harvested by centrifugation (7150g, 10 min, 4°C) and resuspended with 13 mL PBS, evenly divided into six 2 mL reaction tubes ($\approx$ 25 mL culture per tube), and disrupted as described before (see “Bead mill tube”). The cryogenic cell powder was resuspended in 1 mL lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% IGEPAL CA-630, 0.1% SDS, 0.5% sodium deoxycholate, 2 M urea, 2 $\times$ Complete protease inhibitor EDTA-free [Merck, 11873580001], 1 mM DTT, 1 mM PMSF [+5 μ L SUPERase-In, Thermo Fisher AM2696]) per 2 mL reaction tube. The homogenate was centrifuged at 16,200g for 10 min at 4°C and the supernatant was transferred to a new tube and centrifuged (16,200g, 5 min, 4°C). The supernatants were pooled into 50 mL tubes. Protein concentrations were determined using Pierce BCA Protein Assay Kits (Thermo Fisher, 23225) and adjusted to match the sample with the lowest concentration (aiming for $\approx$ 2 mg/mL). Cell debris-free supernatants were distributed into three 2 mL tubes, each containing 20 μ L prewashed GFP-Trap beads (ChromoTek; gtma-20). Beads were prewashed twice with lysis buffer without inhibitors using a magnetic separation rack. Immunoprecipitation was conducted for 1 h at 4°C with continuous rotation. The beads were pooled in a 2 mL reaction tube and washed four times with 1 mL high-salt buffer (50 mM Tris-HCl, pH 7.4, 1 M NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 0.1% SDS, 0.5% sodium deoxycholate). This was followed by two washes with PNK buffer (20 mM Tris-HCl, pH 7.4, 10 mM Mg_2Cl , 0.2%

Tween-20). Finally, the beads were transferred into a fresh 1.5 mL reaction tube and washed three more times with PNK buffer. Radioactive labeling, RNA recovery, cDNA synthesis, and library preparation, were adopted from the original iCLIP2 protocol including information on oligonucleotide design (Buchbender et al. 2020). Western blot analysis was performed as described before (see above). The membrane was washed with PBS, covered with a transparent plastic bag and exposed to a BAS Storage Phosphor Screen (GE Healthcare) for ~1 h or overnight at -20°C , depending on the signal strength. The screen was visualized by a Typhoon laser scanner (Cytiva), according to the manufacturer's instructions. The iCLIP2 libraries were sequenced on an Illumina NextSeq 500 sequencing machine to generate 92 nt single-end reads, which included a 6 nt sample barcode as well as 5 + 4 nt unique molecular identifiers (UMIs).

RNase I treatment

An optional RNase I (Life Technologies, AM2295) treatment was performed after the immunoprecipitation. Various dilutions (ranging from 1:50 to 1:1500) were prepared in $1\times$ PNK buffer (20 mM Tris-HCl, pH 7.4, 10 mM Mg_2Cl_2 , 0.2% Tween-20) and added to the beads. The beads were incubated for 3 min, 37°C at 1000 rpm, and subsequently washed with cold high-salt buffer and handled on ice.

Processing of iCLIP2 reads and binding site definition

The processing of iCLIP2 reads and the definition of binding sites was conducted mostly as previously described (Busch et al. 2020). In brief, initial quality controls were performed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads with a Phred quality score below 10 in the barcode or UMI regions were discarded. Remaining reads were de-multiplexed based on the sample barcode, which is found on positions 6 to 11, using Flexbar (Dodt et al. 2012, version 3.4.0). Barcode and UMI regions as well as adapter sequences were trimmed from read ends using Flexbar, with a minimal overlap of 1 nt between the read and the adapter, and UMIs were added to the read names. Only reads with a minimum length of 15 nt were retained for subsequent analysis. Trimmed reads were mapped to the *U. maydis* genome (assembly version Umaydis521_2.0) and its annotation based on Ensembl release 51 (Yates et al. 2022) using STAR (Dobin et al. 2013, version 2.7.3a). During mapping, up to 4% mismatched bases were allowed without soft-clipping on the 5' end. Uniquely mapped reads were processed to remove PCR duplicates using UMI-tools (Smith et al. 2017, version 1.0.0). Cross-linked nucleotides were extracted using the BEDTools suite (Quinlan and Hall 2010, version 2.27.1), as described in Chapter 4.2 of Busch et al. (2020).

Processed reads from all five replicates were merged into a single data set and subjected to peak calling using PureCLIP (Krakau et al. 2017, version 1.3.1) with default parameters. The resulting cross-link sites were used to define binding sites with the R/Bioconductor package BindingSiteFinder (10.18129/B9.bioc.BindingSiteFinder, version 1.7.9). Prefiltering steps were implemented to retain only the most significant sites. First, cross-link sites ranked in the lowest 5% by PureCLIP score—a measure of binding affinity strength provided by PureCLIP—were excluded.

Second, to account for gene expression levels and to improve the binding site quality, cross-link sites with the lowest 10% PureCLIP score in each gene were filtered out. For defining the binding sites, processed cross-link sites within a distance closer than 8 nt were merged, requiring at least two out of nine sites to be covered by cross-link events. The centers of the binding sites were iteratively positioned at cross-link events with the highest PureCLIP score and extended by 4 nt on either side, resulting in binding sites with 9 nt width. Furthermore, binding sites were deemed reproducible if they harbored more cross-link events than the fifth percentile of the cross-link distribution across all binding sites, with at least two cross-link events per binding site, observed in four out of five biological replicates.

To assign gene and transcript regions to the binding sites, we utilized the gene annotation file version *U. maydis* 521_2.0.41. Genes in the *U. maydis* genome were manually extended by 300 nt on each side to encompass potential 5' UTR and 3' UTR regions (Sankaranarayanan et al. 2023). As a result of this manual extension, overlapping transcript types were resolved based on a hierarchical rule prioritizing genes in the following order: tRNA > mRNA > rRNA > snRNA > snoRNA. The transcript regions (5' UTR, CDS, intron, 3' UTR) most frequently observed were assigned to the binding sites to resolve overlapping transcript regions.

To visualize the metaprofile of cross-link events around the stop codon, cross-link events within a 61 nt window centered around the stop codon were extracted from the cross-link coverage for both iCLIP data sets. These cross-link events were normalized using the min-max normalization approach, scaling the minimum value to 0 and the maximum value to 1. This normalization accounted for differences in expression level and other variations. A heatmap of cross-link patterns around the stop codon of specific mitochondrial transcripts was generated using the R Bioconductor package ComplexHeatmap (Gu et al. 2016, version 2.18.0).

DATA DEPOSITION

iCLIP2 data are available in the NCBI Gene Expression Omnibus (GEO) under the SuperSeries accession number GSE273496.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

We thank laboratory members and Drs. Heiner Schaal, Alexander Steffen, and Lena Kröninger for critically reading the manuscript. We are grateful to Maria Méndez-Lago and the IMB Genomics Core Facility for their excellent support on iCLIP2 library sequencing and the use of their NextSeq 500 (funded by the Deutsche Forschungsgemeinschaft, DFG, German Research Foundation—P#329045328). In addition, research was funded by grants from the DFG under Germany's Excellence Strategy EXC-2048/1—Project ID 39068111 to M.F. as well as DFG-FOR2333-TP03 to M.F., DFG-FOR2333-TP02 to K.Z. and to J.K., Project-ID 267205415—DFG-SFB 1208 to M.F. (project A09) as well as Project ID 458090666—DFG-SFB1535 to M.F. (project A03).

Author contributions: N.K.S., S.S., K.Z., J.K., and M.F. designed this study and analyzed the data. S.S., A.B., N.K.S., and K.Z. performed the bioinformatic analysis. N.K.S. performed the iCLIP2 experiments supported by N.K. and J.K. N.K.S., K.M., and M.F. drafted and revised the manuscript with input from all co-authors. M.F., Z.K., and J.K. contributed funding and resources.

Received August 6, 2024; accepted November 21, 2024.

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MEET THE FIRST AUTHOR



Nina Kim Stoffel

Meet the First Author(s) is an editorial feature within *RNA*, in which the first author(s) of research-based papers in each issue have the opportunity to introduce themselves and their work to readers of *RNA* and the RNA research community. Nina Kim Stoffel is the first author of this paper, “Microbial iCLIP2: enhanced mapping of RNA–protein interaction by promoting protein and RNA stability.” Nina is a PhD student at Heinrich Heine University, Düsseldorf, Germany, under the supervision of Professor Michael Feldbrügge. Her research focuses on dissecting the functional roles of individual RNA-binding domains in RNA–protein interactions, with a particular emphasis on their significance in endosomal mRNA transport.

What are the major results described in your paper and how do they impact this branch of the field?

I believe this work underscores the critical importance of obtaining high-quality starting material and optimizing it for complex methodologies. It demonstrates that small, well-defined steps can significantly influence the success of sophisticated techniques like iCLIP2, a principle that can be broadly applied to enhance the reliability and performance of other advanced techniques as well.

What led you to study RNA or this aspect of RNA science?

RNA–protein interactions are highly complex and intricately regulated at multiple levels, which often makes them challenging and complicated to study. However, this very complexity is what I find incredibly fascinating and exciting, as it continually pushes me to expand my knowledge and redefine my understanding of molecular biology.

During the course of these experiments, were there any surprising results or particular difficulties that altered your thinking and subsequent focus?

One of the most memorable moments during this process was when I had just finished optimizing protein integrity, only to realize that my optimizations had simultaneously improved RNA integrity to such an extent that I had to start over to determine the optimal RNase conditions. I remember being unsure whether to be excited or frustrated. In hindsight, it was a perfect example of how solving one problem often reveals another—an experience that truly embodies the essence of scientific research.

What are some of the landmark moments that provoked your interest in science or your development as a scientist?

Attending the conference “Ribosome heterogeneity and specialization” was a landmark moment that elevated my interest in science to a new level. I am fascinated by the complexity of ribosomes and the concept that they are more than just uniform molecular machines. The idea of specialized ribosomes and defined hotspots of translation, along with their distinct functions within the cell, captivated me. This experience reinforced my realization that there is still so much left to discover in this field, further fueling my passion for scientific exploration.

Microbial iCLIP2: Enhanced mapping of RNA-Protein interaction by promoting protein and RNA stability

Nina Kim Stoffel¹, Srimeenakshi Sankaranarayanan¹, Kira Müntjes¹, Nadine Körtel², Anke Busch², Kathi Zarnack³, Julian König², Michael Feldbrügge¹

¹ Institute of Microbiology, Heinrich Heine University Düsseldorf, Cluster of Excellence on Plant Sciences, 40204 Düsseldorf, Germany

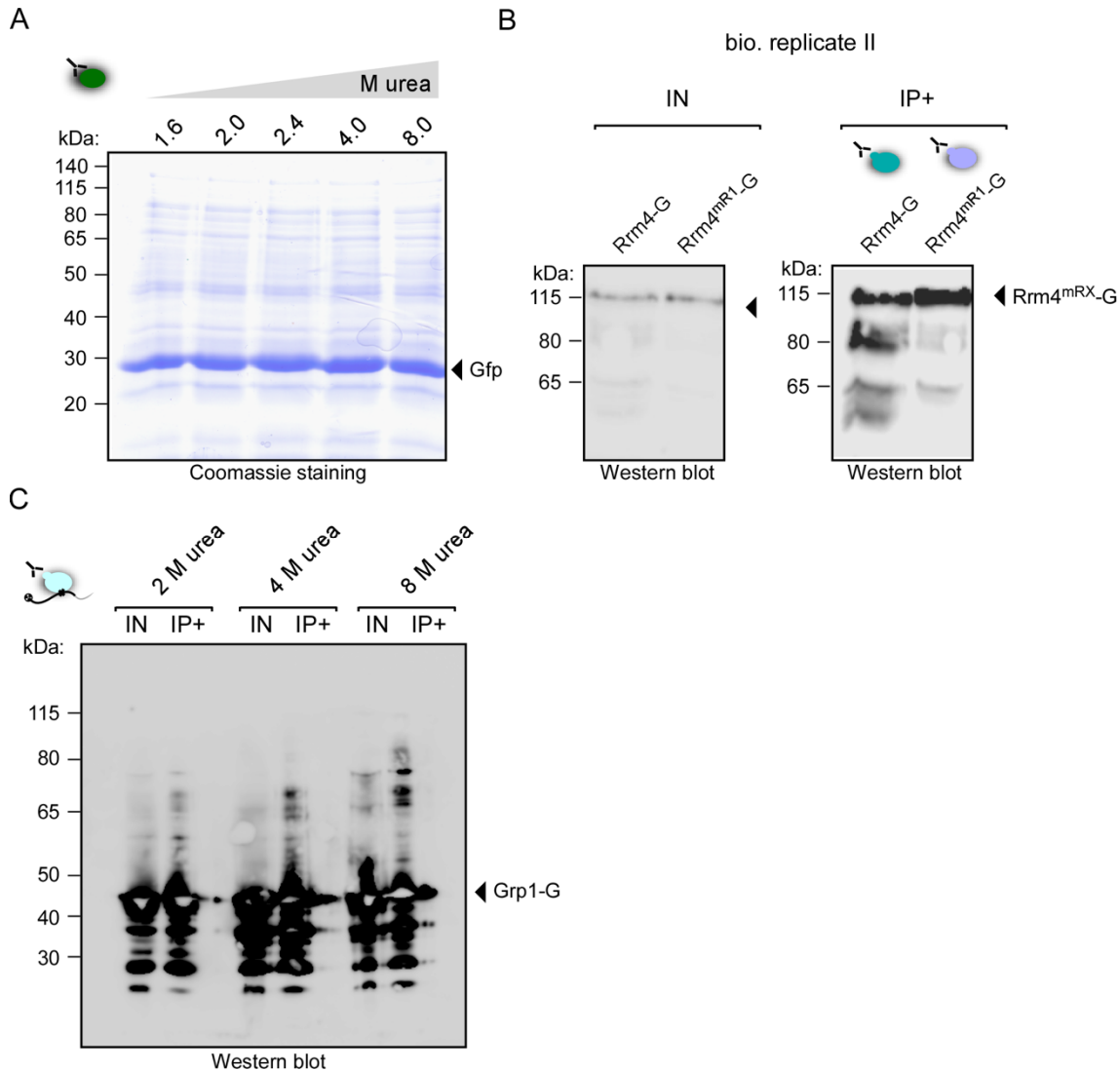
² Institute of Molecular Biology gGmbH, Ackermannweg 4, 55128 Mainz, Germany

³ Buchmann Institute for Molecular Life Sciences (BMLS), Goethe University Frankfurt, Max-von-Laue-Straße 15, 60438 Frankfurt am Main, Germany

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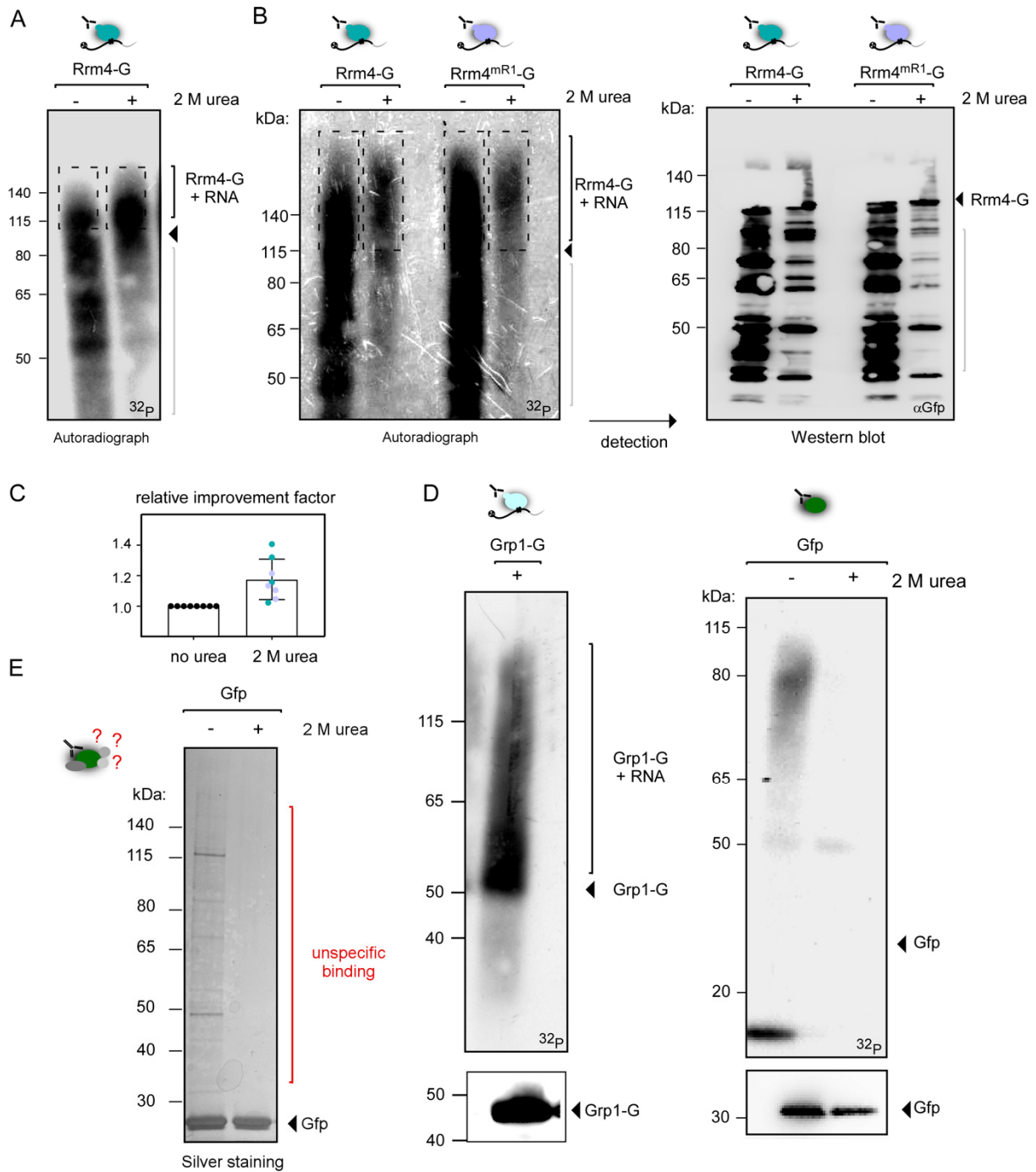
Supplemental Figure S1	2
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Supplemental Figure S1



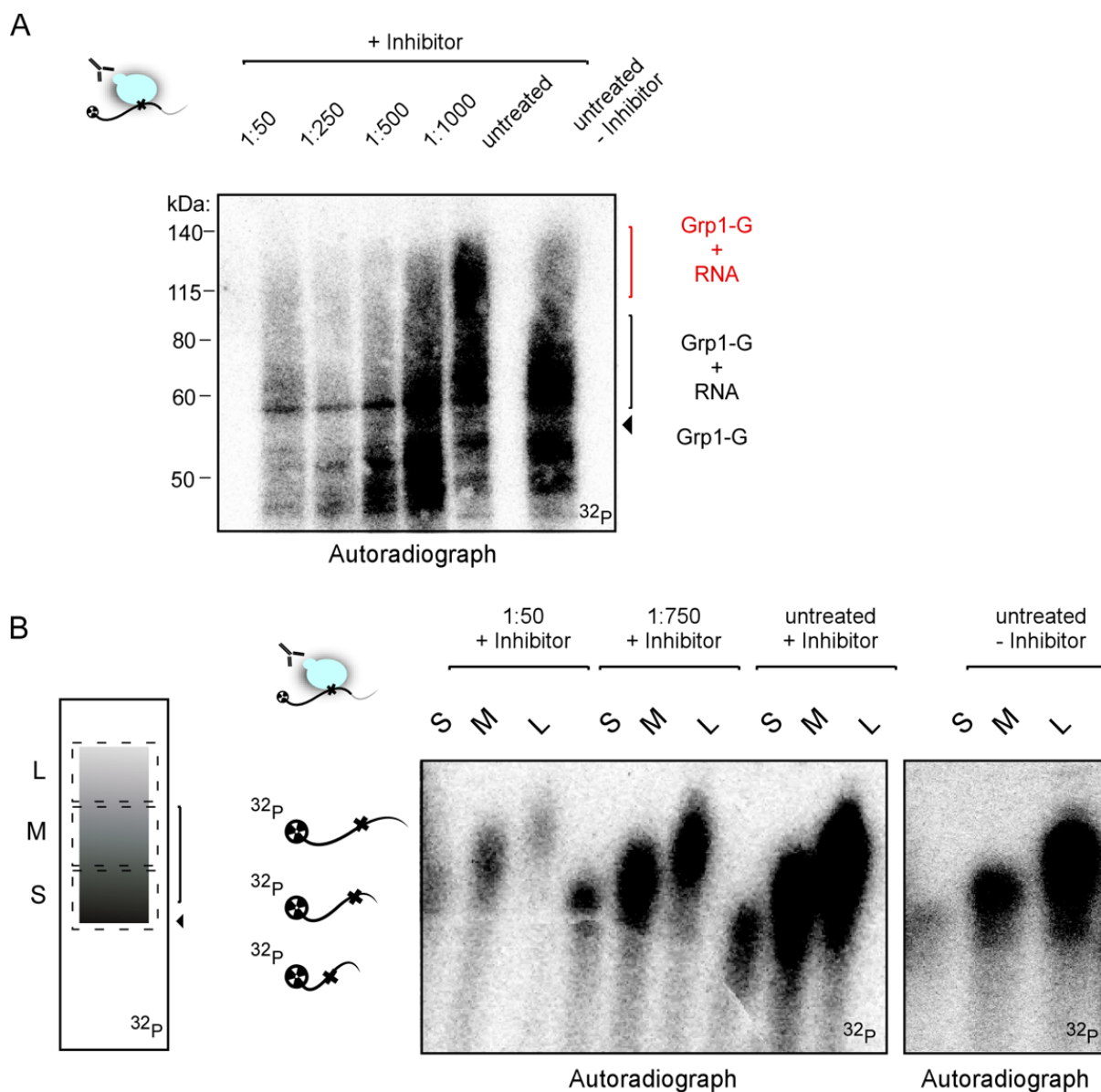
Supplemental Fig. S1. Increased protein stability by improved iCLIP2 protocol. (A) Coomassie-stained SDS-gel of Gfp IP, purified under different urea molarities conditions. Gfp was expressed in *E.coli* and the respective cell lysate was used as starting material for IP. (B) Western Blot analysis Rrm4-Gfp IP (IP+) by the usage of lysing buffer containing 8 M urea. Cell lysate (Input = IN) (C) Western blot of Grp1-Gfp iCLIP2 (IP+) experiments and respective cell lysate (IN) were produced under different urea conditions (2-8 M urea) - the respective autoradiograph is depicted in Fig. 3B. cell lysate (IN)

Supplemental Figure S2



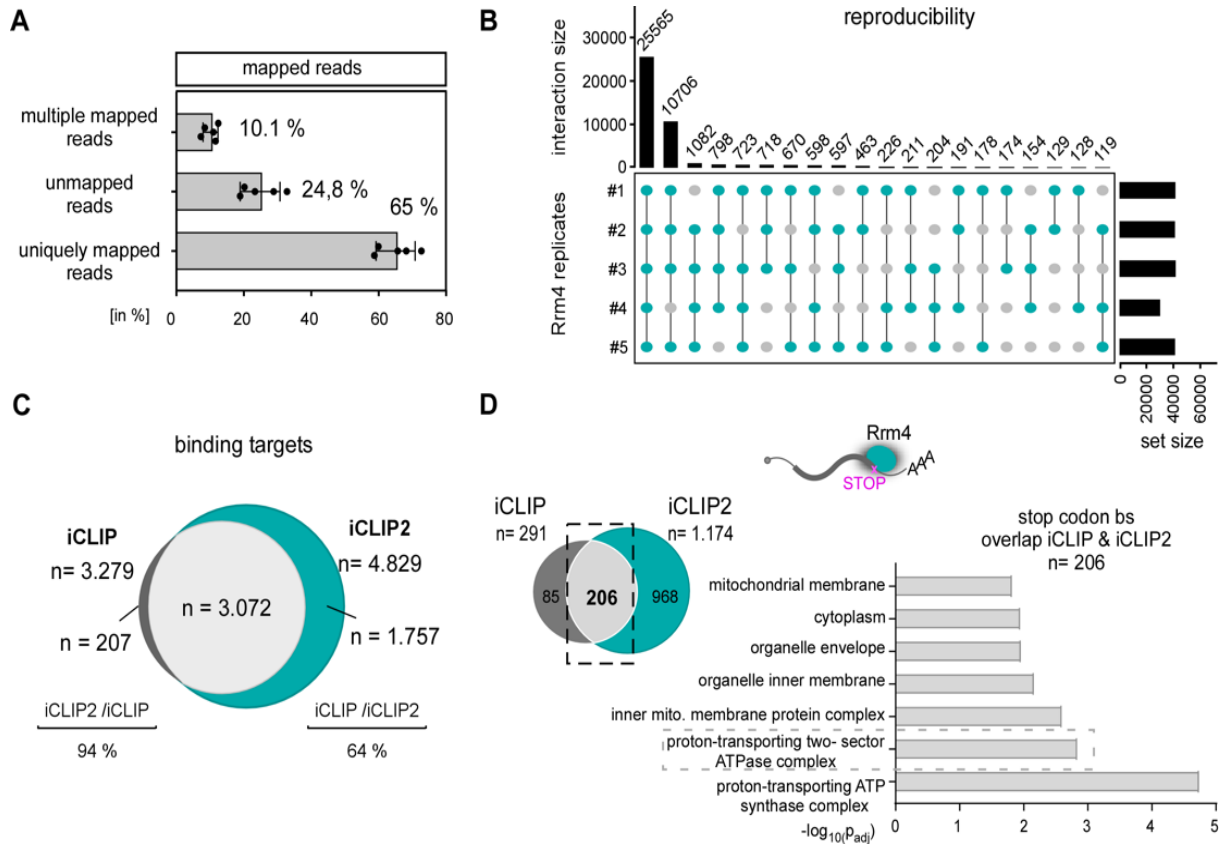
Supplemental Fig. S2. 2 M urea increases protein stability. (A -B) Autoradiograph of Rrm4-Gfp-RNA complexes by the usage of IPs +/- 2 M urea containing lysis buffer (biological replicate). In B the respective Western blot is depicted. (C) The relative improvement factor was calculated by dividing the results of relative area quantification (refer to Figure 3D) with the untreated buffer (+ 2M urea/ - urea). (D) Autoradiograph of Grp1-Gfp-RNA complexes and free cytoplasmic Gfp-RNA complexes, purified +/- 2 M urea conditions. Respective Western blots are below. (E) Silver-stained SDS-gel from IP Gfp samples prepared +/- 2 M urea containing lysis buffer. Unspecific co-purified proteins are indicated in red.

Supplemental Figure S3



Supplemental Fig. S3. Optimal RNase I conditions for microbial iCLIP2 protocol. (A) Autoradiography of RNase I titration series for Grp1-Gfp. In addition, intrinsic RNase digestion was tested (untreated - Inhibitor). The non-adequately-separated Grp1-Gfp-RNA complexes are marked in red. (B) Autoradiograph of RNase I titration for Grp1-Gfp-RNA complexes (1:50, 1:1750), untreated +/- Inhibitor. The respective autoradiograph was separated into short (S), medium (M) and long (L) RNAs. The respective size-selected Grp1-RNA complexes were isolated and separated by a 6 % TBE-Urea-gel as described by Huppertz et al., 2014.

Supplemental Figure S4



Supplemental Fig. S4. Improvements achieved in iCLIP2 data set by optimizing the protocol. (A) Bar chart of mapped reads out of the 5 biological Rrm4 replicates of iCLIP2. The majority of reads are uniquely mapped, as expected. The high number of unmapped reads is due to the low-quality genome of *U. maydis*. Reads within the „unmapped“ group for example could be manually determined as rRNAs of *U. maydis*. (B) UpSet plot of 5 biological replicates of iCLIP2 Rrm4. Indicating high reproducibility of the biological replicates (C) Ven diagram of iCLIP vs. iCLIP2 Rrm4 overall binding targets (D) Bar chart of gProfile analysis of stop codon binding targets determined within both iCLIP and iCLIP2 datasets (overlap n=206)

Table S1. Summary of different cell lysis conditions

Technique:	1 Bead mill jar	2 Bead mill tubes	3 Homogenizer	4 Vibrax
Cryogenic conditions	+	++	++	
Time efficiency		++	++	+
High throughput/ Volumina		++	++	++
Mechanical separation/ chemical lysis		++		

^a For details of the four different techniques see Materials and methods.

Table S2. Mitochondrial respiratory chain complex I and III

Respiratory chain complex I	
N	
NDUFV1	UMAG_11170
NDUFV2	UMAG_00634
NDUFS1	UMAG_10695
NDUFA2	UMAG_10820
Q	
NDUFA5	UMAG_11517
NDUFS2	UMAG_11162
NDUFS3	UMAG_11896
NDUFS7	UMAG_11038
NDUFS8	UMAG_11448
Pp-a	
ND1	mito-DNA
NDUFA3	UMAG_11731
NDUFA8	UMAG_05598
NDUFA13	UMAG_01311
Pp-b	
ND2	mito-DNA
NDUFC1	unkown
NDUFC2	unkown
ND3	mito-DNA
ND4L	mito-DNA
ND6	mito-DNA
PD-a	
NDUFB5	unkown
NDUFB10	UMAG_12001
NDUFB11	UMAG_00153
NDUFB6	unkown
ND4	mito-DNA
PD-B	
NDUFAB1	UMAG_00778
NDUFB7	UMAG_11217
NDUFB3	UMAG_05102
NDUFB8	UMAG_03956
ND5	mito-DNA
NDUFB9	UMAG_05625
NDUFB2	unkown
added at later stage	
NDUFA1	unkown
NDUFA10	unkown
NDUFS5	UMAG_06051
NDUFB1	unkown
NDUFB4	unkown
NDUFA9	UMAG_00381
NDUFA7	unkown
NDUFA11	UMAG_11495
NDUFAB1	UMAG_00778
NDUFA6	UMAG_02437
NDUFS4	UMAG_00512
NDUFV3	Unknown
NDUFA12	UMAG_10847
NDUFS6	UMAG_11583
Respiratory chain complex III	
ISP	UMAG_10507
QCR7	UMAG_04237
QCR10	UMAG_11097
QCR9	UMAG_11212
Cyt1	UMAG_11534
QCR6	UMAG_00940
QCR8	UMAG_11288
QCR2	UMAG_01478
COR1	unkown
Cytb	unkown

4.1 Author contributions

Figure 1

(A–C) Nina Kim Stoffel (NKS) designed the graphics with input from Prof. Dr. Michael Feldbrügge (MF). (D) Western blot performed by NKS.

Figure 2

(A) NKS designed the graphics with input from all co-authors. (A–E) Western blot performed by NKS.

Figure 3

(A–C) Autoradiographs and data quantification performed by NKS.

Figure 4

(A–C) Autoradiographs and PCR data performed by NKS.

Figure 5

(A) iCLIP2 experiments, autoradiographs, and PCR data performed by NKS. (B) iCLIP2 libraries performed by NKS; visual quantification via TapeStation by Nadine Körtel (NK). (C) iCLIP2 libraries and data quantification performed by NKS. (D) iCLIP2 libraries performed by NKS; iCLIP data originally published in Olgeiser et al., 2019; data analysis and visualization performed by NKS. (E) iCLIP2 libraries performed by NKS; data analysis by Anke Busch (AB) and Srimeenakshi Sankaranarayanan (SM); iCLIP data from Olgeiser et al., 2019. (F) iCLIP2 libraries performed by NKS; data analysis by AB and SM; iCLIP data from Olgeiser et al., 2019.

Figure 6

(A) iCLIP2 libraries performed by NKS; data analysis by AB and SM; iCLIP data from Olgeiser et al., 2019; visualization performed by SM. (B) iCLIP2 libraries performed by NKS; iCLIP2 analysis by AB and SM; iCLIP data from Olgeiser et al., 2019; overlap and GO term analysis performed by NKS. (C) iCLIP2 libraries performed by NKS; iCLIP2 data analysis by AB and SM; visualization performed by SM. (D) iCLIP2 libraries performed by NKS; data analysis by AB and SM; visualization performed by NKS. (E) NKS designed the graphic with input from all co-authors.

Supplementary Figure 1

(A–C) Protein biochemistry experiments and data visualization performed by NKS.

Supplementary Figure 2

(A–D) iCLIP2 experiments, protein biochemistry experiments, and data visualization performed by NKS.

Supplementary Figure 3

(A–B) iCLIP2 experiments and data visualization performed by NKS.

Supplementary Figure 4

(A, C, D) iCLIP2 libraries performed by NKS; iCLIP2 data analysis by AB and SM; data comparison and visualization performed by NKS. (B) iCLIP2 libraries performed

by NKS; iCLIP2 data analysis by AB and SM; data visualization performed by SM.

Supplementary Table:

SI-Tab. 1–2: Data analysis compiled by NKS with input from MF.

microbial iCLIP2 protocol

The detailed step-by-step protocol was written by NKS, with input from MF, and is accessible online at RNA Journal (<http://www.rnajournal.org/cgi/doi/10.1261/rna.080193.124>)

Literature research:

NKS and MF conducted the literature review.

Writing:

The manuscript was written by NKS and MF, with input from Drs. Florian Altegoer, Kerstin Schipper, Alexander Steffen, Heiner Schaal, and all co-authors.

5 Dissecting the RNA-binding capacity of the multi-RRM protein Rrm4 essential for endosomal mRNA transport

This manuscript is currently under revision in *Nucleic Acids Research*:
Dissecting the RNA-binding capacity of the multi-RRM protein Rrm4 essential for endosomal mRNA transport
Nina Kim Stoffel*, Srimeenakshi Sankaranarayanan*, Kira Müntjes, Anke Busch, Julian König, Kathi Zarnack, and Michael Feldbrügge
<https://doi.org/10.1101/2025.02.12.636894>

Dissecting the RNA binding capacity of the multi-RRM protein Rrm4 essential for endosomal mRNA transport

**Nina Kim Stoffel^{1,*}, Srimeenakshi Sankaranarayanan^{1,*}, Kira Müntjes¹, Anke Busch²,
Julian König^{2,3}, Kathi Zarnack^{4,5,§} and Michael Feldbrügge^{1,§}**

¹ Institute of Microbiology, Heinrich Heine University Düsseldorf, 40204 Düsseldorf, Germany

² Institute of Molecular Biology gGmbH, Ackermannweg 4, 55128 Mainz, Germany

³ Institute of Biochemistry and RNA Biology, Theodor-Boveri-Institute for Biosciences, Julius Maximilians University Würzburg, Am Hubland, 97074 Würzburg, Germany

⁴ Buchmann Institute for Molecular Life Sciences (BMLS) & Institute of Molecular Biosciences, Goethe University Frankfurt, Max-von-Laue-Str. 15, 60438 Frankfurt, Germany

⁵ Theodor Boveri Institute for Biosciences, Biocenter, Julius Maximilians University Würzburg, Am Hubland, 97074 Würzburg, Germany

* Shared first authors; § Co-corresponding authors

Running title: *Modular RNA recognition code of multi-RRM RNA-binding protein*

Keywords: multi-RRM / RNA binding / functional binding sites / iCLIP2 / mRNA transport

Co-corresponding authors:

Prof. Dr. Kathi Zarnack

Theodor Boveri Institute for Biosciences, Biocenter, Julius Maximilians University Würzburg,
Am Hubland, 97074 Würzburg, Germany

Phone: +49 931 31-81878; kathi.zarnack@uni-wuerzburg.de

Prof. Dr. Michael Feldbrügge

Institute of Microbiology, Collaborative Research Center Microbial Networking

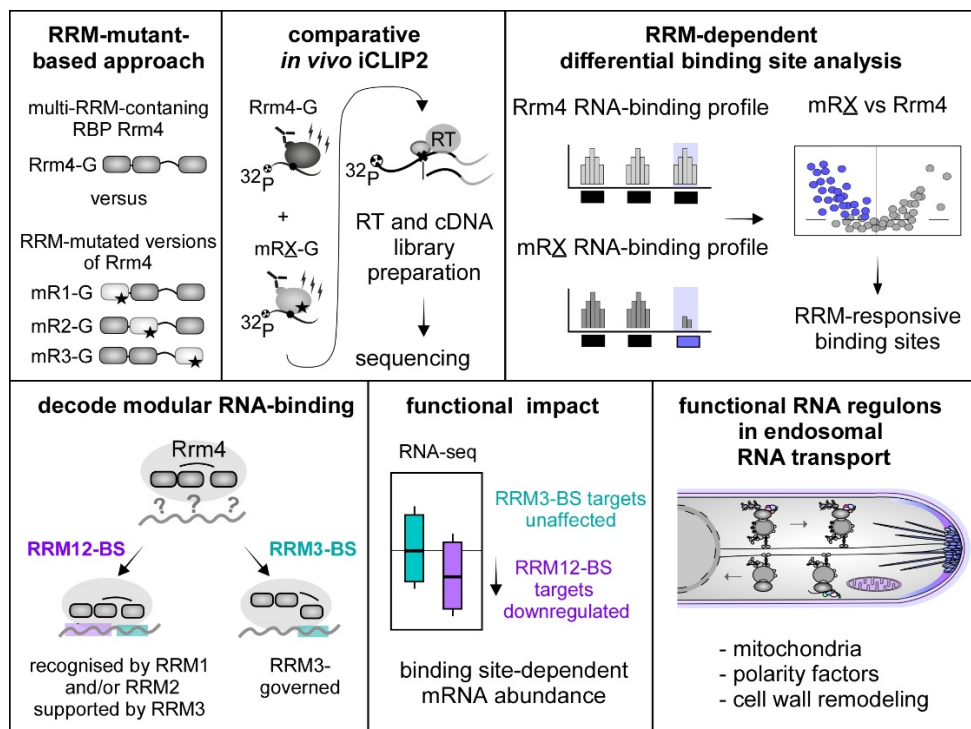
Heinrich Heine University Düsseldorf, 40204 Düsseldorf, Germany

Phone: +49 211 81-15475; feldbrue@hhu.de

Abstract

RNA-binding proteins (RBPs) utilize multiple RNA-binding domains (RBDs) to engage with extensive mRNA networks. Understanding the intricate interplay of modular RBDs is essential for uncovering RBP function. Yet, how individual RBDs shape transcriptome-wide interactions remains poorly understood. Here, we dissect the roles of the three RNA recognition motifs (RRMs) in the endosomal mRNA transporter Rrm4 during polar growth of *Ustilago maydis*. Using a comparative mutant-based iCLIP2 approach, we disclose an extensive inventory of RRM-specific binding sites. Most binding sites are prominently governed by RRM3, however, they are not critical for function. Conversely, functionally essential binding sites are recognized by a more complex RBD interplay, involving RRM1 and/or RRM2 with partial support from RRM3. By integrating transcriptome-wide RNA binding data with transcriptomics, we pinpoint their function as regulatory RNA elements affecting mRNA abundance, linking endosomal transport to stability. The modular RNA binding of Rrm4 defines distinct RNA regulons controlling mitochondrial activity, polarity factors, and cell wall remodeling – processes critical for polar growth. These findings disclose the intricate binding modes of an RBP *in vivo*, emphasizing how multiple RBDs differentiate functional binding sites from accessory ones to determine mRNA fate.

Graphical abstract



Introduction

RNA-binding proteins (RBPs) dynamically regulate the life cycle of each mRNA from synthesis to decay. Their malfunction can lead to death and disease (1,2). Consequently, RBPs have garnered significant interest in drug development and cancer treatment (3-5). Powerful approaches to study the RNA interactome of RBPs include transcriptome-wide UV crosslinking-based methods, such as individual-nucleotide resolution UV crosslinking and immunoprecipitation (iCLIP) and its derivatives (6). In recent years, large-scale binding maps of 150 human RBPs revealed thousands of RNA binding sites (7,8), yet discerning functional binding sites from others within the full spectrum of RNA interactions presents a significant challenge. This is further complicated by the modularity of RNA-binding domains (RBDs) in most RBPs, which often mediates target recognition by binding specific RNA sequences or structures (9-11). Dissecting the intricate RNA binding code is crucial to elucidate the precise role of each RBD in recognizing and modulating functionally critical binding sites.

The RNA recognition motif (RRM) is the most prevalent RBD in eukaryotes with a characteristic $\beta 1-\alpha 1-\beta 2-\beta 3-\alpha 2-\beta 4$ topology (Fig. EV1A) (12,13). Canonical RNA contact regions (RNP1 and RNP2) within the β -strands are crucial for sequence-specific binding (Fig. EV1A). However, single domains are often insufficient to confer specificity and affinity (14). Hence, multiple RBDs frequently occur within RBPs enhancing RNA binding (13). Tandem RRM, for example, provide an expanded RNA-binding surface, promoting binding specificity and avidity (15). A typical example is the ELAV family of multi-RRM RBPs (embryonic lethal abnormal vision, Fig. EV1A), which feature a typical arrangement of tandem RRM separate from a third RRM (16). Members of this family include proteins such as HuD (ELAV-like protein 4, *Homo sapiens*), Rbp9 (*Drosophila melanogaster*), and Rrm4 (*Ustilago maydis*) regulating translation, stability, and transport of mRNAs (Fig. EV1A) (17-19). HuD, for example, is specifically implicated in neurogenesis in motor neuron function, as knock-out mice exhibit motor deficits and its loss leads to increased self-renewal of neuronal stem cells and progenitors (20,21).

We investigate the long-distance transport along microtubules, focusing on the co-transport of mRNAs on the cytoplasmic surface of early endosomes. This vesicle hitchhiking process was initially discovered in fungi but has recently been observed in other systems, including plants, mammalian cells, and neurons (22-27). In the fungal microorganism *U. maydis*, the highly polarized infectious hyphae rely on the key endosomal mRNA transport protein Rrm4 (28-30). Besides its three N-terminal RRM, Rrm4 harbors three C-terminal Mademoiselle domains that serve as a binding platform for interaction with the endosomal anchor protein Upa1 (Fig. 1A, Fig. EV1A) (31). Additionally, Upa2 functions as a scaffold protein, stabilizing higher-order messenger ribonucleoprotein particle (mRNP) complexes, including the RNA chaperone Grp1 and the poly(A)-binding protein Pab1 (Fig. EV1B) (32).

Loss of Rrm4 results in aberrant hyphal polarity, linking endosomal mRNA transport to unipolar growth (Fig. 1B, Fig. EV1D-E) (33). One function of endosomal transport is the widespread distribution of bulk mRNAs and associated ribosomes throughout the cell (34,35). Additionally, Rrm4 specifically targets all four septin mRNAs (*cdc3*, *cdc10*, *cdc11*, *cdc12*) and is required for their endosomal transport, a process crucial for the formation of heteromeric septin complexes and their proper localization at the cell tip, essential for defined polarity (32,36,37). Previous iCLIP analysis of Rrm4 identified hundreds of mRNA targets preferentially bound in the 3' untranslated region (UTR) (34). The motif UAUG was enriched in Rrm4 target mRNAs, and yeast-3-hybrid assays suggested that the third RRM (RRM3) is essential for binding UAUG (34). However, mutational analyses indicated that RRM3 is dispensable for unipolarity, while RRM1 is crucial, indicating that each RRM contributes differently to the role of Rrm4 in polar growth (33). Here, we integrated a comprehensive

iCLIP2 analysis of mutated Rrm4 variants with transcriptomics and live-cell imaging to decode the modular RRM recognition code and unveil its functional significance.

Material and methods

U. maydis strain generation, cultivation of yeast cells and induction of hyphal growth

The laboratory strain AB33 of *U. maydis* (homotypic synonym of *Mycosarcoma maydis*, NCBI taxonomy ID 5270) was used to synchronize hyphal growth by altering the nitrogen source. In brief, cell suspensions were grown in a complete medium containing 1% glucose at 28°C with constant agitation at 200 rpm until an OD₆₀₀ of 0.5 was reached. Hyphal growth was induced by transferring the cells to a nitrate minimal medium supplemented with 1% glucose and incubating them at 28°C with agitation at 200 rpm for 6 hours post-induction (h. p. i.) (38). Further details regarding media composition, growth conditions, and general strategies for strain generation in *U. maydis* were previously described (38,39). Strains, plasmids and DNA oligonucleotides used for strain generation are given in the supplementary information (Supplementary Tables S1-S3). UMAG identifiers for selected Rrm4 target genes are given in Supplementary Table S4. Strain names are typically written in italics and are designated based on the construct they carry (*rrm4-g*, *mR1-g*, *mR2-g*, *mR3-g*) or, in cases where specific genes are absent, marked with a delta symbol (*rrm4Δ*). For example, *rrm4-g* refers to a strain expressing the Rrm4-G protein. Protein names are written without additional tags when referring to them in a general sense.

Microscopy

For standard microscopy, cells were grown in 20 mL cultures to an OD₆₀₀ of 0.5. Hyphal growth was induced and cells were imaged 6 h. p. i. To assess unipolar and bipolar growth, more than 100 hyphae (6 h. p. i.) per strain were analyzed across three biological replicates (n=3). To quantify the number of shuttling particles, their velocity, and the endosomal attachment of fluorescently labeled fusion proteins (Gfp, mKate2, Rfp, mCherry), movies were recorded with an exposure time of 150 ms for a total of 150 frames (32). For generating kymographs, 15 or 30 μm sections of hyphal cells starting from the cell tip or perinuclear region were used. Only processively moving particles that traveled >5 μm were considered shuttling particles. Each experiment involved more than 24 hyphae per strain (n=3). Rrm4-G signal intensity of shuttling particles was measured using kymograph intensity relative to the cytoplasmic background (relative fluorescent units, RFU). All movies, kymographs, and images were processed and analyzed using Metamorph software (Version 7.7.0.0, Molecular Devices, Seattle, IL, USA). For co-localization experiments of shuttling particles, a two-channel imaging system (DV2; Photometrics, Tucson, AZ, USA) was utilized. Co-localization efficiency was assessed by calculating the ratio of Pab1-Gfp particles to Rrm4-mKate2 particles. Data analysis and visualization were conducted in RStudio (version R 4.4.2).

Western blot analysis

Protein concentrations of cell extracts were measured using either the BCA assay (Thermo Fisher, Pierce™ 23225) or the Bradford assay (BioRad). Samples were adjusted to 1 mg/mL in NuPAGE LDS containing DTT according to the manufacturer's instructions (Thermo Fisher, NP0007). Samples were boiled for 5 minutes and loaded onto a 4-12% NuPAGE™ Bis-Tris gel (Thermo Fisher, NP0321). Electrophoresis was carried out at 180 V for 1.5 hours. Proteins were transferred onto a nitrocellulose membrane (Amersham Protran) via wet blotting using a NuPAGE™ transfer buffer supplemented with 15% methanol (Thermo Fisher, NP00061) for

1.5 hours at 30 V. For Western blot analysis, primary antibodies α -GFP (1:2000 dilution; Roche, 11814460001) and α -Actin (1:2000; MP Biomedicals, 0869100) were applied for 1 hour at room temperature under continuous rotation. Subsequently, the membrane was washed three times with TBS-T for 15 minutes each. The secondary antibody, α -mouse HRP conjugate (1:5000 dilution; Promega, W4021), was applied for 1 hour. All antibodies were diluted in TBS-T containing 5% milk powder. The detection was performed using LAS400 (GE Healthcare) and ECLTM Prime (Amersham, RPN2232) following the manufacturer's guidelines.

Comparative microbial iCLIP2

The comparative iCLIP2 approach used 150 mL hyphae cultures ($OD_{600}=0.5$, 6 h. p. i.) as the starting material. All experiments were conducted as detailed in the microbial iCLIP2 protocol tailored for Rrm4-G and mutated versions (40). Rrm4-G and the comparative mutants (mR1-G, mR2-G, mR3-G) were prepared simultaneously in at least five biological replicates for high-resolution analysis. The iCLIP2 libraries were sequenced in two independent runs (1: Rrm4-G, mR1-G; 2: Rrm4-G, mR2-G, and mR3-G) to obtain at least 25 million reads per sample using an Illumina NextSeq 500 platform to produce 92 nt single-end reads. These reads included a 6 nt sample barcode and unique molecular identifiers (UMIs, Supplementary Table S3) of 5+4 nt (41).

Processing of iCLIP reads

The processing of iCLIP2 reads as well as the binding site definition (see below) were carried out mostly as previously described (42). In brief, FastQC (version 0.11.8; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was utilized for initial quality controls. Reads with a Phred quality score below 10 in the barcode or UMI regions were removed from further analysis using the FASTX-Toolkit (version 0.0.14; https://github.com/agordon/fastx_toolkit) and seqtk (version 1.3; <https://github.com/lh3/seqtk/>). Flexbar (version 3.4.0) (43) was used to de-multiplex the reads based on the sample barcode on positions 6 to 11 and to trim the barcodes, UMI regions, and adapter sequences from the ends of the reads, while ensuring a minimal overlap of 1 nt between the read and the adapter. UMIs were added to the read names and only reads with a minimum length of 15 nt were retained. Trimmed reads were mapped to the *U. maydis* genome and its annotations (Ensembl release 51; assembly version Umaydis521_2.0) (44) using STAR (version 2.7.3a) (45). During mapping, up to 4% mismatched bases were accepted and soft-clipping was prohibited on the 5' end. Reads directly mapped to the chromosome ends were removed since they do not have an upstream position and, consequently, no crosslink position can be extracted. PCR duplicates were removed from uniquely mapping reads using UMI-tools (version 1.0.0) (46). Crosslinked nucleotides were extracted using the BEDTools suite (version 2.27.1) (47) as described in Chapter 4.2 of (42).

iCLIP2 binding site definition

Peak calling was performed using PureCLIP (version 1.3.1) (48) with default parameters on the merged, preprocessed alignments of the individual replicates. The resulting crosslink sites were used to define binding sites with the R/Bioconductor package BindingSiteFinder (version 1.7.9) (42); (<https://doi.org/doi:10.18129/B9.bioc.BindingSiteFinder>) (49). Focusing on the most significant sites, we excluded the lowest 5% of crosslink sites ranked by the PureCLIP score. Additionally, a gene-wise filter was applied to remove the bottom 10% of crosslink sites based on PureCLIP scores for each gene. Analysis of crosslink event coverage revealed that binding sites of width 5 nt more effectively captured the central peak in iCLIP2 read coverage than the previously used 9 nt width. Consequently, crosslink sites located within a 5 nt distance were

merged into binding regions. Isolated crosslink sites and binding sites of width 2 nt were removed. Binding site centres were defined at crosslink events with the highest PureCLIP scores and extended by 2 nt on either side. For binding sites longer than 5 nt, the binding regions were divided into 5 nt regions by iteratively identifying the maximum signal and extending by 2 nt on either side, ensuring no overlap between binding regions. Reproducibility was assessed by retaining binding sites that contained more crosslink events than the 5th percentile of the crosslink distribution across all binding sites, with a minimum of two crosslink events per site. Sites were considered reproducible if detected in at least $n-1$ replicates, where n is the total number of replicates. To assign gene and transcript regions to the binding sites, we utilized the gene annotation file version *U. maydis* 521_2.0.41 (http://ftp.ensemblgenomes.org/pub/fungi/release-53/fasta/ustilago_maydis/dna/) (50). Genes in the *U. maydis* genome were manually extended by 300 nt on each side to encompass potential 5' UTR and 3' UTR regions (18). To resolve overlapping transcript types, we applied the following hierarchy: tRNA > mRNA > rRNA > snRNA > snoRNA. For overlapping transcript regions, binding sites were assigned to the transcript regions (5' UTR, CDS, intron, 3' UTR) that were most frequently observed.

Differential binding analysis

The differential binding analysis was performed using the package BindingSiteFinder (version 1.7.9) (51). The binding sites for each iCLIP2 dataset were defined independently as mentioned above. For the differential binding analysis, the binding sites from the Rrm4 iCLIP2 data were used as control. Binding sites from all datasets (Rrm4-G, mR1-G, mR2-G, and mR3-G iCLIP2 data) were combined to enable differential testing. For pairwise comparisons, the binding sites corresponding to the Rrm4 control and the corresponding mutant condition were extracted from the merged dataset. Differential testing was performed by dividing crosslink events for each gene into two categories: the binding site bins, containing crosslink events overlapping the binding sites, and the background bin, encompassing all crosslink events within the gene that do not overlap with binding sites or the adjacent 2 nt offset regions flanking the binding sites. The inclusion of the offset regions ensures that any signal near the binding sites is excluded from the background bin, allowing for a more accurate representation of the background bin. Additionally, the background bin accounts for variations in RNA expression levels. Subsequently, the crosslink counts for each binding site and the corresponding background bin were extracted for each gene. The background bin counts were used to normalize and correct for expression level changes. Genes were excluded from the analysis if they met any of the following criteria: fewer than 100 crosslink events across all samples, more than 30% of crosslink events attributed to binding sites, more than 70% of crosslink events attributed to the background, or more than 95% of total crosslink events attributed to a single condition. Finally, fold change for each binding site between the two conditions was calculated using the DESeq2 likelihood ratio test (52), separating transcriptional changes from alterations at the binding site level.

RNA isolation and sequencing

25 mL of hyphal cell culture with an OD₆₀₀ of 0.5 was used to isolate total RNA. Cells were harvested by centrifugation (7,150 g, 10 min, 4°C) and resuspended in 2 mL of PBS (phosphate buffered saline) divided and transferred into 2 mL reaction tubes. Cells were harvested by another round of centrifugation (16,200 g, 5 min, 4°C) and tubes containing the cell pellets were frozen in liquid nitrogen. A steel bead (5 mm) was added to each cell pellet and placed in the pre-cooled TissueLyser Adapter (Qiagen). The samples underwent two rounds of mechanical disruption for 30 seconds at 30 Hz each (Retsch, M400), with a cooling step in

liquid nitrogen between rounds. The cryogenically frozen cell powder was resuspended in 500 μ L of TriZol (Invitrogen) and incubated, shaking at room temperature for 5 minutes at 1000 rpm. Next, 500 μ L of RNase-free water was added, and the mixture was manually shaken for 30 seconds. Subsequently, 400 μ L of chloroform/isoamylalcohol (24:1; BioChemica) was added. The samples were shaken vigorously by hand for 3 minutes, then incubated at room temperature for another 3 minutes. Following this, the samples were centrifuged for 10 minutes at 16,200 g. The aqueous phase was transferred to a fresh 1.5 mL tube pre-filled with 250 μ L of chloroform/isoamylalcohol (24:1; BioChemica). This extraction process was repeated, followed by centrifugation at 16,200 g for 5 minutes at 4°C. The aqueous phase was transferred to a tube containing 40 μ L of sodium acetate (3 M, pH 5.2) and 1 mL of ice-cold 100% ethanol. Samples were incubated at -20°C for 2 hours, and RNA was pelleted by centrifugation at 16,200 g for 30 minutes at 4°C. The pellet was washed twice with 75% ice-cold ethanol, centrifuged for 5 minutes at 16,200 g and 4°C, briefly dried, resuspended in 50 μ L of RNase-free water, and stored at -80°C. RNA concentration was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). For each sample, 2 μ g of RNA was subjected to DNase digestion using the dsDNase Kit (Thermo Fisher, EN0771) at 37°C for 10 minutes. The RNA was then precipitated overnight by adding 1 mL of ice-cold 100% ethanol and 40 μ L of sodium acetate (3 M, pH 5.2). The RNA pellet was collected by centrifugation at 16,200 g for 30 minutes at 4°C, washed twice with 75% ice-cold ethanol and centrifuged again for 5 minutes at 16,200 g and 4°C. The pellet was briefly dried, resuspended in 10 μ L of RNase-free water, and stored at -80°C. RNA concentration was quantified using the Qubit RNA BR Assay (Invitrogen by Thermo Fisher Scientific), and RNA integrity was verified with the Bioanalyzer RNA Nano Assay (Agilent). NGS library prep was performed with Illumina's Stranded mRNA Prep Ligation Kit following Stranded mRNA Prep Ligation ReferenceGuide (April 2021, Document 1000000124518 v02). Libraries were prepared with a starting amount of 500 ng and amplified in 11 PCR cycles. Two post-PCR purification steps were performed to exclude residual primer and adapter dimers. Libraries were profiled in a High Sensitivity DNA chip on a 2100 Bioanalyzer (Agilent Technologies) and quantified using the Qubit dsDNA HS Assay Kit, in a Qubit 4.0 Fluorometer (Invitrogen by Thermo Fisher Scientific). All samples were pooled together in equimolar ratio aiming for at least 10 million reads per sample and sequenced on a NextSeq 2000 P3 (100 cycles) FC, SR for 1x116 cycles plus 2x10 cycles for the index reads and one dark cycle upfront R1.

Differential gene expression analysis

Basic quality control of RNA-seq libraries was conducted using FastQC (version 0.12.1; <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Prior to mapping, RNA-seq reads were processed using Trimmomatic (version 0.39) (53) to remove adapter sequences and enhance sequence quality. Trimming was performed under the following criteria: Reads were truncated when the average Phred score within a 4 nt sliding window fell below 15, reads were trimmed at the start or end of reads when the Phred score dropped below 30 and reads shorter than 36 nt were discarded. The processed reads were aligned to the *U. maydis* genome using STAR (version 2.7.11b) (45), allowing up to 4% mismatches. Reads per gene were counted with featureCounts (version 2.0.1) (54) using default parameters. Differential gene expression analysis between the wild-type (*rrm4-g*) and mutant conditions (*rrm4Δ*, *mR1-g*, *mR2-g*, *mR3-g* and *mR123-g*; Supplementary Table S1) were performed using DESeq2 (version 1.42.0) (52) in the R environment (version 4.3.1; <https://www.R-project.org/>).

Motif analysis

De novo motif discovery analysis was conducted using the XSTREME tool from the MEME suite (55). A 15 nt window encompassing binding sites extended by 5 nt to either side was used as input. Shuffled input sequences served as a background control. XSTREME analysis was conducted with default parameters. For each enriched motif, its relative enrichment ratio was compared against the absolute percentage of binding sites with this motif. The motif logo was created using the package ggseqlogo (version 0.2) (56) in R. For *k*-mer analysis, the frequency of all possible pentamers within the same 15 nt windows was computed using the R/Bioconductor package Biostrings (version 2.70.1, <https://bioconductor.org/packages/Biostrings>). Additionally, pentamer frequency was calculated within randomly selected 15 nt regions from the genome, which served as a control. Motif enrichment was determined as the ratio of pentamer frequency in binding sites to its total frequency across both the binding sites and background regions. Since the antisense and scrambled sequences of UAUG contain parts of the motif, we chose the random GCAU motif as a control.

Positional analysis of binding sites

RRM12-BS and RRM3-BS were each extended by 150 nt from their centres to obtain a 301 nt window. For relative positional analysis, the overlaps of RRM12-BS within the 301 nt RRM3-BS window, and *vice versa*, were identified. The relative position of RRM12-BS within the RRM3-BS window (or RRM3-BS within the RRM12-BS window) was determined by calculating the distance from the start position of the binding site to the centre of the window.

GO term analysis

For the functional enrichment analysis, we stratified the mRNA targets (RRM12-BS or RRM3-BS targets) into groups based on the number of binding sites they possess. The mRNA targets were initially defined as those containing at least one binding site ([1, >1], with subsequent groups defined as progressively more stringent [2, >2] and [3, >3]). Gene Ontology (GO) term analysis was conducted using the R package gProfiler2 (version 0.2.3) (57), applying a significance threshold at *P* value ≤ 0.05 and the *g_SCS* correction method. The results of the GO term analysis were visualized using heatmaps generated with the R/Bioconductor package ComplexHeatmap (version 2.18.0) (3,58,59).

Gene set enrichment analysis (GSEA)

The GSEA analysis was performed using the R/Bioconductor package ClusterProfiler (v 4.10.1) (60). For input data, the mRNA targets of RRM12-BS and RRM3-BS were ranked by their mRNA expression levels ($\log_2$ fold change) in the *rrm4Δ* vs. *rrm4-g* RNA-seq data in descending order. Ensembl annotation for GO term Cellular Component was retrieved from the web server gProfiler (<https://biit.cs.ut.ee/gprofiler/gost>) (57) and was used as pre-defined gene sets. GSEA was performed with an adjusted *P* value cutoff of < 0.05 , applying the Benjamini-Hochberg correction. The distribution of statistically significant gene sets in the pre-ranked target mRNA list was visualized using the R/Bioconductor package enrichplot (version 1.22.0, DOI: 10.18129/B9.bioc.enrichplot).

Structural prediction of RRM3-UAUG

The structural modeling was performed using AlphaFold3 (3) with default settings, employing the full-length Rrm4 (A0A0D1DWZ5) and the RNA motif sequence

[5'-UAUG-3']. PyMOL (The PyMOL Molecular Graphics System, version 2.1) was utilized for visualization, focusing on the RRM3-UAUG interaction, which was vividly highlighted to enhance clarity.

RT-PCR

Total RNA was isolated as previously described. RNA was reverse transcribed using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher), following the manufacturer's instructions. The resulting cDNA was diluted 1:10 and used for standard PCR with the GoTaq Green Master Mix (Promega). The primers used for PCR are listed in Supplementary Table S3.

Protein sequence comparison

To identify mammalian homologs of FERRY-associated targets (22), protein sequence comparisons were performed using the BLASTp program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (61). The analysis aimed to identify *U. maydis* orthologs by querying the non-redundant protein sequences (nr) database. The default parameters used included an E-value threshold of 10, the BLOSUM62 substitution matrix, a word size of 6, and gap costs set to 11 for existence and 1 for extension. The analysis was conducted bidirectionally, from human to *U. maydis* and *vice versa* (Supplementary Table S5).

Results

The three RRM domains differentially impact hyphal growth and mRNA localization

To dissect the function of the three individual RRM domains of Rrm4 during unipolar growth, we exploited Rrm4 versions carrying four amino acid mutations in the respective RNP1 regions of the three RRMs (Fig. 1A; Fig. EV1A; mR1-3-G) (33). We generated strains in which the *rrm4* gene was replaced with variants encoding the mutated Rrm4 versions, each C-terminally fused to Gfp (e.g., mR1-G; Fig. 1A; Fig. EV1A; Supplementary Tables S1-2). Importantly, neither mRNA nor protein amounts were altered due to the mutations (Fig. EV1C).

Analyzing hyphal growth revealed that strains expressing mR1-G and mR2-G, with mutations in RRM1 and RRM2, respectively, resembled the *rrm4* deletion (*rrm4Δ*) strain, exhibiting an increased amount of bipolarly growing cells (Fig. 1B; Fig. EV1D-E). Both mutant versions, however, are still shuttled on endosomes (Fig. EV1F). The RRM2 mutation differed from the RRM1 mutation, as the rate of bipolar hyphae with septa was increased (Fig. EV1E), while the ratio of endosome-associated-to-cytoplasmic mR2-G intensity was decreased, indicating less efficient attachment (Fig. 1C). By contrast, mutations in RRM3 (mR3-G) did not significantly affect the rate of unipolar growth, the number of shuttling particles nor the signal intensity of Rrm4 on shuttling endosomes (Fig. 1B-C; Fig. EV1D-F) (33). Interestingly, all three RRM mutations increased the velocity of endosomes (Fig. 1D). A similar effect was observed in the mutant lacking the scaffold protein Upa2 (*upa2Δ*), suggesting elevated motility due to the reduced burden of transporting mRNA ribonucleoprotein (mRNP) cargo and associated ribosomes (32).

To study mRNP trafficking, we used the poly(A)-binding protein Pab1-G as a reporter for bulk mRNA transport (Fig. 1E-F; functional C-terminal eGfp fusion) (36). Pab1 is associated with the poly(A) tail of most mRNAs (62,63) and its visualization can hence be used as a proxy for the localization of mRNAs. As expected, co-localization was almost completely abolished in the mR123-K-expressing strain (C-terminal mKate2 fusion carrying mutations in all three RRM domains of Rrm4; Fig. 1E, H; Supplementary Tables S1-2). This is consistent with previous observations that mRNA association with Pab1 on endosomes is RRM-dependent (36). As expected, the co-localization of Pab1-G was reduced in strains with mutations in single RRMs (Fig. 1E, H). Comparing the subcellular localization of Pab1-G at the growth apex with the perinuclear region revealed an almost equal distribution of Pab1-G signals in Rrm4 wild-type cells (Fig. 1F-G, Fig. EV1G). However, in *rrm4Δ* or *mR123-k* strains, Pab1-G localization was reduced at the growth pole and increased in the perinuclear region (Fig. 1F-G; Fig. EV1G). Individual RRM mutations led to intermediate effects on Pab1-G localization (Fig. 1G, Fig. EV1G), suggesting that mRNA transport is still operational but to various extents.

In summary, the RRM domains play distinct roles: RRM1 and RRM2 are essential, while RRM3 is largely dispensable for unipolar growth. Although all mutants affect Pab1-associated mRNA localization, the impact of the loss of RRM3 is the least severe, highlighting the dominant roles of RRM1 and RRM2 in mRNA localization and hyphal polarity.

A comparative iCLIP2 approach uncovers specific signatures for all three RRMs

To study the contribution of the individual RRMs to target RNA recognition, we conducted a comparative iCLIP2 analysis using Rrm4-G and its mutants (Fig. 2A). As expected, quantification of *in vivo* UV-crosslinked RNA revealed reduced binding in all three mutants compared to Rrm4-G (Fig. 2B). In line with earlier UV crosslink experiments (33), the most drastic reduction was observed for mR3-G (Fig. 2B). To ensure sufficient recovery of UV-crosslinked RNA, particularly for RRM mutants, we optimized our iCLIP2 protocol and

sequenced at least five biological replicates per experiment (40,41). The binding landscapes exhibited high reproducibility among biological replicates and across datasets (Fig. EV2A). Peak calling (48) and binding site definition (42,49) yielded a total of 50,410 binding sites across all datasets. To assess the binding specificity of the RRM domains, we compared the background-normalized crosslink counts of the mutant binding sites with those of Rrm4 wild-type. Using a differential binding analysis, we identified binding sites responsive to mutating each of the three RRM domains, i.e., showing a significant reduction in binding compared to wild-type ($\log_2$ -transformed fold change [$\log_2FC$] < -1 , adjusted P value < 0.05 [Benjamini-Hochberg correction], and mean of normalized crosslink events [baseMean] > 15). These sites were strongly affected by mutation and likely directly contacted by the corresponding RRM (defined as RRM1-, RRM2-, or RRM3-responsive sites; Fig. 2C; Supplementary Table S6). RRM3 has the highest number of responsive binding sites, followed by RRM1 and RRM2 (Fig. 2C). Additionally, we observed binding sites with increased binding following RRM domain mutation ($\log_2FC > 1$, adjusted P value < 0.05 , and baseMean > 15), likely indicating aberrant binding of the mutated Rrm4 variants.

Conducting a *de novo* motif discovery and k -mer analysis in the vicinity of responsive sites revealed a clear motif only for RRM3-responsive sites (Fig. EV2B-C). Here, the motif UAUG and its derivatives were most enriched (Fig. EV2B-C). More than 80% of UAUG-containing sites showed decreased binding upon RRM3 mutations compared to sites lacking the motif (Fig. 2D; EV2D). This is consistent with our previous yeast three-hybrid assays showing the interaction of RRM3 with UAUG (34). Zooming in the crosslinking landscape of mR3 reveals a clear absence of the prominent UAUG-containing sites observed in mR1, mR2, and Rrm4 (Fig. 2E). In essence, RRM3 binds the UAUG motif *in vivo* in a transcriptome-wide, sequence-specific manner (Fig. 2F) and confirms that comparative iCLIP2 enables the dissection of individual RRM binding sites.

The tandem RRMs conjointly bind to RNA and are further supported by RRM3

Given the modular multi-RRM architecture, we analyzed whether the three RRMs influence each other's RNA binding. While most RRM3-responsive sites were unique to the third RRM, a substantial amount of RRM1- and RRM2-responsive sites were also responsive to RRM3 (Fig. EV3A). To address this link, we compared the quantitative changes in crosslink events at all binding sites following RRM mutations (Fig. 3A). As expected, the majority of RRM3-responsive sites showed only a minimal reduction upon RRM1 and RRM2 mutation (Fig. 3A, right, compare #9, with #7 and #8). In contrast, most RRM1- and RRM2-responsive sites displayed a marked decrease in binding upon RRM3 mutation, in addition to a reduction from mutations in their respective domains (Fig. 3A, #3 and #6). Furthermore, the binding at RRM1-responsive sites decreased upon mutation in RRM2, and *vice versa* (Fig. 3A, #2 and #4), highlighting an additional interdependency within the tandem RRM domains. Thus, for a large subset of RRM1- and RRM2-responsive sites, RRM3 further supported RNA binding, and RRM1 and RRM2 also affect each other's binding.

Based on these results, we defined two distinct sets of binding sites for Rrm4: RRM12-BS and RRM3-BS (Fig. 3B-C, Fig. EV3A). In the case of RRM3-BS, binding is mainly governed by the third RRM domain, with the other two contributing only marginally (Fig. 3A-C; Fig. EV3A; $n=10,654$). In contrast, for RRM12-BS, binding strongly relies on RRM1 and/or RRM2 of the tandem RRM domains, with RRM3 often supporting the interaction (Fig. 3A-C, $n=2,594$). Mutations in RRM1 show a stronger effect on RRM12-BS than RRM2, underscoring the critical role of RRM1 in recognizing these targets (Fig. 3B).

To test whether the RRMs bind in a defined order on the target mRNAs, we analyzed the positioning of RRM12-BS and RRM3-BS relative to each other in a 301 nt window. Of note,

48% of the RRM12-BS contained at least one RRM3-BS within this window, suggesting that the RRMs bind in the vicinity of each other. Moreover, RRM12-BS tended to accumulate upstream of UAUG-containing RRM3-BS (Fig. 3D-E). This pattern suggests that the RRMs of Rrm4 are positioned in a defined arrangement, with the tandem RRM1 and 2 placed upstream of RRM3 (Fig. 3C). Consistently, the RRM12-BS were enriched predominantly in coding sequences (CDS), whereas RRM3-BS were increasingly found in 3' UTRs (Fig. EV3B).

In essence, our transcriptome-wide comparative binding analysis highlights the coordination between the RRMs in facilitating Rrm4 binding. Specifically, RRM3 plays a key role in recognizing RRM3-BS, while RRM1 and RRM2 bind together at composite RRM12-BS, which are further supported by RRM3.

The composite RRM12-BS function as regulatory RNA elements

Consistent with the notion that RRM3 supports the binding of RRM1 and RRM2 at RRM12-BS, most mRNAs with RRM12-BS also harbored at least one RRM3-BS, whereas the latter also frequently occurred alone (Fig. EV4A). Based on this, we categorized target mRNAs as either RRM12-BS targets (n=1,630) or RRM3-BS targets, whereby the latter contained only RRM3-BS but no RRM12-BS (n=2,211; Fig. EV4A). To analyse the regulatory impact of Rrm4 binding to these targets, we performed RNA sequencing (RNA-seq) to measure mRNA levels in the Rrm4-G strain and the corresponding mutant strains. A quantitative comparison revealed that *rrm4Δ*, mR1, mR2, and mR123 mutations resulted in significant changes in mRNA levels, while the mR3 mutation showed only marginal effects (Fig. EV4B). Of note, we observed a clear correlation between targets carrying RRM12-BS and a reduction in relative transcript levels across all mutants with impaired tandem RRM binding (Fig. 4A; Fig. EV4C). Strikingly, this decrease was proportional to the number of RRM12-BS present in target transcripts, indicating an additive effect. Moreover, this pattern was absent in mR3 (Fig. 4A). In contrast, the RRM3-BS targets exhibited no significant changes or additive effects following *rrm4* deletion or any RRM domain mutation (Fig. 4A). This demonstrates that RRM12-BS are functional regulatory RNA elements that link RNA transport and RNA abundance, further underscoring the importance of RRM1 and RRM2. Importantly, despite their functional relevance, the RRM12-BS are less prominent than the RRM3-BS (Fig. EV4F, EV5A-B) and hence easily overlooked in the wild-type Rrm4 iCLIP2 data, underlining the advantage of the comparative iCLIP2 approach in discriminating the effects of the individual RRM.

Following up on the functional relevance of tandem RRM binding, an analysis of Gene Ontology (GO) term enrichment revealed that terms related to polar growth, such as cell periphery and plasma membrane, were significantly overrepresented in RRM12-BS targets, whereas no significant enrichment was observed in RRM3-BS targets (Fig. EV4D-E). A clear functional distinction between RRM12-BS and RRM3-BS targets was further supported by a gene set enrichment analysis, which incorporated mRNA level changes following *rrm4* deletion into gene sets based on cellular compartments (Fig. 4B). Importantly, RRM12-BS targets, which exhibited reduced levels in the respective mutants, were strongly enriched for compartments related to cell wall synthesis, cell periphery, and plasma membrane. This was not observed in the case of RRM3-BS targets. Conversely, mRNAs encoding ribosomal proteins were enriched in both RRM12-BS and RRM3-BS targets and showed increased levels in the absence of Rrm4, suggesting a potential compensatory mechanism due to disturbed RNA transport (Fig. 4B). In summary, RRM12-BS exhibit characteristic features of regulatory RNA elements important for Rrm4-dependent transport of cognate target mRNAs during polar growth.

RRM12-BS targets encode polarity factors, cytoskeletal proteins, and cell wall enzymes

Rrm4 has a well-established role during unipolar hyphal growth. To elucidate the contribution of RRM12-BS to cell polarity – as suggested by the GO analysis – we examined the 1,630 RRM12-BS target mRNAs in greater detail. Generally, hyphal growth is initiated by the subcellular accumulation of polarity factors at the growth pole. These regulators reorganize the cytoskeleton to orchestrate efficient expansion of plasma membrane and cell wall at defined sites (64). Interestingly, within the relevant GO terms *cell periphery*, site of polar growth, and growing tip, we observed several key polarity factors such as small GTPases Cdc42, Rac1, and Rho1/2/4 (Fig. 5A-B, Supplementary Table S7). Consistently, mRNAs encoding cytoskeletal proteins such as F-actin and septin proteins (Cdc3, Cdc11, Cdc12) were RRM12-BS targets (Fig. 5B, Fig. EV5A, Supplementary Table S7). Septin-encoding mRNAs are established Rrm4-dependent cargos, and their endosome-coupled translation is crucial for both the assembly of heterotetrameric complexes and the formation of a septin gradient at the growing pole (36,37).

In line with polar cell wall expansion as a critical process in hyphal growth, RRM12-BS targets were strongly enriched for mRNAs encoding enzymes for cell wall synthesis and remodeling. This included all chitin synthases, Chs1-7 and Mcs1, which are involved in the synthesis of cell wall chitin (Fig. 5C, Fig. EV5B, Supplementary Table S7). Furthermore, transcripts coding for enzymes acting in cell wall remodeling (Fig. 5A-C) included 1,3- β -glucan synthase Gls1 (65,66), three chitin deacetylases (Cda1, Cda6-7), chitinases Cts1 and Cts2, and three glycoside hydrolases (Gla5-7) (67) were also RRM12-BS targets. Noteworthy, we could previously show that *cts1* mRNA is a known Rrm4 target and the unconventional secretion of Cts1 is Rrm4-dependent (68). Together, Rrm4-mediated mRNA transport appears to coordinate cytoskeletal dynamics and cell wall remodeling required for polar growth preferentially via the coordinated action of the tandem domains RRM1 and 2 at RRM12-BS.

For experimental verification of these observations, we chose septin Cdc3 (Fig. 5A). The corresponding mRNA contains one RRM12-BS and three RRM3-BS, providing an ideal target to investigate how variations in RRM-dependent interactions affect the subcellular localization of functional Cdc3 protein (Fig. 5D-E). Consistent with previous reports, Cdc3 fused to Gfp (Cdc3-G, Supplementary Table S1) was barely detectable on endosomes in *rrm4* Δ and RRM1 mutants (mR1), and the gradient at the tip was lost (Fig. 5D-E) (32,36,37). Mutations in RRM3 (mR3) caused a slight but significant reduction in endosomal shuttling and gradient formation (Fig. 5D-E), supporting the idea that RRM3 enhances RRM1 binding, while the tandem RRMs are essential for RNA transport. Accordingly, cell length that depends on cell wall synthesis was reduced in strains expressing mR1, but not mR3 (Fig. 5F). In essence, RRM12-BS targets encode factors contributing to polar growth at all levels, from initial regulation to cell wall remodeling, including a novel extensive link to cell wall synthesis.

A core set of endosomal transport target mRNAs is evolutionarily conserved from fungi to humans

We leveraged evidence from mammalian cells to investigate the evolutionary conservation of endosomal mRNA transport and hence its relevance beyond *U. maydis*. In neurons, the FERRY complex links mRNAs to early endosomes and using it as a bait in pull-down experiments, 252 target mRNAs were identified in human HEK293 cells (22). Reciprocal BLAST analysis identified 122 unambiguous orthologs in *U. maydis*, the majority of which was bound by Rrm4 (n=105, 86%; Fig. EV5C-D, Supplementary Table S5). When dissecting the overlap in Rrm4's binding sets (RRM12-BS vs RRM3-BS), the shared pool of targets was approximately evenly divided, with 40% bound by RRM12-BS and 40% by RRM3-BS (Fig. EV5D, Supplementary Table S5). However, RRM3-BS targets were enriched for endocytic vesicles and intracellular

structures. In contrast, RRM12-BS targets were enriched for the GO terms plasma membrane and mitochondrial proteins such as Atp16 and Mdh1. Interestingly, this data set also contained small GTPases such as Rho1 and Rac1 (Fig. 5G, Supplementary Table S5). In essence, a comparison of mRNA targets of the endosomal transport machinery from fungi and humans identified an evolutionarily conserved subset of targets involved in mitochondrial homeostasis and determination of polarity.

Discussion

RNA-binding proteins often use multiple RBDs to recognize defined RNA regulons. To understand their precise regulatory capacity, it is essential to dissect how individual RBDs distinguish essential from accessory interactions (Fig. 6A) (8,9,64). Here, we established a multifaceted comparative iCLIP2 approach to decipher the interplay of multiple RRM s present in the endosomal mRNA transporter Rrm4. We succeeded in differentiating functional from accessory binding sites and thereby disclosed new RNA transport regulons. Our unique study combining single-nucleotide UV crosslinking of RBD mutants with transcriptomics and cell biology might serve as a blueprint to tackle the RNA binding code in general and advance *in vivo* RNA biochemistry.

RRMs recognize defined sets of binding sites to distinguish accessory from main functions

Currently, most transcriptome-wide RNA binding studies, which use iCLIP2 and variations, employ wild-type RBPs and provide a wealth of information concerning binding motifs, binding sites, and target mRNAs (7,8). For instance, PAR-CLIP/CRAC approaches of the multi-RRM RBPs, HuR and HuD, revealed AU-rich binding motifs within hundreds of target mRNAs (17,69). Similarly, our previous iCLIP study of wild-type Rrm4 identified *bona fide* mRNA targets and revealed UAUG as the dominant binding motif (34). However, the ultimate challenge is to unravel how individual RBDs drive the specific recognition of functionally relevant regulatory RNA elements (Fig. 6A) (10,11,70).

An attractive approach is the comparison of wild-type and RBD mutants. For example, iCLIP2 was used to compare the RNA-binding ubiquitin E3 ligase TRIM25 with its RBD mutants, unraveling their importance in RNA binding (71). Here, we advanced this concept further. By analyzing RBD mutant variants of the multi-RRM protein Rrm4, we determined their distinct binding sites and functions with high resolution. We refined our iCLIP2 protocol to achieve high-quality data (40), enabling precise detection of even subtle changes in RBD mutants with up to 60% reduced binding.

Advanced differential binding analysis (51) revealed two distinct Rrm4 binding sets validated by target transcript expression profiling: functionally critical sites primarily recognized by the tandem RRM s RRM1 and/or RRM2 (RRM12-BS, Fig. 6A) differ from accessory sites predominantly bound by RRM3 (RRM3-BS, Fig. 6A). Intriguingly, RRM3 supported the binding activity of RRM1 and RRM2, emphasizing its auxiliary role in overall RNA binding (Fig. 6A). Mutations in RRM3 caused a subtle yet significant shift in bulk mRNA distribution as well as Cdc3 shuttling and localization but did not disrupt unipolar growth. This indicates that while RRM3 is supportive, RRM1 and RRM2 mediate the functionally critical RNA contacts (Fig. 6A). These findings align with *in vitro* studies of the multi-RRM RNA transporter HuD, where RRM1 and RRM2 mediate RNA binding, and RRM3 enhances binding affinity (21,72).

While solving the multi-RRM code, we discovered that the binding of RRM3 to UAUG resulted in the most prominent UV crosslinks, despite their limited functional significance as seen for septin Cdc3. A plausible explanation is a quantitative UV crosslinking bias due to π -stacking interactions between aromatic amino acids and predominantly U or G nucleotides (73). RRM3 contains four aromatic amino acids (Fig. EV1A) known for efficient crosslinking to uracil (74). Consistently, an AlphaFold3 prediction indicates potential π -stacking interactions between the phenylalanine side chains of RRM3 and both uracils of UAUG (Fig. 6B) (3). This nicely illustrates that pronounced UV crosslink events may not reflect the most functionally significant binding sites *in vivo*.

RRM12-BS exhibit characteristic features of regulatory RNA elements

Our transcriptome-wide binding analysis successfully distinguished functionally relevant Rrm4 binding sites (RRM12-BS) from accessory sites (RRM3-BS), emphasizing the central role of RRM1 and RRM2 in target recognition and functional regulation. Loss-of-function mutants showed a significant reduction in mRNA levels of RRM12-BS targets and an increasing number of RRM12-BS sites amplified this effect, indicating a direct link between binding site abundance and mRNA levels. This highlights RRM12-BS as a hallmark regulatory RNA element. Our results are consistent with current views reporting that vesicle-mediated transport safeguards mRNAs from degradation during transit in mammals (22,24). Such a protective function was also described for the RBP Oskar from *D. melanogaster* that shields *nanos* mRNA during localization in embryos (75). We, therefore, propose that Rrm4-mediated transport protects RRM12-BS-containing mRNAs from cytoplasmic degradation, linking this process to changes in mRNA abundance. This is in accordance with a global link between mRNA stability and defined subcellular localization that was recently disclosed in neurons (29,76,77). Overall, we uncovered distinct functional and spatial specificities of RRM1 and RRM2 compared to RRM3 (Fig. 6A), providing new insights into the nuanced regulation of RNA binding, transport, and stability.

Target mRNAs encode factors orchestrating polar growth

mRNA translocation by vesicle hitchhiking is a widespread mechanism and mRNAs encoding mitochondrial proteins have already been identified as evolutionarily conserved cargos of this process (Fig. 6C) (3,22,24,26,27,30,34,78). Consistently, RRM12-BS targets encode subunits present in all five respiratory chain complexes and as anticipated, human FERRY and *U. maydis* Rrm4 share mitochondrial targets. Intriguingly, a second common mRNA regulon transported by both endosomal transport machinery are mRNAs encoding small GTPases such as Rac1 and polarity factors (22-24). This might be particularly important to determine fungal and mammalian cell polarity (Fig. 6C) (3,79-81).

Besides the FERRY complex, other RBPs are most likely involved in vesicle-mediated mRNA transport. TDP-43-containing mRNPs, for example, are transported by lysosome hitchhiking in axons (26,78) and TDP-43 also binds mRNAs encoding polarity factors such as *RAC1* mRNA (82-84). Thereby, aberrant TDP-43 might contribute to neuronal diseases such as amyotrophic lateral sclerosis and frontotemporal lobar degeneration (85).

Finally, RRM12-BS containing target mRNAs were strongly enriched in cell wall modifying enzymes. These most likely foster local cell wall remodeling (Fig. 6C) (65,66), disclosing a novel link between the coordination of cell wall synthesis and endosomal mRNA transport. Fundamentally, our study not only identifies novel targets involved in cell wall synthesis and modification but also uncovers the core targets of endosomal mRNA transport, evolutionarily conserved across polarized eukaryotic cells.

Conclusions

Our powerful comparative iCLIP2 approach assigned specific functions to individual RRMs. Thereby, accessory sites were distinguished from functional sites, which in turn orchestrate spatiotemporal expression of distinct mRNA regulons. Our findings underscore the complexity of RNA recognition, providing a new perspective beyond simple target identification toward understanding global RNA binding dynamics. Decoding modular RRM binding by iCLIP2 revealed a novel connection between endosomal transport and the subcellular localization of polarity factors – a pathway seemingly conserved in highly polarized cells such as fungal hyphae and neurons. This insight broadens our understanding of membrane-coupled mRNA transport, with broad implications from fighting fungal pathogens to the development of novel therapeutic approaches in neuronal diseases (5).

Data availability

RNA-seq and iCLIP2 data are available in the NCBI Gene Expression Omnibus (GEO). The iCLIP2 data for wild-type Rrm4 can be accessed under GSE273496, while the mutant Rrm4 variants are available under GSE273495 and require the security token xx for access. The RNA-seq data are available under GSE288123, with anonymous reviewer access provided via the security token xx.

Acknowledgments

We sincerely thank Drs. Heiner Schaal, Florian Altegoer, Kerstin Schipper, and lab members for their critical reading of the manuscript and valuable feedback. We are particularly grateful to Dr. Florian Altegoer for his expert guidance on structural prediction and to Dr. Mirko Brüggemann and members of the Zarnack group for insightful discussions on binding site definition and differential binding analysis. We appreciate the dedicated support of Maximilian Neumann, Marie Wittmann, and Caroline Downes in plasmid generation and Ute Gengenbacher and Simone Esch for their exceptional technical assistance regarding strain generation. We extend our thanks to Dr. Maria Méndez-Lago and the IMB Genomics Core Facility in Mainz for their outstanding support with iCLIP2 and mRNA library sequencing, as well as for granting access to their Illumina NextSeq 500 platform.

Author contributions

NKS, SS, KZ, JK, and MF designed this study and analyzed the data. NKS performed wet-lab experiments supported by JK and KM. *U. maydis* strains were planned by NKS, KM, and MF. SS, AB, NKS, and KZ performed the bioinformatic analysis. NKS, SS, KZ, and MF drafted and revised the manuscript with input from all co-authors. MF, KZ, and JK contributed funding and resources.

Funding

This research was generously supported by grants from the DFG under Germany's Excellence Strategy EXC-2048/1 - project ID 39068111 to MF, as well as DFG-FOR2333-TP03 to MF, DFG-FOR2333-TP02 to KZ and JK, DFG-SFB 1208 - project ID 267205415 to MF (project A09), and DFG-SFB1535 - project ID 458090666 to MF (project A03). The Illumina NextSeq 500 platform at IMB Mainz is funded by DFG - project ID #329045328.

Conflict of interest statement

None declared.

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Figures

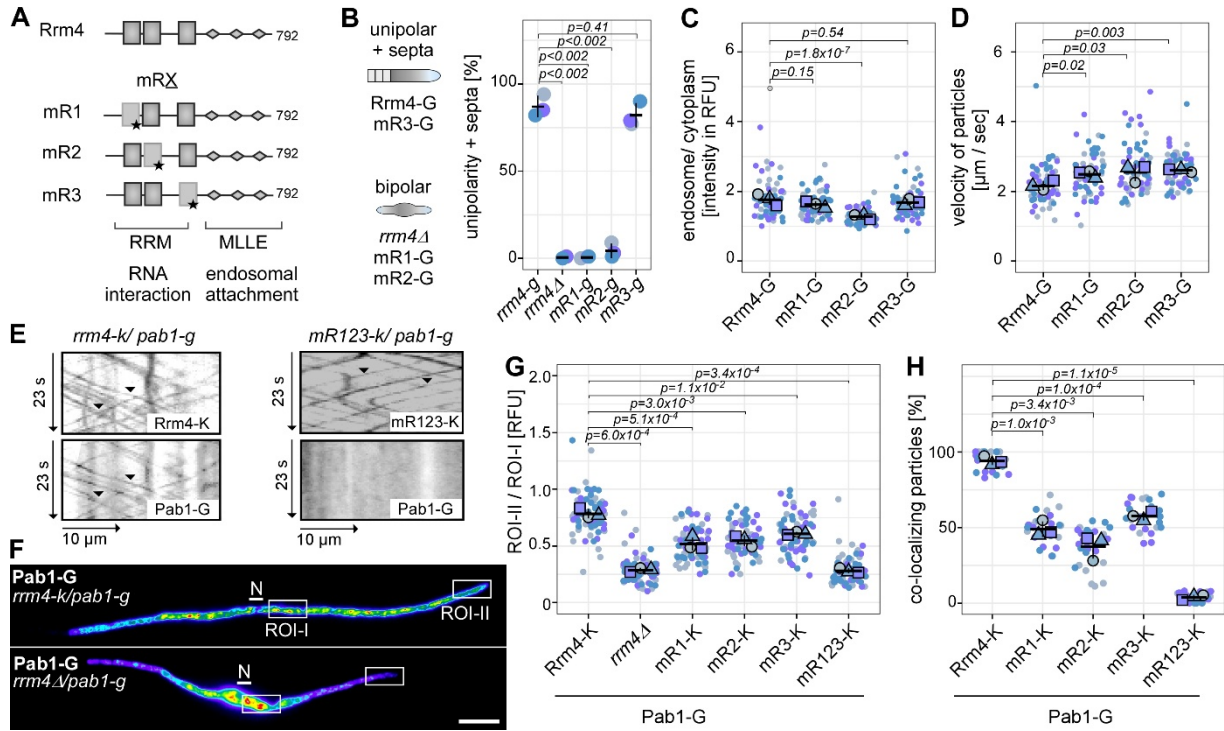


Figure 1. Mutations in RRM domains cause differential defects during polar growth.

(A) Schematic representation of Rrm4 variants (792 amino acids): grey rectangles, RNA recognition motif (star indicate mutation); diamonds, MLLE domains. (B) Hyphal growth (6 h. p. i.) Unipolar hyphae with inserted septa were quantified (Student's *t*-test, unpaired; three biological replicates are shown in different blue shadings, $N > 100$) (C) Ratio of Gfp relative fluorescence unit (RFU) intensity comparing endosomes versus cytoplasm. Blue shading indicates results from three independent experiments (Student's *t*-test, unpaired; based on the means of the three biological replicates). The mean of each replicate is given in different forms and respective colors (replicate 1=circle; replicate 2=triangle, replicate 3=square; $n > 60$). (D) Velocity of Rrm4-containing particles. Shading indicates results from three independent experiments (Student's *t*-test, unpaired; three biological replicates, $N > 60$). (E) Kymographs (inverted fluorescence images) of hyphae (6 h. p. i.) showing mKate2 (-K) and Gfp (-G) signals at the top and bottom. (F) False color imaging of Pab1-G fluorescence in hyphae (6 h. p. i.). Regions of interest (ROI) are marked by rectangles (size bar, 10 μ m). (G) Quantification of Pab1-G distribution comparing ROI-I (perinuclear) versus ROI-II (growth pole). Shading indicates results from three independent experiments (Student's *t*-test, unpaired; $N > 60$). (H) Quantification of Rrm4-K versions co-localising with Pab1-G-containing particles (Student's *t*-test, unpaired; three biological replicates, $N > 30$).

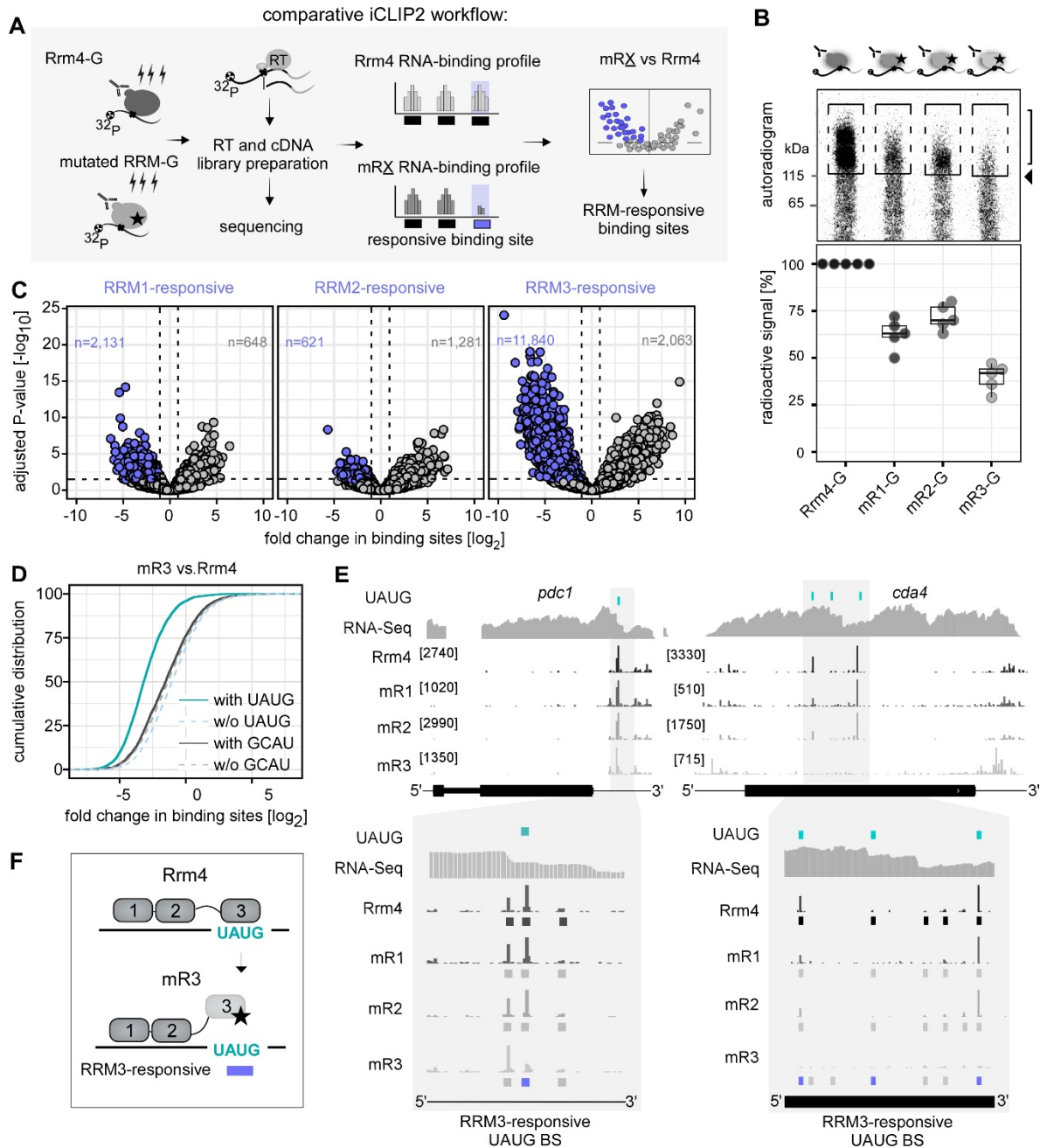


Figure 2. Comparative iCLIP2 approach discloses transcriptome-wide RRM3 / UAUG interaction.

(A) Schematic representation of the comparative iCLIP2 approach. The binding landscape, derived from crosslink events in the Rrm4 mutant iCLIP2 (mRX-G=mutated RRM-G, with mutations in either RRM1, RRM2, or RRM3 domain; bottom), was compared to the wild-type Rrm4-iCLIP2 analysis (Rrm4-G; top). Binding sites dependent on the RRM domain show a significant reduction in crosslink events in the mutant compared to the wild-type (highlighted in purple). These binding sites are designated as responsive binding sites (indicated by a purple rectangle). Binding sites shown in black represent those without significant differences in crosslink events upon RRM mutation (RT, reverse transcription; G, Gfp) (B) Top: representative autoradiogram of UV-crosslinked radioactively labeled RNA bound to Rrm4-G or the respective mutants. Rectangles indicate regions of radioactive signal quantified at the

bottom (arrowhead indicates the estimated position of full-length Rrm4-G protein). **(C)** Volcano plot showing changes in binding for mR1 (left), mR2 (center), and mR3 (right) compared to wild-type Rrm4. Binding sites exhibiting a significant reduction in binding are labeled in blue. The dotted lines indicate the significance cutoffs (absolute fold change $[\log_2] > 1$, adjusted P-value < 0.05 , baseMean > 15). **(D)** Cumulative fraction of binding sites with (solid line) or without (dotted line) UAUG (petrol) or GCAU motifs (negative control motif; dark grey) with given binding site fold change. **(E)** Genome browser view of *pdcl* (14-3-3 gene family) and *cda4* (chitin deacetylase 4) mRNA (UAUG motif and RRM3-responsive binding sites in petrol and blue, respectively). Numbers represent the upper limit of stack heights for iCLIP2 crosslink events. **(F)** Graphical representation of Rrm4 (top) and mR3 interaction with UAUG. Mutation in RRM3 (star) cause loss of UAUG recognition.

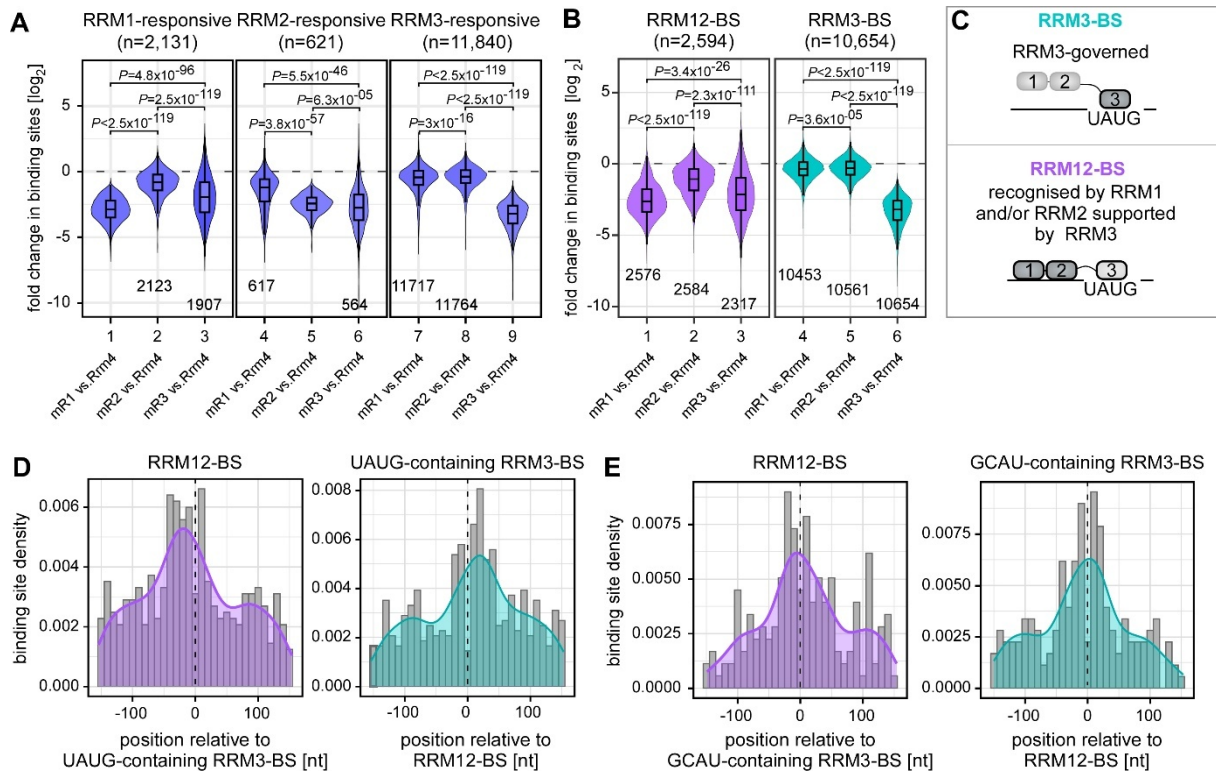


Figure 3. Differential iCLIP2 analysis reveals two types of Rrm4 binding sites.

(A) Violin plots (numbered at the bottom) showing the quantitative changes in crosslink events at binding sites for RRM1- (left box), RRM2- (center box), and RRM3-responsive sites (right box) in response to mutation in RRM1 (mR1 vs. Rrm4), RRM2 (mR2 vs. Rrm4) and RRM3 (mR3 vs. Rrm4). Boxes represent quartiles, with the center line marking the median. The whiskers extend to the most extreme values within the 1.5x interquartile range. The number of responsive sites measured in the mutant datasets is indicated at the bottom. Statistical significance for changes in responsive sites among the mutant datasets was assessed using the Wilcoxon signed-rank test. (B) Violin plots illustrate the quantitative changes in RRM12-BS (purple; left) or RRM3-BS (petrol; right) in mutant datasets. Statistical significance was determined using the Wilcoxon signed-rank test. (C) Graphical representation of Rrm4 binding sites. RRM3-BS: Binding sites governed by the third RRM domain, with minimal influence from the RRM1 and RRM2 domains. RRM12-BS: Binding sites recognized by RRM1 and/or RRM2 with often additional support from RRM3. (D) Histogram with density curve illustrates the positional distribution of RRM12-BS (left panel) within a 301 nt window centered on RRM3-BS with UAUG motifs or *vice versa* (right panel). (E) Same as (D) but using RRM3-BS with GCAU control motifs.

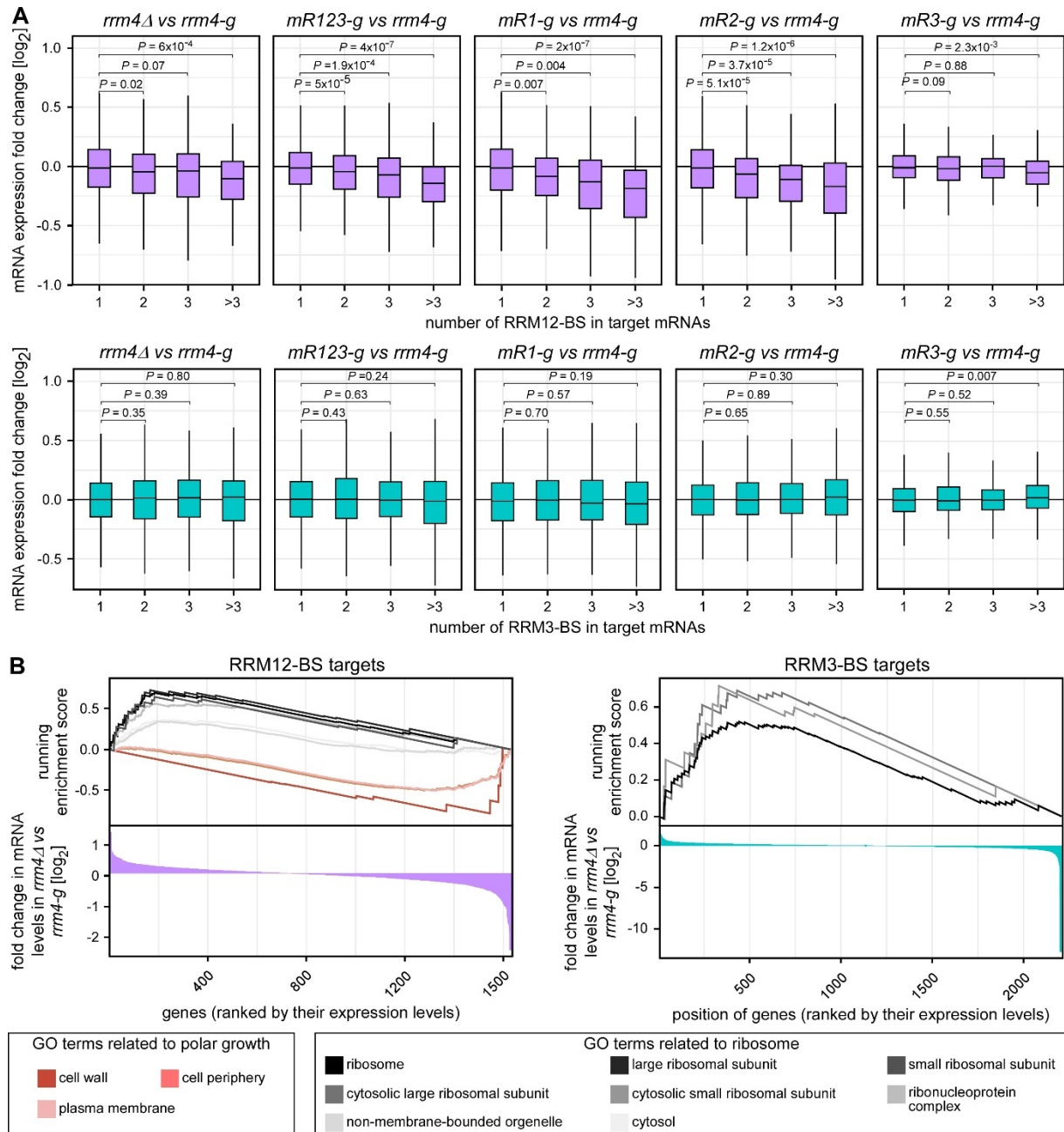


Figure 4. RRM12-BS constitutes functional RNA regulatory elements

(A) Box plots illustrate the fold changes in mRNA levels of RRM12-BS targets (purple; top) and RRM3-BS targets (petrol; bottom) in strains lacking the *rrm4* gene (*rrm4Δ*), or those carrying mutations in RRM domains (*mR1-g*, *mR2-g*, *mR3-g*, and *mR123-g*) compared to wild-type *rrm4* gene (*rrm4-g*). The target mRNAs are grouped based on the number of RRM12-BS (top) or RRM3-BS in them. Statistical significance between groups was assessed using the Wilcoxon signed-rank test. (B) Gene set enrichment analysis showing the enrichment of GO terms related to cellular compartments in RRM12-BS targets (left) and RRM3-BS targets (right). Terms associated with polar growth are colored in red shadings, while those related to ribosomes are colored in shades of black. The colored bars below represent the changes in mRNA levels of RRM12-BS targets (purple) and RRM3-BS targets (petrol), ranked in decreasing order by their fold change.

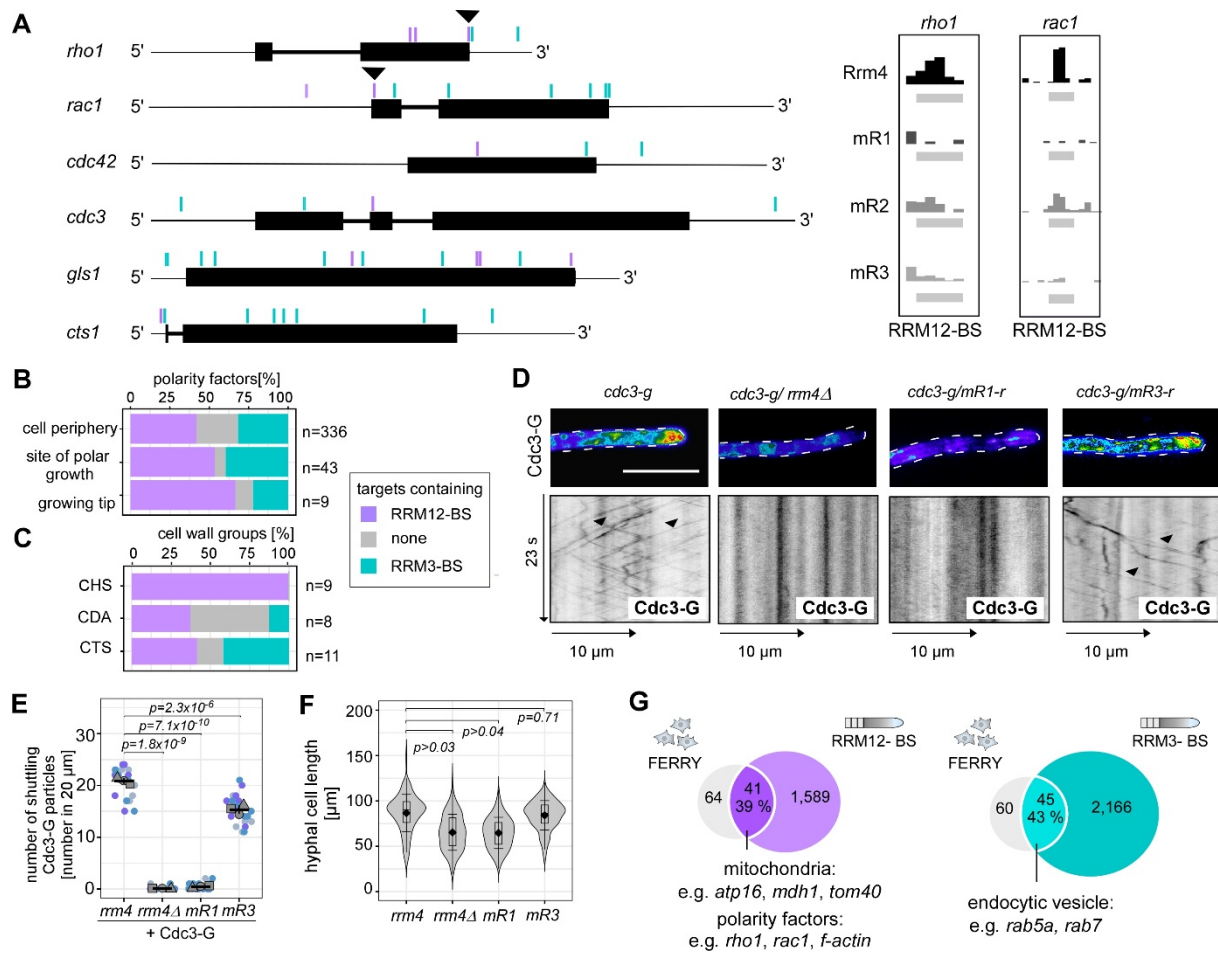


Figure 5. RRM12-BS targets link Rrm4 function to cell wall synthesis, mitochondrial metabolism and polarity

(A) Genome browser visualization of RRM12-BS containing target mRNAs, displaying differential Rrm4 binding site profiles. RRM3-BS (petrol) and RRM12-BS (purple) are highlighted, indicating distinct regulatory contributions of individual RRMs. A zoom-in view of selected RRM12-BS regions (arrowheads) is shown on the right, illustrating the crosslink event landscape for Rrm4, mR1, mR2, and mR3 (group autoscale). (B) Bar diagram showing the percentage of target mRNAs containing RRM12-BS, RRM3-BS or none within GO terms associated with polar growth and polarity factors: *cell periphery* (GO:0071944, n=336), *site of polar growth* (GO:0030427, n=43), and *growing cell tip* (GO:0035838, n=9). The entries (n) of the GO terms are indicated. (C) Bar diagram as in (B) exhibiting cell wall remodeling enzymes categorized in chitin synthase (CHS, n=9), chitin deacetylase (CDA, n=8), and degradation (e.g. chitinases, CTS, n=11). (D) The upper panel represents false color imaging of Cdc3-G fluorescence of the growing cell tip (6. h. i., size bar: 10 μ m) in diverse Rrm4 background strains. The lower panel shows the respective kymographs of shuttling Cdc3-G particles. (E) Quantification of Cdc3-G shuttling particles (Student's *t*-test, unpaired; based on the means of the three biological replicates, replicate 1=circle; replicate 2=triangle, replicate 3=square; N > 20). (F) Violin plot of hyphal cell length (6. h. p. i., Student's *t*-test, unpaired; three biological replicates, N > 200). (G) Venn diagrams of potential FERRY targets and RRM12-BS (purple) and RRM3-BS (petrol) containing targets.

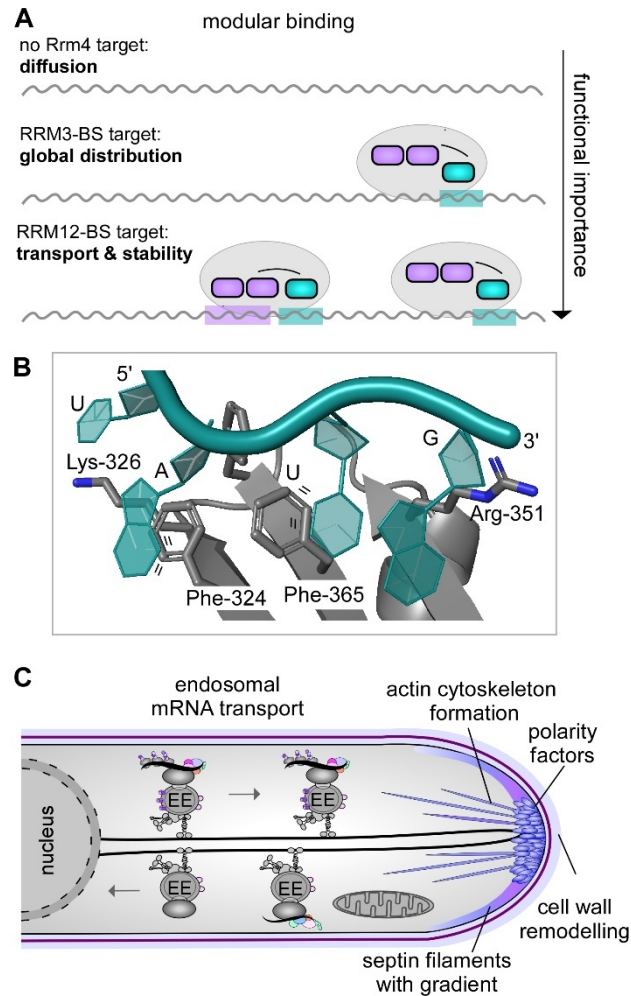


Figure 6. The comparative iCLIP2 approach identifies accessory and key binding sites of the multi-RRM protein Rrm4.

(A) Modular binding mode of the multi-RRM protein Rrm4. RRM3 (petrol) and its specific binding sites (RRM3-BS, petrol) are essential for the uniform distribution of RRM3-BS-containing targets throughout the hyphae. The tandem RRM1 and RRM2 (purple) are crucial in regulating the transport and stability of RRM12-BS-containing mRNAs, which encode proteins that specifically localize to the cell tip, driving cell wall expansion and polarity growth. The regulation of these RRM12-BS targets is primarily controlled by RRM1 and/or RRM2, with additional support from RRM3. Importantly, the abundance of RRM12-BS within a target correlates with a significant effect on its mRNA abundance, highlighting the intricate interplay between these binding modes and cellular architecture. (B) AlphaFold3 model of RRM3/UAUG interaction. For prediction, full-length Rrm4 was offered. Among the three RRM domains, RRM3 was selected. Potential π -stacking events are indicated (lines). (C) Schematic representation of endosomal mRNA transport in *U. maydis*. Functional regulated RRM12-BS containing regulons such as polarity factors, septin-gradient and cytoskeleton formation, cell wall remodeling, as well as mitochondria, are highlighted

Expanded View Figures

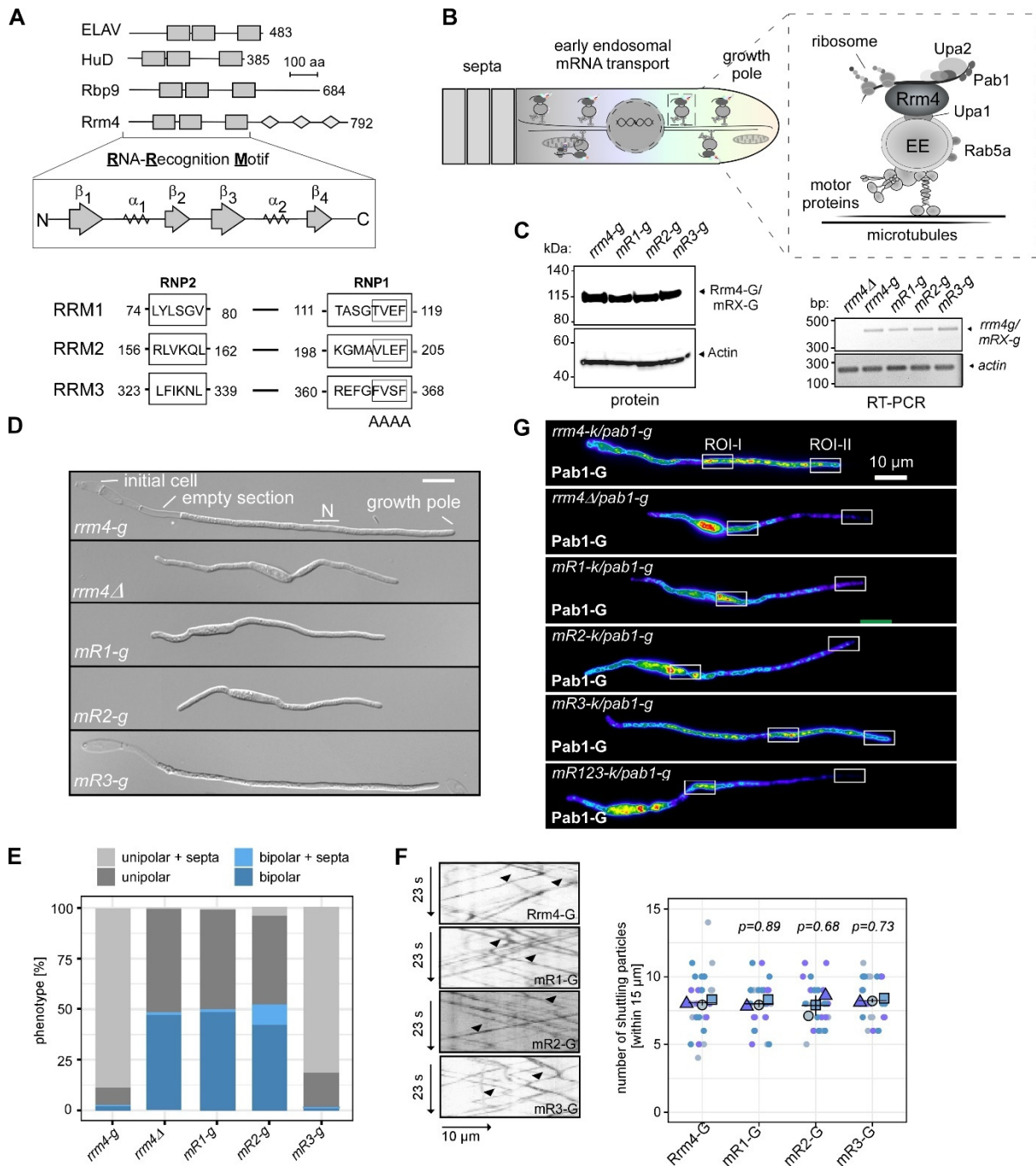


Figure EV1. Mutations in RRM1 and RRM2 cause loss-of-function

(A) Schematic representation of diverse multi-RRM RNA binding proteins: ELAV (*Drosophila melanogaster*), HuD (*Homo sapiens*), Rbp9 (*Drosophila melanogaster*), and Rrm4 (*Ustilago maydis*) and a detailed view of the structural architecture of a typical RNA-recognition motif (RRM). (B) Schematic representation of the current endosomal mRNA transport model in *U. maydis*. The key RBP Rrm4 (RNA recognition motif 4, dark grey), accessory RBP Pab1 (Poly(A) binding protein 1, anthracite) and associated ribosomes are indicated. In addition, the mRNP scaffold protein Upa2, the anchor protein Upa1, and the early endosomal marker protein

Rab5a are labeled. (C) Western blot (left) analysis of Rrm4-G (112 kDa) and the respective RRM mutants (mR1-mR3-G: 112 kDa). Rrm4-G/mRX-G full-length band is indicated by an arrow. Actin protein (45 kDa) serves as a loading control. (right) RT-PCR of *rrm4* mRNA and the respective mutant versions (mR1-mR3). The *actin* mRNA serves as constitutive control for the analysis of equal RNA amounts. (D) DIC pictures of hyphae (6 h. p. i., size bar, 10 μ m). (E) A stacked bar graph of quantified polarity phenotypes of hyphae: unipolarity, bipolarity, and septum insertion were counted (6 h. p. i., three biological replicates, N>100). (F) Kymographs (inverted fluorescence images) of hyphae (6 h. p. i.) showing Rrm4-G and respective mutants (mR1-mR3-G). Right side, a scatter plot of the number of shuttling particles measured within 15 μ m sections (Student's *t*-test, unpaired; three biological replicates indicated by different blue shadings, N=30). (G) False color imaging of Pab1-G fluorescence in hyphae (6. h. p. i.). Regions of interest (ROI) are marked by a rectangle (size bar, 10 μ m).

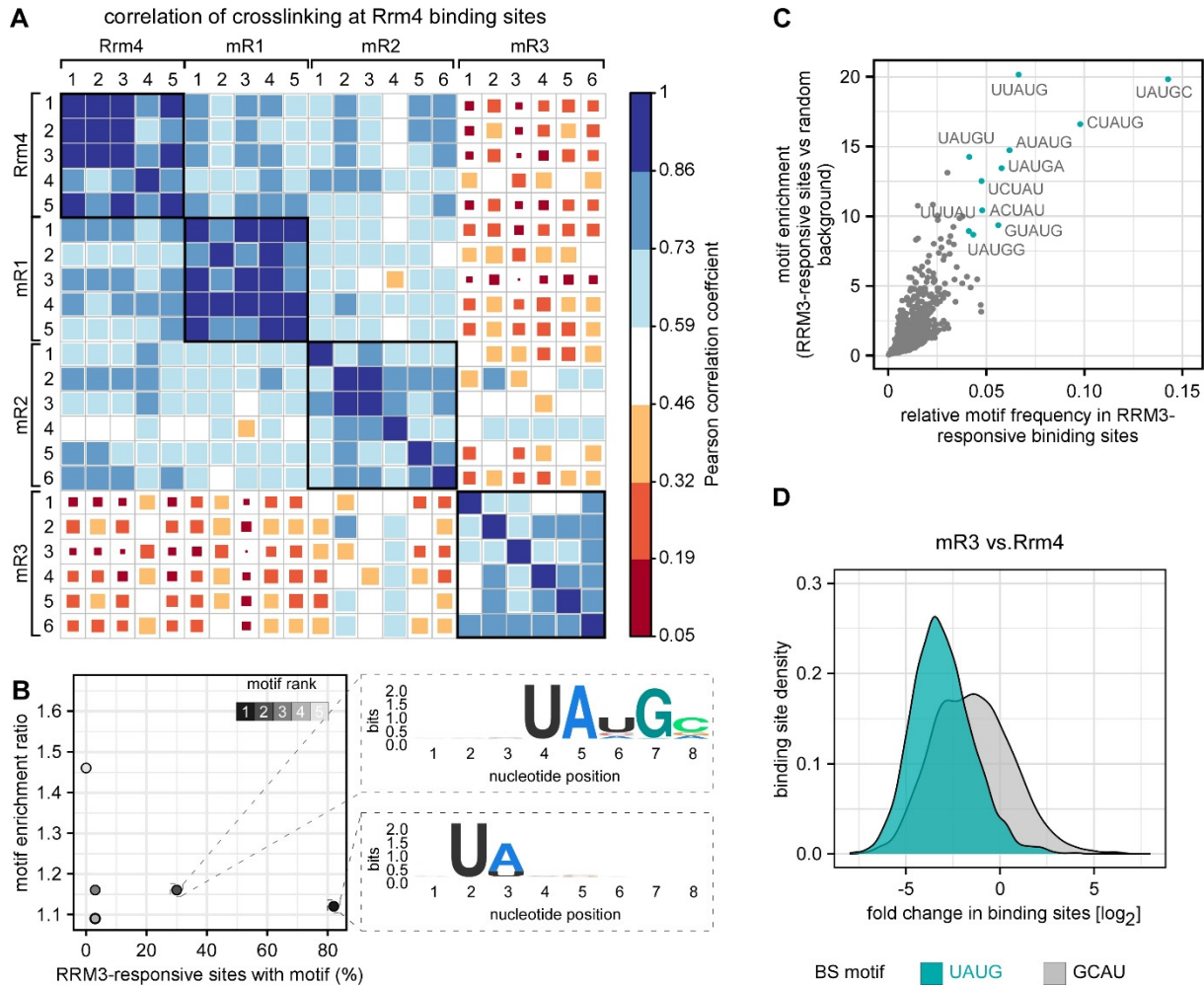


Figure EV2. UAUG is a prominent RRM3 binding motif

(A) Correlation of crosslinking events per Rrm4 binding sites across iCLIP2 (Rrm4; mR1; mR2; mR3) and their respective replicates (minimum of five replicates). The size and color intensity of the square represents the absolute value of the Pearson correlation coefficient. (B) *De novo* motif discovery analysis on RRM3-responsive binding sites. Scatter plot compares the relative enrichment ratio of each motif with its percentage in the RRM3-responsive binding sites. The enlarged section displays the sequence logos of the top two ranked motifs. The motifs were colored according to their ranks. (C) Motif enrichment in the RRM3-responsive sites. Pentamer frequencies were calculated by comparing the RRM3-responsive motifs to a random genome background. Highly enriched motifs were highlighted in petrol. (D) Density plot showing the distribution of binding site fold change [$\log_2$] for UAUG-containing sites (petrol) and GCAU-containing sites (control motif; grey) in the mR1 vs Rrm4 dataset.

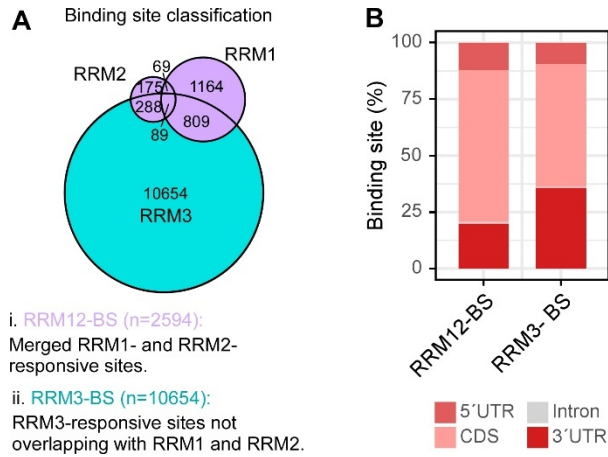


Figure EV3. RRM12-BS differ from RRM3-BS

(A) Overview of Rrm4 binding site classification. RRM1- and RRM2-responsive sites were combined and categorized as RRM12-BS (purple region in the Venn diagram). RRM3-responsive sites that do not overlap with RRM1- and RRM2-responsive sites were classified as RRM3-BS (petrol colored region in the Venn diagram). (B) Distribution of RRM12-BS and RRM3-BS across transcript regions. (CDS- coding sequence; UTR-untranslated region).

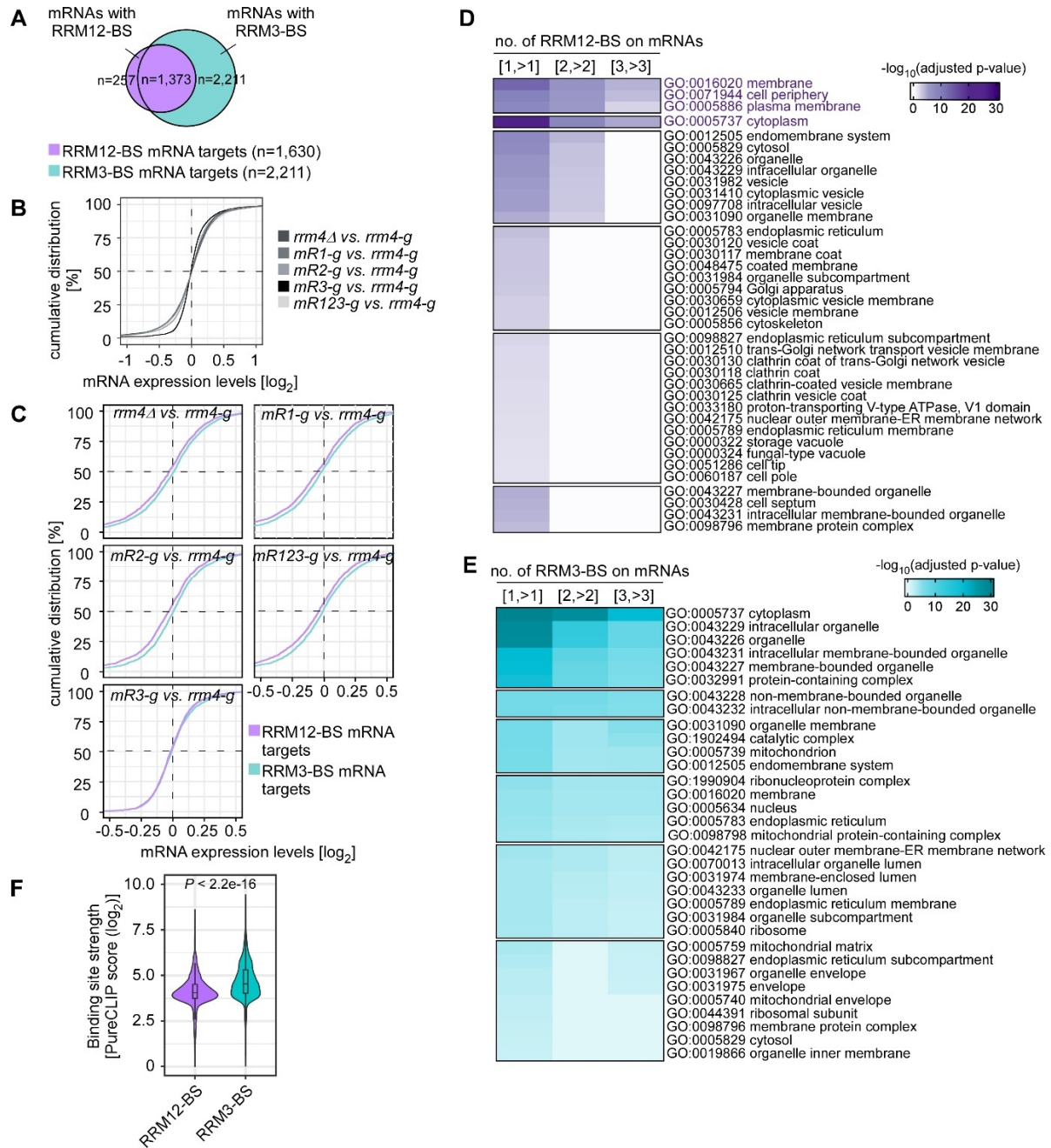


Figure EV4. RRM12-BS target exhibit reduced mRNA amounts if *Rrm4* function is affected

(A) Venn diagram comparing the overlap between RRM12-BS and RRM3-BS mRNA. mRNA targets were assigned hierarchically. (B) Cumulative fraction of all hyphae expressed transcripts (n=6278) with the given difference in mRNA expression levels [log₂] for *rrm4Δ* vs. *rrm4-g*, *mR1-g* vs. *rrm4-g*, *mR2-g* vs. *rrm4-g*, *mR3-g* vs. *rrm4-g* and *mR123-g* vs. *rrm4-g* datasets. (C) Similar to B, for RRM12-BS mRNA targets (n=1630; purple) and RRM3-BS mRNA targets (n=2211; petrol). (D-E) Heatmap of gene ontology term (GO term) analysis (57) for RRM12-BS targets (D) and RRM3-BS targets (E). The color scale represents the -log₁₀ (adjusted p-values). Categories indicate mRNA targets with at least one binding site [1, >1], at least two binding sites [2, >2], or at least three binding sites [3, >3]. (F) Violin plot illustrates the binding site strength (log₂-transformed PureCLIP score) of RRM12-BS (purple) or RRM3-BS (petrol) in the *Rrm4*-iCLIP2 dataset. Statistical significance was calculated using the Wilcoxon signed-rank test.

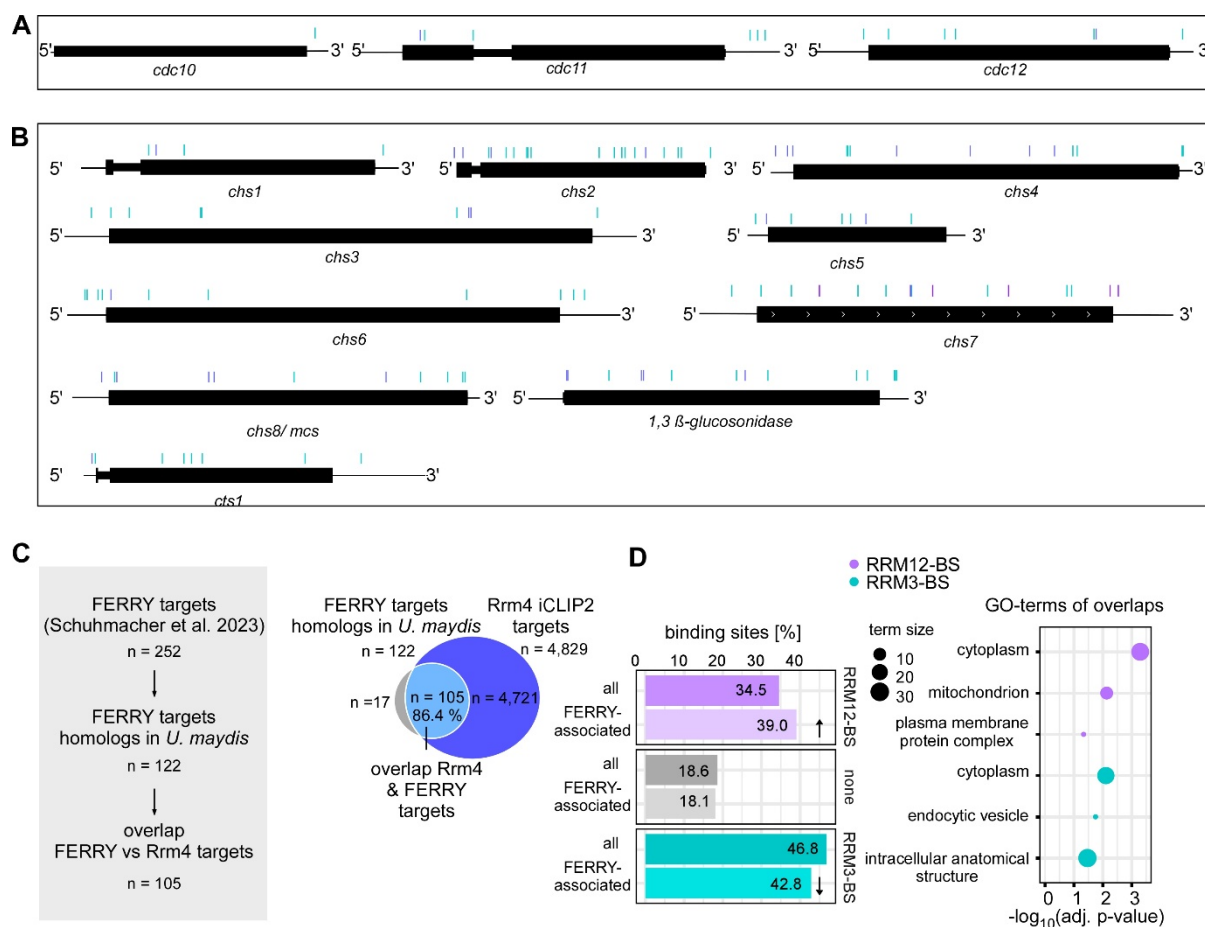


Figure EV5. Endosomal mRNA targets are conserved in fungi and mammals

(A) Differential binding site profile including RRM3-BS (petrol) and RRM12-BS (purple) of septin mRNAs (*cdc10*, *cdc11*, *cdc12*, Supplementary Table S7-8). (B) Genome browser viewer (as in A) of mRNAs encoding enzymes involved in cell wall synthesis (CHS group see Fig. 5, Supplementary Table S7-8). (C) Schematic workflow representation and respective overlap between FERRY-associated mRNA (22) and Rrm4-bound targets (light blue; n=105, Supplementary Table S5). (D) Percentage of distribution of RRM12-BS-, RRM3-BS-, and none BS- containing targets in overlapping category (FERRY-associated) compared to overall Rrm4-binding targets (all). Right side GO term analysis of overlapping targets: RRM12-BS and FERRY (purple, Supplementary Table S5); RRM3-BS and FERRY (petrol, Supplementary Table S5) performed by gProfiler analysis (57).

Supporting information

Dissecting the RNA binding capacity of the multi-RRM protein Rrm4 essential for endosomal mRNA transport

**Nina Kim Stoffel^{1,*}, Srimeenakshi Sankaranarayanan^{1,*}, Kira Müntjes¹, Anke Busch²,
Julian König^{2,3}, Kathi Zarnack^{4,5,§} and Michael Feldbrügge^{1,§}**

¹ Institute of Microbiology, Heinrich Heine University Düsseldorf, 40204 Düsseldorf, Germany

² Institute of Molecular Biology gGmbH, Ackermannweg 4, 55128 Mainz, Germany

³ Institute of Biochemistry and RNA Biology, Theodor-Boveri-Institute for Biosciences, Julius Maximilians University Würzburg, Am Hubland, 97074 Würzburg, Germany

⁴ Buchmann Institute for Molecular Life Sciences (BMLS) & Institute of Molecular Biosciences, Goethe University Frankfurt, Max-von-Laue-Str. 15, 60438 Frankfurt, Germany

⁵ Theodor Boveri Institute for Biosciences, Biocenter, Julius Maximilians University Würzburg, Am Hubland, 97074 Würzburg, Germany

* Shared first authors; § Co-corresponding authors

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Addition files:

Supplementary Table S5 FERRY/ Rrm4 comparison given as a separate Microsoft Excel file

Supplementary Table S6 RRM-responsive side given as a separate Microsoft Excel file

Supplementary Table S7 RRM12-BS given as a separate Microsoft Excel file

Supplementary Table S8 RRM3-BS given as a separate Microsoft Excel file

Table S1. Description of *U. maydis* strains used in this study

Strain name with code	Locus	Progenitor strain	Short description
AB33 (UMa133)	<i>b</i>	FB2	<i>Promotor-nar:bW2bE1</i> , expression of active b heterodimer under control of the <i>nar1</i> promoter, strain grows filamentous upon nitrogen change (Brachmann et al., 2001)
AB33_rrm4Δ (Uma273)	<i>rrm4</i>	AB33	carrying a deletion of <i>rrm4</i> (Becht et al., 2005)
AB33_rrm4-egfp (UMa274)	<i>rrm4</i>	AB33rrm4Δ	expressing Rrm4 C-terminally fused to eGfp, endogenous promotor (Becht et al., 2005)
AB33_mR1-egfp (UMa311)	<i>rrm4</i>	AB33rrm4Δ	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM1: TVEF (116-119 aa) to AAAA, endogenous promotor (Becht et al., 2006)
AB33_mR2-egfp (UMa312)	<i>rrm4</i>	AB33rrm4Δ	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM2: VLEF (201-205 aa) to AAAA, endogenous promotor (this study, plasmid: pUMa636)
AB33_mR3-egfp (UMa313)	<i>rrm4</i>	AB33rrm4Δ	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM3: FVSF (362-368 aa) to AAAA, endogenous promotor (Becht et al., 2006)
AB33_mR123-egfp (UMa276)	<i>rrm4</i>	AB33rrm4Δ	Expressing a triple mutation Rrm4 C-terminally fused to eGfp mutation in RRM1: TVEF (116-119 aa) to AAA; in RRM2: VLEF (201-205 aa) to AAAA, in RRM3: FVSF (362-368 aa) to AAAA, endogenous promotor (Olgeiser et al., 2019)
AB33_rrm4-mKate2/pab1-gfp (UMa3334)	<i>rrm4</i> <i>pab1</i>	AB33 Pab1-egfp-nat/ rrm4Δ (UMa472)	expressing Pab1 C-terminally fused to eGfp and a mutated version of Rrm4 C-terminally fused to mKate2, endogenous promotor (this study; plasmid: pUMa4698)
AB33_mR1-mKate2/pab1-gfp (UMa3268)	<i>rrm4</i> <i>pab1</i>	AB33 Pab1-egfp-nat/ rrm4Δ (UMa472)	expressing Pab1 C-terminally fused to eGfp and a mutated version of Rrm4 C-terminally fused to mKate2; mutation in RRM1: TVEF (116-119 aa) to AAA, endogenous promotor (this study, plasmid: pUMa4569)
AB33_mR2-mKate2/pab1-gfp (UL172)	<i>rrm4</i> <i>pab1</i>	AB33 Pab1-egfp-nat/ rrm4Δ (UMa472)	expressing Pab1 C-terminally fused to eGfp and a mutated version of Rrm4 C-terminally fused to mKate2; mutation in RRM2: VLEF (201-205 aa) to AAAA, endogenous promotor (this study, plasmid: pUL_0192)
AB33_mR3-mKate2/pab1-gfp (UL173)	<i>rrm4</i> <i>pab1</i>	AB33 Pab1-egfp-nat/ rrm4Δ (UMa472)	expressing Pab1 C-terminally fused to eGfp and a mutated version of Rrm4 C-terminally fused to mKate2; mutation in RRM3: FVSF (362-368 aa) to AAAA, endogenous promotor (this study, plasmid: pUL_0193)
AB33_mR123-mKate2/pab1-gfp (UMa3270)	<i>rrm4</i> <i>pab1</i>	AB33 Pab1-egfp-nat/ rrm4Δ (UMa472)	expressing Pab1 C-terminally fused to eGfp and a mutated version of Rrm4 C-terminally fused to mKate2; mutation in RRM1: TVEF (116-119 aa) to AAA; in RRM2: VLEF (201-205 aa) to AAAA, in RRM3: FVSF (362-368 aa) to AAAA, endogenous promotor (this study plasmid: pUMa4571)
AB33_gfp-cdc3 (UMa449)	<i>cdc3</i>	AB33	expressing Cdc3 N-terminally fused to eGfp, endogenous promotor (Baumann et al., 2014)

AB33_gfp-cdc3/rrm4Δ (UMa462)	<i>rrm4</i> <i>cdc3</i>	AB33_gfp-cdc3	expressing Cdc3 N-terminally fused to eGfp in an <i>rrm4</i> Δ background, endogenous promotor (Baumann et al., 2014)
AB33_Gfp-cdc3-/mR1-rfp- (UMa836)	<i>rrm4</i> <i>cdc3</i>	AB33_gfp-cdc3	expressing Cdc3 N-terminally fused to eGfp and a mutated version of Rrm4 C-terminally fused to Rfp; mutation in RRM1: TVEF (116-119 aa) to AAA, endogenous promotor (Baumann et al., 2014)
AB33_Gfp-cdc3/mR3-rfp (UMa940)	<i>rrm4</i> <i>cdc3</i>	AB33_gfp-cdc3	expressing Cdc3 N-terminally fused to eGfp and a mutated version of Rrm4 C-terminally fused to Rfp; mutation in RRM3: FVSF (362-368 aa) to AAAA, endogenous promotor (this study plasmid: pUMa1703)

Table S2. Description of plasmids generated for *U. maydis* strain generation

Plasmid	Plasmid ID	Resistance cassette	Short description
Prrm4:Rrm4_mR2_eGfp_Nat	pUMa636	NatR (nourseothricin resistance)	Plasmid vector for generating a mutant version of <i>rrm4</i> C-terminal fused with eGfp. Mutation in RRM2 -RNP1: VLEF (201-205 aa) to AAAA, construct under control of the endogenous promotor of <i>rrm4</i> .
Prrm4:Rrm4_mR2_mKate2_HA_G418R	pUL192	genitR (G418 resistance)	Plasmid vector for generating a mutant version of <i>rrm4</i> C-terminal fused with mKate2_HA. Mutation in RRM2 -RNP1: VLEF (201-205 aa) to AAAA, construct under control of the endogenous promotor of <i>rrm4</i> .
Prrm4:Rrm4_mR3_mKate2_HA_G418R	pUL193	genitR (G418 resistance)	Plasmid vector for generating a mutant version of <i>rrm4</i> C-terminal fused with mKate2_HA. Mutation in RRM3: FVSF (362-368 aa) to AAAA, construct under control of the endogenous promotor of <i>rrm4</i> .
Prrm4:rrm4-mKate2_HA_G418R	pUMa4698	genitR (G418 resistance)	Plasmid vector for generating <i>rrm4</i> C-terminal fused with mKate2_HA, construct under control of the endogenous promotor of <i>rrm4</i> .
Prrm4:Rrm4_mR1_mKate2_HA_G418R	plasmid: pUMa4569	genitR (G418 resistance)	Plasmid vector for generating a mutant version of <i>rrm4</i> C-terminal fused with mKate2_HA. mutation in RRM1: TVEF (116-119 aa) to AAA, construct under control of the endogenous promotor of <i>rrm4</i> .
Prrm4:Rrm4-mR123-mKate_HA_G418R	plasmid: pUMa4571	genitR (G418 resistance)	Plasmid vector for generating a triple mutant version of <i>rrm4</i> C-terminal fused with mKate2_HA. Rrm4 C-terminally fused to mKate2-HA; mutation in RRM1: TVEF (116-119 aa) to AAA; in RRM2: VLEF (201-205 aa) to AAAA, in RRM3: FVSF (362-368 aa) to AAAA, endogenous promotor of <i>rrm4</i> .
Prrm4:rrm4_mR3_Rfp_HygR	pUMa1703	HygR (Hygromycin resistance)	Plasmid vector for generating a mutant version of <i>rrm4</i> C-terminal fused with Rfp. Mutation in RRM3: FVSF (362-368 aa) to AAAA, construct under control of the endogenous promotor of <i>rrm4</i> .

Table S3: DNA oligonucleotides used in this study

Designation	Nucleotide sequence (5' 3')	Experiment/ Validation of:
MF225	AATTGACCGCACCGTGGG	pUMa636/ pUL192, pUMa1703
MF502	ACGACGTTGTAAAACGACGCCAG	pUMa4571, pUMa4569 pUL172, pUL173, pUMa4698
MF233	TTGAGCTGGCCAACGAGC	pUMa4571
SL56	CCCACATCCGCTCTAACC	pUMa4571, pUMa4569
UM805	TTCCGAGGTGCACTTGAAG	pUMa4569, pUL192, pUL193, pUMa4698
CD503	CATGCCATGGGAGCTAGCGCGGCCGAGCTCCGGTCTGCCTCTCC	pUL193
EF281	CTGGGCGTCTTGTTTCAGAGT	RT-PCR: rrm4
EF282	GCCACCATGACTGACAAGGA	RT-PCR: rrm4
EF283	GGCCTTCTACGTCGCTATCC	RT-PCR: actin
EF284	TCGAGACGCAGAATCGAGTG	RT-PCR: actin
L3-App	/rApp/AGATCGGAAGAGCGGTTCAG/ddC/	iCLIP2
RT_iCLIP2	GGATCCTGAACCGCT	iCLIP2
L01_iCLIP2.0	/5Phos/NNNNATCACGNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L02_iCLIP2.0	/5Phos/NNNNCGATGTNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L03_iCLIP2.0	/5Phos/NNNNTTAGGCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L04_iCLIP2.0	/5Phos/NNNNTGACCANNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L05_iCLIP2.0	/5Phos/NNNNACAGTGNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L06_iCLIP2.0	/5Phos/NNNNGCCAATNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L07_iCLIP2.0	/5Phos/NNNNCAGATCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L08_iCLIP2.0	/5Phos/NNNNACTTGANNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L09_iCLIP2.0	/5Phos/NNNNGATCAGNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L10_iCLIP2.0	/5Phos/NNNNTAGCTTNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L11_iCLIP2.0	/5Phos/NNNNATGAGCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L12_iCLIP2.0	/5Phos/NNNNCTTGTANNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L13_iCLIP2.0	/5Phos/NNNNAGTCAANNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2

L14_iCLIP2.0	/5Phos/NNNNAGTTCCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L15_iCLIP2.0	/5Phos/NNNNATGTCANNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L16_iCLIP2.0	/5Phos/NNNNCCGTCCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L17_iCLIP2.0	/5Phos/NNNNCAACTANNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2
L18_iCLIP2.0	/5Phos/NNNNGTCCGCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	iCLIP2

Table S4. Identifiers of selected RRM12-BS containing target transcripts

Name	UMAG number
Pdc1/Bmh1	UMAG_01366
Cda4	UMAG_01143
Cdc42	UMAG_00295
Rac1	UMAG_00774
Rho1	UMAG_05734
Cdc3	UMAG_10503
Cdc10	UMAG_10644
Cdc11	UMAG_03449
Cdc12	UMAG_03599
Chs8/Mcs1	UMAG_03204
Rax2	UMAG_05194
Atp16	UMAG_01103
Mdh1	UMAG_10276
Tom40	UMAG_00614
F-actin	UMAG_11177
Chs1	UMAG_10718
Chs2	UMAG_04290
Chs3	UMAG_10120
Chs4	UMAG_10117
Chs5	UMAG_10277
Chs6	UMAG_10367
Chs7	UMAG_05480
Chs8/ Mcs1	UMAG_03204
1,3- β -glucan synthase	UMAG_01639
Cts1	UMAG_10419

5.1 Author contributions

Graphical abstract

Designed by Nina Kim Stoffel (NKS) and Prof. Dr. Michael Feldbrügge (MF).

Figure 1

(A–H) Experimental work, data analysis, and visualization were performed by NKS under the guidance of MF.

Figure 2

(A) Graphical visualization was designed by NKS and MF. (B) Autoradiograph and data analysis were performed by NKS under the guidance of Dr. Julian König (JK) (C–D) iCLIP2 experiments were performed by NKS; data analysis was performed by Dr. Srimeenakshi Sankaranarayanan (SM) under the guidance of MF and Prof. Dr. Kathi Zarnack (KZ). (E–F) Genome browser views and graphical figures were created by NKS with input from MF and SM.

Figure 3

(A–D) iCLIP2 experiments were performed by NKS; data analysis was performed by SM under the guidance of MF, JK and KZ.

Figure 4

(A–B) RNA-seq experiments were performed by NKS; data analysis was performed by SM under the guidance of MF, JK and KZ.

Figure 5

(A–C) iCLIP2 analysis was performed by NKS; binding site prediction was carried out by SM; data analysis and visualization were performed by NKS. (D–F) Experimental work, data analysis, and visualization were performed by NKS under the guidance of MF. (G) Experimental work, data analysis, and visualization were performed by NKS under the guidance of MF.

Figure 6

(A) Structural prediction and visualization were performed by NKS with input from Dr. Florian Altegoer and MF. (B) Graphical visualization was designed by NKS, KZ and MF.

Supplementary Figure 1

(A–F) Experimental work, data analysis, and visualization were performed by NKS under the guidance of MF.

Supplementary Figure 2

(A–D) iCLIP2 experiments were performed by NKS; data analysis was performed by SM under the guidance of MF and KZ.

Supplementary Figure 3

(A–B) iCLIP2 experiments were performed by NKS; data analysis was performed by SM under the guidance of MF and KZ.

Supplementary Figure 4

(A–F) iCLIP2 and RNA-seq experiments were performed by NKS; data analysis was performed by SM under the guidance of MF and KZ.

Supplementary Figure 5

(A–F) Experimental work, data analysis, and visualization were performed by NKS under the guidance of MF.

Supplementary Table

SI-Tab. 1–5: Data analysis compiled by NKS with input from MF.

SI-Tab. 6–8: Data analysis compiled by SM with input from MF.

Due to the large amount of data, Supplementary Tables 5–8 are provided separately.

Literature research:

NKS and MF conducted the literature review.

Writing:

The manuscript was written by NKS, SM and MF, with input from Drs. Florian Altegoer, Kerstin Schipper, Heiner Schaal, and all co-authors.

6 The RNA-binding capacity of the multi-RRM protein Rrm4 shapes the endosomal cargo proteome

The RNA-binding capacity of the multi-RRM protein Rrm4 shapes the endosomal cargo proteome

Nina Kim Stoffel*, Thomas Lenz*, Miriam Bäumers, Julian König, Florian Altegoer, Kai Stühler and Michael Feldbrügge, (unpublished)

The RNA-binding capacity of the multi-RRM protein Rrm4 shapes the endosomal cargo proteome

Nina Kim Stoffel,^{1,*} Thomas Lenz,^{2,*} Miriam Bäumers,³
Julian König,⁴ Florian Altegoer,¹ Kai Stühler²
and Michael Feldbrügge^{1,†}

¹Institute of Microbiology, Heinrich Heine University, Düsseldorf, 40204, Germany, ²Molecular Proteomics Laboratory, Bio-Medical Research Center (BMFZ), Heinrich Heine University, Düsseldorf, 40204, Germany, ³Center for Advanced Imaging (CAi), Heinrich Heine University, Düsseldorf, 40204, Germany and ⁴Institute of Biochemistry and RNA Biology, Theodor-Boveri-Institut for Biosciences, Julius Maximilians University Würzburg, Würzburg, 97074, Germany

*Shared first authors †Corresponding author

ABSTRACT

Spatial and temporal control of mRNA transport and translation is fundamental to cellular organization. In hyphae of the plant-pathogenic fungus *Ustilago maydis*, early endosomes act as mobile platforms for long-distance, translationally active mRNA transport, a process essential for polar growth. The RNA-binding protein Rrm4, which harbors three RNA recognition motifs (RRMs), orchestrates mRNA cargo selection and localized translation, as exemplified by septin mRNAs. However, its broader role in shaping the endosome-associated proteome through its RNA-binding capacity remains unresolved. Here, we establish a robust immunoprecipitation–mass spectrometry (endo-IP MS) workflow to isolate early endosomes and define their proteomes across distinct Rrm4 backgrounds, including deletion and RRM-specific mutants. By integrating comparative endo-IP MS with transcriptome-wide, RRM-resolved RNA interactome profiling (iCLIP2), we provide a multidimensional view of Rrm4-mediated transport. Our data reveal that RRM1 and RRM2 predominantly govern mRNA transport and thereby shape the associated protein cargo, while RRM3 contributes auxiliary functions. This integrative approach delineates distinct Rrm4-dependent mRNA regulons encoding key factors required for polarized growth and mitochondrial function. Moreover, our multi-omics analysis, supported by interspecies comparisons, uncovers a conserved yet previously unrecognized transport regulon comprising proteasome core components, linking endosomal hitchhiking to subcellular proteolysis. Together, these findings establish a mechanistic framework for domain-specific mRNA transport and endosomal cargo organization, revealing conserved trafficking regulons from fungi to neurons.

KEYWORDS: endosomal RNA transport, endosome-associated proteome, local proteolysis, local translation, membrane trafficking, mRNA transport, RRM

INTRODUCTION

The transport, localization, and subcellular translation of mRNAs are fundamental post-transcriptional regulatory mechanisms conserved across all kingdoms of life—from microbes and plants to mammals [1, 2, 3]. A striking example of spatial and temporal gene regulation is the hitchhiking of messenger ribonucleoprotein (mRNP) complexes on vesicular structures, such as lysosomes and endosomes, enabling long-distance bidirectional transport along microtubules via motor proteins [1, 4, 5]. In mammals, for instance, nuclear-encoded mitochondrial mRNAs hitchhike over long distances to reach mitochondria—a process critical for neuronal function [6, 7, 8]. Furthermore,

endosomes serve as platforms for localized translation of mitochondrial-targeted mRNAs near their organelle destinations [5, 6, 9, 10]. Beyond mitochondrial transcripts, cytoskeleton-related mRNAs have also been found on early endosomes in a translation-dependent manner [11], expanding the range of cargo targets.

Aberrant endosomal mRNA and/or protein transport have been implicated in neurological disorders, including Alzheimer’s disease, amyotrophic lateral sclerosis (ALS), and Charcot-Marie-Tooth disease [6, 12, 13]. However, despite these links, surprisingly little is known about how mRNA transport and local translation shape the endosomal proteome composition or whether conserved regulatory

cargo regulons exist.

In addition to neurons, endosomal mRNA transport also orchestrates cellular organization in the highly polarized hyphae of the fungal pathogen *Ustilago maydis*, where the hitchhiking mechanism was originally discovered and mechanistically elucidated [1, 3, 4, 14]. It features a well-defined mRNP complex (Müntjes et al., in preparation), characterized cargo mRNAs [15, 16], and established anchoring systems [17, 18]. Central to endosomal RNA trafficking is the RBP Rrm4, which directs the localization of mRNAs and associated ribosomes on the surface of early endosomes [19, 20]. Rrm4 harbors an ELAV-like domain architecture composed of three RNA recognition motifs (RRMs): two tandem N-terminal RRM1 and RRM2 followed by a more distantly spaced third RRM3—all required for RNA binding (SI-Fig. 1A) [4, 15, 21]. Additionally, Rrm4 contains three Mademoiselle domains (MLLE), critical for stable endosomal attachment [17, 18]. Functionally, Rrm4-mediated endosomal mRNA hitchhiking represents a translation-active transport, enabling the localized synthesis of septin proteins during transit, which are essential for higher-order septin assembly [19, 22]. This process is crucial for establishing a septin gradient extending from the hyphal tip, which in turn ensures precise polar growth [14, 19, 22]. Beyond septin mRNAs, Rrm4 binds distinct mRNA regulons encoding key regulators of polarity, cell wall plasticity, and mitochondrial function [15, 23]. Comparative individual-nucleotide resolution UV crosslinking and immunoprecipitation 2 (iCLIP2) analyses using RRM-specific Rrm4 mutants have revealed two modular RNA-binding modes: RRM12-binding sites (RRM12-BS), primarily regulated by RRM1 and/or RRM2, and RRM3-binding sites (RRM3-BS), controlled by RRM3 [23, 24]. While RRM12-BS are essential for mRNA transport and abundance, RRM3-BS appear to have accessory roles. This aligns with phenotypic data: mutants lacking tandem-RRM function exhibit polarity defects, while RRM3 mutants maintain wild-type-like polar growth [21, 25].

Despite these insights, several fundamental questions remain unanswered: How does Rrm4 shape the endosomal proteome? Does it regulate endosomal cargo via RNA binding, and if so, in an RRM-specific manner? Is there additional evidence for local translation on endosomes, and what role does Rrm4 play in this process? Finally, are conserved pools of mRNAs and proteins consistently associated with endosomes across diverse organisms?

RESULTS

Establishment of a robust endosomal immunopurification (endo-IP) protocol preserving protein and RNA integrity in *U. maydis*

Unraveling Rrm4's role in shaping the endosomal proteome requires a precise strategy to isolate early endosomes while preserving protein, lipid, and RNA integrity. To achieve

this, we established a rapid and robust early endosome purification method for *U. maydis* (endo-IP, Stoffel et al., in preparation), adapting immunoprecipitation strategies from mammalian systems [6, 11, 12, 26]. To this end, we evaluated potential marker proteins by assessing the shuttling behavior and subcellular localization of the t-SNARE Yup1-Gfp (Yup1-G) and the small GTPase Rab5a-mKate2 (Rab5a-K) using live-cell fluorescence imaging [18, 27, 28]. While Yup1-G exhibited partially static vacuolar localization (SI-Fig. 1B) [29], Rab5a-K displayed pronounced dynamic movement and precise association with endosomes (Fig. 1A) [27]. Rab5a is stably anchored to the endosomal lipid bilayer via its C-terminal domain, rendering it a particularly suitable bait for endosomal immunoprecipitation (Fig. 1A) [27, 28]. To ensure high accessibility for immunoprecipitation, we generated a constitutively expressed, N-terminally tagged mKate2-Rab5a construct (Rab5a-K) in strains expressing either C-terminally Gfp-tagged Rrm4 (Rrm4-G) under its native promoter or in an Rrm4-deletion background (Rrm4-G/Rab5a-K and *rrm4Δ*/Rab5a-K, respectively; S-Table 1–2, Stoffel et al., in preparation) [21, 27, 30]. These background strains have previously been employed in comparative transcriptomic, iCLIP2, and co-immunoprecipitation (co-IP) studies, ensuring full compatibility with existing datasets [15, 23, 31]. Hyphae induced for 6 hours post-induction (h.p.i.; see Materials and Methods), either expressing Rrm4-G or lacking Rrm4 (*rrm4Δ*), displayed comparable Rab5a-K protein levels and robust endosomal motility (Fig. 1A; SI-Fig. 1C, Stoffel et al., in preparation) [19]. These results validate Rab5a-K as a reliable, Rrm4-independent bait for endosomal immunoprecipitation (Fig. 1A-B).

Given the high RNase and protease activity in fungal extracts [23, 31] and the need to disrupt the rigid fungal cell wall without excessive mechanical damage, we implemented rigorous optimization procedures to preserve RNA, protein, and lipid integrity (Fig. 1B; SI-Fig. 1D-I). To this end, we systematically evaluated mechanical disruption methods (wet vs. cryo-dry bead milling; SI-Fig. 1D), extraction buffer compositions (SI-Fig. 1E-F) [6, 11], and additional parameters such as the use of RNase inhibitors and defined incubation times (SI-Fig. 1G). The adapted *U. maydis* endo-IP approach led to robust enrichment of Rab5a-K and enabled co-immunoprecipitation of the RNA-binding proteins Rrm4 and Poly(A)-binding protein (Pab1), the latter serving as a proxy for bulk mRNA abundance (Fig. 1D, SI-Fig. 1H) [15, 19, 32]. This is consistent with live-cell imaging data demonstrating Rrm4-dependent shuttling of Pab1 on Rab5a-positive endosomes [18, 19, 27]. Simultaneously, cytoplasmic contaminants such as Gapdh and Actin were efficiently depleted compared to input extracts (Fig. 1D, SI-Fig. 1H, Stoffel et al., in preparation). Additionally, preservation of endosome integrity during immunoprecipitation was confirmed via transmission electron microscopy (TEM), which revealed

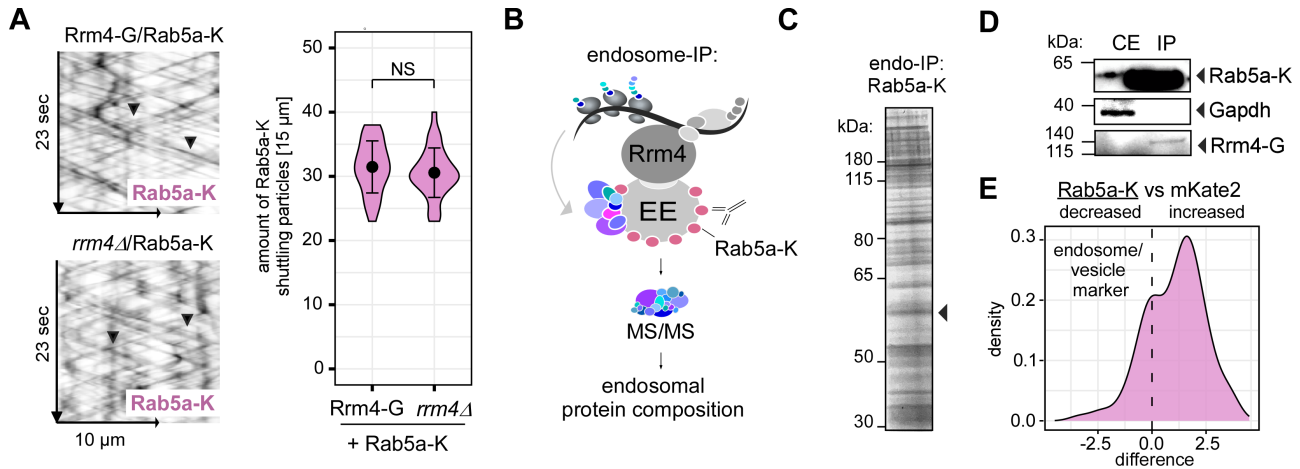


Fig. 1. Establishment of endosome immunoprecipitation followed by mass spectrometry (endo-IP MS) in *U. maydis*

(A) Kymographs illustrating the dynamics of Rab5a N-terminally tagged with mKate2 (Rab5a-K) in different Rrm4 backgrounds, specifically Rrm4-G and *rrm4Δ* *U. maydis* strains at 6 hours post-induction (h.p.i.). Accompanying violin plots quantify the number of Rab5a-K shuttling events (three biological replicates; total cells analyzed > 24). Statistical significance was determined by unpaired two-tailed t-test. (B) Schematic representation of early endosome immunoprecipitation (endo-IP) using Rab5a-K to isolate early endosomes and cargo proteins. (C) Silver-stained gel of endo-IP fraction showing Rab5a-K (indicated by an arrow) and co-immunoprecipitated proteins. (D) Western blot analysis of cell extracts (CE) and endo-IP fractions (IP), demonstrating Rab5a-K and Rrm4-G enrichment alongside the depletion of Gapdh in endo-IP samples. (E) Density plot of vesicle/endosome-associated proteins in Rab5a-K versus free mKate2 fractions ($n=4$; Stoffel et al., *in preparation*). The x-axis represents the *difference* in $\log_2$ intensity values between Rab5a-K and mKate2 samples.

intact vesicle structures (SI-Fig. 1I, Stoffel et al., *in preparation*). To gain a global view of the endosome-associated proteome, we performed mass spectrometry (MS) on at least four biological replicates of endo-IP (Rab5a-K/Rrm4-G) and control samples expressing free mKate2 (Fig. 1B,E, Stoffel et al., *in preparation*). Differential protein abundance analysis based on $\log_2$ -transformed intensity values revealed clear enrichment of endosomal and vesicle-associated markers such as Vps27 and Vps9 [33, 34], validating the successful implementation of an endosomal purification protocol via Rab5a-K immunoprecipitation in *U. maydis* (Fig. 1B–E; Stoffel et al., *in preparation*; S-Tab. 4). Beyond vesicle/endosomal markers (Fig. 1E), the key mRNA transporter Rrm4-G was among the most strongly enriched proteins and was absent from mKate2 MS control samples (Fig. 1D–E, SI-Tab. 4, Stoffel et al., *in preparation*). This observation aligns with prior live-cell imaging data showing that Rrm4 localizes specifically to early endosomes [15, 16, 19, 21].

In summary, we adapted an endosomal immunoprecipitation coupled with mass spectrometry (endo-IP MS) approach to enable detailed analysis of the endosome-associated proteome.

Endosomal cargo composition is defined by mRNA binding through Rrm4's tandem-RRMs

Given that endosomal mRNA transport and local translation are tightly coupled processes conserved from fungi to neurons [3, 4, 6, 19], it is likely that mRNA hitchhiking directly influences the composition of the endosomal cargo proteome. To gain deeper insight into how the endosome-associated proteome is shaped by the RNA-binding capacity of Rrm4 [21], we extended our endo-IP MS approach to include Rrm4 deletion (*rrm4Δ*) and various RRM mutant backgrounds, each carrying a specific amino acid substitution within the RNP1 motif of the respective domain (Fig. 2A–B, SI-Fig. 1C, 2A; 6 h.p.i.; $n=4$) [21].

The comparative analyses revealed that *rrm4Δ*/Rab5a-K, mR1-G/Rab5a-K, and mR2-G/Rab5a-K exhibited pronounced proteomic shifts relative to Rrm4-G/Rab5a-K, whereas mR3-G/Rab5a-K remained largely unaffected (Fig. 2B). Consistently, linear regression analysis demonstrated reproducible patterns in RRM-dependent endo-IP MS profiling (SI-Fig. 2A). These results align with previous findings on the functional contributions of individual RRM domains to polar growth and mRNA transport, which indicated that RRM3 serves an accessory role, whereas RRM1 and RRM2 function as the principal domains [21, 25].

4 • The RNA-binding capacity of the multi-RRM protein Rrm4 shapes the endosomal cargo proteome

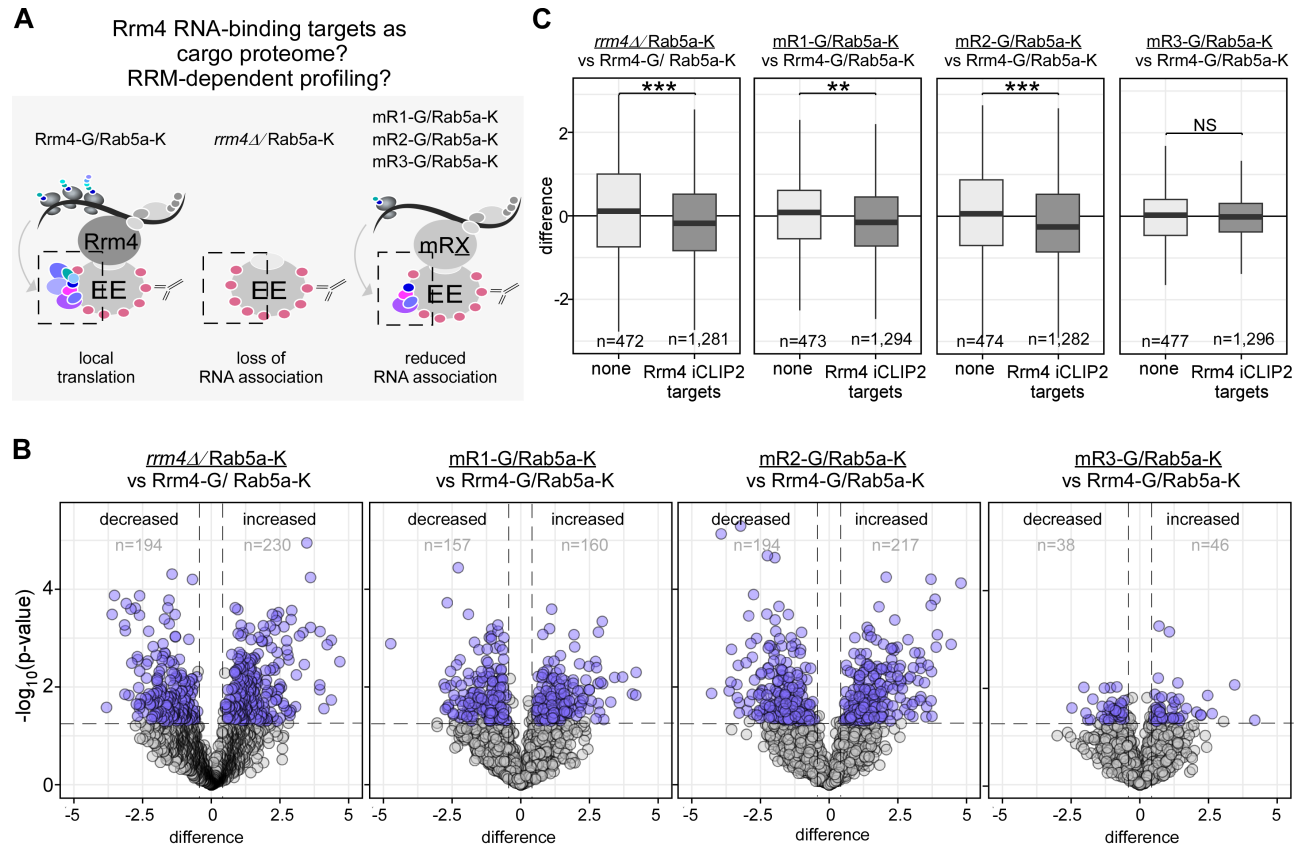


Fig. 2. Tandem-RRMs of Rrm4 regulate endosomal-associated proteome

(A) Schematic illustration of comparative endo-IP approach across Rrm4 background variants (*rrm4*Δ, mR1-G, mR2-G, and mR3-G) indicating how the different mutants potentially shape the endosomal protein composition. (B) Volcano plots of comparative endo-IP analyses (four biological replicates, IP via Rab5a-K) of various Rrm4 mutant background strains (*rrm4*Δ, mR1-G, mR2-G, and mR3-G) relative to Rrm4-G. (C) Boxplots of comparative endo-IP analyses in various Rrm4 mutant strains (*rrm4*Δ, mR1-G, mR2-G, and mR3-G) relative to Rrm4-G, focusing on the behavior of Rrm4 RNA-binding targets (dark grey, $n > 400$) versus non-target proteins (grey, $n > 1,200$). Two-tailed Wilcoxon test with Bonferroni correction was used for statistical analysis. Asterisks indicate statistically significant differences (* p -val < 0.05 , ** p -val < 0.01 , *** p -val < 0.001).

This suggests that the RNA-binding capacity of Rrm4 likely shapes the composition of the endosomal proteome. To investigate whether proteins encoded by Rrm4-bound mRNAs are preferentially affected, we performed a global comparison of proteins encoded by Rrm4-targets versus non-target mRNAs (Fig. 2C). Strikingly, proteins encoded by Rrm4-bound transcripts were significantly reduced in *rrm4*Δ/Rab5a-K, while proteins encoded by non-bound mRNAs remained largely unchanged (Fig. 2C, SI-Tab. 5). A similar profile was observed in mR1-G/Rab5a-K and mR2-G/Rab5a-K, whereas mR3-G/Rab5a-K showed no substantial differences (Fig. 2C). These results establish RRM1 and RRM2 as the primary regulators of endosomal cargo composition, with RRM3 contributing a more accessory role. To further delineate RNA-binding specificity, comparative iCLIP2 analyses identified two distinct target

sets: RRM12-BS (regulated by RRM1 and/or RRM2, SI-Fig. 2B) and RRM3-BS (governed by RRM3, SI-Fig. 2B). Indeed, RRM12-BS-containing targets were significantly reduced in *rrm4*Δ/Rab5a-K, mR1-G/Rab5a-K, and mR2-G/Rab5a-K, but remained unaffected in mR3-G/Rab5a-K (SI-Fig. 2B). In contrast, RRM3-BS-containing targets were only altered in *rrm4*Δ/Rab5a-K and mR2-G/Rab5a-K, likely reflecting indirect effects (SI-Fig. 2B). Notably, mR2-G displayed diminished endosomal association *in vivo*, as shown by live-cell imaging [25], and exhibited lower abundance in endo-IP MS data compared to Rrm4-G/Rab5a-K (difference = -0.88), a reduction not observed for mR1-G or mR3-G (S-Tab. 4).

Collectively, these findings establish RRM1 and RRM2 as the key regulators of the Rrm4-dependent endosomal

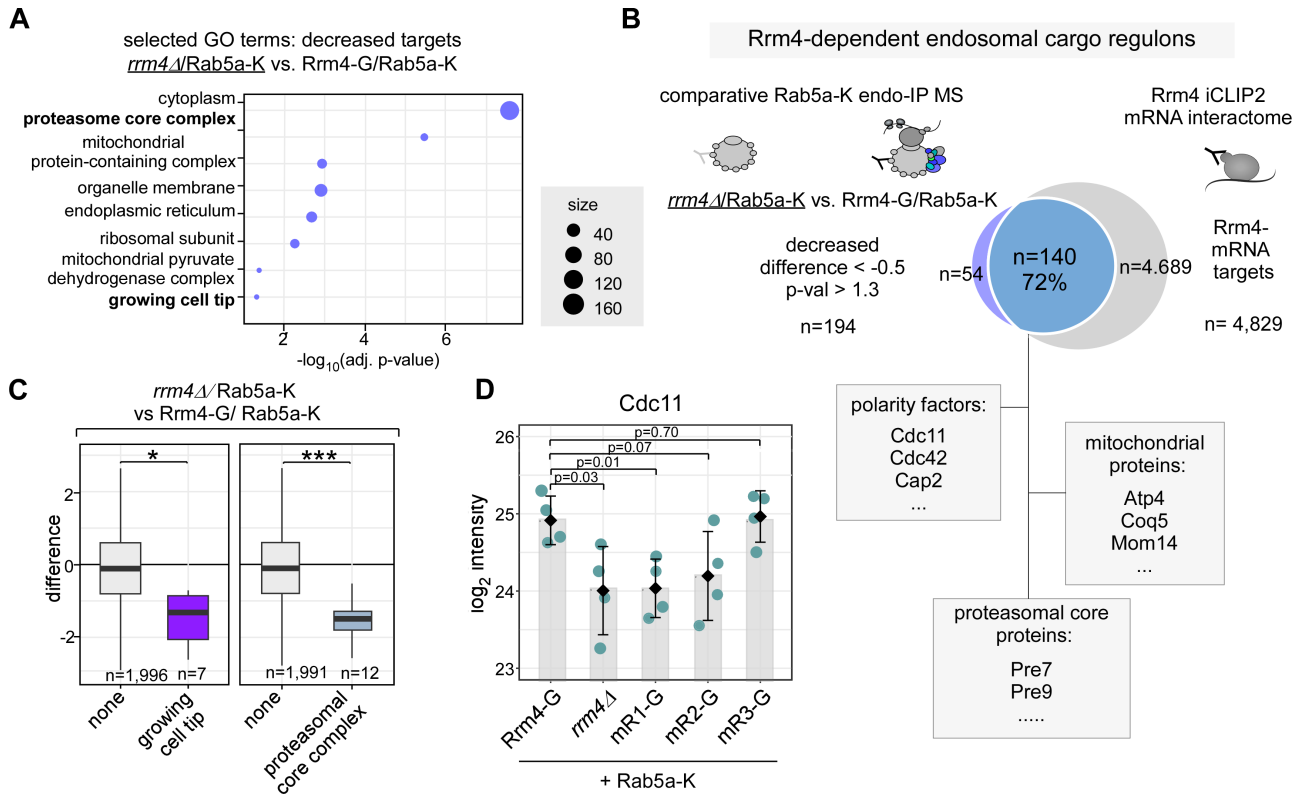


Fig. 3. Endosomal mRNA transport by Rrm4 targets regulons for polarity, mitochondria, and the proteasome

(A) Gene Ontology (GO) term enrichment analysis [35] of proteins significantly reduced in endosomal fractions from *rrm4Δ* compared to *Rrm4-G* background strains (p-value < 0.05). (B) Venn diagram comparing Rrm4 mRNA targets identified by microbial iCLIP2 with proteins depleted in the endosomal proteome (*Rab5a-K/rrm4Δ* versus *Rab5a-K/Rrm4-G*, p-value < 0.05, difference < -0.5; see Fig. 2B). (C) Boxplots showing differential protein abundances between *rrm4Δ* and *Rrm4-G* strains, highlighting GO terms associated with the growing cell tip (purple; 7 of 9 proteins identified) and proteasomal core complex (blue; 12 of 12 proteins identified). (D) Scatter plot displaying $\log_2$ intensity values of the septin protein Cdc11 in endosomal fractions across different Rrm4 mutant backgrounds (*Rrm4-G*, *rrm4Δ*, mR1-G, mR2-G, mR3-G). Statistical significance was assessed using an unpaired two-tailed t-test. Asterisks denote statistically significant differences (*p < 0.05, **p < 0.01, ***p < 0.001).

cargo proteome, particularly by controlling RRM12-BS-containing mRNA targets.

Rrm4 coordinates the endosomal localization of mRNAs and their encoded proteins

Previous live-cell imaging studies established that Rrm4 is essential for the endosomal transport of septin mRNAs and their encoded proteins [14, 19, 22]. To test whether endosomal mRNA localization can be captured using our biochemical approach, we performed endo-IP followed by RNA isolation and RT-PCR (SI-Fig. 3A, see Materials and Methods, Stoffel et al., in preparation). This confirmed the presence of the septin transcripts *cdc10*, *cdc11*, and *cdc12* in endosomal fractions (SI-Fig. 3). Notably, these mRNAs were depleted in endo-IP samples from the triple RRM mutant (mR123-G/*Rab5a-K*) and in

the deletion background (*rrm4Δ/Rab5a-K*, SI-Fig. 3). To extend this analysis beyond septins, we examined additional Rrm4 targets [15, 23]. These included the nuclear-encoded mitochondrial transcripts *atp3* and *atp10*, encoding ATP synthase subunits, and *rep1*, a hyphal tip-localized mRNA encoding the protein Repellent 1, critical for surface hydrophobicity [15, 36, 37]. All tested targets were robustly enriched in endosomal fractions in an Rrm4/RRM-dependent manner, whereas the negative control *tus1*, which is not bound by Rrm4, showed no such enrichment (SI-Fig. 3B–C). These results demonstrate the specificity and robustness of the endo-IP protocol and support the conclusion that Rrm4-bound transcripts are *bona fide* endosomal mRNA cargos (Stoffel et al., in preparation).

Given this, we next asked: What types of proteins are

affected upon loss of Rrm4-mediated mRNA transport? To address this, we performed gene ontology (GO) term analysis on proteins significantly reduced in the *rrm4Δ*/Rab5a-K strain relative to the Rrm4-G/Rab5a-K control (Fig. 3A). This revealed enrichment for proteasomal core subunits, mitochondrial proteins, ribosomal components, and factors implicated in polar growth. These findings are consistent with earlier observations that ribosomes shuttle with early endosomes in an Rrm4-dependent manner [4, 19, 20], and with aberrant polar growth observed when endosomal mRNA transport is disrupted [14, 21]. Conversely, proteins enriched upon Rrm4 deletion were predominantly cytoplasmic or vesicle-associated, likely reflect compensatory responses.

To determine whether the proteins affected in the *rrm4Δ* background are encoded by Rrm4 target mRNAs, we intersected the identified protein pool (n=194) with Rrm4 iCLIP2 targets (Fig. 3B) [15, 23]. Approximately 70% of these proteins are encoded by Rrm4-bound mRNAs, including mitochondrial, proteasomal, and tip-localized proteins (Fig. 3A–B), pointing to a coordinated regulation of endosomal cargo at both the mRNA and protein level. Mirroring findings in mammalian cells, where endosomes serve as platforms for the local translation of nuclear-encoded mitochondrial mRNAs [6, 9], we observed a depletion of mitochondrial membrane proteins in endosomal fractions upon loss of Rrm4 (Fig. 3A, SI-Fig. 4A–B). Moreover, comparison of Rab5a-K/Rrm4-G with cytoplasmic mKate2 control confirmed the selective enrichment of mitochondrial proteins in endo-IP fractions (SI-Fig. 4A–B). In addition to mitochondrial proteins, similar depletion patterns were observed for components involved cell tip growth (e.g., Cdc42) and in proteasome function (e.g., Pre7, Fig. 3C, SI-Fig. 4A). The functional link between endosomal transport and polar growth, particularly involving septins like Cdc11, is well established [14, 19, 22]. As a case in point, quantitative proteomics revealed reduced levels of Cdc11 in *rrm4Δ*/Rab5a-K, mR1-G/Rab5a-K, and mR2-G/Rab5a-K strains, but not in mR3-G/Rab5a-K. This pattern aligns with the presence of RRM12-BS within the *cdc11* transcript and underscores the role of tandem-RRMs in directing Cdc11's localization to endosomes (Fig. 3D). While Rrm4's role in coordinating mRNA transport for polar growth and mitochondrial physiology is well established [4, 15, 19, 22], the identification of proteasomal core subunits as Rrm4-dependent endosomal cargos is unexpected (Fig. 3C). However, these proteins were specifically enriched in Rab5a-K pulldowns compared to mKate2 controls and their mRNAs are direct Rrm4 targets (Fig. 3A–C, SI-Fig. 4A) [15, 23]. This suggests the existence of a previously unrecognized Rrm4-mediated endosomal cargo regulon, potentially involved in spatially restricted proteasome assembly and localized protein turnover.

Rrm4-driven endosomal transport is linked to proteasomes

To further resolve the contribution of individual RRM domains to the endosomal cargo regulons, we performed a refined analysis focusing on selected GO term categories: growing cell tip, transport and localization, and proteasomal core (Fig. 4A–C).

Previous work has shown that motor proteins and endosomal markers are largely unaffected by Rrm4 expression, displaying no discernible differences in their shuttling dynamics between wild-type and *rrm4Δ* strains [18, 19, 27]. Consistently, this group showed no preferential association with specific RRM binding sets (RRM12-BS vs. RRM3-BS). These characteristics designate the transport and localization category as a reliable constitutive reference group. As anticipated, this group remained unchanged across all mutants (Fig. 4A).

As a positive control, we analyzed the growing cell tip category, reflecting the established role of Rrm4 (Fig. 4B) [23]. Comparative iCLIP2 analysis revealed that this group is primarily regulated via RRM12-BS (Fig. 3B). Concordantly, endosomal localization of these proteins in an RRM-dependent manner aligned with both iCLIP2 data and bipolar phenotype (Fig. 3C). These findings underscore the essential role of tandem-RRMs in mediating the subcellular transport of mRNAs and their encoded proteins, exemplified by polarity determinants such as Cdc42.

Beyond established targets, GO term analysis of Rrm4-mediated cargos at both the protein and mRNA levels (Fig. 1E, Fig. 2D–E, SI-Fig. 2A) uncovered an unexpected connection between endosomes and the proteasomal core complex, positioning this group as of particular interest (Fig. 3C). Dissecting this category revealed that the majority of proteasome-related mRNAs are predominantly regulated via RRM12-BS (57 %, Fig. 3C). Protein abundance analysis showed a marked reduction in proteasomal core subunits in all mutants compared to Rrm4-positive endosomes (Fig. 3C–D), with a clear hierarchy: Rrm4-G/Rab5a-K > mR3-G/Rab5a-K > mR2-G/Rab5a-K ≈ mR1-G/Rab5a-K ≈ *rrm4Δ*/Rab5a-K (Fig. 4C–D). This gradient highlights the pivotal role of tandem-RRMs as primary regulators.

Notably, Rrm4 binds to defined sites on mRNAs encoding proteasomal core subunits, including *pre7* and *pre9*, both containing RRM12-BS (Fig. 4E). These binding patterns are consistent with the observed RRM-dependent alterations in endosomal protein abundance (Fig. 4C–D, Fig. 2D–E). Reanalysis of differential RNA-seq data comparing *rrm4Δ* and Rrm4-G strains revealed that mRNA levels encoding proteasomal core components remain largely unchanged (SI Fig. 4C). This likely accounts for their underrepresentation in our previous iCLIP2 study, which focused on RRM12-bound mRNAs exhibiting reduced abundance [25]. Together, these findings reveal a novel link

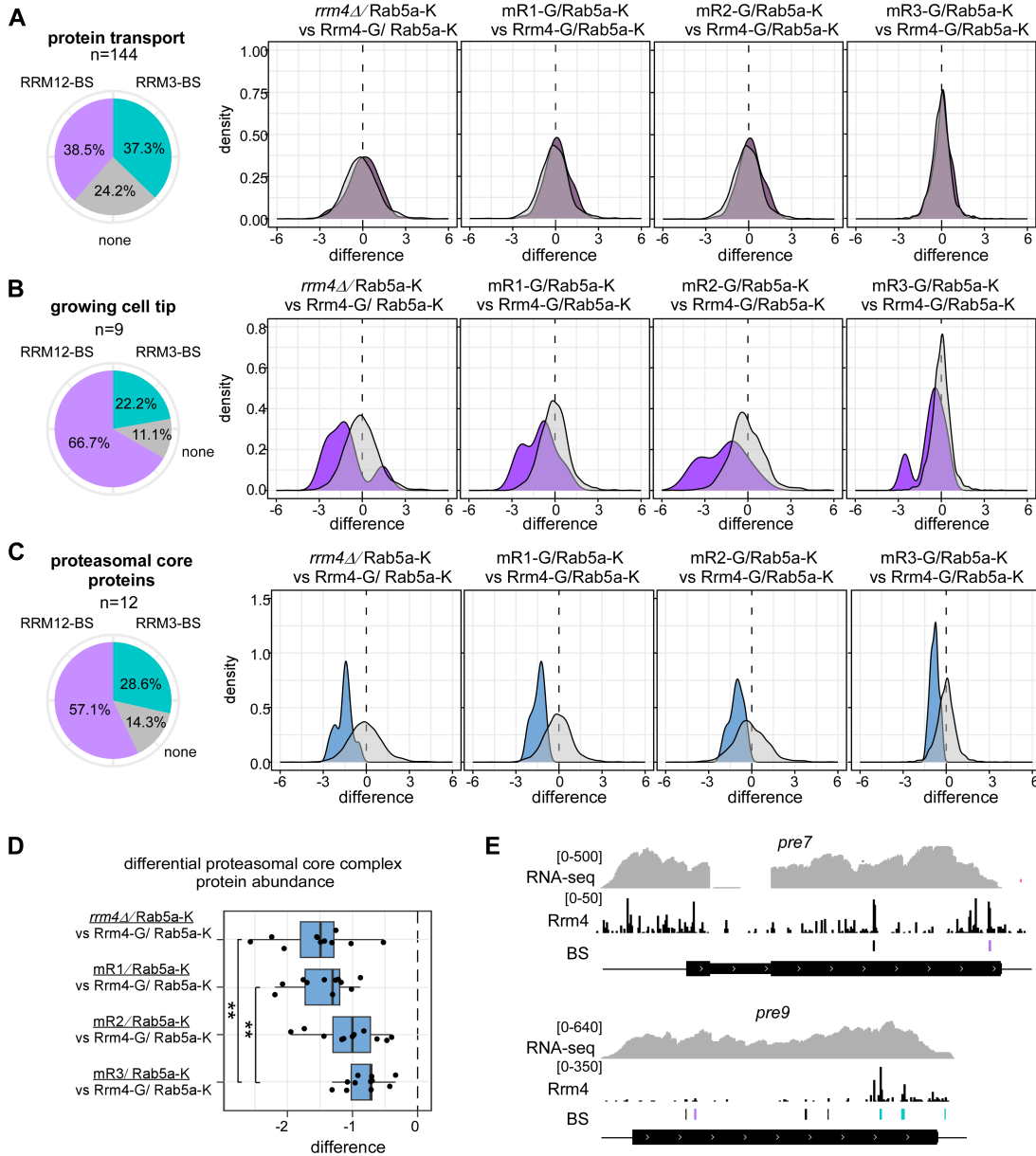


Fig. 4. Rrm4-mediated RNA interactions coordinate endosomal recruitment of cell polarity and proteasomal cargo proteins

(A-C) Pie charts illustrating the distribution of Rrm4 binding modes based on iCLIP2 data (grey=none, purple=RRM12-BS, turquoise=RRM3-BS) alongside density plots depicting protein abundance from comparative endo-IP MS analyses in different Rrm4 mutant backgrounds (*rrm4Δ*/Rab5a-K, *mR1*-G/Rab5a-K, *mR2*-G/Rab5a-K, and *mR3*-G/Rab5a-K relative to Rrm4-G/Rab5a-K). Density plots focus on GO terms related to (A) protein transport (detected in endo-IP MS n=144), (B) growing cell tip (detected in endo-IP MS n=7), and (C) proteasomal core proteins (detected in endo-IP MS n=12). GO terms are color-coded, while control proteins are shown in grey. (D) Comparative scatter plot displaying individual protein abundances of proteasomal core complex components in endo-IP fractions across different Rrm4 backgrounds, illustrating Rrm4-dependent effects on proteasomal protein association. Asterisks indicate statistically significant differences (unpaired Student's t-test, * p-val <0.05, ** p-val <0.01, *** p-val <0.001). (E) Genome browser view of RNA-seq coverage, Rrm4 crosslinking events (iCLIP2), and associated binding sites on proteasomal core target mRNAs *pre7* and *pre9*. Binding site classifications are indicated: black=undefined, purple=RRM12-BS, turquoise=RRM3-BS.

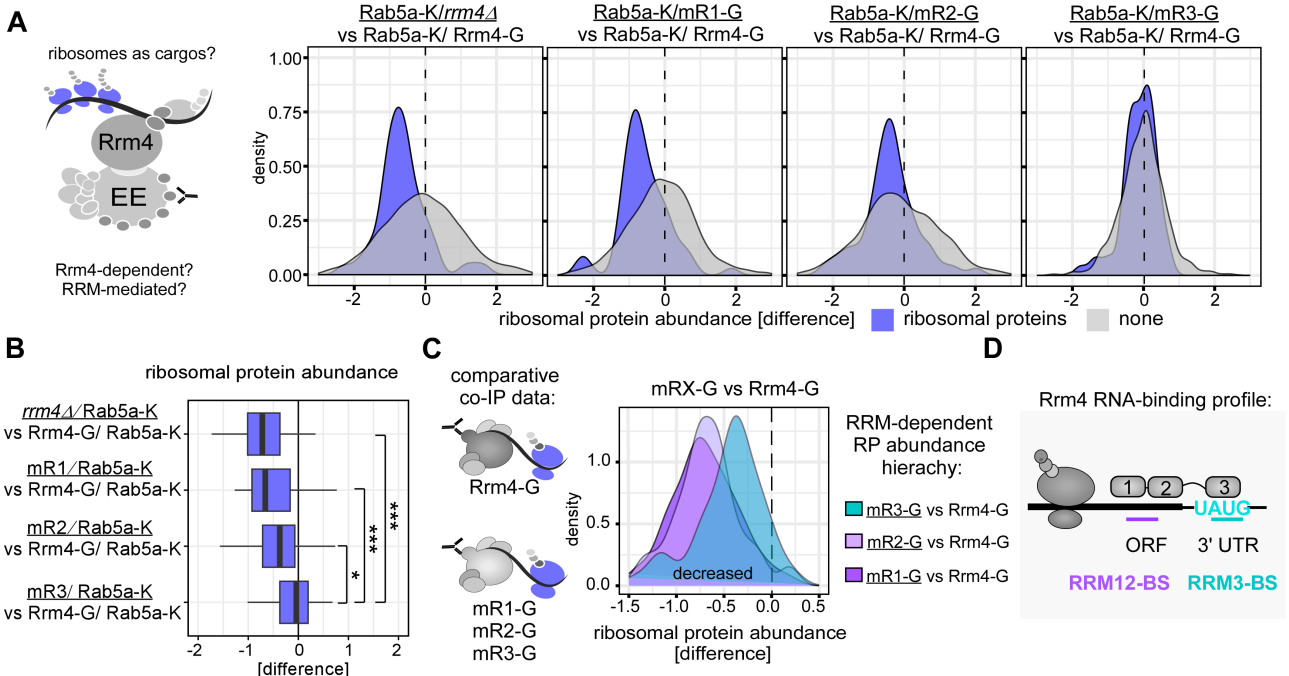


Fig. 5. RRM-mediated mRNA binding controls endosomal ribosome co-localization

(A) Schematic model of endosomal mRNA transport, emphasizing ribosome (blue) association in an mRNA-dependent manner. Density plot comparing the distribution of ribosomal proteins (blue, RPs>60) versus non-RP proteins (grey, n>1,500) from endo-IP fractions (via Rab5a-K) across different Rrm4 mutant backgrounds (*rrm4Δ*, mR1-G, mR2-G, and mR3-G) relative to Rrm4-G. (B) Boxplots comparing ribosomal protein abundance (RPs>60) within different Rrm4 mutant backgrounds (*rrm4Δ*, mR1-G, mR2-G, and mR3-G) relative to Rrm4-G. Two-tailed Wilcoxon test with Bonferroni correction was used for statistical analysis. Asterisks indicate statistically significant differences (* p-val <0.05, ** p-val <0.01, *** p-val <0.001). (C) Schematic overview of the comparative co-IP approach of Rrm4-G and the RRM mutants (mR1-G, mR2-G, mR3-G). Density plot illustrating ribosomal protein abundance from comparative co-IP MS analyses of mR1-G, mR2-G, and mR3-G relative to Rrm4-G, highlighting RRM-specific effects on ribosome association. (D) Illustration of Rrm4 RNA-binding profiles, distinguishing RRM12-BS (purple), primarily bound by RRM1 and RRM2 with contributions from RRM3, and RRM3-BS (petrol), which RRM3 predominantly regulates.

between mRNA transport and the endosomal association of proteasomal core components.

RRM-dependent mRNA binding controls endosomal ribosome association

The endosomal association of both mRNAs (e.g., *cde11*, SI-Fig. 4A–C) and their encoded proteins (e.g., Cdc11, Fig. 3D) in an RRM-dependent manner—together with prior RNA and protein live-cell imaging studies [19, 22]—supports the hypothesis of localized translation on endosomes [3, 4, 6, 9, 11, 38]. Moreover, imaging data indicate that ribosomal proteins associate with endosomes in a manner dependent on both Rrm4 and the translational state [20, 38]. However, only a limited number of ribosomal proteins—such as Rps2-G—have been examined by fluorescence microscopy to date [19, 20, 27].

To gain deeper insight into ribosomal protein (RP) association with endosomes and to determine whether ribosomes are co-transported in an RRM-dependent

manner, RP abundance was examined across the comparative endo-IP MS datasets. Indeed, ribosomal proteins (n>60) were decreased in both the *rrm4Δ* and tandem-RRM mutant backgrounds compared to Rrm4-G (Fig. 5A). Notably, ribosomal protein association with endosomal fractions revealed a clear hierarchical pattern: Rrm4-G/Rab5a-K ≈ mR3-G/Rab5a-K > mR2-G/Rab5a-K > mR1-G/Rab5a-K ≈ *rrm4Δ*/Rab5a-K (Fig. 5A–B). To validate these observations, we reanalyzed data from our recently conducted comprehensive co-immunoprecipitation mass spectrometry (co-IP MS) study of Rrm4-G, mR1-G, mR2-G and mR3-G (Müntjes et al., in preparation; five biological replicates). Consistently, ribosomal proteins showed a stepwise reduction in association, following a comparable order: Rrm4-G > mR3-G > mR2-G > mR1-G (Fig. 5C). These findings establish a functional hierarchy among the Rrm4 domains, identifying the tandem RRM1 and RRM2—as the primary drivers of ribosome localization (Fig. 5D). Collectively, the

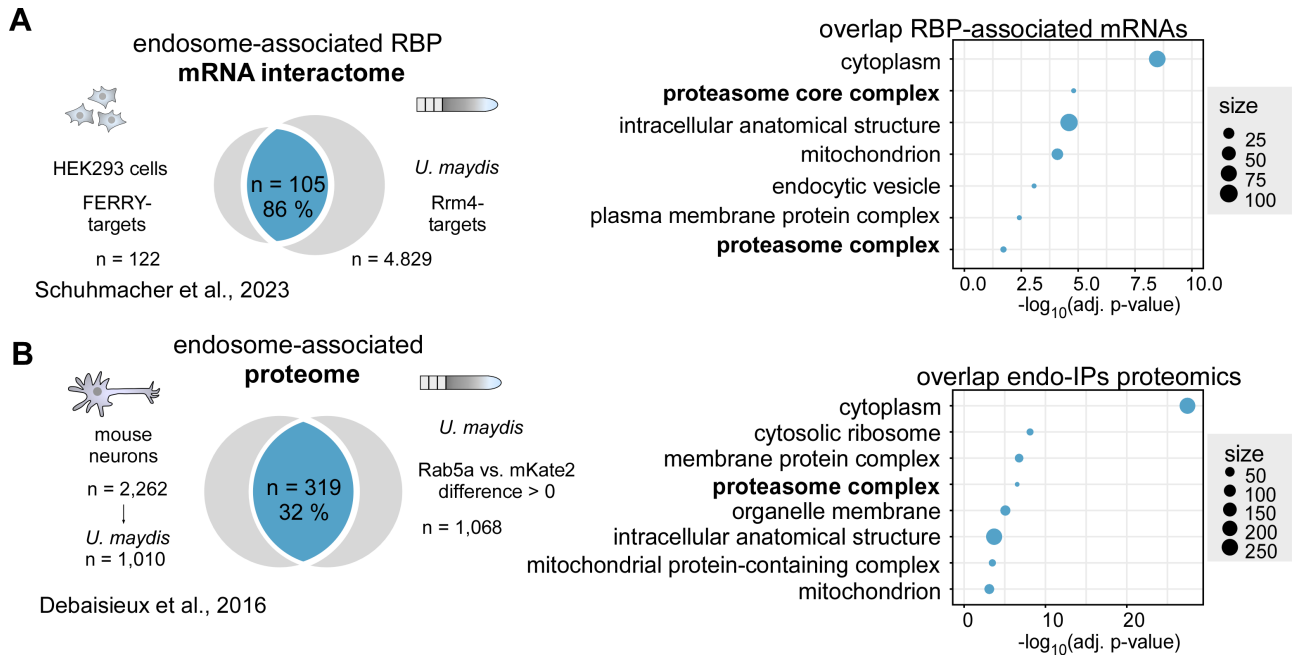


Fig. 6. Interspecies analysis of endosomal cargo components.

(A) Overlap between the mRNA interactome bound by the mammalian FERRY complex [7] and that of *U. maydis* Rrm4, accompanied by GO term analysis of the shared targets. (B) Comparison of endosome-associated proteins identified from purified mouse neuron endosomes [12] (n=2,262; homologs mapped to *U. maydis*, n=1,010) and *U. maydis* hyphal endosomes (Rab5a-K vs mKate2, difference > 0, n=1,068), along with GO term analysis of the overlapping proteins.

data support a model in which Rrm4 facilitates mRNA-dependent recruitment of ribosomes to early endosomes, thereby promoting localized translation at the endosomal surface (Fig. 5A).

Conserved endosomal association of proteasomal, mitochondrial, and polarity regulons

The identification of endosomal cargoes involved in cell polarity, mitochondrial function, and proteasomal activity raises a central question: Are these transport regulons unique to *U. maydis*, or is it conserved across species? To address this, we compared the Rrm4-associated mRNA interactome (Fig. 6A) and the endosome-associated proteome (Fig. 6B) with corresponding datasets from mammalian systems [7, 12].

At the transcriptomic level, intersecting *U. maydis* Rrm4 iCLIP2 targets [15, 23] with mRNAs bound by the mammalian endosomal RNA-binding protein complex FERRY (five-subunit endosomal Rab5 and RNA/ribosome intermediary) [7] identified 105 shared transcripts [25]. These were significantly enriched for GO terms associated with proteasomes, the plasma membrane, endosomes, and mitochondria (Fig. 4A). This overlap indicates that these mRNA regulons are not a fungal-specific feature of Rrm4 but represent an evolutionarily conserved set of endosomal cargo mRNAs (Fig. 6A).

At the proteomic level, mapping *Mus musculus* endosomal proteins (n=2,262) [12] to their *U. maydis* orthologs (n=1,010) and comparing them to our endo-IP dataset (Rrm4-G/Rab5a-K versus mKate2; difference > 0, n=1,068) revealed a conserved subset (n=319; Fig. 6B) enriched for GO terms related to cytoplasmic, ribosomal, membrane-associated, proteasomal, and mitochondrial functions. In addition to canonical polarity regulators (e.g., Cdc42, Rho1, Rac1), this cross-species comparison uncovered a striking conservation of proteasomal core components (e.g. Pre7, Pre9; SI-Tab. 6).

Consistent enrichment of endosome-associated mRNAs and their protein products across fungal and mammalian datasets reveals a conserved molecular organization (Fig. 6), uncovering an evolutionarily preserved link between endosomes and the proteasomal core complex.

DISCUSSION

mRNA transport, localization, and local translation are essential for cellular vitality, with disruptions linked to neurological disorders such as ALS and Charcot-Marie-Tooth disease [1, 3, 6, 10]. In neurons, nuclear-encoded mitochondrial mRNAs hitchhike on endosomes for localized translation near mitochondria [6, 9, 10]. Similarly, in the hyphal pathogen *U. maydis*, septin-encoding mRNAs (e.g.,

cdc11) are recruited onto early endosomes via the multi-RRM RBP Rrm4 and translated in transit—an essential process for polar growth [14, 15, 22]. However, whether mRNA transport shapes the endosomal cargo proteome in a regulon-specific manner, and how this selection is regulated in neurons and fungi, remains unclear.

Modular architecture of the multi-RRM protein Rrm4 shapes endosomal composition

Using a multi-omics approach combining comparative endo-IP proteomics with transcriptomics and iCLIP2, we generated a high-resolution, RRM-dependent map of endosomal cargo composition. As proof of principle, we confirmed that both the septin mRNA *cdc11* and its encoded protein Cdc11 associate with early endosomes in an Rrm4-dependent manner, consistent with previous live-cell imaging data [22].

Expanding to a proteome-wide scale, we identified additional RRM-dependent cargo regulons, including proteins involved in polar growth (e.g., Cdc42), mitochondrial physiology (e.g. Atp4), and proteasomal function (e.g., Pre7). Globally, RRM1 and RRM2 emerged as key regulators of the endosomal cargo proteome, whereas RRM3 contributed only marginally. This correlates with earlier *in vivo* studies showing that tandem-RRM mutants exhibit severe growth defects, aberrant mRNA distribution and abundance, whereas RRM3 mutants remain largely unaffected [21].

Beyond *U. maydis*, the mammalian ELAV-like RNA transporter HuD similarly employs tandem-RRMs for RNA-binding specificity, with RRM3 providing auxiliary affinity enhancement [39, 40]. This points to a conserved modular architecture among ELAV-like RBPs, where tandem-RRMs predominantly define RNA target selection [21, 41, 42]. Together, our findings refine the mechanistic understanding of multi-RBD-containing RBPs, revealing how RRM-dependent target selectivity orchestrates the composition of the endosomal cargo proteome.

Rrm4 as a key coordinator of ribosome-associated mRNP transport

Endosomes as platforms for local translation are conserved from mammals to fungi [4, 6, 11, 19, 38]. In *U. maydis*, fluorescence live-cell imaging has demonstrated the essential role of Rrm4 in recruiting mRNAs and associated ribosomes to early endosomes [4, 19, 20, 22]. While previous microscopy studies primarily focused on individual ribosomal proteins [19, 20], our comparative analysis of endosomal cargo proteomics (endo-IP MS) and Rrm4 protein interactome datasets (co-IP MS, Müntjes et al., in preparation) provides a broader, high-resolution, RRM-dependent profile. These analyses not only reveal an expanded repertoire of ribosomal proteins and translation elongation factors associated with Rrm4 and Rab5a-positive endosomal fractions, but also uncover a clear

RRM-dependent hierarchy. In particular, the tandem-RRMs—especially RRM1—emerged as key regulators of mRNA-mediated ribosome association. These results align with previous studies showing that mutations within the tandem-RRMs, in particular, compromise the RNA-binding capacity of Rrm4, leading to impaired bulk mRNA transport and perinuclear accumulation [15, 21, 25]. In addition, ribosomal proteins were affected in Rrm4 protein interactome studies following RNase treatment (Müntjes et al., in preparation), suggesting that their association is mediated by mRNA.

Although in-depth analysis of the translational profiles of cargo mRNAs during transport lies beyond the scope of this study, future experiments should assess the translational states on endosomes [43]. Combining endo-IP with Ribo-Seq [44, 45] could identify ribosome-associated mRNAs, assess ribosome occupancy and pausing dynamics, and determine translation velocities in dependence on Rrm4 and its individual RRM3 [43]. Additionally, time-course experiments incorporating translational inhibitors (e.g., RocA, puromycin) [20, 46], combined with endo-IP MS, could elucidate how translation modulates the endosomal cargo proteome.

While endo-IP provides high-resolution insight into endosomal cargo composition, it lacks spatiotemporal resolution. Live-cell imaging is therefore essential to visualize endosomal dynamics in real time and assess whether endosomes shuttle, pause, or remain tethered to organelles such as mitochondria, as reported in neurons [5, 6, 9]. Previous studies have shown that translation inhibitors, such as Verticillin A, disrupt ribosome movement with early endosomes in *U. maydis*, indicating that ribosomal association depends on the translational state rather than direct physical transport [20]. Similarly, in mammalian cells, endosomal transport of mRNAs encoding cytoskeleton-associated proteins is translation-dependent [11]. Thus, to further dissect the translational status of endosome-associated ribosomes in *U. maydis*, combining live-cell imaging (e.g., Rps2-Gfp) with puromycin treatment could distinguish actively translating from stalled ribosomes during transport.

Beyond ribosomes, this approach could be applied to cargo proteins (e.g., Pre7-Gfp, Pre9-Gfp, Cdc42-Gfp) to assess whether they undergo local translation on endosomes, similar to septin proteins [14, 19, 22]. Advanced systems such as the SunTag method (SUPERNOVA-tagging system) [47, 48, 49] could offer real-time, target-specific translation readouts; however, attempts to establish this system in *U. maydis* have thus far proven challenging [30]. Thus, the integrative approach of combining endo-IP, Ribo-Seq [43], and RNA/protein live-cell imaging offers a powerful strategy to unravel the translational landscape of cargo mRNAs in a transcriptome-wide and dynamic manner [19, 22, 43, 50]. This will provide critical insights into the mechanistic basis of local translation and its role in subcellular organization [1, 4, 5]. In conclusion, the establishment of endo-IP MS

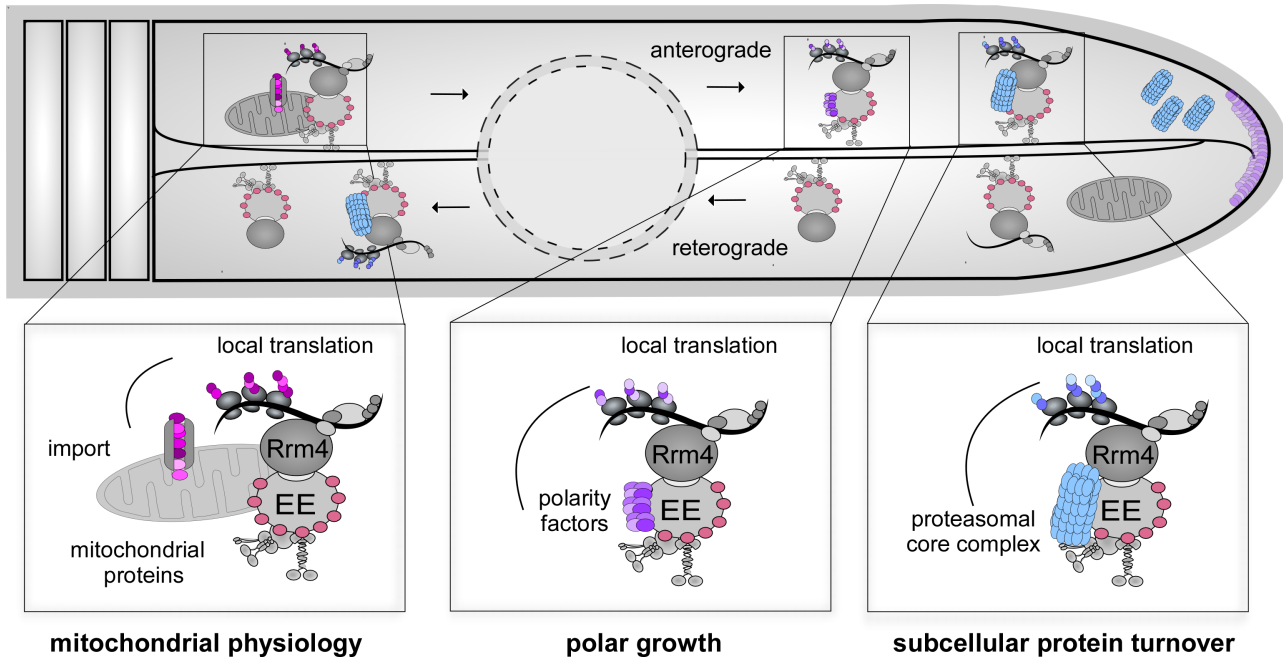


Fig. 7. Endosomes as hubs for distinct mRNA transport regulons in *U. maydis*

Schematic representation of the endosomal mRNA transport model highlighting three distinct transport regulons: Endosomes serve as platforms for the translation of mitochondrial target mRNAs in proximity to mitochondria, enabling localized protein synthesis (left panel). Translational-active endosomal transport of mRNAs decoding polarity factors, essentially for cytoskeletal organization and polar growth (middle panel). A novel model showing endosomal mRNA transport of the proteasomal core complex targets and its local translation, emphasizing the importance of defined subcellular proteolysis in hyphae (right panel).

and RNA-based profiling lays the foundation for future studies aimed at dissecting local translation and mRNP dynamics with unprecedented resolution [1, 2].

From transport to turnover: A new role for endosomal mRNA trafficking

While comparative iCLIP2 and endo-IP analyses, combined with interspecies transcriptome- and proteome-wide comparisons, reinforce the connection between endosomal mRNA transport, cytoskeletal organization [11, 19, 22], and mitochondria [6, 7, 8, 15], these links are already well-established in both fungi and mammals (Fig. 7) [4, 9, 14]. Our study expands this concept by uncovering a previously unrecognized, yet conserved, class of endosomal cargo—proteasome core-related targets—regulated by Rrm4 at both the mRNA and endosome-associated proteome levels (Fig. 7). This discovery reveals an unexpected but potentially functionally coherent regulon under endosomal control. Supporting this idea, an independent study in neurons showed that impaired vesicle-mediated mRNA transport correlates with reduced abundance of mitochondrial and proteasomal transcripts [8]. This is consistent with the notion that aberrant transport mechanisms affect the mRNA abundance of associated

cargo targets [1, 8, 25, 51].

Although live-cell imaging to directly visualize endosomal association of proteasomal components (e.g., Pre7-G) in *U. maydis* was beyond the scope of this study, mechanistic parallels from neuronal systems provide valuable insight. In mammalian cells, proteasomes exhibit microtubule- and motor protein-dependent transport, partly mediated by vesicle hitchhiking mechanisms [52]. The scaffolding protein Ecm29 was shown to tether proteasomes to endosomes and motor proteins [53, 54], providing a framework for how proteasomal subunits could associate with endosomes during intracellular transport (Fig. 7). Given the striking mechanistic parallels between neurons and hyphae in endosomal (mRNA) transport [3, 4], it is plausible that proteasomal proteins in *U. maydis* are similarly tethered to the endosomal surface to facilitate their subcellular localization.

Functionally, the proteasome is essential for maintaining protein homeostasis across eukaryotes [55, 56]. In highly polarized neurons, its precise subcellular localization is critical for synaptic plasticity and renewal [57, 58]. Yet, the regulatory mechanisms underlying its subcellular targeting—and particularly whether mRNA transport plays a key role—remain poorly understood [57, 59]. Thus, our

findings therefore open a new avenue for exploring the molecular link between mRNA transport and subcellular protein turnover, which may represent a conserved strategy critical for maintaining polarity and local proteostasis from filamentous fungi to neurons [56, 57].

MATERIALS AND METHODS

Strains and growth conditions

The laboratory strain *AB33* of *Ustilago maydis* was used as the background strain [60], as it allows the synchronization of filamentous growth by switching the nitrogen source. Pre- and main cultures were grown in complete medium (CM) containing 1% (w/v) glucose at 28°C with constant agitation at 200 rpm until an OD₆₀₀ of 0.5 was reached [60]. For filamentous induction, the cells were shifted to a nitrate minimal medium (NM) supplemented with 1% (w/v) glucose and incubated at 28°C with constant agitation at 200 rpm for 6 hours post-induction (h.p.i) [60].

All *U. maydis* strains used for endo-IP and cell biological analyses are listed in Supplementary Table 1. Plasmids used for strain generation are detailed in Supplementary Table 2.

Microscopy

Cells were grown in 20 mL cultures to an OD₆₀₀ of 0.5, followed by induction of filamentous growth for 6 hours post-induction, as previously described [60]. To quantify the number of shuttling particles (Gfp, mKate2), movies were recorded with an exposure time of 150 ms for a total of 150 frames [17, 61]. Kymographs of Rab5a-mKate2 (Rab5a-K) were generated within a 15 µm section of the hyphal cells starting from the cell tip. Only processive signals that traveled > 5 µm were considered shuttling particles. Each experiment involved three biological replicates and a minimum of > 8 cells per replicate. was used. For statistical analysis, Students-t-Test (unpaired) was applied. Movies, kymographs, and images were processed and analyzed using Metamorph software (Version 7.7.0.0, Molecular Devices, Seattle, IL, USA).

Cell lysis and comparative endo-IP

For the comparative endo-IP experiments, 300 mL filamentous *U. maydis* cultures (OD₆₀₀ = 0.5; 6 h.p.i.) were prepared as described previously. The cultures were split into two 150 mL aliquots and centrifuged at 8,500 rpm for 10 minutes at 4°C. The pellets were resuspended in 50 mL ice-cold PBS, transferred to pre-cooled 50 mL tubes, and centrifuged again under the same conditions. The pellets were resuspended in 10 mL ice-cold PBS, combined, and split into ten 2 mL reaction tubes. After centrifugation at 16,000 rpm for 10 minutes at 4°C, the supernatant was removed, the pellets were frozen in liquid nitrogen, and stored at -70°C.

For mechanical cell disruption [23], frozen cell pellets were mixed with a scoop containing approximately 15 steel

beads (1.5 mm) and then cryo-milled at 2 × 30 Hz, 30 sec, with an intermediate re-freezing step in liquid nitrogen. The pulverized cell material was resuspended in 1 mL endo-IP buffer (2× Complete protease inhibitor EDTA-free [Merck, 11873580001], 1 mM DTT, 1 mM PMSF, 2 µL SUPERase-In [Thermo Fisher AM2696], 3 µL RiboLock [ThermoFisher EO0382], 100 µg/mL cycloheximide, Pierce Protease and Phosphatase Inhibitor [ThermoFisher A32961]) and homogenized at 4°C using a Vibrax. Cell debris were removed by centrifugation at 16,000 ×g for 3 minutes at 4°C, and the lysate was transferred to fresh pre-cooled 50 mL tubes. The lysate was split into five pre-cooled 2 mL tubes and centrifuged for 30 minutes at 5,000 rpm. The supernatant was collected in a pre-cooled 50 mL Falcon tube. The protein concentration of the lysate was measured using a BCA assay (Thermo Fisher, Pierce™ 23225) and adjusted to approximately 2.5-3.0 mg/mL. For immunoprecipitation, 5× 20 µL RFP-magnetic particles (Chromotek, M-270) were pre-washed twice with endo-IP buffer (without inhibitors). 2 mL of the adjusted lysate was added to a pre-cooled 2 mL tube containing RFP-magnetic particles (Chromotek, M-270) and incubated at 4°C for 1 hour under continuous rotation. The magnetic particles were attracted and washed twice with 1 mL endo-IP buffer. The suspension, including particles, was transferred to a fresh, pre-cooled 1.5 mL tube and washed once more. The beads were resuspended in 1 mL endo-IP buffer and stored on ice. 100 µL (10%) of the beads were transferred to a fresh tube, the supernatant was completely removed, and the beads were resuspended in 20 µL 1× NuPAGE LDS buffer, including DTT, according to the manufacturer's instructions (Thermo Fisher, NP0007). The samples were boiled, loaded onto a 4-12% NuPAGE™ Bis-Tris gel (Thermo Fisher, NP0321), and electrophoresed at 180 V for 1 hour. Silver staining (Pierce™ Silver Stain Kit, 24612) was performed according to the manufacturer's instructions.

Liquid Chromatography and Mass Spectrometric Analysis

Liquid chromatography and mass spectrometric analysis were conducted by Dr. Thomas Lenz (Molecular Proteomics Laboratory, BMFZ, Heinrich-Heine University Düsseldorf, Prof. Dr. Kai Stühler).

RNA Isolation and cDNA Synthesis of Endo-IP RNA and Cell Extracts

RNA was isolated as previously described within plant RIP-seq protocol [62] with optimizations tailored for *U. maydis*. For RNA isolation, both the beads and 5% of the cell extract (CE, serving as a positive control) were used as starting material, followed by cDNA synthesis to validate endosome-associated mRNAs.

Samples were mixed with 500 µL TriZol (Invitrogen) and incubated at 50°C with shaking (1,000 rpm) for 5 minutes. Next, 500 µL of RNase-free water was added,

and the mixture was manually shaken for 30 seconds. Subsequently, 400 μ L chloroform/isoamyl alcohol (24:1; BioChemica) was added, and the samples were vigorously shaken for 3 minutes followed by an incubation at room temperature for 3 minutes. The samples were centrifuged at 16,200 g for 10 minutes. The aqueous phase was re-extracted using 250 μ L chloroform/isoamyl alcohol (24:1; BioChemica), and after a second centrifugation step at 16,200 g for 5 minutes at 4°C, the aqueous phases were combined and mixed with 40 μ L 3M sodium acetate (pH 5.2), 1 mL of ice-cold 100% ethanol, and 0.5 μ L of GlycoBlue (Thermo Fisher, AM9516). The samples were incubated overnight at -20°C to facilitate RNA precipitation. The RNA pellet was recovered by centrifugation at 16,200 g for 30 minutes at 4°C, washed twice with 75% ice-cold ethanol, and centrifuged at 16,200 g for 5 minutes at 4°C. The pellet was air dried and resuspended in 8 μ L RNase-free water and either used immediately for cDNA synthesis or stored at -70°C. For quality control, 1 μ L RNA was analyzed using a Bioanalyzer and the Agilent RNA 6000 Pico Kit, following the manufacturer's instructions.

7 μ L RNA was carefully mixed with 3 μ L dsDNase digestion mix (1 μ L 10x DNase buffer, 1.5 μ L dsDNase, and 0.5 μ L RNase-free water; ThermoFisher, EN0771) in a PCR tube and incubated for 10 min at 37°C at 1,000 rpm. Samples were incubated at 65°C for 5 min to inhibit the dsDNase and resolve secondary structures of RNA molecules. Maxima-Kit and random hexamer primers were used for reverse transcription according to the manufacturer's description.

Western Blot Analysis

Protein concentrations of cell lysates were quantified using either the PierceTM BCA assay (Thermo Fisher, 23225) or the standard Bradford assay (Bio-Rad, 1976). To ensure consistency across samples, protein concentrations were adjusted to 1 mg/mL in NuPAGE LDS sample buffer supplemented with dithiothreitol (DTT; Thermo Fisher, NP0007) as per the manufacturer's protocol. Proteins were separated on a 4–12% NuPAGETM Bis-Tris gel (Thermo Fisher, NP0321) at 180 V for 1.1 hours followed by transfer onto a nitrocellulose membrane (Amersham Protran) via wet blotting using 1× NuPAGETM Transfer Buffer supplemented with 15% methanol (Thermo Fisher, NP00061) for 1 hour at 30 V.

Membranes were probed with primary antibodies against RFP (1:2000; Abcam, EPR18992, rabbit), GFP (1:2000 dilution; Roche, 11814460001, mouse), and actin (1:2000; MP Biomedicals, 0869100, mouse). Secondary detection was performed using HRP-conjugated anti-mouse (1:5000; Promega, W4021) or anti-rabbit (1:5000; NEB, 7074S) antibodies. All antibodies were diluted in 1× TBS-T containing 5% milk powder. Detection was carried out using the LAS400 Image Analyzer (GE Healthcare) in combination with ECLTM Prime chemiluminescent reagent

(Amersham, RPN2232), following the manufacturer's instructions.

Bioinformatic Tools and Data Analysis

The prediction of potential protein-interaction networks and GO term analysis was determined by the use of STRING (<https://string-db.org/>, version 12.0, [63]) and g:Profiler [35], considering an adjusted p-value under 0.05 as statistically significant. Additional orthology analyses were performed using the webpage-based g:Profiler [35] and through the respective R package (gprofiler2; [35]).

Data analysis was performed using RStudio (version R 4.4.2). Data visualization and statistical analysis were conducted using the R packages ggplot2, tidyr, readr, dplyr, stringr, purrr, and forcats.

COMPETING INTERESTS

No competing interest is declared.

AUTHOR CONTRIBUTIONS

NKS conceptualized, designed, and validated the study. NKS, in collaboration with KM/MF, generated the *U. maydis*. Comparative endo-IP MS analysis was performed by NKS and TL under the guidance of KST and MF. NKS conducted live-cell imaging and analyzed the data. TEM experiments were carried out by NKS, FA and MB. RNA isolation and other biochemical analyses were performed by NKS under guidance of JK and MF. NKS drafted the manuscript. MF and KST secured funding and provided the research environment.

ACKNOWLEDGMENTS

We sincerely thank Drs. Franziska Kuhnert, Marten Exterkate, Seomun Kwon, and Sainathan Shanmugasundaram for their enthusiastic and supportive discussions regarding endo-IP establishment. In addition, we are particularly grateful for exceptional technical assistance in strain generation provided by Simone Esch and Ute Gengenbacher.

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SUPPLEMENTARY INFORMATION

SI-Fig. 1: Establishment of endosomal immunoprecipitation (endo-IP) in *Ustilago maydis*

SI-Fig. 2: Validation of specificity of endosome-associated targets

SI-Fig. 3: Validation of endosomal immunoprecipitation (endo-IP) in diverse RRM mutant strains

SI-Fig. 4: Differential gene expression of mRNAs encoding proteasomal core components

SI-Fig. 5: Rrm4-dependent mRNA abundance in the endosomal fractions

SI-Table 1: *Ustilago maydis* strains

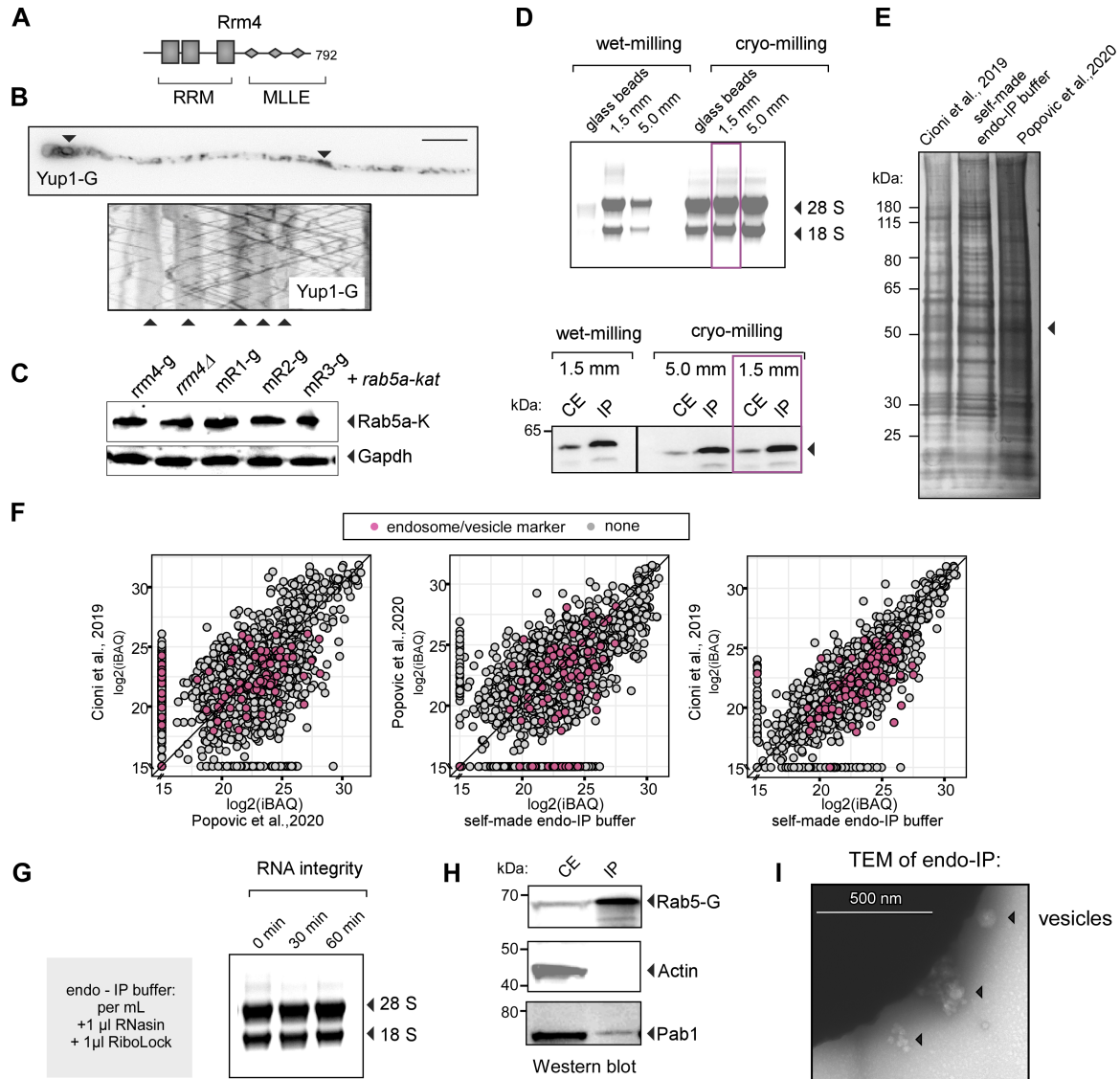
SI-Table 2: Plasmids

SI-Table 3: Primer

SI-Table 4: Comparative endo-IP MS is provided as a separate Excel file due to the large dataset

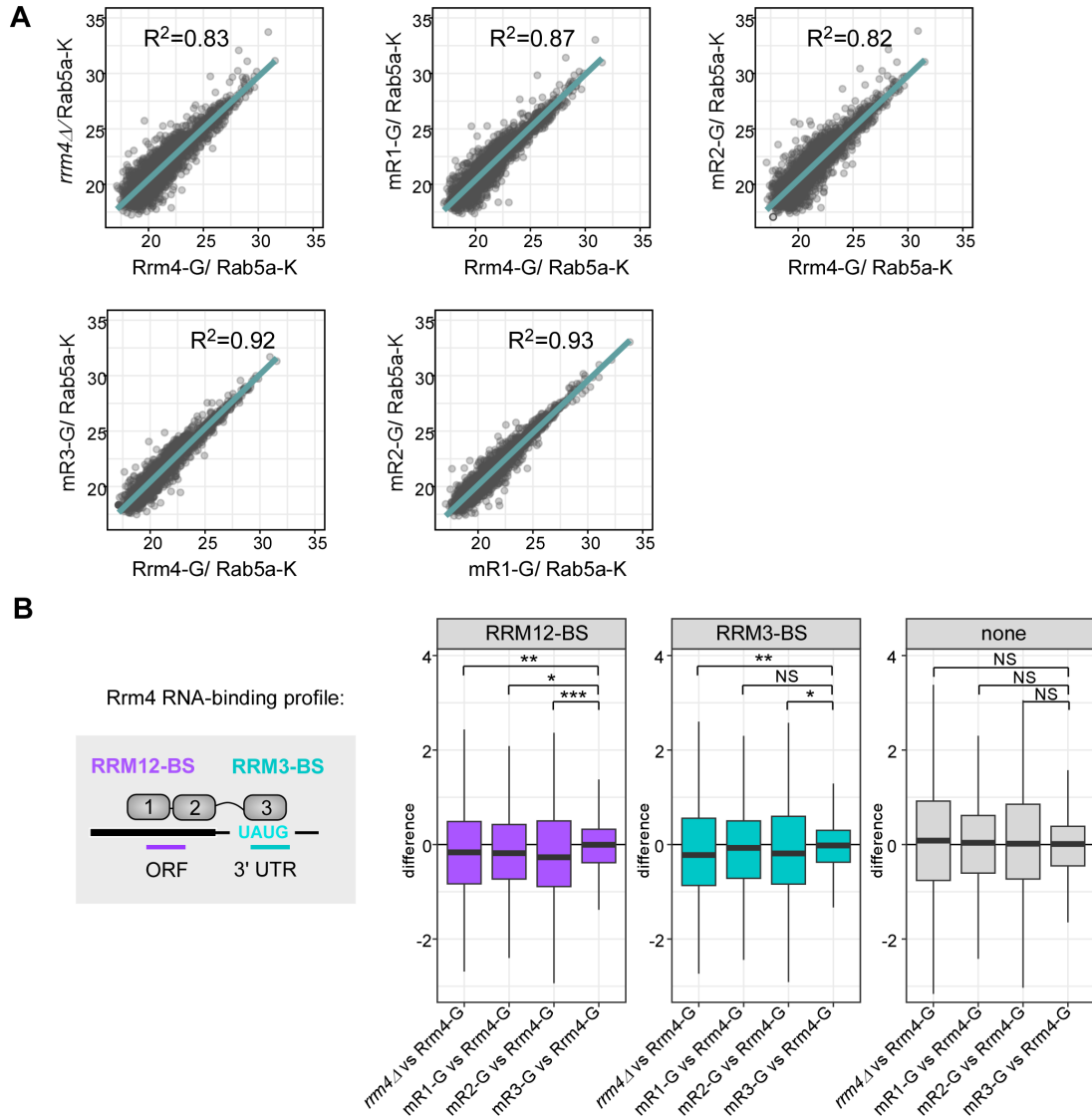
SI-Table 5: Endo-IP MS vs iCLIP2 is provided as a separate Excel file due to the large dataset

SI-Table 6: Ortholog-based neuronal endo-IP vs *U. maydis* endo-IP is provided as a separate Excel file due to the large dataset



SI-Fig. 1. Establishment of endosomal immunoprecipitation (endo-IP) in *Ustilago maydis*

(A) Domain architecture of the RNA-binding protein Rrm4, featuring three RNA recognition motifs (RRMs) and three Mademoiselle (MLLE) domains. (B) Fluorescence image of Yup1-Gfp in *U. maydis* (6 h.p.i.), along with the corresponding kymograph illustrating Yup1-G dynamics. Arrows indicate static fluorescence signals. (C) Western blot analysis of Rab5a-mKate2 across diverse Rrm4 mutant strains, with Gapdh as a loading control. (D) MOPS-formaldehyde RNA gel validating RNA integrity after different cell disruption techniques. Below: Western blot analysis of Rab5a-K in cell extracts (CE) and endo-IP fractions (IP), evaluating protein stability and enrichment across different cell disruption techniques (wet-milling versus cryo-milling; see Materials and Methods) and bead sizes. Frozen cell pellets from the same batch were used as starting material to ensure consistency. (E) Silver-stained SDS-PAGE of endo-IP fractions, testing buffer conditions from Cioni et al. (2019), Popovic et al. (2020), and a self-made endo-IP buffer. An arrow indicates Rab5a-K. (F) MS analysis of endo-IP fractions using different buffers, with endosomal/vesicle markers highlighted in pink. The self-made endo-IP buffer yielded the strongest enrichment for endosomal markers and was used for all subsequent experiments. Samples from the same batch were used as starting material to ensure consistency. (G) Assessment of RNA integrity over time during the endo-IP procedure, ensuring RNA quality remains stable. RNase inhibitors were included in the lysis buffer. (H) Western blot analysis of cell extracts (CE) and endo-IP fractions (IP) showing co-immunoprecipitation of Poly(A)-binding protein (Pab1) with endosomal fractions, while actin serves as a control for the depletion of cytoplasmic proteins. (I) Transmission electron microscopy (TEM) of an endo-IP approach showing vesicles coupled to a magnetic particle.

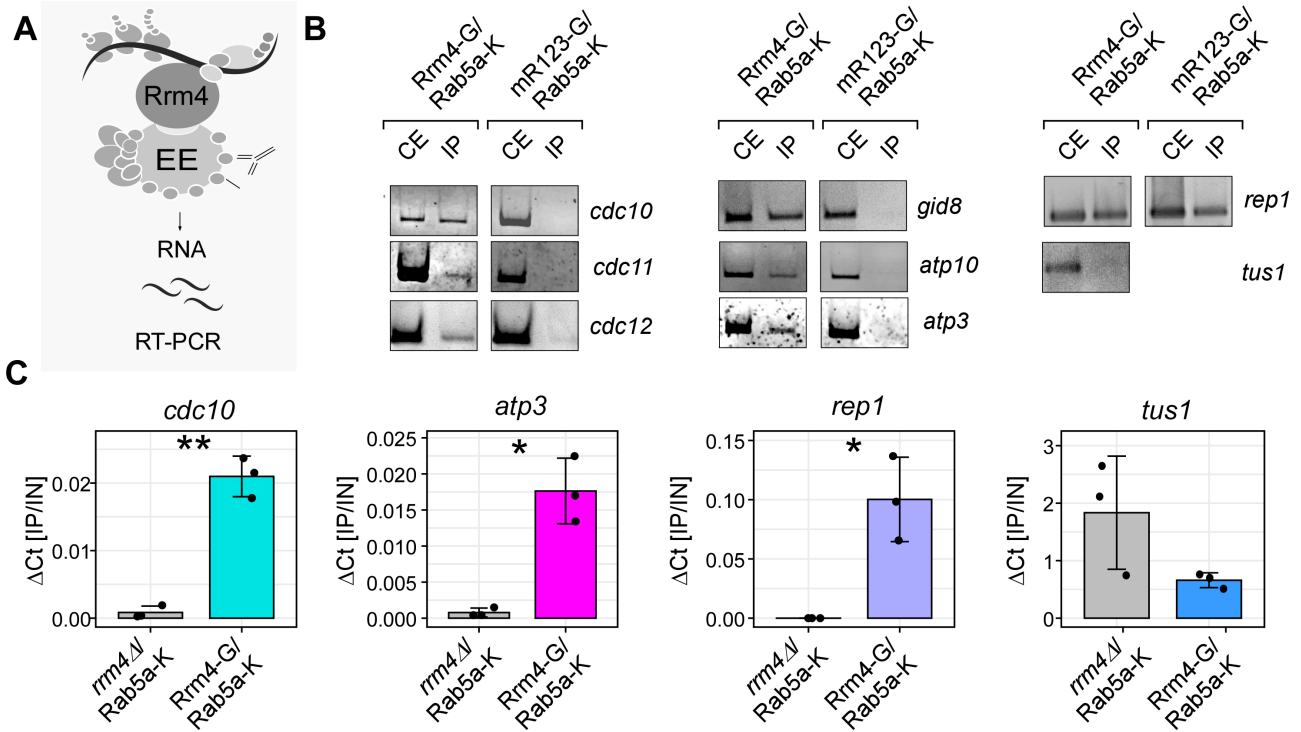


SI-Fig. 2. RRM-dependent endosomal proteome profiling

(A) Reproducibility blots illustrating proteins identified through endo-IP MS log₂(iBAQ) in various Rrm4 background strains (n=4):

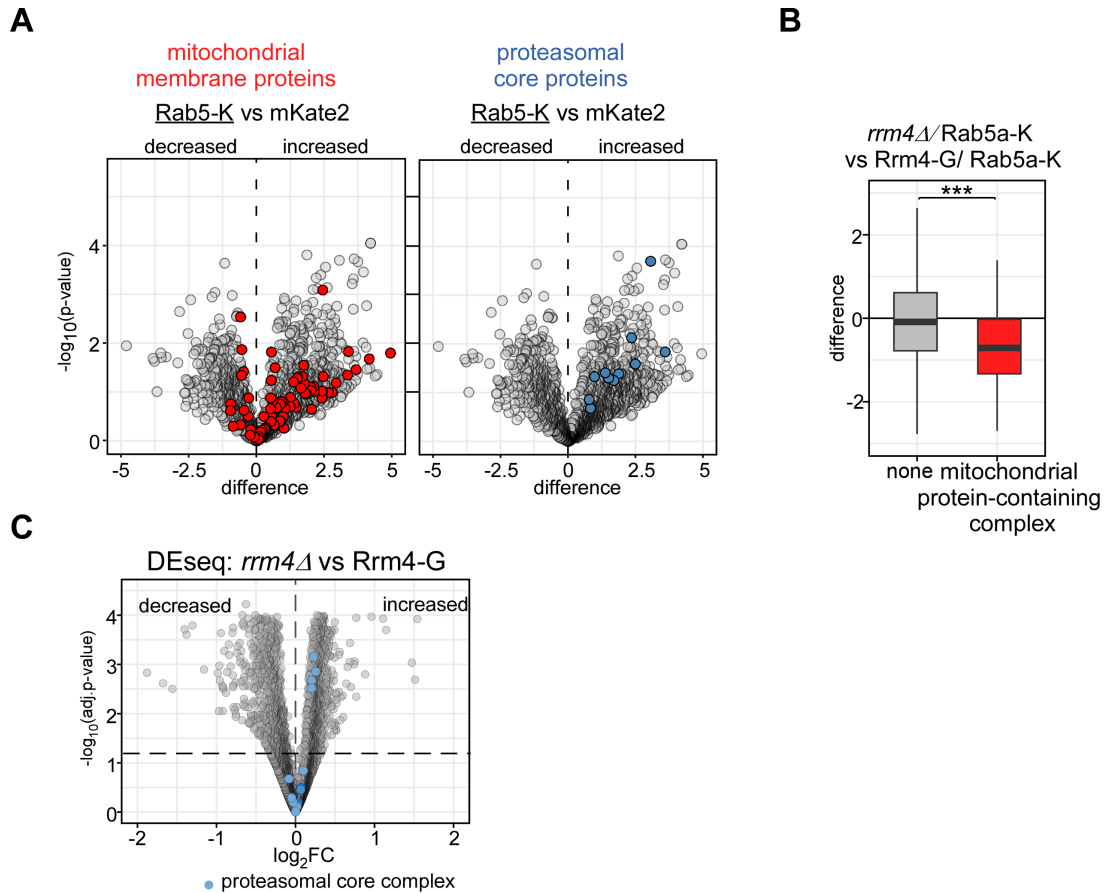
Rrm4-G/Rab5a-K vs *rrm4*Δ/Rab5a-K,
Rrm4 -G/Rab5a-K vs mR1-G/Rab5a-K,
Rrm4-G/Rab5a-K vs mR2-G/Rab5a-K,
Rrm4-G/Rab5a-K vs mR3-G/Rab5a-K,
mR1-G/Rab5a-K vs mR2-G/Rab5a-K.

(B) Rrm4 RNA-binding profile highlighting the two binding sets (RRM12-BS purple; RRM3-BS petrol) and their distribution across mRNA targets. Boxplots comparing RRM12-BS (purple)/ RRM3-BS (petrol) containing targets in endosomal fractions (Rab5a-K purified) of Rrm4 mutant background strains (*rrm4*Δ, mR1-G, mR2-G and mR3-G) relative to Wt Rrm4 strain (Rrm4-G/Rab5a-K). Statistical significance was tested by Dunn's test with Bonferroni correction (* p-val < 0.05, ** p-val < 0.01, *** p-val < 0.001).



SI-Fig. 3. Rrm4 controls mRNA enrichment in Rab5a-positive endosomal fractions

(A) Schematic representation of endo-IP coupled with RNA isolation followed by reverse transcription and PCR (RT-PCR). (B) RT-PCR was performed on endo-IP fractions from Rrm4-G/Rab5a-K and mR123-G/Rab5a-K (an Rrm4 triple mutant with amino acid substitutions in all three RRM). Enrichment of Rrm4-bound targets *cdc10-12*; *atp3*, *atp10*, *gid8* was observed in Rrm4-G/Rab5a-K compared to mR123-G/Rab5a-K endosomal fractions. Although *rep1* was also detected in mR123-G/Rab5a-K fractions due to its high abundance, its endosomal enrichment is strongly Rrm4-dependent (see B). The mRNA *tus1* serves as a negative control. (C) Comparative endo-IP of Rrm4-G/Rab5a-K versus *rrm4Δ*/Rab5a-K strains followed RT-qPCR, normalized to 5/cell extract (IN). The Rrm4-binding targets *cdc10*, *atp3*, and *rep1* were analyzed, while *tus1* served as a negative control. The statistic analysis was performed by using an unpaired, two-tailed student t-test (n=3) (* p-val <0.05, ** p-val <0.01, *** p-val <0.001).



SI-Fig. 4. Validation of mitochondria- and proteasome-related endosomal cargo specificity

(A) Volcano plot comparing Rab5a-K vs mKate2 approaches (n=4). Mitochondrial membrane proteins (red, n=44) and proteasomal core complex targets (blue, n=12) are highlighted (B) Boxplots comparing the differential protein abundances of mitochondrial protein complexes (red, n=44) of endo-IP approaches *rrm4*Δ/Rab5a-K versus Rrm4-G/Rab5a-K. The two-tailed Wilcoxon test with Bonferroni correction was used for statistical analysis. Asterisks indicate statistically significant differences (* p-val < 0.05, ** p-val < 0.01, *** p-val < 0.001). (C) Volcano plot of differential gene expression RNA-seq data comparing *rrm4*Δ vs. Rrm4-G background strains (n > 3). mRNAs encoding proteasomal core complex components are highlighted in blue. .

Table 1. *Ustilago maydis* strains

Name/Identifier	Description	Publication
AB33_rrm4- egfp/rab5a-mKate2 (UL174)	expressing Rrm4 C-terminally fused to eGfp, endogenous promotor and Rab5a N-termianlly fused to mKate2 under the overexpression promotor potef in the ectopic <i>ip</i> locus, using carboxin resistance for selection (plasmid pUMa4237)	this study, UG/SE/NKS
AB33_rrm4Δ/rab5a- mKate2 (UL175)	rrm4 deletion background and Rab5a N-termianlly fused to mKate2 under the overexpression promotor potef in the ectopic <i>ip</i> locus, using carboxin resistance for selection (plasmid pUMa4237)	this study, UG/SE/NKS
AB33_mR1- egfp/rab5a-mKate2 (UL176)	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM1: TVEF (116-119 aa) to AAAA, endogenous promotor and Rab5a N-termianlly fused to mKate2 under the overexpression promotor potef in the ectopic <i>ip</i> locus, using carboxin resistance for selection (plasmid pUMa4237)	this study, UG/SE/NKS
AB33_mR2- egfp/rab5a-mKate2 (UL177)	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM2: VLEF (201-205 aa) to AAAA, endogenous promotor and Rab5a N-termianlly fused to mKate2 under the overexpression promotor potef in the ectopic <i>ip</i> locus, using carboxin resistance for selection (plasmid pUMa4237)	this study, UG/SE/NKS
AB33_mR3- egfp/rab5a-mKate2 (UL178)	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM3: FVSF (362-368 aa) to AAAA, endogenous promotor and Rab5a N-termianlly fused to mKate2 under the overexpression promotor potef in the ectopic <i>ip</i> locus, using carboxin resistance for selection (plasmid pUMa4237)	this study, UG/SE/NKS
AB33_mR123- egfp/rab5a-mKate2 (UL194)	expressing a triple mutation Rrm4 C-terminally fused to eGfp mutation in RRM1: TVEF (116-119 aa) to AAA; in RRM2: VLEF (201-205 aa) to AAAA, in RRM3: FVSF (362-368 aa) to AAAA, endogenous promotor and Rab5a N-termianlly fused to mKate2 under the overexpression promotor potef in the ectopic <i>ip</i> locus, using carboxin resistance for selection (plasmid pUMa4237)	this study, UG/SE/NKS

Table 2. Plasmid

Name/Identifier	Description	Publication
Potef:mKate2_Rab5a_CbxR (pUMa4237)	plasmid vector for generating mutant expressing rab5a N-terminal fused with mKate2 under control of the overexpression promotor potef for insertion in the <i>ip</i> locus	this study, plasmid was generated by UG/SE/KM

Table 3. Primer

Name/Identifier	Sequence (5'→ 3')
EF261_Cdc10_fwd	AAGCCTCACACCGTTTTCCA
EF262_Cdc10_rev	TCGTGGTTCAGTATGACGGC
EF263_Cdc11_fwd	AGGACATTGAGCACTACGGC
EF264_Cdc11_rev	AGTCGTGCGTGATCTCCTTG
EF265_Cdc12_fwd	ACGGATACGCTGCTTGAACA
EF266_Cdc12_rev	CAAGAACAGCAACCCGAACG
EF267_Atp3_fwd	CCTTGGTGACATCGTCCTCC
EF268_Atp3_rev	CTTTGCCCAAGAACCTTGCC
EF269_Atp10_fwd	TAGGTAGCTCTTGAGCGGGT
EF270_Atp10_rev	TGAGCGACAAAGCAAAGTGC
EF277_Rep1_fwd	ATCGATCTCTCGGACCTGCT
EF278_Rep1_rev	CTTAGGAGGAGTGGGCTTGC
EF317_Umag_05399_fwd	AGGAAGCGACTTTGACCTCG
EF318_Umag_05399_rev	GGCAAGAGGGGTTTCGTGATT

6.1 Author contributions

Figure 1

(A–B) Microscopy and data analysis performed by Nina Kim Stoffel (NKS). (C) Graphical visualization designed by NKS. (C–D) Endo-IP performed by NKS. (E) Endo-IP MS reused from (Stoffel et al., in preparation; see Section 8), performed by NKS in collaboration with Dr. Thomas Lenz (TL).

Figure 2

(A) Graphical visualization designed by NKS. (B–C) Comparative endo-IP MS performed by NKS and TL. Data analysis and visualization by NKS.

Figure 3

(A–D) Comparative endo-IP MS performed by NKS and TL. Data analysis and visualization by NKS.

Figure 4

(A–D) Comparative endo-IP MS performed by NKS and TL. Data analysis and visualization by NKS. (E) Genome browser views analyzed by NKS.

Figure 5

(A–C) Comparative endo-IP/co-IP MS performed by NKS and TL. Data analysis and visualization by NKS. (D) Graphical visualization designed by NKS.

Figure 6

(A–B) Data analysis and visualization by NKS.

Figure 7

Graphical visualization designed by NKS.

SI-Figure 1

(A) Graphical visualization designed by NKS. (B) Microscopy data acquired by NKS. (C–E) Biochemical validation and optimization of endo-IP conducted by NKS. (F) Endo-IP MS and data analysis performed by NKS and TL. (G–H) Biochemical validation performed by NKS. (I) TEM microscopy conducted by NKS in collaboration with Miriam Bäumers.

SI-Figure 2

(A–B) Comparative endo-IP MS performed by NKS and TL. Data analysis and visualization by NKS.

SI-Figure 3

(A–B) Comparative endo-IP and RNA abundance analysis performed by NKS.

SI-Figure 4

(A–B) Comparative endo-IP MS performed by NKS and TL. Data analysis and visualization by NKS. (C) Reanalysis of differential RNA-seq data conducted by NKS.

Supplementary Tables:

SI-Tables 1–3: Data analysis compiled by NKS.

SI-Table 4: Data analysis compiled by NKS and TL.

SI-Table 5: Data analysis compiled by NKS.

SI-Table 6: Data analysis compiled by NKS and TL.

Due to the large amount of data, Supplementary Tables 4–6 are provided separately.

Literature Research:

NKS conducted the literature review.

Writing:

The manuscript was written by NKS, with proofreading provided by Drs. Kerstin Schipper and Franziska Kuhnert.

7 Dissecting the RRM-dependent mRNP composition of the endosomal RNA transporter Rrm4

Part of this subsection will likely be published in:

**Dissecting the RRM-dependent protein interactome of the endosomal RNA
transporter Rrm4 uncovers the recruitment of the translation repressor Ssd1**

Kira Müntjes*, **Nina Kim Stoffel***, Markus Tulinski, Johannes Postma, Thomas Lenz, Kai
Stühler, Lasse van Wijlick and Michael Feldbrügge (manuscript submitted to *PNAS*)

and

Upa2 scaffolds higher-order mRNP assembly during endosomal RNA transport

Sainath Shanmugasundaram, **Nina Kim Stoffel** et al., (planned)

Dissecting the RRM-dependent mRNP composition of the endosomal RNA transporter Rrm4

ABSTRACT

Polarized growth and cellular organization depend on precisely regulated mRNA transport and localized translation. In hyphae of the fungal pathogen *Ustilago maydis*, mRNAs are dynamically trafficked on motile endosomes, enabling the spatiotemporal control essential for polarity. This process is orchestrated by the RNA-binding protein (RBP) Rrm4, which contains three RNA recognition motifs (RRMs) that govern mRNA delivery and global transcript distribution. Yet, the protein composition and interaction logic of Rrm4-mediated transport messenger ribonucleoprotein complexes (mRNPs) remain poorly defined. A comparative co-immunoprecipitation mass spectrometry approach—integrating RRM-specific mutants and RNase treatment—enabled systematic dissection of the modular mRNP architecture. This analysis identified a core set of Rrm4-associated factors, including the mRNP scaffold protein Upa2 and the RBPs Ssd1, Rrm71, and Upf1, each exhibiting distinct RRM- and/or RNA-dependent interaction modes. Proteomic data combined with live-cell imaging demonstrated that the conserved translational repressor Ssd1 interacts with Rrm4 in an RNA-independent, but RRM1-dependent, manner. An ortholog-based RNA interactome analysis revealed that Ssd1 and Rrm4 likely co-target a conserved mRNA regulon critical for cell wall plasticity. These findings challenge the prevailing model of constitutive translation during endosomal transport and instead support regulon-specific translational regulation, analogous to mechanisms observed in neurons. Additionally, a direct interaction between the GWW motif of Upa2 and RRM1 of Rrm4 was shown to be essential for Upa2 motility. Thus, RRM1 functions as a central anchoring hub, coordinating both RNA and protein interactions to ensure the stable and translational-regulated endosomal mRNA transport. This integrative approach—combining proteomics, structural modeling, RNA interactome mapping, and live-cell imaging—establishes a robust framework for RRM-centric mRNP profiling and reveals fundamental principles underlying interaction modes and spatiotemporal regulation.

KEYWORDS: mRNA transport, mRNP composition, RBP network, RRM-mediated protein interaction

INTRODUCTION

Post-transcriptional gene regulation relies on highly coordinated RNA–protein interactions, essential for cellular homeostasis and adaptability [1, 2]. While RNA–protein interactions are central to the assembly and function of messenger ribonucleoprotein particles (mRNPs), protein–protein interactions equally shape their structural organization and stability [3, 4, 5].

Advanced proteomic approaches—such as co-immunoprecipitation (co-IP) and proximity labeling combined with mass spectrometry [6, 7]—have been widely employed to decipher RNA-binding protein (RBP) interactomes [8, 9]. Moreover, large-scale analyses involving dozens of RBPs, integrating co-IP MS with RNase treatment, have been successfully applied in *Saccharomyces cerevisiae* and *Homo sapiens* to resolve RBP networks at unprecedented resolution [10, 11]. When complemented by structural modeling, biochemical validation, and phenotypic characterization, these strategies form a powerful framework for dissecting mRNP composition and defining distinct

interaction modes, thereby providing crucial insights into the spatiotemporal regulation of mRNA fate [3, 12, 13].

In the fungal pathogen *Ustilago maydis*, the RBP Rrm4 orchestrates endosomal mRNA transport along microtubule bundles within hyphae (Fig. 1A) [14, 15]. Rrm4 harbours three C-terminal Mademoiselle domains (Fig. 1A, MLE), with MLE3 critically mediating endosomal anchorage via interaction with the PAM2-like motif of the adaptor protein Upa1 [16, 17]. Upa1, in turn, binds directly to early endosomal membranes through its FYVE zinc finger domain [17]. The RNA-binding landscape of Rrm4 is defined by three N-terminal RNA recognition motifs (RRMs), which together shape its mRNA interactome (Fig. 1A) [14]. These interactions enable Rrm4 to mediate the transport and spatial localization of distinct mRNA regulons, particularly those governing cell polarity [18], cell wall plasticity [19, 20], and mitochondrial function [21, 22]. Rrm4 operates within a dynamic mRNP network comprising the glycine-rich RNA-binding protein Grp1 [21], the poly(A)-binding protein Pab1 [18] and, based on emerging evidence, the translational

repressor Ssd1 [23, 24]. The scaffold protein Upa2 further stabilizes transport mRNPs by directly interacting with Pab1 (Fig. 1A) [25]. Moreover, Rrm4 mediates endosome-coupled, translationally active transport, as exemplified by all four septin mRNAs, which are essential for polarity establishment in growing cells [18, 26]. Consistent with this model, ribosomal proteins co-localize with motile endosomes in an Rrm4- and translation-dependent manner [22, 27], reinforcing the mechanistic link between mRNA transport and local translation (Fig. 1A).

Despite these insights, a high-resolution, proteome-wide map of the Rrm4 interactome remains elusive (Fig. 1A). Several key questions remain unresolved: Are additional, yet unidentified, RBPs involved in endosomal mRNA transport? How is Rrm4's association with its interactors regulated? Do cooperative RBP interactions fine-tune mRNA target selection and local translation? How do Rrm4's individual RRMs shape the composition of endosomal mRNPs, and are these interactions direct or RNA-mediated?

More broadly, a systematic dissection of how a multi-RBD protein such as Rrm4 organizes its protein interactome—particularly with respect to RNA dependence and domain-specific direct interactions—remains lacking [28, 29]. Both RNA-binding and non-RNA-associated proteins likely contribute to the spatial and temporal organization of transport mRNPs [5, 12, 25], a process fundamental to cellular architecture and polarized growth [30, 31].

RESULTS

A proteome-wide atlas of the Rrm4 interactome

To map the proteome-wide interactome of Rrm4, a C-terminally Gfp-tagged version of Rrm4 (Rrm4-G, Fig. 1B, SI-Tab. 3) was expressed in *U. maydis* hyphae under native promoter conditions (6 h.p.i.; see Materials and methods) [14, 23]. The Gfp tag enabled specific immunoprecipitation (IP) of Rrm4-G along with its co-associated proteins and RNAs (Fig. 1B) [21, 23]. To control for non-specific protein binding to the beads and/or the Gfp tag, hyphal cells expressing free Gfp were used as a negative control (Fig. 1B) [21, 23]. Five biological co-IP experiments followed by mass spectrometry-based proteomic analyses were conducted (Fig. 1B, SI-Tab. 3). To quantify target protein enrichment in Rrm4-G versus Gfp controls, the difference in average $\log_2$ intensities between Rrm4-G and Gfp samples was used to measure protein abundance changes (*difference*, x-axis in Figure 1B). Statistical significance was determined using a two-tailed paired Student's t-test, with p-values per protein displayed as their negative decadic logarithm ($-\log_{10}$ p-value, y-axis in Figure 1B).

The comparative co-IP MS approach identified robust enrichment of proteins in Rrm4-G samples relative to the free Gfp control (Fig. 1A,B, 177 proteins; difference > 1; $-\log_{10}(\text{p-value}) > 1.3$; paired t-test; SI-Tab. 3). Among the

top candidates were established interactors, including the endosomal anchor Upa1 [17], the mRNP scaffold protein Upa2 [25], and the translational repressor Ssd1 [23, 32]. In addition, novel components (*vide infra*) such as the RBPs Upf1 (an ATP-dependent RNA helicase associated with nonsense-mediated decay, also known as Nam7; Fig. 1B, SI-Fig. 1A) [33] and Rrm71 (RRM-containing protein with unknown function, Fig. 1B, SI-Fig. 1A) were highly enriched. Additionally, α - and β -tubulin (Tub1/2) likely co-immunoprecipitate due to their proximity to Rrm4, reflecting local background from its subcellular localization in microtubule-dependent endosomal mRNA transport [15, 34]. Comparison of previous co-IP MS data performed by Markus Tulinski (283 significantly enriched proteins in Rrm4-G vs. Gfp, SI-Fig. 1A) [23] with this study (177 proteins significantly enriched in Rrm4-G vs Gfp, Fig. 1B, SI-Tab. 3) reveals an overlap of core components (55 proteins, including Upa1, Upa2, Ssd1, and Upf1; SI-Fig. 1A). Despite variations in enrichment levels, particularly among RBPs, the overlap highlights the robustness and reproducibility of both dataset. Moreover, the present study identified novel RNA-associated factors, such as Rrm71, the chaperone Hsp70 [35], and a set of ribosomal proteins (Fig. 1B, SI-Tab. 3), while also achieving stronger enrichment of RNA-associated proteins like Upf1 (SI-Fig. 1A) [23]. These improvements likely result from optimized experimental conditions, including refined mechanical cell disruption and a co-IP buffer specifically formulated to preserve RNA integrity and ribosome associations (see Materials and methods) [36].

To determine the composition and binding modes of Rrm4-associated mRNP complexes (Fig. 1A,B), determining local background proteins was essential for accurately mapping Rrm4-specific interactors (Fig. 1C). To this end, co-IP MS analyses were performed using Upa1-G as bait in an Rrm4-interaction-deficient strain (Upa1-Gfp/mMLE3-mKate2, Fig. 1C,D; five biological replicates) [16]. In this context, Upa1-G remains endosome-associated but excludes the Rrm4-mediated mRNP complex (Fig. 1C,D). Comparative analysis of Rrm4-G versus Upa1-G/mMLE3-K co-IP datasets delineated Rrm4-specific interactors from the local endosomal background (Fig. 1C,D). The interactomes of Rrm4-G and Upa1-G overlapped substantially, reflecting a shared endosomal protein pool, including α - and β -tubulin (Fig. 1C,D). As expected, Rrm4 itself was strongly enriched in Rrm4-G samples relative to Upa1-G, along with the scaffold protein Upa2 [25] and the translational repressor Ssd1 [23, 37, 38], consistent with prior live-cell imaging showing Rrm4-dependent shuttling of Ssd1 and Upa2 [23, 25]. The novel RBPs Upf1 and Rrm71 exhibited marked enrichment, supporting their specific association with Rrm4 rather than general endosomal background (Fig. 1D,E). Additionally, the majority of ribosomal proteins ($n=55$; Fig. 1E) were enriched in Rrm4-G samples relative to Upa1-G/mMLE3-K, in agreement with previous observations that ribosomal protein shuttling is mediated by Rrm4

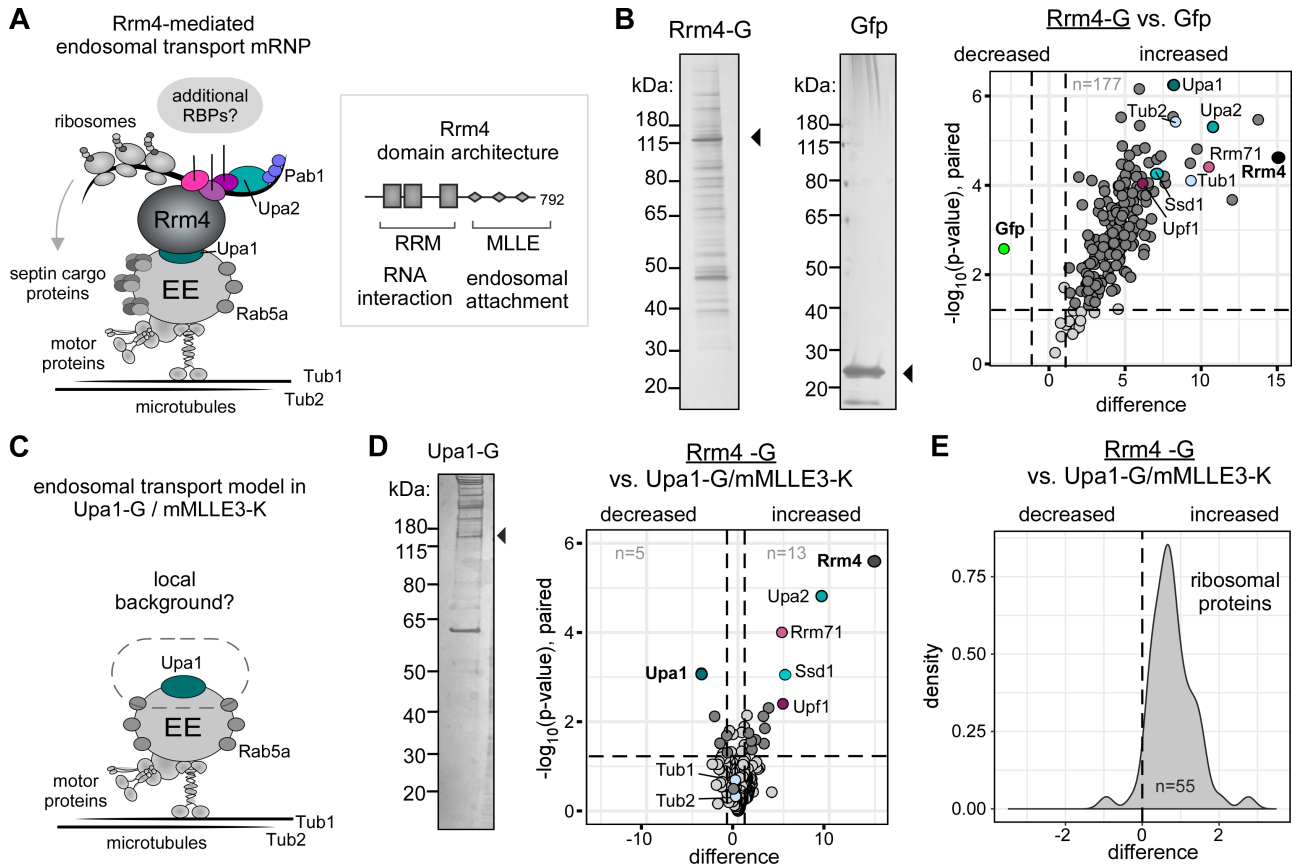


Fig. 1. Uncovering novel components of Rrm4-mRNP complexes

(A) Schematic representation of the translation-active, Rrm4-dependent endosomal transport model in *U. maydis*. Core components include the endosomal adaptor Upa1, the mRNP scaffold protein Upa2, and the poly(A)-binding protein Pab1. (B) Co-immunoprecipitation of Rrm4-Gfp (Rrm4-G; ~112 kDa, arrow) and free Gfp (~28 kDa, arrow), visualized via silver-stained SDS-PAGE. (Right) Volcano plot comparing Rrm4-G versus Gfp control (five biological replicates). The *difference* represents the change in average log₂ protein intensities between Rrm4-G and the Gfp control, serving as a measure of protein enrichment. Significantly increased protein abundance (difference > 1; -log₁₀(p-value) > 1.3, paired) and significantly decreased protein abundance (difference < -1; -log₁₀(p-value) > 1.3, paired) are highlighted in dark grey. Key interactors include Rrm4 (black), Upa1 (deep petrol), Upa2 (light petrol), Ssd1 (turquoise), Rrm71 (light pink), Upf1 (dark pink), and Tub1/2 (light blue). (C) Schematic illustrating the endosomal protein landscape in the mMLLE3 mutant background, where the interaction between Rrm4 and Upa1 is disrupted [16]. (D) Silver-stained SDS-PAGE of the Upa1-Gfp pulldown in the mMLLE3-K background strain (Upa1-G/mMLLE3-K). The volcano plot shows the comparative co-IP MS analysis of Rrm4-G versus Upa1-G/mMLLE3-K (five biological replicates), highlighting key candidates as in panel (B). (E) Density plot of ribosomal proteins (n=55) in the comparative co-IP MS approach of Rrm4-G versus Upa1-G/mMLLE3-K (see panel D).

[18, 27]. In summary, this comparative co-IP MS study refined and expanded the current model of Rrm4-mediated mRNP composition in *U. maydis* (Fig. 1A,B; SI-Fig. 1A) [22].

RRM1 shapes the Rrm4-mRNP protein interactome

Rrm4 contains three RRM domains (Fig. 1A), yet their individual contributions to mRNP composition remain unresolved. To delineate their roles, Gfp-tagged Rrm4

variants carrying four point mutations in the conserved RNP1 motif of each RRM (mR1-G, mR2-G, mR3-G; Fig. 2B, SI-Fig. 1B) were used [14, 20]. In agreement with previous studies, phenotypic analysis showed that mR3-G supported wild-type-like hyphal growth, whereas mR1-G and mR2-G phenocopied the *rrm4Δ* deletion strain, highlighting essential roles for RRM1 and RRM2 in hyphal development (SI-Fig. 1C) [14, 20, 21]. All three mutant proteins exhibited endosomal movement, maintained protein stability, and displayed unaltered

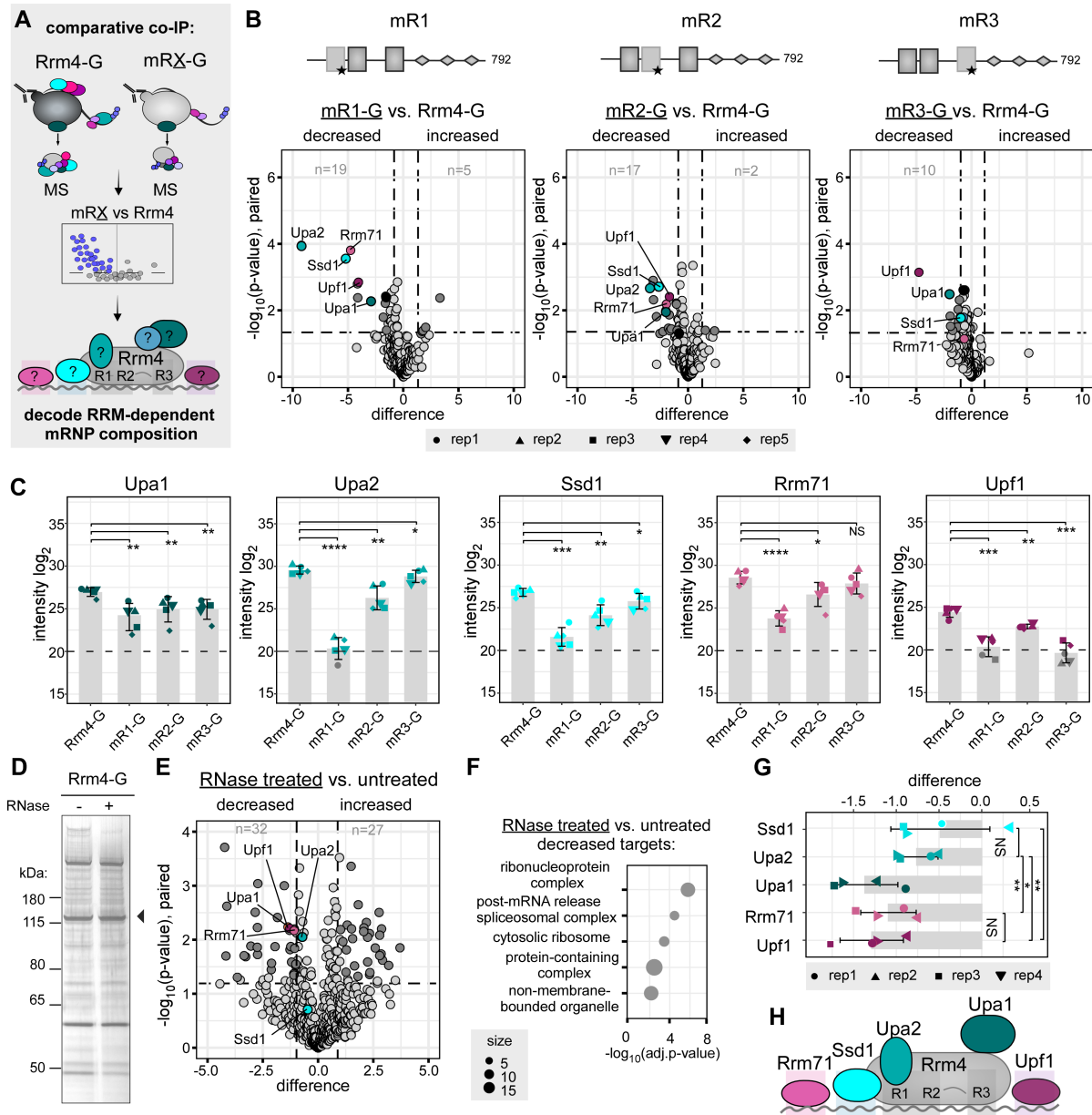


Fig. 2. Dissecting RNA- and RRM-specific interactions within the Rrm4-mRNP complex

(A) Schematic representation of the comparative co-IP MS approach to dissect the RRM-dependent protein interactome of Rrm4. Gfp-tagged RRM mutant variants (mRX-G, light grey), were analyzed relative to Rrm4-G (dark grey). (B) Domain architecture of RRM mutants (mutations are indicated by an asterisk, mRX). Volcano plots illustrating differential protein enrichment in co-IP MS for mR1-G, mR2-G, and mR3-G relative to Rrm4-G (five biological replicates). Significantly enriched (difference > 1; $-\log_{10}(\text{p-value}) > 1.3$, paired) and decreased (difference < -1; $-\log_{10}(\text{p-value}) > 1.3$, paired) proteins are indicated. Key interactors are color-coded: Rrm4 (black), Upa1 (deep petrol), Upa2 (light petrol), Ssd1 (turquoise), Rrm71 (light pink), and Upf1/Nam7 (dark pink). (C) Scatter plot showing individual intensity values for selected Rrm4 interactors (Upa2, Rrm71, Ssd1, Upf1, and Upa1). Grey dots below the dashed line represent imputed missing values (paired Student's t-test). (D) Co-IP of Rrm4-G with and without RNase treatment, visualized by silver-stained SDS-PAGE and (E) a corresponding volcano plot (four biological replicates, paired Student's t-test). (F) GO cellular component analysis of decreased interactors upon RNase digestion (difference < -1; $-\log_{10}(\text{p-value}) > 1.3$, paired). (G) Scatter plot of individual differences (Rrm4-G $\pm$ RNase, paired Student's t-test, four biological replicates). (H) Schematic summary of the RRM-dependent interaction modes of Rrm4.

immunoprecipitation efficiency (SI-Fig. 2D-E) [14, 20].

To examine how each RRM domain influences mRNP composition, comparative co-IP MS using Rrm4-G and its RRM mutants (mRX-G) were performed (Fig. 2A-B, SI-Fig. 1B-E). The comparative analysis revealed that mR1-G exhibited the most affected protein interactome, followed by mR2-G and mR3-G (Fig. 2B-C, SI-Tab. 3). The Rrm4-specific cohort, including Upa2, Ssd1, Rrm71, Upf1, and the endosomal anchor protein Upa1, showed differential association across the mutants.

Upa1 showed a moderate but consistent decrease across all Rrm4 variants ($\text{Rrm4-G} > \text{mR1-G} \approx \text{mR2-G} \approx \text{mR3-G}$). This reduction likely reflects a response to diminished RNA binding upon mutation of individual RRMs [14]. The decreased RNA-binding capacity may induce a conformational change in Rrm4 that weakens the interaction between its C-terminal MLLE3 domain and the PAM2L motif of Upa1. This effect is likely accentuated due to extensive washing steps during co-IP procedures (Fig. 2A-C).

Interestingly, Upa2, Ssd1, and Rrm71 exhibited a similar dependency pattern, following the order $\text{Rrm4-G} \approx \text{mR3-G} > \text{mR2-G} > \text{mR1-G}$ (Fig. 2B-C). Notably, their binding was only marginally affected in mR3-G, moderately reduced in mR2-G, and strongly impaired in mR1-G compared to Rrm4-G (Fig. 2B-C). These results suggest that the tandem RRM1 and RRM2 domains govern the association of Upa2, Ssd1, and Rrm71, with RRM1 serving as the principal determinant.

In contrast, Upf1—a key factor in nonsense-mediated decay and RNA quality control—displayed a distinct interaction pattern ($\text{Rrm4-G} > \text{mR2-G} > \text{mR1-G} \approx \text{mR3-G}$), consistent with previous radioactive UV-crosslinking studies showing a similar hierarchy in RNA-binding efficiency across the RRMs (Fig. 2B-C) [14, 20]. This observation supports the notion that Upf1 associates with Rrm4 in an RNA-dependent manner. In summary, comparative co-IP analyses of Rrm4 mutants demonstrate that RRM1 plays a primary role in shaping mRNP composition.

Mapping RNase-sensitive interactions in the Rrm4-mRNP complex

To gain deeper insight into the interaction modes, co-IP of Rrm4-G, followed by RNase digestion was performed (Fig. 2D-E; SI-Fig. 1F-G, SI-Tab. 3, see Materials and methods) [10]. This approach enabled the distinction of two major interaction types: (I) RNase-sensitive interactions, where protein association was significantly reduced upon RNase treatment (difference < -1 ; $-\log_{10}(\text{p-value}) > 1.3$, paired t-test), indicating that RNA is essential for maintaining these interactions; and (II) RNase-resistant interactions, which persisted despite RNA digestion. The latter can be further subdivided into two categories: direct protein-protein interactions, which were never dependent

on RNA, and RNA-induced protein-protein interactions, where RNA was initially required to mediate the interaction. A recent comprehensive RBP-interactome study in *S. cerevisiae*, analyzing 40 RBPs with and without RNase A treatment, demonstrated a broad spectrum of affected proteins, ranging from 5 (co-IP of Upf3) to 143 (co-IP of Upf1) targets (median 26.5) [10]. In this study, the number of significantly altered Rrm4-associated proteins upon RNase treatment was relatively modest (32 proteins; difference < -1 ; $-\log_{10}(\text{p-value}) > 1.3$, paired t-test; Fig. 2G,H), but within the expected range. The targets affected by RNase treatment were enriched for RNA-binding proteins and ribosomal proteins, confirming both efficient RNA digestion and successful detection of RNA-mediated proteins (Fig. 2D-G, SI-Fig. 1F-G).

The RBPs Rrm71 and Upf1, as well as the endosomal anchor protein Upa1, exhibited a reduction after RNase treatment (Fig. 2E,G; SI-Fig. 1G), suggesting that both RBPs are likely RNA-dependent interaction partners of Rrm4. In contrast, Ssd1 and Upa2 showed lower sensitivity to RNase treatment (Fig. 2E,G; SI-Fig. 1G) but displayed a marked reduction in the mR1-G mutant relative to Rrm4-G (Fig. 2B-C). This indicates that these factors likely engage in RNA-independent, yet RRM1-dependent manner with Rrm4. In summary, both RNase-resistant (e.g., Ssd1) and RNase-sensitive (e.g., Upf1, Rrm71, Hsp70) interactions underscore the diverse interaction modes of Rrm4 and its pivotal role in modulating mRNP composition (Fig. 2H).

Direct protein-protein interaction between the GWW motif of Upa2 and RRM1 of Rrm4

Upa2 was previously identified as a core component of the endosomal mRNP complex, where it functions as a scaffold protein essential for stabilizing Rrm4-mediated mRNP transport (Fig. 1-2) [25]. Consistent with previous reports, fluorescence live-cell imaging showed that Upa2-G co-shuttles with Rrm4-positive early endosomes, and that this movement is completely abolished in both *rrm4Δ* and *mR1* mutant backgrounds (Fig. 3A-B) [23, 25].

Although this phenotype was previously attributed to impaired RNA binding by mR1 [23, 25], the proteomic data challenge this RNA-dependent model: (I) Upa2 is only marginally affected in the mR3-G mutant, despite this variant exhibiting the strongest reduction in RNA binding as shown by UV crosslinking [14]; and (II) Upa2 association is only slightly impacted by RNase treatment (Fig. 2G, SI-Fig. 1G).

To further dissect the contribution of individual RRM domains to Upa2 motility, live-cell imaging was extended to mR2-K and mR3-K mutant backgrounds (Fig. 3A-B). In mR2-K mutants, Upa2-G movement remained detectable, although the number of shuttling particles was significantly reduced (Fig. 3A-B). In contrast, Upa2-G motility in mR3-K mutants was indistinguishable from wild type, in full agreement with the co-IP MS results, and supports

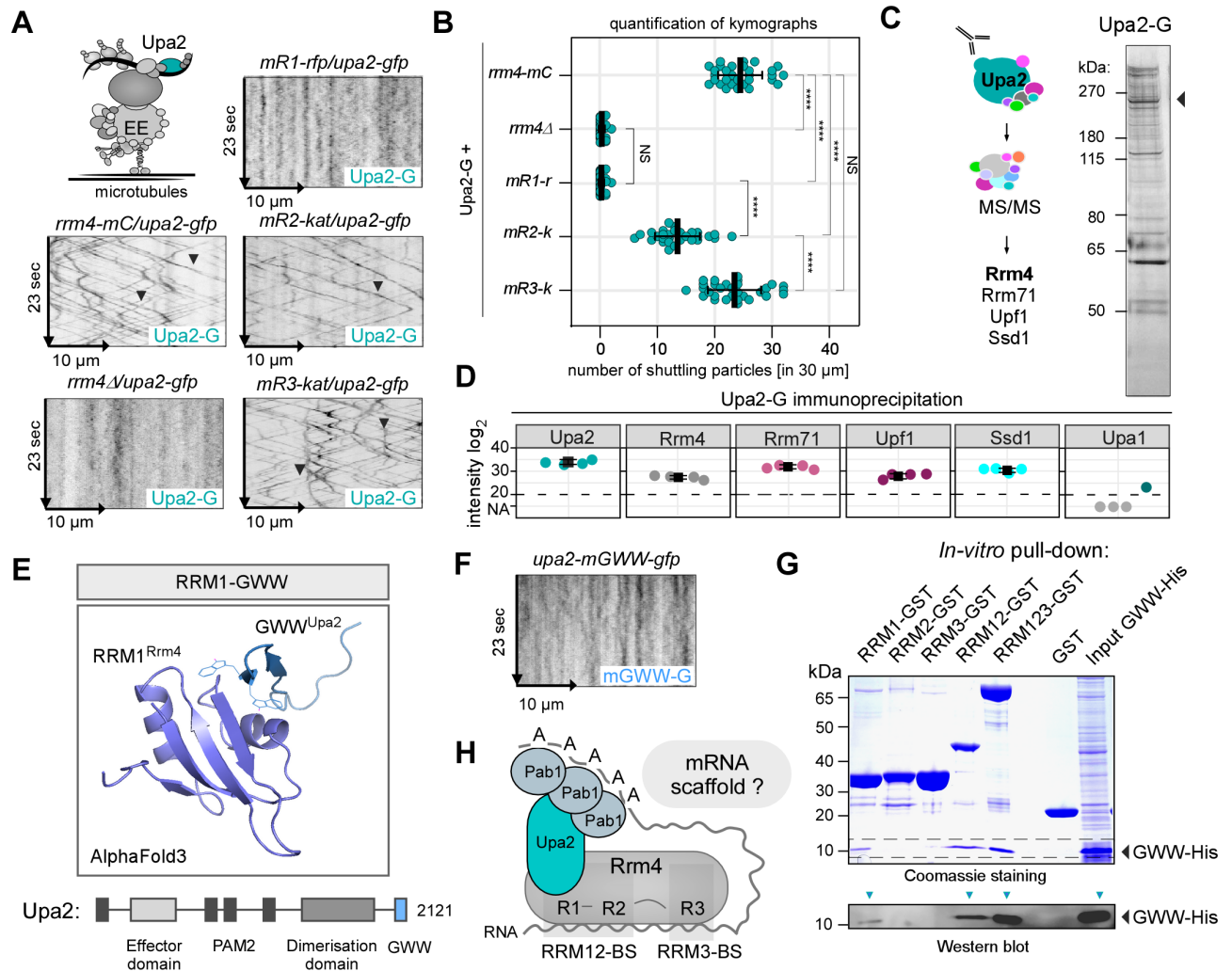


Fig. 3. *In vivo* and *in vitro* validation of the protein-protein-interaction between RRM1 of Rrm4 and the GWW motif of Upa2

(A) Kymographs of shuttling Upa2-G particles in various Rrm4 background strains at 6 h.p.i. (B) Quantification of shuttling particles over a 30 μm distance (three biological replicates, N ≥ 30; unpaired two-tailed Student's t-test). (C) Silver-stained SDS-PAGE of co-immunoprecipitated Upa2-G (258 kDa), highlighting top MS-identified interaction partners. (D) Scatter plot of intensity values from Upa2-G pull-down experiments (four biological replicates); proteins not detected (NA) are shown in light grey below the dashed line. (E) AlphaFold3 model predicting the interaction between RRM1 of Rrm4 (purple) and the GWW motif of Upa2 (light blue). Model generated in collaboration with Dr. Florian Altegoer. (F) Representative kymograph of mGWW-G, Upa2 including the GWW motif mutated to AAA (6 h.p.i.) [25]. (G) *In vitro* GST pull-down assays using *E. coli*-expressed GST-tagged RRM variants (bait) incubated with *E. coli* extracts containing GWW-His (prey; 14 kDa, arrow). (H) Advanced model of Rrm4-mediated mRNP composition, highlighting the direct interaction between Upa2, Rrm4, and Pab1.

an RNA-independent recruitment mechanism. Together, these findings suggest that the tandem RRMs—particularly RRM1—are essential for endosomal Upa2 movement *in vivo* (Fig. 2; Fig. 3A-B).

To validate the Rrm4-Upa2 interaction from the reciprocal perspective, co-IP MS experiments using Upa2-G as bait were performed (Fig. 3C-D, four biological replicates).

Indeed, the core set of RBPs, including Rrm4, Rrm71, Ssd1, and Upf1, were co-purified (Fig. 3D). Upa1, by contrast, was detected in only one of four replicates, consistent with its peripheral localization as an endosomal anchoring protein within the mRNP structure [17].

To pinpoint the Upa2-Rrm4 interaction surface, AlphaFold3-based structure prediction was conducted (in collaboration

with Dr. Florian Altegoer [39]. The model suggests that the upper portion of RRM1 interacts with two tryptophan residues (W2119 and W2120) located within the N-terminal GWW motif of Upa2 (Fig. 3E) [25]. Strikingly, previous work identified this motif as essential and sufficient for Upa2-G shuttling (Fig. 3F), though its direct interaction partner remained undefined [25].

Here, the combined structural and proteomic data now suggest a specific, RNA-independent interaction between the GWW motif and RRM1. To directly test this, *in vitro* GST pull-down assays, under stringent RNase and DNase treatment, were conducted (Fig. 3G, see Materials and methods, in collaboration with Dr F. Altegoer and S. Shanmugasundaram). These experiments confirmed that the GWW motif of Upa2 interacts specifically with RRM1, as well as the tandem domains RRM12 as well as RRM123 of Rrm4. No interaction was detected with solely RRM2, RRM3, or GST, underscoring the specificity of the RRM1-GWW interaction *in vitro* (Fig. 3E,G).

In conclusion, the integration of comparative co-IP MS, *in vivo* imaging, structural prediction, and *in vitro* binding assays reveals a direct, RNA-independent interaction between the GWW motif of Upa2 and the RRM1 domain of Rrm4. These findings indicate that RRM1 not only mediates RNA specificity [14, 36], but also plays a central role in stabilizing the Rrm4-mediated mRNP complex through direct protein-protein interaction with the scaffold protein Upa2 (Fig. 3H).

Ssd1 and Rrm4 co-target mRNAs involved in cell wall plasticity

Previous co-IP MS analysis (Rrm4-G vs. Gfp) obtained by Markus Tulinski identified Ssd1 as a prominent Rrm4 interactor [23]. Building on these findings, mutant-based comparative co-IP MS revealed an RNase-resistant interaction between Ssd1 and Rrm4, mediated by RRM1 (Fig. 2).

To gain deeper insight into the domain architecture of *U. maydis* Ssd1, comparative structural analyses were performed by superimposing the crystal structure of *S. cerevisiae* Ssd1 [40] with the AlphaFold3-predicted model of *U. maydis* Ssd1 [39]. The structural superposition revealed a high degree of conservation (RMSD = 1.514 over 3,694 atoms), particularly within the RNB domain (RMSD = 1.114 over 1,524 atoms; Fig. 4A). Key structural features, including two conserved β -barrel Cold Shock Domains (CSD1 and CSD2) and a β -barrel S1 domain, were retained [40]. In addition, a PAM2 motif and a nuclear localization signal (NLS) were identified, consistent with previous reports [23], and incorporated into the refined domain architecture (Fig. 4B). Thus, the combined use of manual sequence analysis, structural modeling, and complementary prediction tools enabled precise annotation of the domain organization of *U. maydis* Ssd1 (Fig. 4A-B) [23, 39, 40].

In line with previous fluorescence live-cell imaging data [23], Ssd1-G exhibits endosomal motility in an Rrm4- and RRM1-dependent manner (Fig. 4C, Muntjes et al., in preparation), corroborating the findings from comparative co-IP MS analyses (Fig. 2B-C).

To gain deeper insights molecular function of Ssd1, comparative co-IP MS was performed (Ssd1-G, 6 h.p.i., five biological replicates). Direct comparison with Rrm4-G revealed specific enrichment of endosome-associated proteins (e.g., Tub1, Tub2) and a distinct cohort of mRNP components (e.g., Upf1, Rrm71, Upa2, Upa1; Fig. 4D) for Rrm4-G. GO term analysis further supported these findings, showing significant enrichment for categories such as endosomes, microtubules, cytoskeleton, mRNA transport, and RNA localization (Fig. 4E; SI-Fig. 2A), consistent with the known function and localization of Rrm4 [14, 18, 22]. Additionally, enrichment of polymerases (e.g., Rpb3) was observed, likely reflecting non-specific interactions or co-purification artefacts (SI-Fig. 2A).

In contrast, Ssd1-G showed a strong enrichment for translation-associated factors, particularly cytoplasmic ribosomal subunits (Fig. 4E), in line with co-IP MS data for GUL-1, the *Neurospora crassa* homolog of Ssd1, which similarly associates with ribosomal components [24, 41]. Ssd1 is a well-characterized translational repressor of mRNAs involved in fungal cell wall remodeling across diverse species [37, 40, 41, 42]. To assess whether Ssd1 binds RNA in *U. maydis*, a CLIP-PNK assay was performed, visualizing UV-crosslinked Ssd1-RNA complexes via radioactive labeling (Fig. 4F). Autoradiographs from both sporidia and hyphal cells (6 h.p.i.) confirm *in vivo* RNA-binding activity and underscore the suitability of Ssd1-G for microbial iCLIP2 approaches [36, 43].

RNA interactome reports of Ssd1/GUL-1 across fungal species have revealed a conserved mRNA regulon governing cell wall biosynthesis and remodeling, closely paralleling the recently defined Rrm4 interactome [24, 40, 42, 44, 45]. This functional convergence prompted investigation of shared target transcripts (Fig. 4G). GUL-1/Ssd1 RIP-seq data from *N. crassa* were leveraged (Fig. 4G-H) [24]; of the 1,048 GUL-1/Ssd1-associated transcripts, 487 have orthologs in *U. maydis* (see Materials and methods) [46]. Notably, 86% (n=420) of these overlapped with Rrm4 iCLIP2 targets [36], and 38% (n=187) contained functionally relevant RRM12-binding sites (RRM12-BS; Fig. 4G; SI-Tab. 4) [20]. GO term analysis of this conserved core set (n=187) revealed significant enrichment for components of the cell periphery, plasma membrane, and sites of polarized growth (Fig. 4G-H), consistent with the established roles of GUL-1/Ssd1 in cell wall regulation [24, 40, 42, 44, 45]. This overlap includes key biosynthetic enzymes such as β -1,3-glucan synthase (Gsc1), chitin synthase 1 (Chs1), and the glucosidases Glu2 and Glu6, supporting the existence of a conserved, functionally coherent mRNA regulon critical for cell wall plasticity [24, 37, 42].

Collectively, these cell biological, biochemical, and

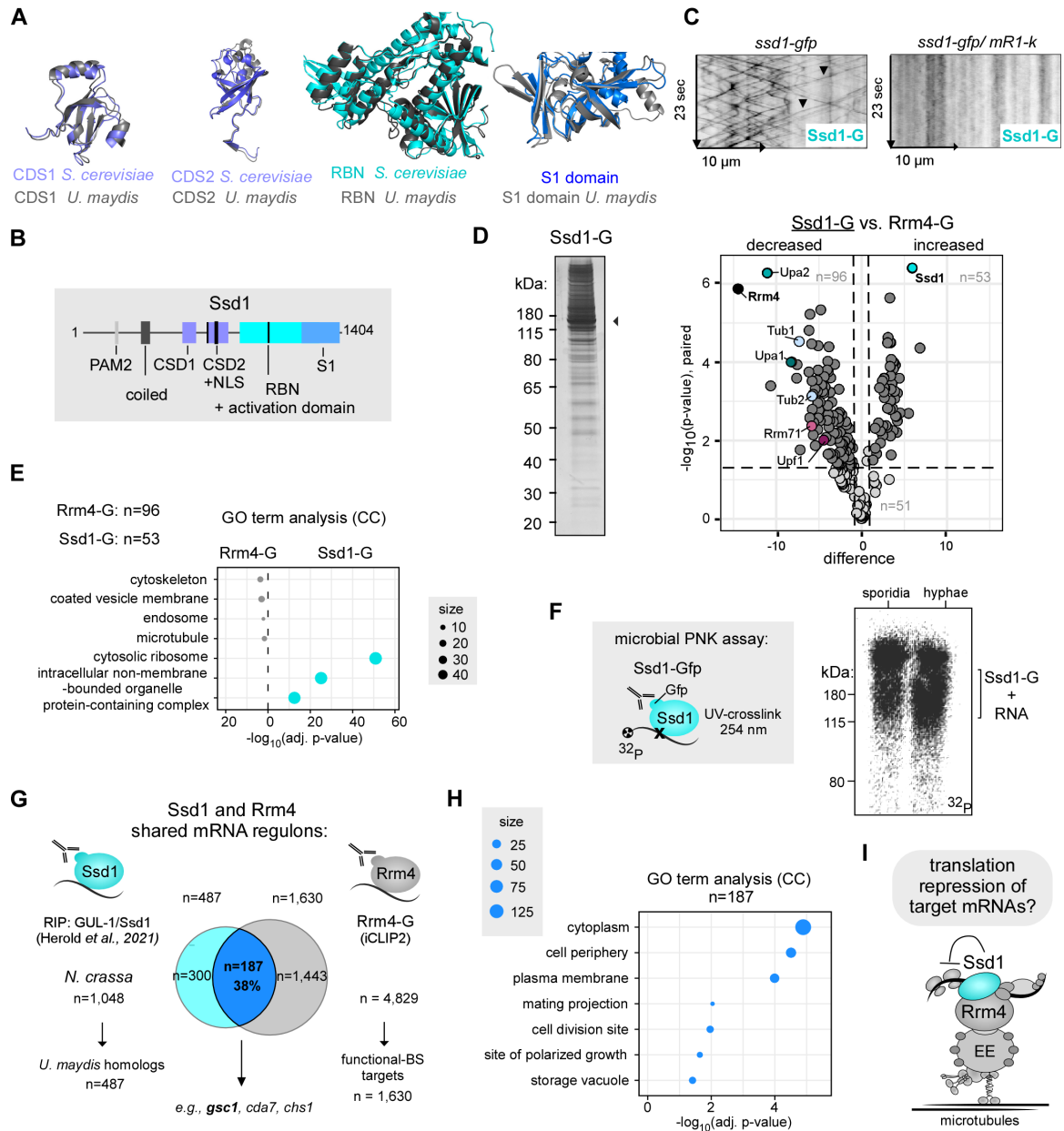


Fig. 4. The RBP Ssd1 is a component of the Rrm4-mediated endosomal mRNP complexes

(A) Superposition of domain architectures of *S. cerevisiae* Ssd1 (colored) [40] and predicted *U. maydis* Ssd1 (grey; AlphaFold3) [39]. (B) Refined domain organization of *U. maydis* Ssd1. (C) Representative kymographs of shuttling Ssd1-G particles in Rrm4-K and mR1-K mutant backgrounds (6 h.p.i.). (D) Silver-stained SDS-PAGE of immunoprecipitated Ssd1-G (180 kDa, arrow) and volcano plot of co-IP MS comparing Ssd1-G versus Rrm4-G (n=5). Significance cutoffs: enrichment (difference > 1; $-\log_{10}(\text{p-value}) > 1.3$, paired), decreased (difference < -1; $-\log_{10}(\text{p-value}) > 1.3$, paired). (E) GO term analysis of proteins enriched with Ssd1-G (turquoise) or Rrm4-G (grey). Displayed GO:CC terms have adjusted p < 0.05. (F) Autoradiograph of UV-crosslinked, radioactively-labeled Ssd1-G-RNA complexes from yeast and hyphal cells (6 h.p.i.). (G) Venn diagram comparing *N. crassa* GUL-1 RIP-seq targets [24] with *U. maydis* Rrm4-G iCLIP2 targets containing functional important RRM12-BS [20]. (H) GO term analysis of overlapping targets (n=187) from (G). (I) Model of endosomal mRNA transport highlighting the potential role of Ssd1 in fungal mRNP dynamics.

bioinformatic data identify Ssd1 as an RNA-binding protein that likely functions as a translational regulator within the

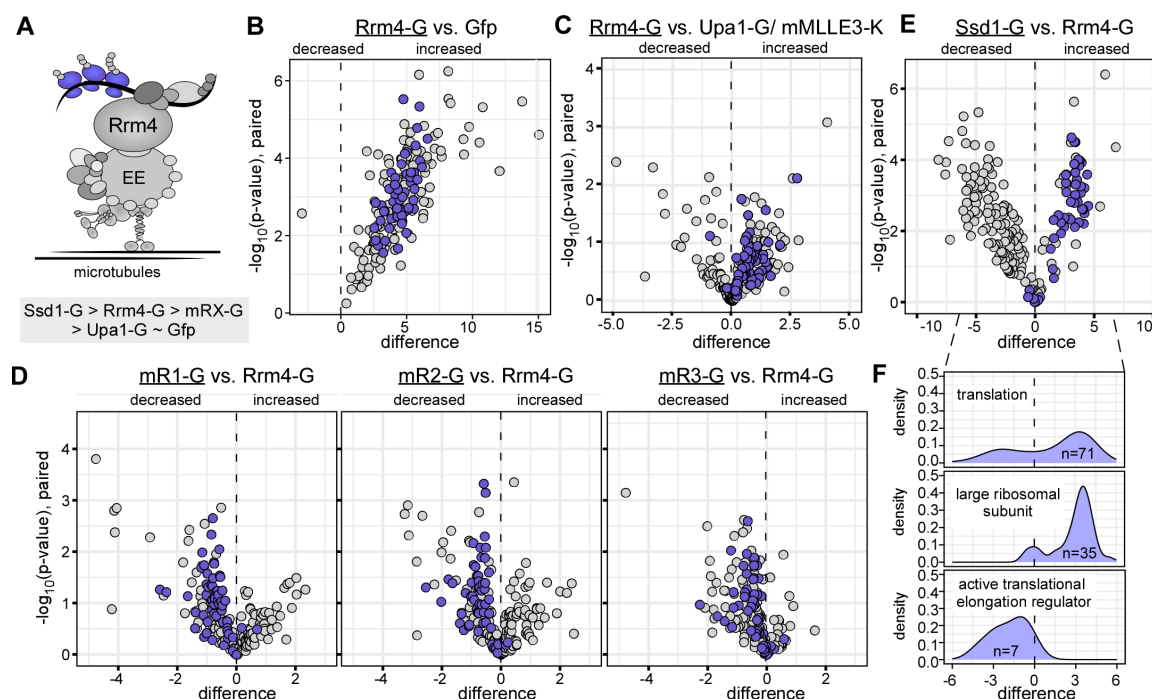


Fig. 5. RRM-dependent ribosome association of Rrm4

(A) Model of Rrm4-mediated endosomal mRNA transport in *U. maydis*, illustrating ribosomes (slate blue) (B-E) Enlarged volcano plots of co-IP MS data from Figures 1,2 and 4, with ribosomal proteins (RPs) highlighted in slate blue. (B) Comparison of Rrm4-G versus Gfp (C) Rrm4-G versus Upa1-G/mMLE3-K (D) mR1-G, mR2-G, and mR3-G versus Rrm4-G and (E) Ssd1-G versus Rrm4-G, respectively. (F) Density plot comparing the difference of protein abundance in Ssd1-G versus Rrm4-G, with focus on translation (n=71), ribosomal proteins of the large subunit (n=35) and active translation elongation factors (n=7). The numbers of detected proteins are indicated.

Rrm4-mediated endosomal mRNA transport machinery, with a conserved role in regulating cell wall dynamics (Fig. 4I; Müntjes et al., in preparation).

RRM-dependent ribosome association

The hypothesis of translationally active endosomal mRNA transport is reinforced by live-cell imaging studies demonstrating Rrm4-dependent endosomal hitchhiking of ribosomal proteins, such as Rps2-G (Fig. 5A) [17, 18, 22, 27]. However, only a limited subset of ribosomal proteins has been tested, and direct biochemical evidence remains scarce. To systematically dissect ribosomal protein (RP) association, comparative co-IP MS datasets were analyzed, revealing a defined enrichment hierarchy: $Ssd1-G > Rrm4-G > mRX-G > Upa1-G$ ($Upa1-G/mMLE3-K$) $\approx$ Gfp (Fig. 5A-F). These findings corroborate the model of Rrm4-associated ribosomes during endosomal transport observed by live-cell imaging [17, 18, 27]. Notably, all RRM mutants exhibited progressively reduced ribosomal protein association relative to Rrm4-G, suggesting that the RNA-binding capacity of Rrm4 indirectly mediates ribosomal protein recruitment (Fig. 5A-E).

In contrast, Ssd1-G exhibited markedly higher ribosomal

protein abundance compared to Rrm4-G (Fig. 5E-F). Detailed categorization—encompassing small (Rps; SI-Fig. 2B) and large (Rpl; Fig. 5F) ribosomal subunits—revealed preferential association of Ssd1 with Rpl components (Fig. 5E-F, SI-Fig. 1E). Notably, factors essential for translation elongation, including eEF23 and eEF2, showed reduced association with Ssd1-G relative to Rrm4-G (n=7; Fig. 5F). The combination of enhanced Rpl interaction and diminished association of elongation factors supports a model in which Ssd1 acts as a repressor of translation elongation [24, 38].

Together, these data provide compelling evidence for Rrm4-dependent local translation on early endosomes [18, 27], highlighting the critical roles of RRM1 and RRM2 in facilitating RNA-dependent ribosome association within the mRNP complex [20]. Additionally, it underlines Ssd1 as a putative translational elongation regulator modulating Rrm4-mediated endosomal mRNA transport (Fig. 5A).

Bioinformatic characterization of the novel RNA-binding proteins Rrm71 and Upf1

In addition to the established mRNP components Upa2 and Ssd1 [23, 25], two previously uncharacterized RBPs, Rrm71

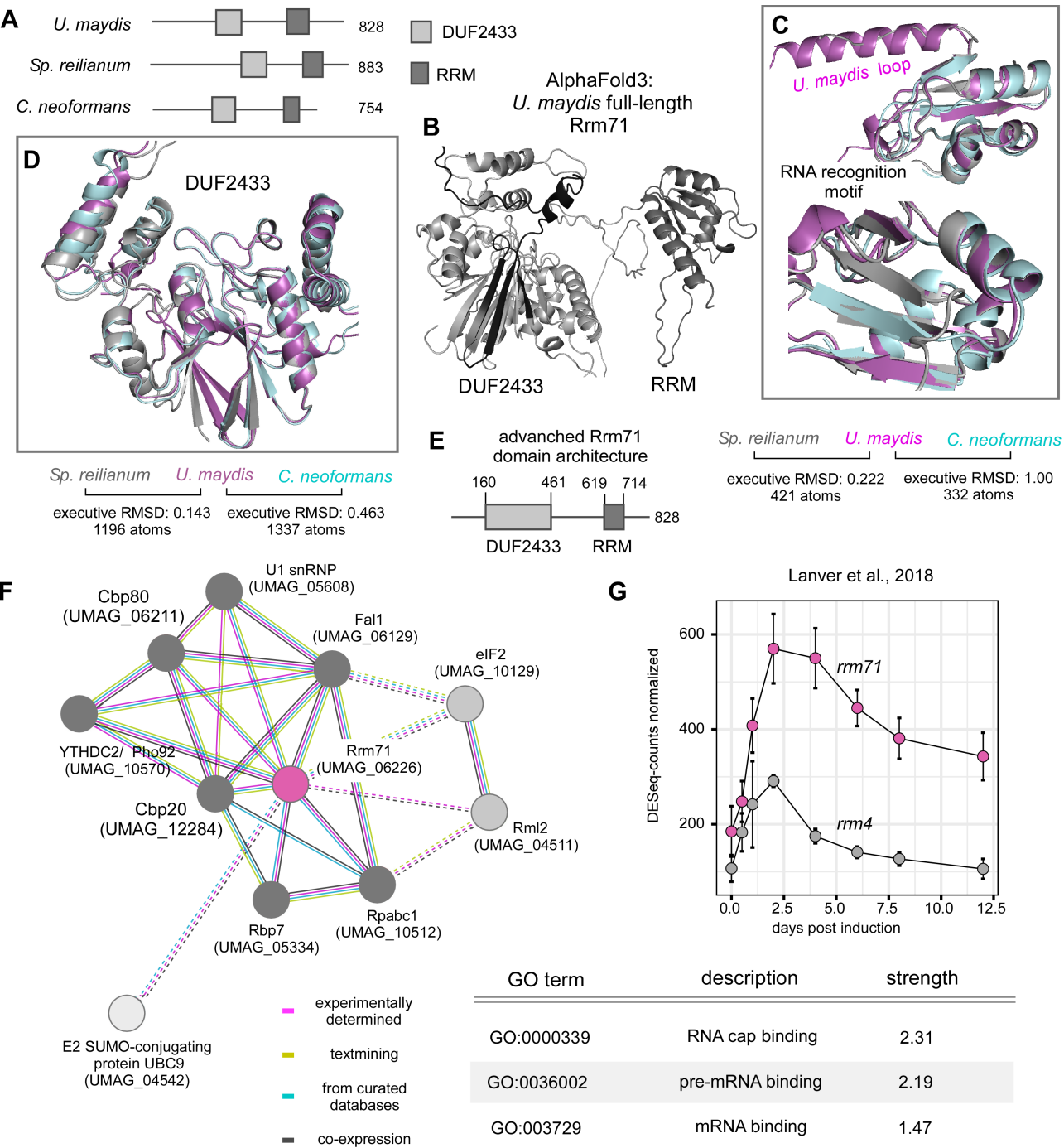


Fig. 6. Bioinformatic characterization of Rrm71

(A) Domain architecture of Rrm71 from *U. maydis*, *Sp. reilianum* and *C. neoformans* [47]. (B) AlphaFold3 prediction of full-length *U. maydis* Rrm71 [39]; DUF2433 domain prediction based on SMART (see A) analysis is highlighted in black. (C-D) Superposition of the (C) RRM domain and (D) the extended DUF2433 across *U. maydis*, *Sp. reilianum*, and *C. neoformans*. (E) Advanced domain architecture of *U. maydis* Rrm71 based on panels A–D, including extended DUF2433 domain. (F) Protein interaction network of Rrm71 (light pink) as determined by STRING [48]. (G) Time-course RNA-seq analysis of infectious SG200 *U. maydis* from Lanver et al., (2018) [49], showing normalized differential gene expression (DESeq counts) of *rrm4* (grey) and *rrm71* (light pink) over time.

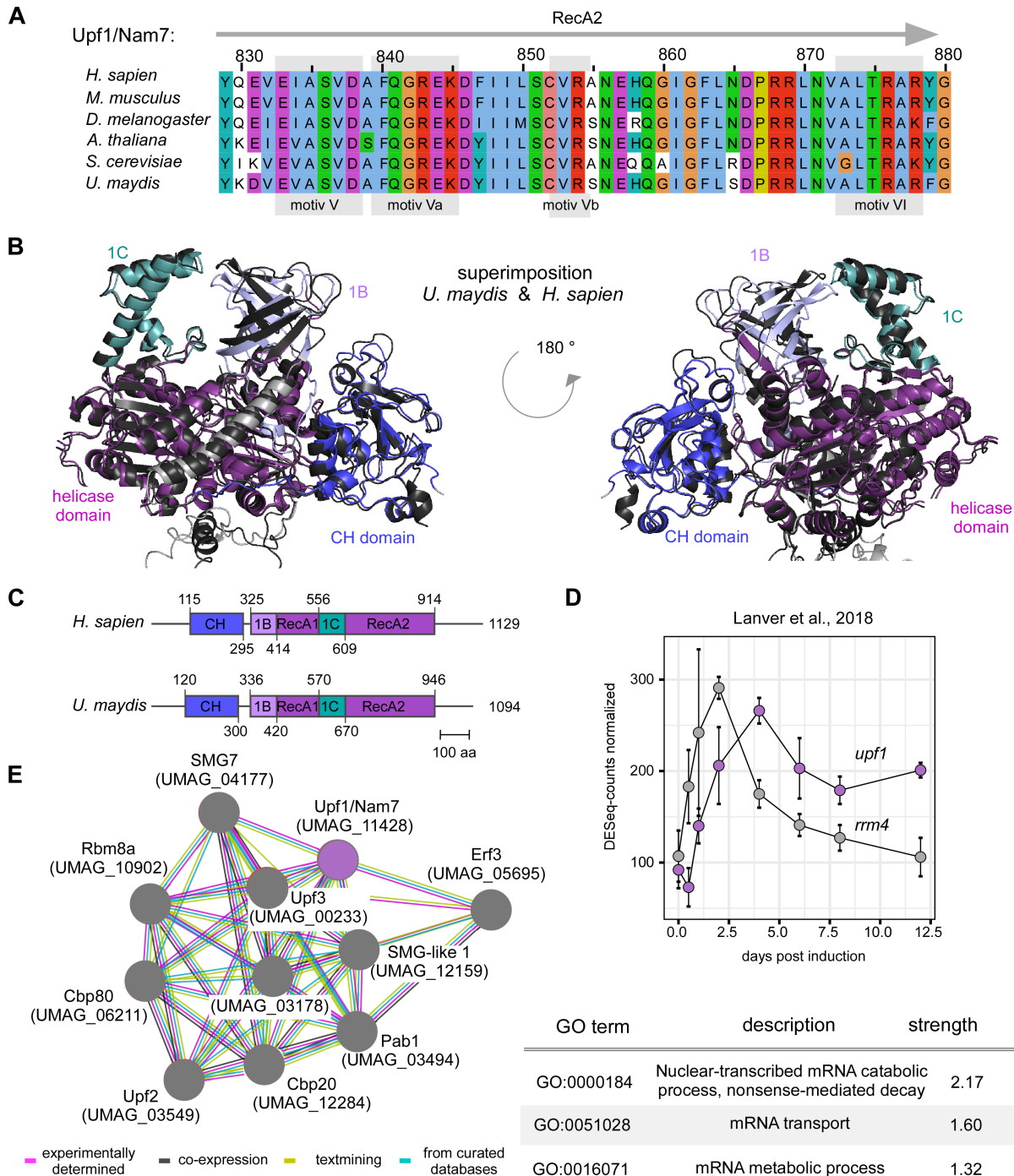


Fig. 7. Bioinformatic characterization of the nonsense-mediated decay factor Upf1

(A) Multiple sequence alignment of the RecA2 domain of Upf1/Nam7 from various organisms. (B) Superposition of *H. sapiens* UPF1 adapted from Langer et al., (2024) [50] and *U. maydis* Upf1, predicted via AlphaFold3 [39]. (C) Domain architecture comparison between *U. maydis* Upf1 and *H. sapiens* UPF1, adapted from Langer et al., (2024) [50]. (D) RNA-seq time-course analysis from infectious SG200 *U. maydis* Lanver et al., (2018) [49], showing normalized differential gene expression (DESeq counts) of *rrm4* (grey) and *upf1* (dark pink). (E) Predicted interaction network of *U. maydis* Upf1 (dark pink) determined by STRING [48].

and Upf1, were identified as components of the Rrm4-mediated mRNP complex (Fig. 1–2). Both associations are RNase-sensitive and the individual RRMs of Rrm4 differentially regulate their association: Rrm71 is primarily diminished in mR1-G and mR2-G, with no effect in mR3-G, whereas Upf1 binding is predominantly reduced in mR3-G, followed by mR1-G and mR2-G (Fig. 2).

Rrm71 is conserved across basidiomycetes [51, 52], indicating a specialized fungal role. Domain annotation using established bioinformatic tools [52, 53, 47] revealed a canonical RNA recognition motif (RRM) and a fungal-specific DUF2433 domain (Fig. 6A). Comparative analysis showed that DUF2433 shares similarity with the corresponding domain of the phosphatase DipA from *Aspergillus nidulans*, a protein implicated in early endosomal trafficking and peroxisome motility [54]. These observations implicate DUF2433 in endosomal transport, although functional validation is pending.

Structural predictions for *U. maydis* Rrm71 confirmed a well-defined RRM and a large domain partially overlapping DUF2433 (aa 416–502; Fig. 6A–B) [39]. This domain architecture (RRM + DUF2433) is conserved in *Cryptococcus neoformans* and *Sporisorium reilianum* (Fig. 6A). Structural superposition demonstrated high conservation (*U. maydis* vs. *C. neoformans*: RMSD=0.203 over 2,373 atoms; *U. maydis* vs. *S. reilianum*: RMSD=0.539 over 1,932 atoms). In details, the RRM domain is especially conserved (Fig. 6C), although *U. maydis* Rrm71 features an additional, non-conserved loop of unknown function (Fig. 6C). The extended DUF2433 domain (aa 160–460) also displays structural conservation (Fig. 6D).

To elucidate the interaction network of Rrm71, *in silico* STRING analysis was performed [55], predicting associations with the cap-binding complex components Cbp20 and Cbp80—core factors in mRNA processing, nuclear export, and localization (Fig. 6F) [56]. GO term enrichment via STRING further linked Rrm71 to mRNA and RNA-cap binding, implicating it in RNA metabolism (Fig. 6F). To investigate its potential functional connection with Rrm4, RNA-seq time-course data from infectious *U. maydis* SG200 [49] were reanalyzed. Both *rrm71* and *rrm4* transcripts exhibited co-expression, peaking during hyphal growth and early plant infection before declining at 4 days post-infection (Fig. 6F). Importantly, *rrm71* mRNA levels remained unchanged in *rrm4*Δ mutants [20], indicating Rrm4 does not regulate *rrm71* expression. Given Rrm71's association with Rrm4-mRNPs (Fig. 1–2), its subcellular localization was assessed via enrichment in Rab5a-positive endosomes using endosomal immunoprecipitation followed by mass spectrometry (endo-IP MS, SI-Fig. 1F; Stoffel et al., unpublished). Rrm71 was detected in endo-IP fractions and was reduced in the mR1 and mR2 mutant backgrounds, but remained unaffected in mR3. These findings establish Rrm71 as a compelling candidate for further functional analysis (see Discussion, Fig. 8).

In contrast, the RBP Upf1/Nam7 is a highly conserved, well-characterized ATP-dependent RNA helicase and ATPase, known for its roles in nonsense-mediated mRNA decay (NMD), regulation of translation termination, and protein ubiquitination [33, 57, 58]. As expected, multi-sequence alignment revealed strong conservation of Upf1/Nam7 across kingdoms (Fig. 7A; see Materials and methods). Structural superposition of predicted *U. maydis* Upf1 with human UPF1 further confirmed high structural conservation [50]. Leveraging the well-resolved human UPF1 architecture [50], the domain organization of *U. maydis* Upf1 was annotated (Fig. 7C). The CH domain, critical for Upf2 interaction, as well as the RNA-binding domains 1B and 1C, were highly conserved (Fig. 7C–D).

To investigate potential co-expression of *upf1* and *rrm4* during the hyphal growth phase, time-course RNA-seq data were reanalyzed [49] and revealed co-expressed, with *upf1* showing a slight temporal delay (Fig. 7D). In addition, comparative RNA-seq analysis between wild-type and *rrm4*Δ strains revealed no significant differences in *upf1* mRNA abundance, suggesting that Rrm4 does not regulate *upf1* transcript levels [20].

STRING-based predictions [48] identified Upf1 interaction network consistent with conserved NMD machinery, including Upf2, Upf3, Smg1, and Smg7 (Fig. 7E) [33, 50, 58], along with cap-binding complex components Cbp20 and Cbp80 (Fig. 6E) [56]. Gene ontology term analysis supports Upf1 involvement in NMD, mRNA transport, and metabolism (Fig. 7E), positioning it as a putative quality control factor within the Rrm4-associated mRNA transport unit (see Discussion).

In summary, comparative proteomics and bioinformatic analyses position the RBPs Rrm71 and Upf1 as novel components of the endosomal mRNP complex.

DISCUSSION

RBPs are pivotal regulators of the RNA life cycle, orchestrating processes such as RNA transport, localization, and mRNA stability [1, 2, 31, 59]. Given their essential roles, dysregulated RBPs are implicated in a broad spectrum of human diseases, ranging from cancer to neurological disorders [60, 61]. RBPs form dynamic mRNPs complexes through interactions with both RNAs and proteins [3, 5, 29, 62]. While CLIP and related approaches [63, 64] provide high-resolution RNA-binding profiles, they do not capture the complexity of RBP networks [3, 11, 13, 29]. To fully understand spatiotemporal gene expression, dissection of *in vivo* mRNP protein composition is essential [4, 12]. Moreover, many RBPs contain multiple RNA-binding domains, which can influence interactome networks through both direct protein-protein and RNA-mediated associations [65, 28, 29, 66, 67]. However, the individual contributions of these domains to the composition and structural organization of mRNP complexes at the proteome-wide scale remain largely unresolved [3, 68, 28].

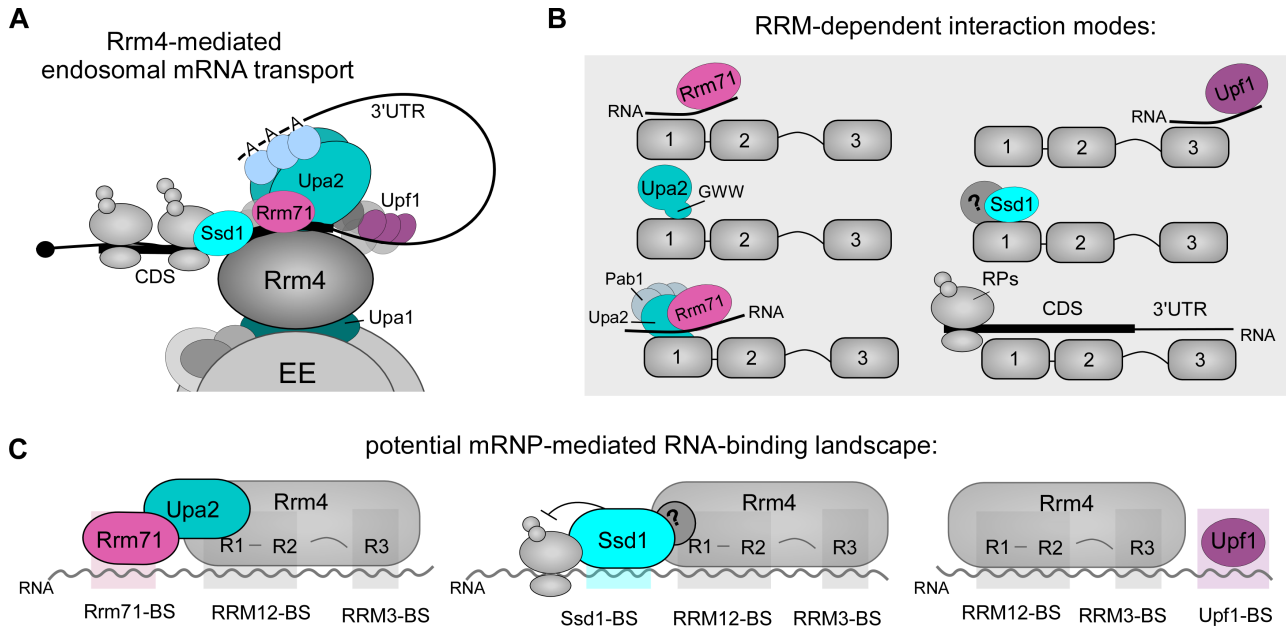


Fig. 8. Rrm4-mediated mRNP architecture: RRM-dependent interaction logic and cooperative RNA binding

(A) Refined model of Rrm4-driven endosomal mRNA transport, revealing a novel composition of the mRNP transport unit. (B) Schematic of RRM-dependent interaction modes of Rrm4. (C) Potential cooperative RNA-binding landscape of Rrm4 with the RBPs Rrm71, Ssd1, and Upf1, highlighting the interplay of mRNP networks that coordinate precise RNA attachment and spatially regulated translation during endosomal transport.

The multi-RRM RBP Rrm4 is the central regulator of endosomal RNA transport in *U. maydis* hyphae; however, the precise protein composition of the transport mRNP and the specific contributions of its three RRM domains have remained unclear [20].

A proteome-wide atlas of RRM-directed interactions within Rrm4-containing mRNPs was established using a comparative co-IP MS strategy. By combining RRM-mutant variants of Rrm4 and RNase treatment, interaction networks were resolved at high resolution, revealing two principal interaction modes: (I) RRM-dependent, RNase-resistant interactions, indicative of direct RRM–protein contacts, exemplified by the transport scaffold Upa2 (Fig. 8) [25]; (II) RNA-mediated interactions, where the RNA-binding capacity of specific RRMs modulates association strength with other RBPs, as illustrated by the RNA quality control factor Upf1 or Rrm71 (Fig. 8) [57]. Integrated biochemical, cell biological, bioinformatic, and structural analyses delineate how individual RRMs shape mRNP composition and function. Together, these approaches provide a blueprint for dissecting the interaction modes of multi-RBD proteins on a proteome-wide scale, offering mechanistic insights into the modular organization of RRM-mediated mRNP networks (Fig. 8)

The dual function of RRM1 in RNA and protein interaction: insights into mRNA shuttling and scaffold stability

Comparative mutant-based co-IP MS and live-cell imaging indicate that the scaffolding protein Upa2 is specifically affected in the Rrm4 variant mR1, which carries a mutation in the first RRM [14], whereas mutations in the third RRM (mR3) do not alter Upa2 movement. Furthermore, structural predictions and biochemical assays pinpoint RRM1 as the direct interaction interface for the GWW motif of Upa2. This interaction corroborates previous findings identifying the GWW motif as essential for Upa2 co-shuttling *in vivo* (Fig. 8) [25]. The tryptophan residues within the GWW motif specifically interact with RRM1. Interestingly, a comparable interaction mode has been reported for the RRM of spliceosome-associated protein Snu17p, which binds the tryptophan residues of Bud13p [69]. Direct superposition of RRM1-GWW and Snu17p-Bud13p confirmed the similarity in binding modes [69], suggesting a common principle in RBP interaction networks. Moreover, the respective Snu17p-Bud13p *in vivo* studies indicate that the RRM interacts with RNA and Bud13p simultaneously [69]. Multivalent functionality of RBDs in mediating both RNA and protein interactions has been reported for mammalian RBPs, including the KH domain of NOVA-1 [70] and RRM2 of the splicing factor U2AF2 in complex with FUBP1 [71].

Hence, it is likely that RRM1 of Rrm4 also plays a dual role: binding RNA [14] and mediating protein-protein interactions *in vivo* (Fig. 8). This raises intriguing questions: Can RRM1 bind RNA while interacting with Upa2? Furthermore, does Upa2 merely scaffold the interaction, or does it actively modulate the RNA-binding specificity or strength of Rrm4? These questions underscore the necessity of further exploring how RNA binding and protein-protein interactions cooperate during mRNP assembly and transport stability.

To deepen the understanding of how Upa2 influences the RNA-binding capacity and specificity of Rrm4, several experimental approaches could be employed. Electrophoretic Mobility Shift Assays (EMSA) [72] could determine whether RRM1 can simultaneously bind RNA and GWW of Upa2 *in vitro*. Additionally, high-resolution structural techniques like Nuclear Magnetic Resonance (NMR) [73, 74] and cryo-electron microscopy (cryo-EM) [75, 76], combined with *in vitro* iCLIP2 [77], could provide insights into how the RNA recognition of RRM1 is modulated by its interaction with Upa2 *in vitro*. Considering the interaction between the PAM2 motifs of Upa2 and the MLLE domains of Pab1 [25], including poly(A)-tailed mRNAs and Pab1, is valuable for a comprehensive understanding of how Upa2 influences Rrm4 binding behavior and affinity (Fig. 8). However, the molecular size of the proteins and the mRNP complex could be challenging.

While these techniques would provide valuable insights into biochemical interactions *in vitro*, they may not fully capture the biological function of Upa2. Critical questions remain: Does Upa2 influence the RNA-binding affinity of Rrm4 solely through scaffolding, or does it also modulate specificity *in vivo*?

Since all previously tested mRNAs by RNA live-imaging (e.g., *ubi1*, *rho3*, *cdc3*, *cdc12*) exhibit reduced, but not abolished, shuttling in the *upa2Δ* mutant background [25], it is more likely that the interaction affinity—rather than the RNA-binding specificity of Rrm4—is affected. However, to address this question in a transcriptome-wide and precise manner, comparative *in vivo* iCLIP2 experiments of Rrm4 in the *upa2Δ* background would be highly beneficial.

Moreover, recent Upa2 pulldown assays and AlphaFold3 structural predictions of Upa2 (mutants) suggest a potential direct interaction between the effector domain of Upa2 and the novel RNA-binding protein Rrm71 (Fig. 8; S. Shanmugasundaram and Dr. T. Lenz, personal communication). This reveals a previously unrecognized regulatory dimension in endosomal mRNP formation. Interestingly, deleting the effector domain of Upa2 leads to bipolarity [25], suggesting that this interaction—and possibly Rrm71—plays a critical role in endosomal mRNA transport (Fig. 8). It is well established that RBPs enhance their RNA-binding affinity, interaction surface, and specificity through cooperative interactions with other RBPs, often mediated by their RNA-binding domains

[3, 65, 68]. Here, RRM1 of Rrm4 mediates recruitment of the mRNP scaffold protein Upa2, potentially bridging three RNA-binding proteins—Rrm4, Pab1, and Rrm71—within the same complex (Fig. 8).

In the absence of Upa2 (*upa2Δ*), the movement of Pab1—which serves as a proxy for bulk mRNAs—is reduced but remains detectable, whereas Pab1 shuttling is completely abolished in the *rrm4Δ* background, highlighting distinct dependency relationships within the transport mRNP (Fig. 8) [25]. Thus, it is plausible that all components—including Rrm71—remain present, albeit at reduced abundance, in the absence of Upa2. This suggests that Upa2 primarily functions as a stabilizer of the mRNP complex rather than as a determinant of specificity (Fig. 8). This aligns with the concept that RBPs involved in transport require exceptionally stable interactions and attachments [3].

To determine how Upa2 stabilizes mRNA transport, an *in vitro* reconstitution approach combined with Total Internal Reflection Microscopy (TIRF-M) [78] could quantitatively measure shuttling distances and dynamics in the presence or absence of Upa2 and additional factors. Such experiments would offer mechanistic insight into how scaffolding enhances mRNP transport efficiency and complex stability. This scaffolding principle likely extends to other mRNP transport systems, including neuronal late endosomal transport [79], FERRY complex-mediated early endosomal transport in HEK cells [80], and Annexin-linked processes on lysosome-like structures in neurons [81], where stable attachment and complex integrity are equally critical. Disruptions in these systems are linked to neurodegenerative diseases like Charcot-Marie-Tooth disease and ALS [79, 81] highlighting their functional significance.

Ssd1 as a conserved translational repressor during endosomal transport

The translational repressor Ssd1/GUL-1 has been characterized as a microtubule-dependent, shuttling RBP [23, 24, 37]. Its dynamic localization has been documented in various fungal systems: in *Neurospora crassa*, where GUL-1/Ssd1 forms motile aggregates along hyphae [41], and in *Sordaria macrospora*, where it co-traffics with Rab5a-positive endosomes [82]. In *U. maydis*, Rab5a-positive endosomal movement of Ssd1 is mediated by Rrm4 [23]. However, the precise anchoring mechanism and the transported mRNA regulon remained elusive.

RRM-dependent interactome profiling and live-cell imaging reveal that RRM1 of Rrm4 is critical for Ssd1 incorporation into the transport mRNP (Müntjes et al., in preparation). Furthermore, RNase treatment did not significantly disrupt the Ssd1-Rrm4 interaction, challenging the previous notion that their association is exclusively RNA-dependent [23]. Supporting this, a Ssd1-RNBΔ mutant—lacking the RNA-binding domain—retained endosomal mobility, reinforcing

the concept of an RNA-independent attachment mechanism (Müntjes et al., in preparation). While multiple lines of evidence underscore an RRM1–Ssd1 interaction, the precise molecular determinants at amino acid resolution remain elusive. In-depth structural and biochemical validation, including *in vitro* GST pulldown assays, will be essential to define the mechanistic nature of the Ssd1–Rrm4 interface (Fig. 8).

Nevertheless, a comparable functional interaction was described in *Drosophila melanogaster*, where the translational regulator dFMRP interacts with the qRRM2 domain of the multi-qRRM protein Glorund to repress translation elongation of *nanos* mRNA [83]. The RBP dFMRP is also involved in RNA transport and neuronal polarity [84], closely paralleling the mRNA transport model proposed here, and indicating a common mechanism of cooperative RBP interaction and translational regulation [4, 85, 86].

An interspecies RNA interactome comparison between *U. maydis* Rrm4 iCLIP2 data and *N. crassa* GUL-1 RIP-seq data [24] revealed a defined overlap in mRNA targets, particularly transcripts encoding proteins involved in cell wall plasticity (e.g., *gsc1*; Fig. 4). Additionally, quantitative proteomics demonstrated that Ssd1 interacts predominantly with the large ribosomal subunit and that translation elongation factors are notably reduced, indicating that Ssd1 functions as a translational elongation repressor in *U. maydis*. This aligns with its conserved role in *S. cerevisiae*, *C. albicans*, and *N. crassa*, where Ssd1 binds and represses the translation of mRNAs encoding cell wall enzymes [37, 40, 41, 42, 45, 87]. Moreover, evidence from aneuploid yeast strains lacking *SSD1*, which show hypersensitivity to the elongation inhibitor nourseothricin, highlights the regulatory role of Ssd1, particularly in translation elongation [42, 44, 45]. It is therefore highly plausible that Ssd1 regulates a subset of Rrm4-bound mRNAs, including cell wall-related targets, during transport (Fig. 8, Müntjes et al., in preparation). Furthermore, growing evidence from diverse fungal systems suggests that the cell tip functions as a hotspot for translation, particularly for mRNAs encoding secreted proteins and cell wall-related enzymes [88, 89, 90, 91]. This supports the notion that cell wall-related mRNAs are transported in a translationally regulated state and become locally translated upon arrival at the hyphal tip [37, 88, 90, 91]. A similar transport mechanism has been described for *ASH1* mRNA in *S. cerevisiae*, where the transcript is conveyed in a translationally repressed state and activated only upon arrival at the bud tip, crucial for cellular polarity [92, 93].

To gain deeper insight into the cooperative interplay between Ssd1 and Rrm4 in regulating local translation, advanced transcriptome-wide approaches—such as polysome profiling [94], translating ribosome affinity purification (TRAP) [95], or ribosome profiling (Ribo-Seq) [96, 97]—should be systematically applied across well-defined

genetic backgrounds. Moreover, subcellular techniques, including proximity-based Ribo-Seq [98] or endosome immunoprecipitation (endosome-IP)-coupled Ribo-Seq, are particularly suited to uncovering the spatial regulation of translation during endosomal mRNA transport.

To dissect the cooperative RNA-binding landscape of Ssd1 and Rrm4 in greater detail, iCLIP2 analyses across defined mutant contexts—such as Rrm4-G in Ssd1 knockdown, overexpression, and RNB domain deletion (Ssd1-RNBΔ), as well as Ssd1-G in *rrm4*Δ and RRM1 mutant backgrounds—would provide high-resolution, nucleotide-level insights into RNA-binding recognition and their coordination *in vivo* (Fig. 8C) [4, 43, 85, 99].

Together, these approaches support an expanded model of endosomal mRNA transport (Fig. 8). Rather than focusing solely on the translocation of translationally active mRNAs such as septin transcripts [18, 26], this refined framework incorporates regulon-specific translational control, particularly of mRNAs involved in cell wall plasticity [24, 37, 38, 41]. This shift enhances the mechanistic understanding of how mRNA transport and localized translation is spatially and temporally regulated during fungal growth (Fig. 8) [88, 91, 100].

The emerging role of Upf1 in endosomal mRNA surveillance

In addition, Upf1 and Rrm71, were discovered, and preliminary bioinformatic analyses further indicate their potential as novel components of the endosomal transport mRNP (Fig. 6-7). Upf1, is highly conserved across kingdoms and is a well-established nonsense-mediated decay (NMD) factor [33]. The mammalian UPF1 also localizes to the endoplasmic reticulum surface, where it participates in co-translational import and mRNA surveillance [101], suggesting that its membrane-associated localization is subject to regulatory control. Since translation and quality control mediated by UPF1 are closely coupled processes [33], studying its role in the context of endosomal translation control would be highly valuable (e.g., proximity Ribo-seq, RNA-seq, iCLIP2). Intriguingly, Upf1 association is significantly altered across all RRM mutants and upon RNase treatment, suggesting that Upf1 may function as a more global RNA-mediated interactor (Fig. 8). This observation aligns with large-scale co-IP MS studies in *S. cerevisiae*, where Upf1/Nam7 was shown to interact with a broad array of RBPs in an RNA-dependent manner [10]. Moreover, UPF1 iCLIP data from mice demonstrate predominant binding within 3' UTRs [102]—a pattern reminiscent of RRM3 of Rrm4 [20]. Consistently, mutation of RRM3 leads to the strongest reduction in Upf1 association compared to the other mutants. This raises a key question: Does the spatial distribution of RNA-binding preferences across individual RRMs influence mRNP composition? Exploring this distribution-dependent

effect may reveal fundamental principles underlying mRNP architecture and cooperative RNA-binding landscapes. Such insights would significantly deepen the mechanistic understanding of how RBP networks orchestrate mRNA recognition and regulation [5, 11, 12, 30].

Exploring the RNA-mediated interactions between Rrm4 and Rrm71

The fungal-specific RBP Rrm71 remains largely uncharacterized, underscoring the need for further investigation into its role in mRNA transport. Comparative endosomal immunoprecipitation (endo-IP MS) revealed reduced association of Rrm71 with endosomes in the mR1, mR2, and *rrm4Δ* backgrounds, with only marginal effects observed in the mR3 mutant (SI-Fig. 1, Stoffel et al., in preparation). This supports the notion that Rrm71 functions as an endosomal mRNP component and corroborates comparative co-IP MS data indicating RRM-mediated association (Fig. 2D).

Future studies should aim to clarify the subcellular localization of Rrm71 and its co-localization with Rrm4 (e.g., Rrm71-K/Rrm4-G strains), including potential dependencies on RRM1 and the Upa2 effector domain. Moreover, integrative multi-omic approaches—such as RNA-seq (e.g., *rrm71Δ*), co-IP, and iCLIP2 e.g., Rrm71 in *rrm4Δ* or *mR1* backgrounds; Rrm4 in *rrm71Δ*)—are essential to define molecular functions and interaction networks. Mapping shared targets and binding site distributions between Rrm71 and Rrm4 will help delineate RNA regulons, uncover recognition motifs, and advance understanding of mRNP transport and condensation dynamics [5, 12, 103].

Rrm4-dependent ribosomal protein recruitment: insights into local translation on endosomes

Defined mRNA transport and local translation are widely conserved processes, from yeast to neurons [30, 31, 104]. Most characterized transport mechanisms operate in a translationally silent mode [103, 105], exemplified by She2p/She3p-mediated transport of *ASH1* mRNA in yeast [93, 106] or ZBP1-associated *ACTB* in neurons [103, 107, 108]. In contrast, translationally active endosomal mRNA transport—as demonstrated for septin mRNAs in *U. maydis*—represents an emerging and underexplored field. Beyond *U. maydis*, endosomes have also been implicated as translational platforms in mammals, particularly for nuclear-encoded mitochondrial mRNAs and cytoskeleton-related targets [79, 109, 110]. Moreover, a link between endosomal mRNA transport and ribosomes has been demonstrated across diverse systems—including neurons, HEK cells, and *U. maydis* hyphae—using approaches ranging from microscopy to mass spectrometry [18, 27, 79, 80, 111, 112]. Here, the comparative co-IP MS approach provides mechanistic insights into how Rrm4 associates with ribosomal proteins, revealing an RRM-dependent

hierarchical pattern ($\text{Rrm4-G} > \text{mR3-G} > \text{mR2-G} > \text{mR1-G}$; Fig. 5A–D). Strikingly, this hierarchy closely mirrors the abundance patterns of ribosomal proteins observed in endosome immunoprecipitation mass spectrometry (endo-IP MS) samples across various Rrm4 mutant backgrounds, highlighting a dependency on Rrm4 and its individual RRMs ($\text{Rrm4-G} \approx \text{mR3-G} > \text{mR2-G} > \text{mR1-G} \approx \text{rrm4}\Delta$; Stoffel et al., unpublished).

This defined hierarchy likely reflects the distinct RNA-binding site preferences of the individual RRMs: RRM1 predominantly binds coding sequences, positioning it in proximity to ribosomes, whereas RRM3 shows higher affinity for 3'UTR regions (Fig. 8B) [20]. The proposed link between RRM-specific binding site distribution and ribosome association is unprecedented. Alternatively, RRM1 and RRM2 recognize functionally critical binding sites (RRM12-BS) [20], suggesting that mRNAs containing RRM12-BS represent prioritized transport targets more frequently undergoing local translation during transit (Fig. 8). Both scenarios may contribute to the observed pattern. Beyond its central role in recruiting mRNAs and coordinating local translation on the surface of early endosomes [18], Rrm4 operates alongside the translational repressor Ssd1 [23, 37, 82]. Comparative co-IP MS analyses and ortholog RNA interactome studies of Ssd1 and Rrm4 indicate that mRNA transport encompasses a spectrum of translational states—ranging from repressed regulons, such as those encoding cell wall enzymes [88], to transcripts undergoing active translation, including septins [26]. This model resonates with recent multi-omics studies in neuronal axons, which argue for a nuanced and dynamic regulation of translation during mRNA transport, rather than a simple binary on/off system [113].

To unravel the functional interplay between Rrm4, its RRM domains, Ssd1, ribosomal association, and the stages of translation during endosomal mRNA transport, subcellular translatoome profiling is essential [114]. Additionally, live-cell imaging of ribosomal proteins (e.g., Rpl25-G) combined with treatments using translation inhibitors—such as RocA, puromycin, nourseothricin, and cycloheximide—will allow differentiation between active and inactive ribosome populations during transport [18, 27]. This approach promises to reveal fundamental principles of local translation during endosomal trafficking, with broad implications for mitochondrial homeostasis [36, 79, 80, 109, 115] and polarized growth [18, 30, 90] in both fungal and neuronal systems.

Limitations of this study

One key limitation of this study is the incomplete characterization of Upa2's functional role within the mRNP complex. Although the data suggest that Upa2 serves as a scaffold stabilizing mRNAs through interactions with Rrm4, Pab1, and potentially Rrm71, its precise mechanistic contribution remains unresolved. Loss of

Upa2 (*upa2Δ*)—similar to *rrm4Δ* and the mR1 and mR2 mutants—leads to an increased frequency of bipolar cells [14, 25]. The prevailing model proposes that Upa2 loss weakens mRNP attachment and stability, thereby reducing mRNA abundance without altering RRM1 RNA-binding specificity. However, this hypothesis remains untested. Moreover, the interaction between Upa2 and Rrm4 has only been partially explored; it remains unclear whether the Upa2 GWW motif, RRM1, and RNA can interact simultaneously.

Conclusion and outlook

This study provides mechanistic insights into how a multi-RRM RBP utilizes its domains to orchestrate mRNP composition, integrating scaffolding functions, translational regulation, and quality control for precise endosomal mRNA transport. Future research on the cooperative RNA-binding landscapes of RBPs—critical for target specificity and regulatory dynamics—will advance our understanding of mRNA localization and local translation from fungi to neurons [2, 11, 12, 30, 31, 104, 105].

Furthermore, defining domain-specific RNA and protein interactomes of RBPs is essential for developing highly specific pharmacological strategies to target dysregulated RBPs in diseases such as neurological disorders and cancer [60, 61, 116].

MATERIALS AND METHODS

Strains and growth conditions

The laboratory strain *AB33* of *Ustilago maydis* was used as the background strain [117], as it allows the synchronization of filamentous growth by switching the nitrogen source. Pre- and main cultures were grown in complete medium (CM) containing 1% (w/v) glucose at 28°C with constant agitation at 200 rpm until an OD₆₀₀ of 0.5 was reached. For filamentous induction, the cells were shifted to a nitrate minimal medium (NM) supplemented with 1% (w/v) glucose and incubated at 28°C with constant agitation at 200 rpm for 6 hours post-induction (h.p.i) [117].

All *U. maydis* strains used for co-IP and cell biology experiments are listed in Supplementary Table 1; corresponding plasmids for strain generation are provided in Supplementary Table 2.

Microscopy

Cells were grown in 20 mL cultures to an OD₆₀₀ of 0.5, followed by induction of filamentous growth for 6 hours post-induction, as previously described. To quantify the number of shuttling particles (Gfp, mKate2, Rfp, mCherry), movies were recorded with an exposure time of 150 ms for a total of 150 frames [25]. Kymographs were generated within a 30 μm section of the hyphal cells starting from the cell tip. Only processive particles that traveled above 5 μm were counted as moving particles. Each experiment involved three biological experiments and a minimum of >8

cells per replicate. For processing and analysis of movies, kymographs, and images Metamorph software (Version 7.7.0.0, Molecular Devices, Seattle, IL, USA) were used. Statistic analyses were performed via RStudio (version R 4.4.2).

Comparative co-IP

For comparative co-IP experiments, OD₆₀₀=0.5 100 mL filamentous *U. maydis* cultures (6 h. p. i.) were produced. Harvesting and cryogenic cell disruption were performed as described in the microbial iCLIP2 protocol (resulting in 4 x 2 mL tubes, 25 mL per tube). The cryogenic cell pulver was resuspended in 800 μL co-IP lysis buffer (50 mM Tris-HCl, pH 7.4; 100 mM NaCl; 0.5% IGEPAL CA-630; 5 mM EGTA pH 8). Inhibitors were added freshly immediately before use to 2 mL lysis buffer (2× Complete protease inhibitor EDTA-free (Merck, 11873580001), 1 mM DTT, 1 mM PMSF, 2 μL SUPERase-In, Thermo Fisher AM2696, 100 μg/ml cycloheximide). The homogenous suspension was centrifuged for 10 min at 16,200 g and 4°C. The supernatant was centrifuged in a fresh, pre-cooled tube (16,200 g, 5 min, 4°C). The clear supernatants were pooled into a pre-cooled 50 mL tube. Protein concentrations of the cell lysate were measured using BCA assay (Thermo Fisher, Pierce™ 23225) after manufacturer descriptions and adjusted to match the sample with the lowest concentration (aiming for app. 2 mg/mL). 2 mL of the cell debris-free, adjusted lysate was added into a pre-cooled 2 mL tube, containing 30 μL pre-washed GFP-Trap beads (Chromotek; gtma-20). The beads were pre-washed twice with lysis buffer without inhibitors using a magnetic separation rack before use. Immunoprecipitation was conducted for one hour at 4°C with continuous rotation. Beads were attracted and washed twice with lysis buffer (without inhibitors). Beads were transferred into a fresh pre-cooled 1.5 mL tube and washed once more. The beads were resuspended in 1 mL lysis buffer (without inhibitors) and stored on ice at 4°C. 150 μL (15%) beads were added to a fresh tube, the supernatant was removed and the beads were resuspended in 20 μL 1x NuPAGE LDS including DTT according to the manufacturer's instructions (Thermo Fisher, NP0007). The boiled samples were loaded onto a 4-12% NuPAGE™ Bis-Tris gel (Thermo Fisher, NP0321) and electrophoresed at 180 V for 1.5 hours. Silver staining (Pierce™ Silver Stain Kit, 24612) was performed according to the manufacturer's description. The remaining beads (850 μL) were stored at 4°C until submission.

Optional RNase treatment

For a comparative co-IP under RNase-treated and untreated conditions, 200 mL OD₆₀₀=0.5 cultures were grown and filamentous growth was induced, and procedure as described before. The co-IP buffer (described above) was prepared without RNase inhibitor to prevent interference with subsequent RNase digestion. To preserve RNA integrity in

the untreated samples, all handling steps were performed at 4 °C and completed as quickly as possible. After immunoprecipitation and washing, the bead-solution were splitted into two test and attracted. Afterwards the beads of one of the test tubes (RNase treated sample) were resuspended in 50 µL in 1x PNK buffer (20 mM Tris-HCl, pH 7.4, 10 mM Mg₂Cl, 0.2% Tween-20) and 10 µL RNase I (Life Technologies, AM2295) and 10 µL RNase A (10 mg/ml, Boehringer Ingelheim). Control samples (untreated) were processed identically, except that no RNase was added, ensuring comparability between conditions. The beads were incubated at 37°C for 10 minutes at 1000 rpm, then washed twice with 1 mL of lysis buffer. Subsequent steps followed the standard co-IP procedure as described above.

Liquid Chromatography and Mass Spectrometric Analysis

Liquid chromatography and mass spectrometric analysis were conducted by Dr. Thomas Lenz (Molecular Proteomics Laboratory, BMFZ, Heinrich-Heine University Düsseldorf, Prof. Dr. Kai Stühler).

Western blot analysis

Protein concentrations of cell lysates were quantified using either the PierceTM BCA assay (Thermo Fisher, 23225) or the standard Bradford assay (Bio-Rad, 1976). To ensure consistency across samples, protein concentrations were adjusted to 1 mg/mL in NuPAGE LDS sample buffer supplemented with dithiothreitol (DTT; Thermo Fisher, NP0007) as per the manufacturer's protocol. Proteins were separated on a 4–12% NuPAGETM Bis-Tris gel (Thermo Fisher, NP0321) at 180 V for 1.1 hours followed by transfer onto a nitrocellulose membrane (Amersham Protran) via wet blotting using 1× NuPAGETM Transfer Buffer supplemented with 15% methanol (Thermo Fisher, NP00061) for 1 hour at 30 V. Membranes were probed with primary antibodies against RFP (1:2000; Abcam, EPR18992, rabbit), GFP (1:2000 dilution; Roche, 11814460001, mouse), and actin (1:2000; MP Biomedicals, 0869100, mouse). Secondary detection was performed using HRP-conjugated anti-mouse (1:5000; Promega, W4021) or anti-rabbit (1:5000; NEB, 7074S) antibodies. All antibodies were diluted in 1× TBS-T containing 5% milk powder. Detection was carried out using the LAS400 Image Analyzer (GE Healthcare) in combination with ECLTM Prime chemiluminescent reagent (Amersham, RPN2232), following the manufacturer's instructions.

GST-pull-down experiments

In vitro GST-pull-down was conducted following the protocol described by Devan et al. (2022) [16]. In brief, N6xHis-GST-RRM variants of Rrm4 and N6xHis-Gb1-GWW of Upa2 were expressed in *E. coli* BL21(DE3) LOBSTR cells (Kerafast EC1002). Cell pellets from a 50 ml *E. coli* culture (OD₆₀₀ = 2.5) were resuspended first

in 10 mL of buffer A (20 mM HEPES, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% IGEPAL CA630) and 5 mL of cell lysis buffer (20 mM HEPES, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% IGEPAL CA630, 1 mM PMSF, 0.1 mg/ml lysozyme, 10 µL DNase and 10 µL RNase A) with an increased amount of RNase and DNase were added. The cells were lysed through sonication (5x 30 sec on and 60 sec off, 25% amplitude) on ice and centrifuged at 16,000 × g for 20 minutes at 4 °C. The supernatant was transferred into pre-cooled fresh tubes. For GST pre-purification (bait loading), 1.2 ml of the supernatant was incubated with 100 µL of glutathione sepharose (GS) resin (GE Healthcare), pre-equilibrated in buffer A (see above), at 4 °C with continuous rotation for 1 hour. The GS resin suspension was then washed three times with 1 mL of buffer and followed by centrifugation for 1 min at 4°C and 13,000 rpm. The supernatant was soaked off. Next, the N6xHis-Gb1-GWW cell lysate was added to the N6xHis-GST-RRM variant-bound resin and incubated for 1 hour at 4°C with continuous rotation (co-precipitation). Afterward, the resins were three times washed with buffer A and centrifuged (1 min, 4 °C, 13,000 rpm). The washed co-complex resin (RRM variants and GWW) was resuspended in 100 µL of 2x Laemmli loading buffer, boiled at 95°C for 10 minutes, centrifuge for 1 min, room temperature at 4,000 rpm, and analyzed via SDS-PAGE and subsequently Coomassie staining and/or Western blot analysis [16, 36]. For Western blot analysis, mouse antibodies α -His (1: 4000, Sigma, H1029) and HRP conjugate α -mouse (1: 5000 dilution; Promega; W4021) were used as secondary antibody.

Radioactive PNK assays of Ssd1-G and Rrm4-G

For UV-crosslinking of Ssd1-Gfp-RNA or Rrm4-Gfp-RNA complexes, 150 mL sporidia (OD₆₀₀=0.5) and/or 150 mL hyphae culture (OD₆₀₀=0.5; 6 h.p.i) were used. The PNK assays as well as RNase treatment and RNA-recovery of Rrm4-Gfp-RNA complexes, are detailed in the microbial iCLIP2 protocol for Rrm4 [36]. Minor adaptations were made regarding radioactive labeling and NuPAGE running/blotting conditions for Ssd1-Gfp-RNA. (I) The complete samples were radioactively labeled. (II) The samples were loaded onto a 4-12% NuPAGETM Bis-Tris gel (Thermo Fisher, NP0321) and run for 2 hours at 170 V. The gel was wet-blotted onto a nitrocellulose membrane (Amersham Protran) using 1x NuPAGETM Transfer buffer + containing 20% Methanol (Thermo Fisher; NP00061) for 2 hours at 30 V. The nitrocellulose membrane was washed with ice-cold 1x PBS buffer, covered with a transparent plastic bag, and exposed to a BAS Storage Phosphor Screen (GE Healthcare) overnight at - 20°C. The screen was visualized by a TyphoonTM laser scanner (Cytiva), according to the manufacturer's instructions.

RIP-seq Ssd1-G versus iCLIP2 Rrm4-G

Neurospora crassa GUL-1 RIP-seq data from Herold et al., 2021 [24], restricted to significantly enriched mRNAs (n=1,048), were used to identify clear *U. maydis* orthologs via g:Profiler and the associated R package gprofiler2 [46, 118], resulting in ortholog 487 targets. These identified targets (n=487) were subsequently compared to functional binding site (RRM12-BS; n=1,630) – containing Rrm4 iCLIP2 targets [20].

mRNA co-expression analysis of infectious *U. maydis* from Lanver et al., (2018)

Ustilago maydis RNA-seq data from the infectious strain SG200, covering a time course of up to 12.5 days post-induction, were generated by Lanver et al. (2018) [49]. The normalized DESeq counts for the mRNAs *rrm4* and *ssd1* were plotted over time to assess their co-expression behavior. The respective standard deviation is indicated.

Structural prediction by AlphaFold3

The structural modeling was generated by AlphaFold3 [39], using default settings and the following protein identifiers: Rrm4 (A0A0D1DWZ5), Ssd1 (A0A0D1EAA3), Upa2 (A0A0D1E015), Upa1 (A0A0D1DRJ3), Rrm71 (A0A0D1CFN7), Upf1 (A0A0D1EBS5). Superpositions and visualization were performed using the Python-based program PyMol (The PyMOL Molecular Graphics System, Version 2.1.) for Ssd1 and the yeast homolog (P24276) with domain description adapted from Bayne et al., (2022) [40]. For superpositions of *U. maydis* Upf1 (A0A0D1EBS5) the human homologous UPF1 (Q92900) with a detailed description of domain annotation adapted from Langer et al., (2024) [50] were used.

Bioinformatic tools and data analysis

The prediction of potential protein-interaction networks and GO term analysis was determined by the use of STRING (<https://string-db.org/>, version 12.0, [55]) and g:Profiler [46], considering an adjusted p-value under 0.05 as statistically significant. Additional orthology analyses were performed using the webpage-based g:Profiler [46, 118] and through the respective R package (g:profiler2; [46]).

Data analysis was performed using RStudio (version R 4.4.2). Data visualization and statistical analysis were conducted using the R packages ggplot2, tidyr, readr, dplyr, stringr, purrr, and forcats.

COMPETING INTERESTS

No competing interest is declared.

AUTHOR CONTRIBUTIONS

NKS (Nina Kim Stoffel), TL (Thomas Lenz), FA (Florian Altegoer), and MF (Michael Feldbrügge) designed the study.

NKS and TL performed co-IP MS experiments and analyzed the data under the guidance of KST (Kai Stühler). NKS conducted wet in-vitro GST experiments with support from SS (Sainathan Shanmugasundaram) and FA. *U. maydis* strains were planned by MKT (Markus Tulinski), KM (Kira Müntjes), and NKS, with support from MF. NKS performed microscopy and data analysis of Upa2 movement. MKT discovered Ssd1. NKS and TL discovered Rrm71 and Upf1. NKS, SS, MF and FA discovered the GWW Upa2 and RRM1 Rrm4 interaction. NKS performed structural and bioinformatic analyses under the guidance of TL and FA. NKS drafted and revised the manuscript with input from TL and FA. MF, FA, and KST contributed funding and resources.

ACKNOWLEDGMENTS

Thanks to Drs. Alexander Steffen, Franziska Kuhnert, Marten Exerkate, Senthil Devan, and Sainathan Shanmugasundaram for valuable input and support regarding co-IP MS analysis and *E. coli* protein expression. Grateful acknowledgment to Caroline Downes, Maximilian Neumann, and Marie Wittmann for contributions to the phenotypic analysis of the RRM mutants and plasmid generation.

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SUPPLEMENTARY INFORMATION

SI-Fig. 1: RRM mutants.

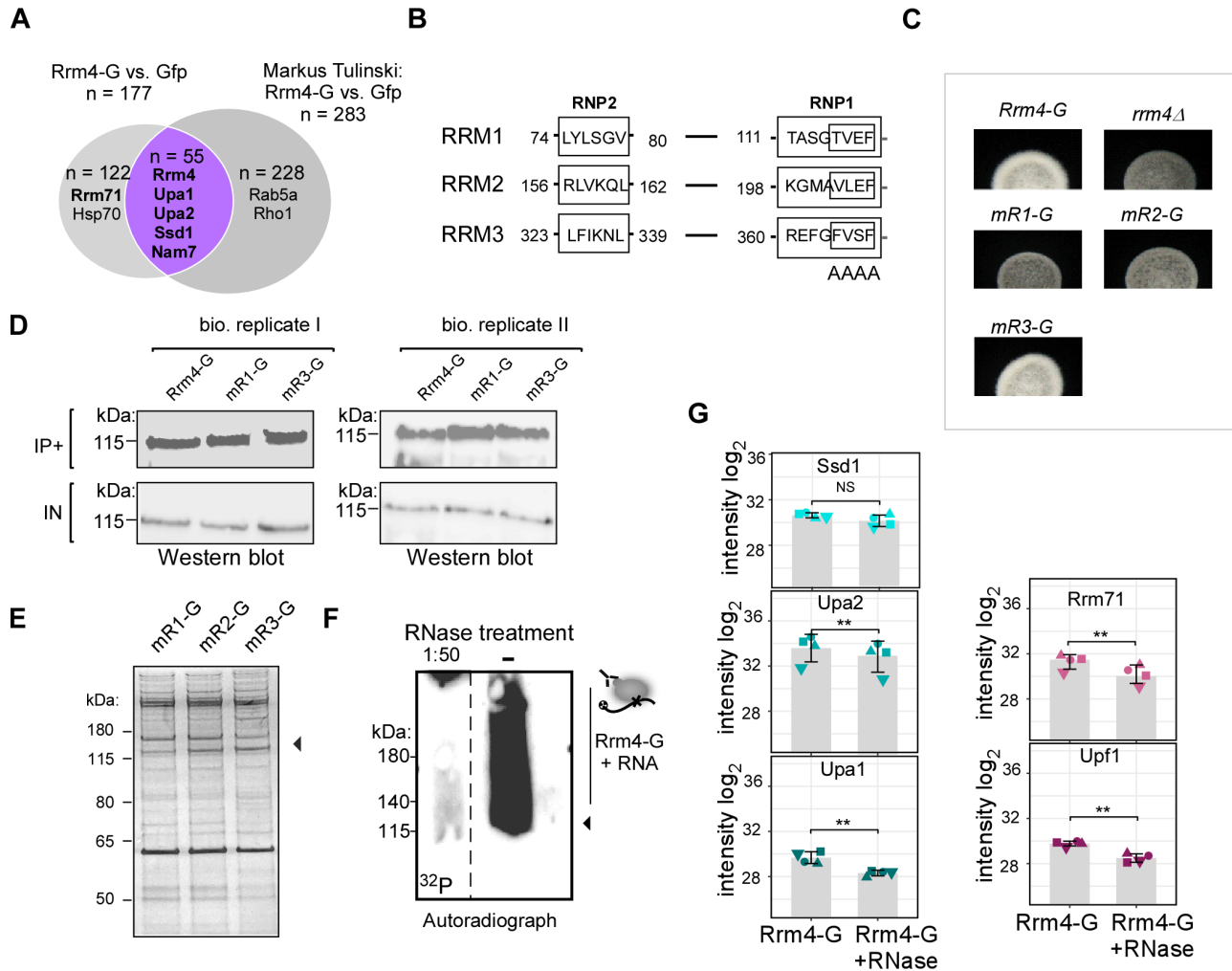
SI-Fig. 2: Specificity of Rrm4-mediated interactions

SI-Table 1: *Ustilago maydis* strains.

SI-Table 2: Plasmids.

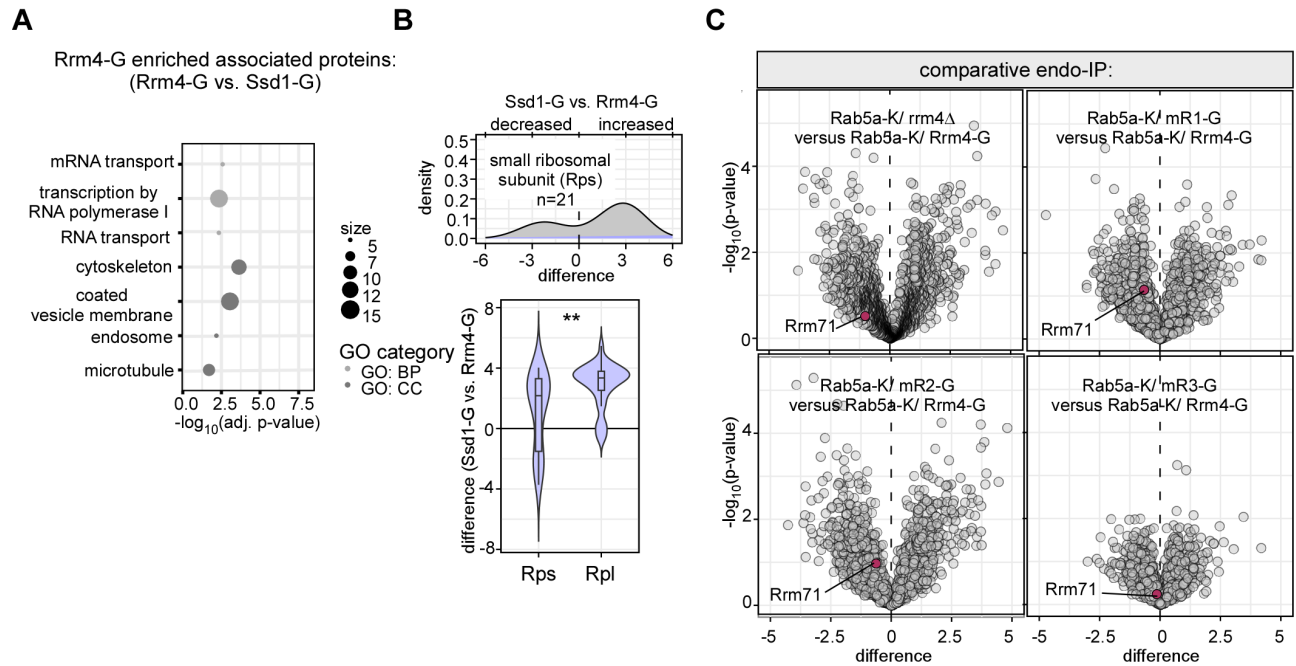
SI-Table 3: Comparative co-IP MS data for Rrm4-G, mR1-G, mR2-G, mR3-G, Ssd1-G, GFP, Upa1-G/mMLLE3-K and RNase-treatment are provided as a separate Excel file due to the large dataset.

SI-Table 4: Ortholog-based Ssd1 interactome study vs Rrm4-BS are provided as a separate Excel file due to the large dataset.



SI-Fig. 1. RRM mutants

(A) Venn diagram displaying the overlap of significantly enriched co-immunoprecipitated proteins ($n=177$; difference > 1 ; $-\log_{10}(\text{p-value}) > 1.3$; paired) identified in Rrm4-G vs. Gfp pull-down datasets from this study (left) and prior co-IP dataset conducted by Markus Tulinski (right) [23]. (B) Schematic of the RNP2 and RNP1 motifs within RRM1–3 of Rrm4. Alanine mutations ($\rightarrow$ AAAA) in the respective motifs are indicated. (C) Colony morphology on NM-charcoal plates displaying hyphal growth of *U. maydis* strains expressing Rrm4-G or its RRM mutants. Wild-type-like filamentation (white, fluffy colonies) is observed in strains expressing Rrm4-G and mR3-G, whereas impaired hyphal growth (transparent colonies) is evident in strains expressing mR1-G, mR2-G, or in the absence of Rrm4 (*rm4 Δ*). In collaboration with C. Downes, M. Neumann and M. Wittmann. (D) Western blot analysis of Rrm4-G, mR1-G, and mR3-G from immunoprecipitated (IP+) samples and input cell extracts (IN). (E) Silver-stained SDS-PAGE of immunoprecipitated mR1-G, mR2-G, and mR3-G, showing co-purified proteins. (F) Autoradiograph of UV-crosslinked Rrm4-G–RNA complexes with (1:50) and without RNase treatment (-). (G) Scatterplots of log₂ intensity values from co-IP MS analysis comparing Rrm4-G with and without RNase treatment (four biological replicates).



SI-Fig. 2. Specificity of Rrm4-mediated interactions

(A) GO term analysis (g:Profiler) [46, 118] of proteins enriched in Rrm4-G co-IPs (Rrm4-G vs. Ssd1-G; difference > 1; $-\log_{10}(\text{p-value}) > 1.3$). (B) Density plot of difference values comparing Ssd1-G and Rrm4-G, focusing on small ribosomal subunit (Rps) proteins. The violin plot compares Rps and large ribosomal subunit (Rpl; see Fig. 5F) protein abundances in Ssd1-G versus Rrm4-G. (C) Volcano plots from comparative endo-IP MS highlighting enrichment patterns of the novel RBP Rrm71 (Stoffel, et al., unpublished).

Table 1. *Ustilago maydis* strains

Name/Identifier	Description	Publication
AB33_rrm4-egfp (UMa274)	expressing Rrm4 C-terminally fused to eGfp, endogenous promotor, endogenous locus	[14]
AB33_mR1-egfp (UMa311)	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM1: TVEF (116-119 aa) to AAAA, endogenous promotor,, endogenous locus	[14]
AB33_mR2-egfp (UMa312)	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM2: VLEF (201-205 aa) to AAAA, endogenous promotor, endogenous locus	[14]
AB33_mR3-egfp (UMa313)	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM3: FVSF (362-368 aa) to AAAA, endogenous promotor, endogenous locus	[14]
AB33_upa1-egfp/mMLLE3-mkate2 (UL167)	Upa1 C-terminally fused to eGfp, endogenous promotor, endogenous locus; in an mLLE3 mutated variant of Rrm4 C-terminally fused to mKate2	[16]
AB33_egfp (UMa487)	expressing eGfp	[21]
AB33_ssd1-egfp (UMa2700)	expressing Ssd1 C-terminally fused to eGfp, endogenous promotor, endogenous locus	[23], Müntjes et al., in preparation
AB33_ssd1-egfp/mR1-mkate2 (UMa3219)	expressing Ssd1 C-terminally fused to eGfp, endogenous promotor, endogenous locus; in an mR1 C-terminally fused to mKate2 mutant strain	Müntjes et al., in preparation
AB33_upa2-egfp (UMa453)	expressing Upa2 C-terminally fused to eGfp, endogenous promotor, endogenous locus	[25]
AB33_upa2-egfp/rrm4Δ (UMa515)	expressing Upa2 C-terminally fused to eGfp, endogenous promotor, endogenous locus in an rrm4 deletion background	[25]
AB33_upa2-egfp/mR1-rfp (UMa896)	expressing Upa2 C-terminally fused to eGfp, endogenous promotor, endogenous locus; in an mR1 C-terminally fused to Rfp mutant strain	[25]
AB33_upa2-egfp/mR2-mkate2	expressing Upa2 C-terminally fused to eGfp, endogenous promotor, endogenous locus; in an mR2 C-terminally fused to mKate2 mutant strain	this study, plasmid pUL192, UG/NKS
AB33_upa2-egfp/mR3-mkate2	expressing Upa2 C-terminally fused to eGfp, endogenous promotor, endogenous locus; in an mR3 C-terminally fused to mKate2 mutant strain	this study, plasmid pUL193, UG/NKS

Note: plasmid descriptions are listed in Table 2

Table 2. Plasmids

Name/Identifier	Description	Publication
Prrm4:Rrm4_mR2_mKate2_HA_G418R (pUL192)	Plasmid vector for generating a mutant version of <i>rrm4</i> C-terminal fused with mKate2_HA. Mutation in RRM2 -RNP1: VLEF (201-205 aa) to AAAA, construct under control of the endogenous promotor of <i>rrm4</i> .	[20]
Prrm4:Rrm4_mR3_mKate2_HA_G418R (pUL193)	Plasmid vector for generating a mutant version of <i>rrm4</i> C-terminal fused with mKate2_HA. Mutation in RRM3: FVSF (362-368 aa) to AAAA, construct under control of the endogenous promotor of <i>rrm4</i>	[20]

7.1 Author contributions

Figure 1

(A) Graphical visualization designed by NKS. (B–E) Comparative co-IP MS analysis performed by NKS and TL. Data analysis and visualization by NKS with input from Prof. Dr. Michael Feldbrügge (MF).

Figure 2

(A) Graphical visualization designed by NKS. (B–E) Comparative co-IP MS analysis performed by NKS and TL. Data analysis and visualization by NKS with input from MF. (H) Graphical visualization designed by NKS.

Figure 3

(A–B) Microscopy analysis performed by NKS. (C–D) co-IP MS analysis performed by NKS and TL. Data analysis and visualization by NKS. (E) Structural modeling performed by NKS in collaboration with Dr. Florian Altegoer (FA). (F) Microscopy analysis performed by NKS. (G) In vitro analysis performed by NKS with input from Sainath Shanmugasundaram (SS) and FA.

Figure 4

(A–B) Domain analysis performed by NKS. (C) Microscopy analysis performed by NKS. (D–E) co-IP MS analysis performed by NKS and TL. Data analysis and visualization by NKS. (F–I) RNA interactome study performed by NKS.

Figure 5

(A) Graphical visualization designed by NKS. (B–F) Comparative co-IP MS analysis performed by NKS and TL. Data analysis and visualization by NKS.

Figure 6

(A–G) Bioinformatic analysis performed by NKS.

Figure 7

(A–E) Bioinformatic analysis performed by NKS.

Figure 8

Graphical visualization designed by NKS.

SI-Figure 1

(A) Comparative analysis and visualization performed by NKS. (B) Graphical visualization designed by NKS. (C) Morphology analysis conducted by C. Downes, M. Neumann, and M. Wittmann under the guidance of NKS. Data visualized by NKS. (D–F) Biochemical analysis performed by NKS. (G) Comparative co-IP MS analysis performed by NKS and TL. Data analysis and visualization by NKS with input from MF.

SI-Figure 2

(A–C) Comparative co-IP/endo-IP MS analysis performed by NKS and TL. Data analysis and visualization by NKS with input from MF.

Supplementary Tables:

SI-Tables 1–2: Data analysis compiled by NKS.

SI-Table 3: Data analysis compiled by NKS and TL.

SI-Table 4: Data analysis compiled by NKS.

Due to the large amount of data, Supplementary Tables 3–4 are provided separately.

Literature Research:

NKS conducted the literature review.

Writing:

NKS wrote the manuscript, with proofreading support from FA and TL. Text passages and figures related to comparative co-IP MS (Figures 1–2, SI Figure 1), which will be published in Müntjes et al. (manuscript submitted to *PNAS*), were prepared with input from MF.

8 Endosomal pre-tRNA trafficking promotes subcellular splicing at mitochondria

This manuscript is submitted to *Nucleic Acids Research*:

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Endosomal pre-tRNA trafficking promotes subcellular splicing at mitochondria

Nina Kim Stoffel¹, Melissa Vázquez-Carrada¹, Thomas Lenz^{2,3}, Kira Müntjes¹, Johannes Postma¹, Srimeenakshi Sankaranarayanan^{1,4,5}, Kai Stühler^{2,3}, Kathi Zarnack^{4,5}, Julian König^{5,6}, Michael Feldbrügge¹

¹Institute of Microbiology, Heinrich Heine University Düsseldorf, 40204 Düsseldorf, Germany.

²Molecular Proteomics Laboratory, Biological Medical Research Center, Heinrich-Heine-University Düsseldorf, 40225 Düsseldorf, Germany.

³Institute for Molecular Medicine, Proteome Research, University Hospital and Medical Faculty, Heinrich-Heine-University Düsseldorf, 40225 Düsseldorf, Germany.

⁴Buchmann Institute for Molecular Life Sciences (BMLS) & Institute of Molecular Biosciences, Goethe University Frankfurt, Max-von-Laue-Str. 15, 60438 Frankfurt, Germany.

⁵Theodor Boveri Institute for Biosciences, Biocenter, Julius Maximilians University Würzburg, Am Hubland, 97074 Würzburg, Germany.

⁶Institute of Molecular Biology gGmbH, Ackermannweg 4, 55128 Mainz, Germany.

Running title: endosomal pre-tRNA^{iMET} transport and splicing

Keywords: isodecoder / pre-tRNA / tRNA binding / tRNA localization / tRNA splicing / tRNA transport

Corresponding author:

Dr. Michael Feldbrügge

Institute of Microbiology,

Heinrich Heine University Düsseldorf, 40204 Düsseldorf, Germany

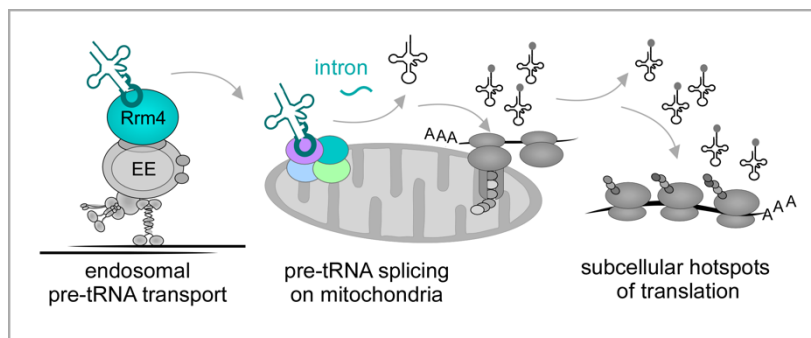
Phone: +49 (211) 81-15475

feldbrue@hhu.de

Abstract

Nuclear-encoded transfer RNAs (tRNAs) are essential for translation, and their maturation is tightly regulated. Disruptions in pre-tRNA processing are linked to diverse cellular dysfunctions, ranging from neurological disorders to fungal growth defects. A crucial maturation process is intron removal by the conserved tRNA splicing endonuclease (SEN) complex. Despite the well-established splicing function, its subcellular localization is remarkably diverse, occurring in the nucleus, cytoplasm, and at mitochondrial surfaces. However, how pre-tRNAs traverse the crowded cytoplasm to reach spatially distinct processing sites has remained unclear. Here, we uncover active long-distance pre-tRNA^{iMET} transport in highly polarized cells. In hyphae of the fungal pathogen *Ustilago maydis*, the RNA-binding protein Rrm4 - previously known for mediating endosomal mRNA transport - binds sequence-specifically to intronic regions of both unspliced initiator tRNA isodecoders (pre-tRNA^{iMET}). The pre-tRNAs thereby hitchhike on motile endosomes, ensuring their precise delivery to mitochondria-associated SEN complexes. Loss of Rrm4 disrupts endosomal association of pre-tRNAs, compromises their splicing efficiency, and perturbs translational homeostasis. Together, these findings reveal that mRNAs and pre-tRNAs share a common trafficking pathway, uncovering an unexpected layer of spatiotemporal regulation of translation through the coordinated transport, localization, and processing of tRNAs.

Graphical abstract



Introduction

Nuclear-encoded transfer RNAs (tRNAs) represent one of the most abundant noncoding RNAs, indispensable for cytoplasmic translation and fundamental to cellular organization. Traditionally viewed as ubiquitous housekeeping molecules uniformly distributed throughout the cell, this paradigm is currently being redefined. Emerging evidence reveals that tRNA abundance is finely regulated and dynamically modulated during development (1), in response to cellular stress (2), and in a cell-type-specific manner (3,4), underscoring its broad roles in cellular adaptation and regulatory complexity. Defects in tRNA abundance (1), tRNA biogenesis factors (5), or their aminoacylation (6) are linked to a range of neural disorders and cancer (7), highlighting the importance of precise regulatory mechanisms.

tRNA biogenesis begins in the nucleus with transcription by RNA polymerase III (8) and proceeds through a highly orchestrated maturation pathway. This includes CCA addition at the 3' acceptor stem (9), extensive nucleotide modifications (10), intron removal when present (11), potential additional modifications (12), and aminoacylation (13) to generate fully functional, translation-competent tRNAs.

Although often overlooked, intron-containing precursor-tRNAs (pre-tRNAs) undergo splicing, a critical step in their maturation (11,14,15). The proportion of intron-containing pre-tRNAs varies markedly across species, accounting for approximately 22% of the tRNA repertoire in yeast and 5–7% in mammals such as mouse and human (16,17). Regardless of its species-specific prevalence, pre-tRNA splicing is indispensable for cellular fitness, as introns disrupt the anticodon stem-loop that is essential for mRNA decoding (11,14-16,18-20). However, unlike pre-mRNA introns, which are removed by the major spliceosome, the introns of the nuclear pre-tRNAs are exclusively removed by proteinaceous enzymes, the tRNA splicing endonucleases (TSEN or SEN complex, in human or yeast, respectively) (11,21,22). The highly conserved (T)SEN complex consists of the four subunits Sen2, Sen15, Sen34, and Sen54, which precisely excise introns positioned between the nucleotides 37 and 38 of the mature tRNA (11,23). Splice site recognition is not based on specific sequence motifs but instead determined by the spatial positioning of the intron, following a ruler mechanism (22,24-26).

Despite evolutionary conservation, the (T)SEN complex displays divergent localizations across eukaryotes (16,19,21). In plants, the tRNA splicing machinery localizes to both the cytoplasm and the nucleus (27). Although the human complex has long been considered primarily nuclear (15), emerging evidence suggests that both TSEN and pre-tRNAs are also present in the cytoplasm (18). In budding and fission yeasts, the SEN complex is anchored to the cytosolic surface of mitochondria (23), highlighting differences in subcellular localization.

Given that the tRNA splicing machinery is at least partially localized outside the nucleus, efficient delivery to these sites is essential. However, in contrast to extensive knowledge of mRNA transport (28-32), the mechanisms guiding pre-tRNAs to their destination remain largely unresolved. Are pre-tRNAs actively transported?

A prominent mRNA transport mechanism is endosomal hitchhiking, as described in polar-growing hyphae of the fungal pathogen *Ustilago maydis* (33-36). Rab5a-positive endosomes function as dynamic platforms for bidirectional mRNA transport along microtubules, a pivotal process for maintaining polarized growth and sustaining mitochondrial function (34,37,38).

Key endosomal mRNA cargos include septin transcripts, e.g., *cdc12*, which are crucial for establishing cell polarity, and nuclear-encoded mitochondrial mRNAs like *atp3*, which encode ATP synthase subunits vital for mitochondrial bioenergetics (33,38). The key endosomal mRNA transporter is the RNA-binding protein Rrm4, which contains three C-terminal MLLE domains for endosomal attachment and three N-terminal RNA recognition motifs (RRMs) for mRNA interaction (Fig. 1A) (36,39). Our mutant-based comparative iCLIP2 analysis revealed that the third RRM domain (RRM3) binds UAUG motifs within target mRNAs with high specificity (38,40). However, this interaction appears to primarily confer binding stability rather than exerting a direct functional effect. In contrast, binding sites (BS) recognized by RRM1 and RRM2 and supported by RRM3, termed RRM12-BS, are essential for mRNA transport and abundance. These interactions regulate key cellular processes, including unipolarity, cell wall plasticity, and mitochondrial physiology (33,34,38,40).

In recent years, evidence of endosomal mRNA hitchhiking has extended beyond *U. maydis* to plants and mammalian neurons, frequently linked to mitochondrial supply (38,41-44). Besides mRNAs, non-coding RNAs such as pre-microRNAs are also transported on endosomes in neurons (45), suggesting that endosomal hitchhiking is not exclusive to mRNA trafficking. This raises the intriguing question: Do tRNAs similarly to mRNAs exploit cytoplasmic transport pathways to achieve spatial regulation and function?

Materials and methods

Strains and cultivation

Laboratory strain *U. maydis* AB33 was used as a background strain, allowing synchronized hyphal induction by switching the nitrogen source (46). Sporidia cells were grown in complete medium (CM) containing 1% (w/v) glucose at 28°C, 200 rpm, until reaching an OD₆₀₀ of 0.5 (46). For hyphal induction, cells were shifted to a nitrate-minimal medium (NM) (47) containing 1% glucose and incubated at 28°C with constant agitation at 200 rpm for 6 hours post-induction (h.p.i.) (46). Strains expressing Rab5a-K were generated using an integrative plasmid encoding an N-terminal mKate2–Rab5a fusion protein (Rab5a-K) under control of a constitutively active promoter (SI-Table S1) (48). The construct was inserted at the ectopic *ip* locus, and transformants were selected using carboxin resistance (49). For C-terminal green fluorescent protein (Gfp) tagging of Sen54 (Sen54-G) and Sen15 (Sen15-G), integrative plasmids were constructed for the *sen54* and *sen15* loci, respectively (SI-Table S1). Transformants were selected via hygromycin resistance. Insertions were confirmed through PCR and Southern blot analysis. All *U. maydis* strains used or generated in this study, along with corresponding plasmids, oligonucleotides, and progenitor strains required for their construction, are listed in SI-Tables S1–3.

Microscopy

Laser-based epifluorescence microscopy was conducted using a Zeiss Axio Observer.Z1. Sporidia cells were grown in 20 mL cultures to an OD₆₀₀ of 0.5, followed by induction of filamentous growth for 6 h.p.i (35,46). To quantify the number of shuttling fluorescent particles (Gfp, mKate2), movies were recorded with an exposure time of 150 ms for a total of 150 frames (39,50). Kymographs of signals originating from Rab5a-K were generated within a 15 µm section of the hyphal cells starting from the cell tip (50). Only signals that traveled >5 µm were considered progressively moving particles. Each procedure was performed in three biological replicates with a minimum of 8 cells per replicate. For statistical analysis, an unpaired t-test was applied. Processing and analysis of movies, kymographs, and images were conducted with Metamorph software (Version 7.7.0.0, Molecular Devices, Seattle, IL, USA).

TEM:

Endo-IP samples (see below) were diluted 1:20 in endo-IP buffer, applied to grids, negatively stained with 2% uranyl acetate and lead citrate, and examined using a transmission electron microscope (Zeiss TEM 902, Germany) operated at 80 kV.

Mitochondrial staining:

For mitochondrial staining, 1 mL of hyphal culture (OD₆₀₀=0.5; 6 h.p.i.) was transferred to a light-protected test tube, and 1 µL of 15 nM TMRE (Thermo Fisher Scientific, T669) was added. The culture was incubated for 20 minutes at 28 °C with constant rotation. Cells were

then harvested by centrifugation (5 min, 5,000 g, room temperature), washed twice with 1 mL PBS (phosphate-buffered saline), and resuspended in PBS before microscopy. Three independent biological experiments were performed, each including at least eight hyphal cells

Translation inhibitor assay:

U. maydis sporidia (SI-Table S1) cells were grown in 20 mL to an OD₆₀₀ of 0.5 and shifted to NM for hyphal induction, as described previously (46). After 2 h.p.i., 0.5 μ M rocaglamide A (RocA, MedChemExpress, HY-19356) or 0.5 μ M cycloheximide (CHX, Merck KGaA, 239763-M) was added. Incubation was continued for an additional 4 h before microscopic analysis (6 h.p.i.). Three independent biological experiments were performed, and hyphal length was measured for 50 cells in each experiment.

Comparative endo-IP

For the comparative endo-IP experiments, 300 mL filamentous *U. maydis* cultures were prepared as described previously (OD₆₀₀=0.5; 6 h.p.i.). The cultures were harvested by centrifugation at 7,150 g, for 10 min at 4 °C. Cells were resuspended in 10 mL pre-cooled PBS and split into ten 2 mL reaction tubes (51). After centrifugation at 7,150 g, for 5 min at 4 °C, the supernatant was removed, and the tubes were placed in liquid nitrogen and stored at -70 °C.

For mechanical cell disruption, frozen cell pellets were mixed with a scoop containing approximately 15 steel beads (1.5 mm) and then disrupted in a cryo-mill at 2× 30 Hz, with an intermediate freezing step in liquid nitrogen. The pulverized cell material was resuspended in 1 mL endo-IP buffer (2× Complete protease inhibitor EDTA-free [Merck, 11873580001], 1 mM DTT, 1 mM PMSF, 2 μ L SUPERase-In [Thermo Fisher Scientific, AM2696], 3 μ L RiboLock [Thermo Fisher Scientific EO0382], 100 μ g/mL cycloheximide, Pierce Protease and Phosphatase Inhibitor [Thermo Fisher Scientific A32961]) and homogenized at 4 °C using a Vibrax. Cell debris was removed by centrifugation at 7,150 g for 3 min at 4 °C, and the lysate was transferred to fresh pre-cooled 50 mL tubes. The lysate was split into five pre-cooled 2 mL tubes and centrifuged at 5,500 g for 30 min at 4°C. The supernatant was collected within a pre-cooled 50 mL Falcon tube. The protein concentration of the lysate was measured using a BCA assay (Thermo Fisher Scientific, Pierce™, 23225) and adjusted to approximately 2.5–3.0 mg/mL (cell extract, CE). Five percent of the adjusted cell extract was frozen and stored for use as an input control.

For immunoprecipitation, 5x 20 μ L RFP-magnetic particles (Chromotek, M-270) were pre-washed twice with endo-IP buffer (without inhibitors). 2 mL of the concentration-adjusted lysate was added to a pre-cooled 2 mL tube containing RFP-magnetic particles (Chromotek, M-270) and incubated at 4 °C for 1 h under continuous overhead rotation.

The magnetic particles were collected and washed twice with 1 mL endo-IP buffer using a magnetic rack. The suspended magnetic particles were transferred to a fresh pre-cooled 1.5 mL tube and washed once more. The beads were resuspended in 1 mL endo-IP buffer and stored on ice. 150 μ L (15 %) of the beads were transferred to a fresh tube, the supernatant was completely removed, and the beads were resuspended in 20 μ L 1x NuPAGE LDS containing DTT according to the manufacturer's instructions (Thermo Fisher Scientific, NP0007). The

samples were heated (10 min, 70°C), loaded onto a 4-12% NuPAGE™ Bis-Tris gel (Thermo Fisher Scientific, NP0321), and subjected to gel electrophoresis (180 V, 1 h). Silver staining (Pierce™ Silver Stain Kit, 24612) was performed according to the manufacturer's instructions. The remaining beads (850 µL, sample: endo-IP) were stored at 4°C until analysis via liquid chromatography mass spectrometry (LC-MS) based proteomics or used for RNA preparation (see below).

Liquid chromatography-mass spectrometry-based proteomics

Sample preparation:

The beads were magnetically collected, and the supernatant was removed. The beads were washed four times by resuspension and magnetic collection using 200 µL 80% ethanol, respectively, followed by a single wash step using 200 µL HPLC grade acetonitrile. On-bead digestion of proteins was performed by keeping the beads suspended by overhead rotation in 20 µL of 50 mM triethylammonium bicarbonate (TEAB) containing 0.1 µg trypsin for 18 h at 37°C and, after the addition of an extra 2 µL 50 mM TEAB containing 0.1 µg trypsin, for a further 3 h at 37°C. The beads were magnetically retained, and the supernatants containing the peptides were transferred to new tubes. To analyze the same amount of peptides per LC-MS run, 2.5% of the respective peptide solutions were considered as quality control and analyzed by LC-MS, and, by considering the median MaxQuant protein group intensity per run, the volumes of the peptide solutions to be analyzed by LC-MS were adjusted to contain the same peptide amount. The peptides were dried (SpeedVac concentrator) and reconstituted in 17 µL 0.1% trifluoroacetic acid for injection into the LC-MS system.

LC-MS analysis:

NanoLC-MS/MS in data-dependent acquisition (DDA) mode was used for label-free peptide/protein identification and quantification: Peptides were separated on a liquid chromatography system (Ultimate 3000, Thermo Fisher) by, first, concentrating the peptides on a trap column (Acclaim PepMap100, 3 µm C18 particle size, 100 Å pore size, 75 µm inner diameter, 2 cm length, Thermo Fisher Scientific, Dreieich, Germany) using a flow rate of 6 µL/min for 10 min with 0.1% TFA as mobile phase. Actual peptide separation was then performed on the analytical column (Acclaim PepMapRSLC, 2 µm C18 particle size, 100 Å pore size, 75 µm inner diameter, 25 cm length, Thermo Fisher Scientific, Dreieich, Germany) heated to 60 °C using a flow rate of 300 nL/min and a 1 hour gradient from 4 to 40% solvent B (solvent A: 0.1% (v/v) formic acid in water; solvent B: 0.1% (v/v) formic acid, 84% (v/v) ACN in water).

Eluted peptides were processed via a nano-source electron spray ionization (ESI) interface equipped with distally coated SilicaTip emitters (New Objective, Woburn, MA, USA) into the online-coupled Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany; Software: Xcalibur 4.7, SII 1.7, Orbitrap Fusion Lumos 4.7), which was equipped with a field asymmetric ion mobility spectrometry (FAIMS) interface (standard resolution, 4.6 total carrier gas flow, compensation voltage (CV): -50 V) and operated in positive mode with a static spray voltage and advanced peak determination enabled. Precursor

mass spectra were recorded in the Orbitrap analyzer within a scan range of 375–1500 m/z and a resolution of 120,000 (maximum injection time 60 ms, automatic gain control target value 400,000, profile mode). Precursors with charge states +2 to +7 and a minimum intensity of 20,000 were isolated with a 1.6 m/z isolation window and were fragmented via higher energy collisional-induced dissociation with a normalized collision energy of 35%. Precursors that were chosen for fragmentation were excluded from further isolation for the next 60 s. MS2 spectra were recorded with a resolution of 15,000 in the Orbitrap in centroid mode with a maximal injection time of 22 ms and a target value for the automatic gain control set to 50,000. The desired minimum points across the peak were set to six for all MSn (n = 1–2) levels.

Data analysis:

MaxQuant (version 2.5.0.0, Max Planck Institute for Biochemistry, Planegg, Germany) was used for peptide/protein identification and quantification, employing an *Ustilago maydis* sequence database (UniProtKB, downloaded on 30/08/2023, 6805 entries) extended with mutant specific sequences (fusion proteins were represented by separate sequences for the fused proteins) using tryptic cleavage specificity (C-terminal to K and R) and a maximum of two missed cleavage sites. Methionine oxidation and N-terminal acetylation were considered as variable modifications. Match between runs was applied. A false discovery rate of 1% on protein and peptide levels was set as the identification threshold using the reversed decoy database approach. Statistical analysis was performed based on experiment- median log₂ (fold change) normalized (against experiment "Rab5a-mKate2/Rrm4-Gfp" replicate 4 as reference) MaxQuant log₂-transformed protein group intensities using the "R" (v4.4.0) programming language after removing potential contaminants, reverse hits, and proteins only identified by modified peptides (1,919 protein groups). Principal component analysis (PCA) was performed using the *prcomp()* function, and cluster analysis using the *heatmap()* function with ward.D2 as the hclust method on protein groups with a complete set of valid values over all samples. Based on these quality controls, experiments "Rab5a-mKate2/Rrm4-Gfp" replicate 2 and "mKate2" replicate 1 were identified as outliers and excluded from further statistical analyses. Missing values were filled in (imputed) with random values from a downshifted normal distribution (0.3 S.D. width, 1.8 s.d. downshift) derived from all valid normalized log₂ values over all samples. Statistical comparison between two groups (comprised of n=4 for groups "Rab5a-mKate2/Rrm4-Gfp" and "mKate2"), respectively, was performed by the difference of means (difference, x-axis of volcano plots) and the negative decadic logarithm of Student's t-test (unpaired, homoscedastic) p-values (-log₁₀(p), y-axis of volcano plots) of the log₂ intensities for each protein group that contained a minimum of four valid (not imputed) values in at least one of the two groups.

RNA isolation, cDNA synthesis, and qPCR analysis

For RNA isolation, both the endo-IP sample (bead-coupled) and 5% of the pre-saved cell extract (CE, serving as input) were used as starting material. The RNA isolation protocol was adapted from *in vivo* RNA immunoprecipitation (52,53). To this end, 500 µL of TRIzol (Invitrogen) was added to the samples and incubated at 50 °C with shaking (1,000 rpm) for 5 min. Next, 500 µL of RNase-free water was added, and the mixture was manually shaken for 30 s. Subsequently, 400 µL of chloroform/isoamyl alcohol (24:1; BioChemica) was added, and the

samples were vigorously shaken by hand for 3 min before being incubated at room temperature for an additional 3 min. The samples were then centrifuged at 16,200 g for 10 min, 4 °C. The aqueous phase was transferred to a fresh 1.5 mL tube containing 250 µL of chloroform/isoamyl alcohol (24:1; BioChemica), and the extraction step was repeated. Following a second centrifugation at 16,200 x g for 5 min at 4 °C, the aqueous phase was collected and mixed with 40 µL of sodium acetate (3 M, pH 5.2), 1 mL of ice-cold 100% ethanol, and 0.5 µL of GlycoBlue (Thermo Fisher Scientific, AM9516). The samples were incubated overnight at -20°C to facilitate RNA precipitation. The RNA pellet was recovered by centrifugation at 16,200 g for 30 min at 4 °C, washed twice with 75% ice-cold ethanol, and centrifuged again at 16,200 g for 5 min at 4 °C. After brief air-drying, the pellet was resuspended in 8 µL of RNase-free water and either used immediately for cDNA synthesis or stored at -70 °C. For quality control, 1 µL of RNA was analyzed using a Bioanalyzer and the Agilent RNA 6000 Pico Kit, following the manufacturer's instructions.

The remaining 7 µL RNA sample was carefully combined with 3 µL dsDNase digestion mix (1 µL 10× DNase buffer, 1.5 µL dsDNase, and 0.5 µL RNase-free water [Thermo Fisher Scientific, EN0771]). The solution was gently mixed and incubated for 10 min at 37 °C with shaking at 1,000 rpm. To inactivate the dsDNase and resolve secondary structures in tRNA molecules, a subsequent incubation step was performed for 10 min at 70 °C. Immediately afterward, reverse transcription was carried out using the Maxima Kit (Thermo Fisher Scientific) with random hexamer primers, following the manufacturer's instructions. The reverse transcription thermocycler program was optimized to minimize tRNA secondary structures (54): (I) primer annealing at 70 °C for 10 min, 5 °C for 2 min, (II) followed by the addition of the enzyme mix, (III) reverse transcription at 55 °C for 30 min, and (IV) inactivation at 80 °C for 5 min. cDNA libraries were diluted 1:4 with DNase-free water. Quantitative PCR (qPCR) was performed in 20 µL reaction volumes using 2 µL of endo-IP sample per reaction, with technical duplicates for each condition. Reactions were carried out using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions, on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific). Primer sequences are listed in SI-Table S3.

Co-immunoprecipitation (co-IP)

100 mL filamentous *U. maydis* cultures (6 h.p.i) were produced for co-IP experiments. Harvesting and cryogenic cell disruption were performed as described in the microbial iCLIP2 protocol (resulting in 4 x 2 mL tubes from initially ~25 mL culture per tube) (51) The cryogenic cell powder was resuspended in 800 µL co-IP lysis buffer (50 mM Tris-HCl, pH 7.4; 100 mM NaCl; 0.5 % IGEPAL [CA-630]; 5 mM EGTA pH 8). Inhibitors were added freshly before use (2× Complete protease inhibitor EDTA-free [Merck, 11873580001], 1 mM DTT, 1 mM PMSF, 2 µL SUPERase-In [Thermo Fisher Scientific AM2696], 100 µg/mL cycloheximide [Merck KGaA, 239763-M]). The homogeneous suspension was centrifuged for 10 min at 16,200 g and 4°C. The supernatant was transferred into a fresh, pre-cooled tube and centrifuged again (16,200 g, 5 min, 4 °C). The clear supernatants were pooled in a pre-cooled 50 mL tube. Protein concentrations of the cell lysate were determined using a BCA assay (Thermo Fisher Scientific, Pierce™ 23225) following manufacturer's descriptions and adjusted to match the sample with the lowest concentration (aiming for ≈ 2 mg/mL). 2 mL of the adjusted lysate was added into

a pre-cooled 2 mL tube, containing 30 μ L pre-washed GFP-Trap beads (Chromotek, gtma-20). The beads were pre-washed twice with lysis buffer without inhibitors using a magnetic rack before use. Immunoprecipitation was conducted for 1 h at 4 °C with continuous rotation. Beads were washed twice with lysis buffer (without inhibitors) using the magnetic rack. Beads were transferred into a fresh, pre-cooled 1.5 mL tube and washed once more. The beads were resuspended in 1 mL lysis buffer (without inhibitors) and stored on ice.

Western blot analysis

Protein concentrations of cell lysates were quantified using either the BCA assay (Thermo Fisher Scientific, Pierce™ 23225) or by Bradford assay (Bio-Rad 1976). To ensure consistency across samples, protein concentrations were adjusted to 1 mg/mL in NuPAGE LDS sample buffer supplemented with dithiothreitol (DTT; Thermo Fisher Scientific, NP0007) according to the manufacturer's protocol. Proteins were separated on a 4–12% NuPAGE™ Bis-Tris gel (Thermo Fisher Scientific, NP0321) at 180 V for 1.5 h. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (Amersham Protran) via wet blotting using 1× NuPAGE™ Transfer Buffer supplemented with 15% methanol (Thermo Fisher Scientific, NP00061) for 1.5 h at 30 V. Western blot detection was carried out using primary antibodies against RFP (1:2000; Abcam, EPR18992, rabbit), GFP (1:2000; Roche, 11814460001, mouse), TSEN2 (1:2000; Proteintech, 13103-2-AP, mouse), GAPDH (1:4000; Proteintech, 10494-1-AP, rabbit) and actin (1:2000; MP Biomedicals, 0869100, mouse). Secondary detection was performed using respective HRP-conjugated anti-mouse (1:5000; Promega, W4021) or anti-rabbit (1:5000; NEB, 7074S) antibodies. All antibodies were diluted in 1× TBS-T containing 5% milk powder. Detection was carried out using the LAS400 Image Analyzer (GE Healthcare) in combination with ECL™ Prime chemiluminescent reagent (Amersham, RPN2232), following the manufacturer's instructions.

RNA-seq analysis

50 mL hyphal culture (OD_{600} = 0.5, 6 h.p.i.) was used, and total RNA was isolated. RNA-sequencing was performed and analyzed as detailed in (40,55).

In situ sequence and structure analyses

Protein homologs and the domain architecture were identified using the web tools BLASTP (56), InterPro (57), and SMART (58). Multiple sequence alignments in ClustalW format were generated using the web-based tool MUSCLE (<https://www.ebi.ac.uk/jdispatcher/msa/muscle?stype=protein>) (59) and the SEN proteins listed in SI-Table S4. The alignments were processed and visualized using Jalview (version 2.11.4.0) (60).

AlphaFold3 predictions (61) were performed for the *U. maydis* SEN proteins (SI-Table S4). The *U. maydis* SEN complex model was run using the multimer mode, allowing for sequence-based interaction predictions (61). Structure ranking was based on the predicted alignment error (PAE) and pLDDT confidence scores (61). The highest-confidence model was selected for further structural analyses and superposition with the human TSEN complex cryo-EM structure (26). Structural superposition of single proteins or the SEN complex was performed in PyMOL

(PyMOL Molecular Graphics System, version 2.1, Schrödinger, LLC) using the *super* command with default parameters (cutoff=2.0 Å, cycles=5, gap=-10, extend=5, max_gap=50). RMSD values were used to evaluate structural similarity. Structural prediction of RRM3 of Rrm4 (A0A0D1DWZ5) and 5'-ACCCCCUAUGAAUC-3' was performed by AlphaFold3 predictions (61), and the highest-confidence model was selected.

tRNA analysis and iCLIP2 binding sites

To analyze the *U. maydis* tRNA repertoire, the tRNA database [GtRNAdb](#) (17) served as a reference for sequences and annotations. For the Rrm4 binding landscape, iCLIP data for Rrm4-G, Grp1-G, and Gfp were obtained from Olgeiser *et al.* (2019) (38), while iCLIP2 data for Rrm4-G, mR1-G, mR2-G, and mR3-G were obtained from microbial iCLIP2 (40,51). Binding sites were defined as the collective set of wild-type and mutant Rrm4 binding sites identified through the comparative iCLIP2 approach (40). Binding sites within tRNAs were manually counted and categorized based on their distribution.

Data visualization and statistical analysis

RStudio (version R 4.4.2) was used for data analysis, visualization, and statistical analysis by employing the packages ggplot2, tidyr, readr, dplyr, stringr, purrr, ggpubr, beeswarm, ggrastr, FSA, pheatmap, and forcats. PyMOL (PyMOL Molecular Graphics System, version 2.1, Schrödinger, LLC). Canvas 15 and Affinity Designer 2.0 were used for graphical design.

Results

The tRNA repertoire of *Ustilago maydis*

The number of genes encoding tRNAs is highly variable between organisms. To initiate research on tRNA biology in *U. maydis*, we first analyzed its tRNA repertoire, consisting of 110 tRNA genes (SI-Fig.1A, [GtRNAdb](#), SI-Table S5) (17). In direct comparison to *Homo sapiens* (n= 429), *Arabidopsis thaliana* (n= 580) or *Saccharomyces cerevisiae* (n= 275), *U. maydis* (n= 110) and its close relatives possess a relatively small tRNA repertoire (Fig. 1A) (21). Also, the proportion of intron-containing tRNAs varies widely across species. Comparing the intron content revealed that most tRNAs in *U. maydis* contain introns (90%), whereas this occurs in only 7% of human tRNAs (Fig. 1A-B). Globally, we observed a correlation between the percentage of intron-containing pre-tRNAs and the overall number of tRNA genes across the selected organisms (Fig. 1A-B, SI Table S6).

Our analysis further revealed substantial intron length variability (10–132 nt, mean: 29 nt, coefficient of variation: 55%; SI-Fig. 1B) and sequence divergence among isodecoders (i.e. tRNAs that share the same anticodon, Fig. 1C-D), while mature tRNAs are highly conserved (SI-Fig. 1C). Similarly, human tRNA introns exhibit high sequence variability, whereas their spliced isodecoder counterparts display markedly lower sequence divergence (SI-Fig. 1D). In summary, *U. maydis* possesses a compact tRNA repertoire that is highly enriched in intron-containing pre-tRNAs.

Rrm4 binds pre-tRNA^{iMET} *in vivo*

Previously, we generated a transcriptome-wide map of *in vivo* RNA-binding sites for the endosomal mRNA transporter Rrm4 using individual-nucleotide resolution UV crosslinking and immunoprecipitation approaches (iCLIP and iCLIP2) (38,51,62). Moreover, to dissect the RNA-binding features of the individual RRM domains, we compared the RNA-binding profiles of Rrm4-G (C-terminal fusion with the green fluorescent protein, Gfp) with RRM mutant variants (mR1-G, mR2-G, mR3-G; Fig. 1A–B). Using visual inspection, we unexpectedly discovered that Rrm4 displayed strong binding sites within tRNAs. This suggested a broader and previously unrecognized RNA-binding spectrum that extends beyond mRNAs.

To investigate this, we analyzed the collective Rrm4-G binding sites (Fig. 2A, see Methods section). Indeed, the majority of annotated tRNAs (77%; n= 85 out of 110; SI-Fig. 1C, SI-Table S5) harbored at least one binding site (Fig. 2B), supporting a widespread interaction of Rrm4 with tRNA species. Binding sites were mainly found in exon I and the intronic region, while exon II sites were most likely underrepresented due to the depletion of very short RNA fragments during the iCLIP2 procedure (51,62) (SI-Fig. 2A, SI-Table S5). More than half of the binding sites were located in the intronic regions (Fig. 2B, SI-Table S5), suggesting that pre-tRNAs are common targets of Rrm4. Furthermore, we previously identified a sequence-specific interaction between the third RRM domain, RRM3, and the UAUG motif in mRNA targets (38,40). Based on this, we investigated RRM3-specific binding sites within tRNAs, identifying both initiator methionine isodecoders, pre-tRNA^{iMET-1} and pre-tRNA^{iMET-2} (Fig. 2C-D, SI-Fig. 1B). These distinct intronic UAUG crosslink events were consistently detected in

Rrm4-G, mR1-G, and mR2-G, but were absent in mR3-G (Fig. 2D). This indicates that RRM3 recognizes UAUG not only in mRNAs (40), but also within intronic regions of pre-tRNA^{iMET} isodecoders (Fig. 2C-D). These results were further supported by our previous iCLIP data (38), which revealed Rrm4-specific binding in both pre-tRNAs, while such signals were absent in control conditions, highlighting the specificity and reproducibility (Fig. 2D).

Given the substantial sequence divergence within the intronic regions of isodecoders (Fig. 1), we investigated whether binding events aligned with defined isodecoder-specific sequences. Indeed, Rrm4 binding sites overlap with variable regions, often coinciding with UAUG motifs (Fig. 2E-F). Rrm4 exhibited a significant binding preference for pre-tRNA^{iMET-2} over pre-tRNA^{iMET-1} (Fig. 2E-F), despite comparable expression levels (Fig. 2G). Thus, Rrm4 specifically binds to intronic regions of both initiator pre-tRNAs, potentially linking endosomal mRNA transport machinery to long-distance pre-tRNA trafficking and subcellular maturation.

The *U. maydis* SEN complex resides on mitochondria

To elucidate the functional relevance of pre-tRNA binding by the endosomal transporter Rrm4, we aimed to investigate the subcellular localization of the tRNA splicing endonuclease complex SEN (TSEN in humans). In *S. cerevisiae* and *Schizosaccharomyces pombe*, the SEN complex is located on the mitochondrial surface (23,63,64). Where does pre-tRNA splicing occur in polarized hyphae?

As a first step, we identified protein orthologs of all four SEN complex components, Sen2, Sen15, Sen34, and Sen54, in *U. maydis* (Fig. 3A-B, SI-Table S4). Multiple-sequence alignments and structural modeling confirmed their conservation (Fig. 3A-B, SI. 3A-B). Moreover, superposition of the AlphaFold3 prediction (61) for the *U. maydis* SEN complex revealed high structural similarity to the recently determined cryo-EM structure of the human TSEN complex (Fig. 3B) (26).

To study the subcellular localization of the SEN complex in *U. maydis*, we generated strains expressing C-terminal Gfp fusions of the structural subunits Sen54 and Sen15 at their native locus (Sen54-G, Sen15-G; Fig. 3C, SI-Fig. 3C). Fluorescence imaging of hyphae confirmed the mitochondrial localization of Sen54-G (94%) and Sen15-G (97%) using the mitochondrial dye tetramethylrhodamine ethyl ester (TMRE, Fig. 3D-E) (30). This degree of co-localization was comparable to the positive control, Mts-G (97%; a mitochondrial-targeting signal fused to Gfp), while no overlap was observed with the negative control, Nls-G (nuclear localization signal fused to Gfp; Fig. 3D-E). To assess the integrity of the SEN complex in *U. maydis*, we performed native co-immunoprecipitation (co-IP) using a Sen54-G-expressing strain (SI-Fig. 3C), followed by Western blot analysis. The core subunit Sen2 (detected using an antibody raised against the human TSEN2, see Methods section) co-precipitated with Sen54-G, confirming successful complex assembly despite the presence of the C-terminal Gfp fusion.

In summary, we uncovered that the SEN complex is conserved in *U. maydis* and localizes to the mitochondrial surface in highly polarized cells, mirroring its reported localization in yeast. Hence, the spatial organization of pre-tRNA splicing is conserved across fungi and present in both yeast and hyphal growth modes.

Endosomal pre-tRNA transport depends on Rrm4

The SEN complex localizes to the mitochondrial surface, including those situated far from the nucleus—raising the question of how pre-tRNAs are delivered to these distal sites. We hypothesize that efficient processing and spatial distribution of pre-tRNA^{iMET} is mediated by Rrm4-dependent transport on Rab5a-positive endosomes (35).

To test whether pre-tRNAs co-associate with endosomes, we established a purification protocol tailored for capturing early endosomes from *U. maydis* (endo-IP) (41,43). As bait for immunoprecipitation, we chose the marker protein Rab5a, which is stably anchored by its C-terminus to the lipid bilayer of early endosomes. Rab5a exhibits characteristic bidirectional endosomal movement along microtubules (35). To enable selective isolation, we generated strains co-expressing Rrm4-G (36) and Rab5a fused N-terminally with the monomeric red fluorescent protein mKate2 (Rab5a-K), driven by a constitutively active promoter (48) (SI-Table S1). Live-cell imaging confirmed that both proteins display coordinated, bidirectional dynamics throughout hyphae (6 h.p.i.; Fig. 4A), consistent with previous results (35).

Our endo-IP approach allowed the isolation of intact Rab5a-K-positive endosomes along with their associated proteins and RNAs (Fig. 4B, SI-Fig. 4A). This was followed by mass spectrometry (MS)-based proteomics comparing Rab5a-K-expressing cells against a control strain expressing cytoplasmic mKate2 (Fig. 4C, SI-Fig. 4B, four biological replicates SI-Table S7). The endo-IP samples were significantly enriched in vesicle-associated proteins and endosomal markers (e.g., Vps9 and Vps27) compared to the control (Fig. 4C-D, SI-Table S7). Additionally, Rrm4 was exclusively detected in the endo-IP fractions and was absent in the control samples (Fig. 4C-D, SI-Fig. 4C, SI-Table S7).

Next, we isolated co-purified endosomal RNAs and performed reverse transcription under optimized conditions to ensure accuracy and minimize secondary structures of tRNAs (54) (Fig. 4D, see Materials and methods). Comparing the endo-IP samples with the corresponding control revealed the presence of known Rrm4 targets like the septin mRNA *cdc12* (33) and the nuclear-encoded mitochondrial complex V subunit *Atp3*, *atp3* (38). Moreover, pre-tRNA^{iMET-1} was exclusively detectable in the endo-IP fractions (Fig. 4E).

To determine whether the endosomal association of pre-tRNA^{iMET} is Rrm4-dependent, we first verified that Rab5a-K shuttling and protein expression remained consistent by loss of Rrm4 (*rrm4Δ*, Fig. 4F). Next, we performed comparative endo-IP experiments in the absence or presence of Rrm4 (*rrm4Δ*/Rab5a-K vs. Rrm4-G/Rab5a-K), followed by RT-qPCR to quantify the relative enrichment of target RNAs normalized to the respective cell extracts (Fig. 4G). As expected, the control mRNA *atp3* exhibited Rrm4-dependent enrichment (Fig. 4G) (38). Importantly, both pre-tRNA^{iMET} isodecoders showed reduced levels in *rrm4Δ* compared to Rrm4-G (Fig. 4G), confirming that their endosomal association is mediated by Rrm4. In line with Rrm4's isodecoder binding preference (Fig. 2E–G), pre-tRNA^{iMET-2} exhibited a more pronounced reduction upon loss of Rrm4 (Fig. 4G–H).

In essence, both pre-tRNA^{iMET} isodecoders associate with Rab5a-positive endosomes in an Rrm4-dependent manner, supporting the notion that endosomal pre-tRNA trafficking serves SEN complexes on distant mitochondria.

Loss of Rrm4 causes defects in pre-tRNA splicing

We hypothesize that Rrm4 binds both pre-tRNA^{iMET} isodecoders and facilitates their endosomal hitchhiking to mitochondria for splicing. To assess the function of Rrm4 during pre-tRNA splicing, we isolated total RNA from strains expressing Rrm4-G and *rrm4Δ* and monitored pre-tRNA^{iMET} and mature tRNA^{iMET}. Due to their high sequence similarity in exonic regions, this approach only allows for a combined analysis of both isodecoders. Indeed, we observed that the splicing of pre-tRNA^{iMET} was significantly reduced in *rrm4Δ* (Fig. 5A). Furthermore, testing RRM mutants (mR1-G, mR2-G, and mR3-G) revealed an intermediate splicing phenotype, indicating that all RRMs contribute to pre-tRNA binding (SI-Fig. 5A). Consistently, the levels of both unspliced pre-tRNA^{iMET} isodecoders exhibited a slight increase upon *rrm4* deletion, supporting impaired pre-tRNA processing upon loss of Rrm4 (Fig. 5B).

Beyond pre-tRNA^{iMET}, loss of Rrm4 affected the splicing of some but not all tested tRNAs (SI-Fig. 5B-C). For instance, the elongator methionine pre-tRNA (pre-tRNA^{eMET}: UMAG_16084, SI-Fig. 5B) showed neither significant splicing defects nor increased pre-tRNA levels (SI-Fig. 5B). In contrast, for pre-tRNA^{ILE}, we observed an altered splicing pattern but no increase in pre-tRNA levels (SI-Fig. 5C), indicating that Rrm4 differentially affects the splicing of selected tRNAs. These findings highlight that the functional impact of Rrm4 is not restricted to initiator pre-tRNA^{iMET} but extends to other tRNA species, underscoring the complexity of Rrm4's role in tRNA regulation and the necessity for a case-by-case approach. In summary, we discovered that the endosomal RNA transporter Rrm4 impacts the precise processing of the prime cargo pre-tRNA^{iMET}.

Loss of Rrm4 causes defects in translation

Our previous RNA-seq approach revealed elevated levels of mRNAs encoding proteins involved in translation upon deletion of Rrm4 (40). To refine this observation, we conducted an in-depth analysis of transcripts encoding aminoacyl-tRNA synthetases, ribosomal proteins (Rpl and Rps families), and eukaryotic translation initiation factors (eIFs), comparing *rrm4Δ* mutants to wild-type (Fig. 5C, SI-Fig. 5D). Indeed, we observed increased mRNA amounts of these translation-related genes, while transcripts encoding polymerases remained unchanged. This selective response underscores a translation-associated adaptation triggered by loss of Rrm4 (Fig. 5C, SI-Fig. 5D). In addition, we examined the global polysome profile of Rrm4-G and *rrm4Δ* hyphae using sucrose density gradient analysis. Notably, *rrm4Δ* strains exhibited aberrant profiles marked by a general reduction in ribosome abundance and increased levels of free 40S and 60S subunits (SI-Fig. 5E). Together, these findings indicate that deletion of Rrm4 impairs translation homeostasis.

Finally, to assess the functional consequences on cellular fitness, we performed *in vivo* growth assays using rocaglamide A (RocA), a translation inhibitor targeting the translation initiation factor eIF4A1, and cycloheximide (CHX), which interferes with elongation (Fig. 5D, SI-Fig. 5F) (65). We applied inhibitor concentrations that preserved hyphal growth but reduced cell length by approximately 30%. Consistent with previous observations (38), *rrm4Δ* strains exhibit reduced hyphal length and an increased frequency of aberrant bipolar growth compared to predominantly unipolar growth of wild-type hyphae (Fig. 5F, 6 h.p.i.). CHX treatment led to

a clear reduction in cell length in both wild-type and *rrm4Δ* hyphae (32% and 28% inhibition, respectively; Fig. 5D, SI-Fig. 5F), suggesting that both strains exhibit comparable sensitivity to stress during translation elongation. In contrast, treatment with the inhibitor of translation initiation RocA caused a marked reduction only in hyphal length of wild-type cells (27%), whereas *rrm4Δ* hyphae were largely resistant to the inhibitor (0% inhibition; Fig. 5D). Since RocA impairs translation initiation by targeting eIF4A, this reduced sensitivity in *rrm4Δ* suggests an adaptive or compensatory mechanism in the mutant. One possible explanation is the observed upregulation of *eIF4A* mRNA in *rrm4Δ* strains, potentially buffering the increased initiation stress. Together, these findings implicate Rrm4 in maintaining translational homeostasis, possibly mediated through its role in pre-tRNA splicing and maturation.

Discussion

The tRNA lifecycle is tightly regulated by RNA-binding proteins (RBPs) (66), encompassing their transcription (67), modification (10), nuclear export/import (68), splicing (16), amino acetylation (13), and decay (69). It is well established that dysregulation of tRNAs is associated with a range of neurological disorders and cancer (7,70). However, knowledge of their transport and localization, and the associated RBP interaction networks, remains limited.

Despite growing recognition of their importance, protein–tRNA interactions remain systematically underexplored in transcriptome-wide approaches, hindered by the exceptional abundance, compact size, complex secondary structures, and pervasive post-transcriptional modifications of tRNAs; all of which pose significant challenges to their analysis (54). One of the few reported examples is an advanced PAR-CLIP study showing that the RBP HDLBP binds sequence-specifically to the D-loop and variable region of leucine-type tRNAs, enhancing decoding efficiency (71,72). Unexpectedly, our *in vivo* Rrm4 iCLIP(2) analysis revealed distinct binding sites within the intron of pre-tRNA^{iMET}. Furthermore, we could demonstrate domain-specific contributions of individual RRM domains and successfully determine a significant isodecoder specificity.

Endosomal pre-tRNA transport for delivery to distant SEN complexes

tRNA maturation depends on a well-regulated spatiotemporal choreography involving extensive base modifications and precise intron removal to ensure the generation of fully functional tRNAs for translation (21,63,73). Although the heterotetrameric tRNA splicing machinery (T)SEN is evolutionarily conserved in eukaryotes, its subcellular localization diverges across species, ranging from the nucleus to the cytoplasm and the mitochondrial surface (63,74,75).

Here, we find that in *U. maydis*, the SEN complex is localized at mitochondria throughout the entire long-polarized cell. This is reminiscent of localization patterns observed in yeasts (21,23,64). Notably, the *U. maydis* tRNA repertoire is exceptionally rich in intron-containing tRNAs (> 90%), implying a particularly efficient splicing processing. This exceptional feature, combined with its long polarized cellular architecture, positions *U. maydis* as a powerful model for dissecting the mechanisms of subcellular pre-tRNA transport and splicing in eukaryotes.

How do pre-tRNAs reach their destination at the mitochondria-associated SEN complexes? In *S. cerevisiae*, mutations in actin and a class V myosin motor led to the accumulation of unspliced pre-tRNAs (76). In analogy with the well-characterized short-distance transport of *ASH1* mRNA (77,78), the authors hypothesized that actin-dependent pre-tRNA transport to mitochondria is required for accurate processing (76). Nevertheless, the molecular machinery governing pre-tRNA transport, as well as the precise mechanisms underlying its regulation, remain largely unresolved.

Our findings uncover a conceptual advance in tRNA biology, demonstrating that long-distance endosomal hitchhiking of pre-tRNAs functions to supply the mitochondria-associated SEN complexes. We identified Rrm4 as a key RNA transporter, disclosing its dual function in mRNA and tRNA trafficking. Recognition is mediated by binding to pre-tRNA intron regions, and the initiator pre-tRNA^{iMET} isodecoders are prime targets for transport (Fig. 6). The endosomal translocation mirrors the “sushi-belt” model proposed for mRNA transport, where transcripts are distributed across the cell to spatially control protein synthesis (79,80). Also, Rrm4 binds a broad spectrum of mRNAs, yet selectively enriches transcripts encoding polarity factors (e.g., *cdc12*) and mitochondrial proteins (e.g., *atp3*) (34,38). Strikingly, pre-tRNA transport appears to follow a similar hierarchical logic: although numerous pre-tRNAs harbor Rrm4 binding sites, initiator pre-tRNA^{iMET} isodecoders are bound with preference. Notably, pre-tRNA^{iMET-2} emerges as a particularly favored target, highlighting a striking isodecoder selectivity. The emerging field of isodecoder specificity represents a new and complex frontier in RNA biochemistry (67,81,82), poised to elucidate how distinct isodecoders exhibit differential subcellular localization, regulate local translation, and orchestrate diverse biological functions.

To date, pre-tRNA trafficking and the spatial organization of the mitochondria-associated SEN complex have been described primarily in fungi (23,63,76). In mammals, the TSEN complex has previously been mainly reported in the nucleus (15). Have tRNA transport and cytoplasmic splicing been lost in evolution? Recent studies have shown that human TSEN subunits are preferentially enriched in cytoplasmic fractions compared to nuclear fractions (18). Additionally, evidence from neuronal systems suggests bidirectional microtubule-dependent tRNA granule movement independent of translation (83,84). This suggests that tRNA trafficking and extranuclear pre-tRNA processing may not be limited to fungal systems and could likewise take place in mammalian cells (19).

Mitochondria as strategic hubs for tRNA splicing and localized translation

What is the functional relevance of pre-tRNA transport, and why are mitochondria the final destination? We propose that cytoskeleton-mediated pre-tRNA delivery targets the SEN complex located on mitochondria (76), enabling localized splicing and possibly coupling tRNA maturation to sites of local translation (Fig. 6). Indeed, loss of the endosomal RNA transporter Rrm4 leads to (i) elevated pre-tRNA levels, (ii) reduced splicing efficiency, and (iii) decreased mature tRNA^{iMET} abundance for some tRNAs, causing translation dysregulation.

tRNA maturation at mitochondria might be favorable because they are distributed along hyphae and thereby might serve as strategic hub, enabling efficient dissemination of newly spliced tRNAs throughout cells (Fig. 6). In addition, the spatial organization may also support local translation of nuclear-encoded mitochondria-targeted mRNAs on the mitochondrial

surface (30,85,86), tightly coupling proximal tRNA supply with local translation (Fig. 6). Notably, the cytoplasmic translation initiation of nuclear-encoded mitochondrial mRNAs constitutes a key cross-talk mechanism with mitochondrial physiology in neurons, contributing to regulatory complexity (87).

Conclusion

Since the discovery in *U. maydis*, endosomal transport has emerged as a conserved mechanism across diverse organisms and tissues, including plants and neurons (41-44,88). Here, we reveal a previously unrecognized hitchhiking mechanism in which both initiator pre-tRNA^{iMET} isodecoders are co-transported on motile endosomes to the mitochondria-associated tRNA splicing machinery, thereby linking RNA transport, processing, and translational regulation. Given the involvement of dysregulated tRNA metabolism in human disease, including tRNA^{iMET} overexpression in breast cancer (89) and pre-tRNA splicing defects in neurodevelopmental disorders (7,90), understanding the regulatory networks that connect RNA-binding proteins and tRNAs is critically important. These findings open a new conceptual avenue in the emerging field of local translation hotspots (37,91-94), positioning tRNA dynamics at the heart of spatial gene expression control and highlighting a new chapter in the complex life of (t)RNA.

Data availability statement

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE (95) partner repository with the dataset identifier PXD064386. Reviewer access details: Log in to the PRIDE website <https://www.ebi.ac.uk/pride/login> using the following account details:

Username: reviewer_pxd064386@ebi.ac.uk,

Password: iwi9j2SEXWYV

Acknowledgments

We sincerely thank Drs. Marina Chekulaeva, Anne Ephrussi, Dierk Niessing, and Heiner Schaal for excellent input regarding RNA transport, splicing, and translation regulation. Drs. Franziska Kuhnert, Marten Exterkate, Seomun Kwon, Lea Geißl, and Sainath Shanmugasundaram for their enthusiastic discussions regarding endo-IP and mass spectrometry. Drs. Dierk Niessing, Heiner Schaal, Alexander Steffen, Kerstin Schipper, and Florian Altegoer for critical proofreading and support. We thank Drs. Miriam Bäumers and Stefanie Weidtkamp-Peters of the Center for Advanced Imaging at Heinrich Heine University for their excellent support and valuable input regarding TEM microscopy. Exceptional technical assistance in strain generation was provided by Simone Esch and Ute Gengenbacher.

This research was generously supported by grants from the DFG under Germany's Excellence Strategy EXC-2048/1 - project ID 39068111 to MF; FOR2333-TP03 to MF, DFG-FOR2333-TP02 to KZ and to JK - project-ID 267205415 as well as DFG-SFB1535 - project ID

458090666 to MF (project A03) and DFG-SFB 1208 - project ID 267205415 to MF (project A09) and to KS (central project Z01; MS / proteome core facility). Furthermore, the Graduate School of Molecules of Infection MOI-V provided funding to MF and JP. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

NKS, JK, KZ, and MF designed this study and analyzed the data. NKS and TL performed endo-IP MS and data analysis under the guidance of KS. NKS performed biochemical experiments with support from JK. *U. maydis* strains were generated by MVC and NKS with the support of KM. NKS and KM performed and analyzed microscopy experiments on SEN and mitochondria co-localization experiments. JP, MVC, and KM performed the translational inhibitor assay and analyzed the data. NKS performed microbial iCLIP2 and discovered tRNA binding together with JK, KZ, and MF. Bioinformatic analysis of iCLIP2 BS and RNA-seq was performed by SS and NKS, under the guidance of KZ. NKS and MF drafted and revised the manuscript with input from co-authors. MF, KS, KZ, and JK contributed funding and resources.

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Figures

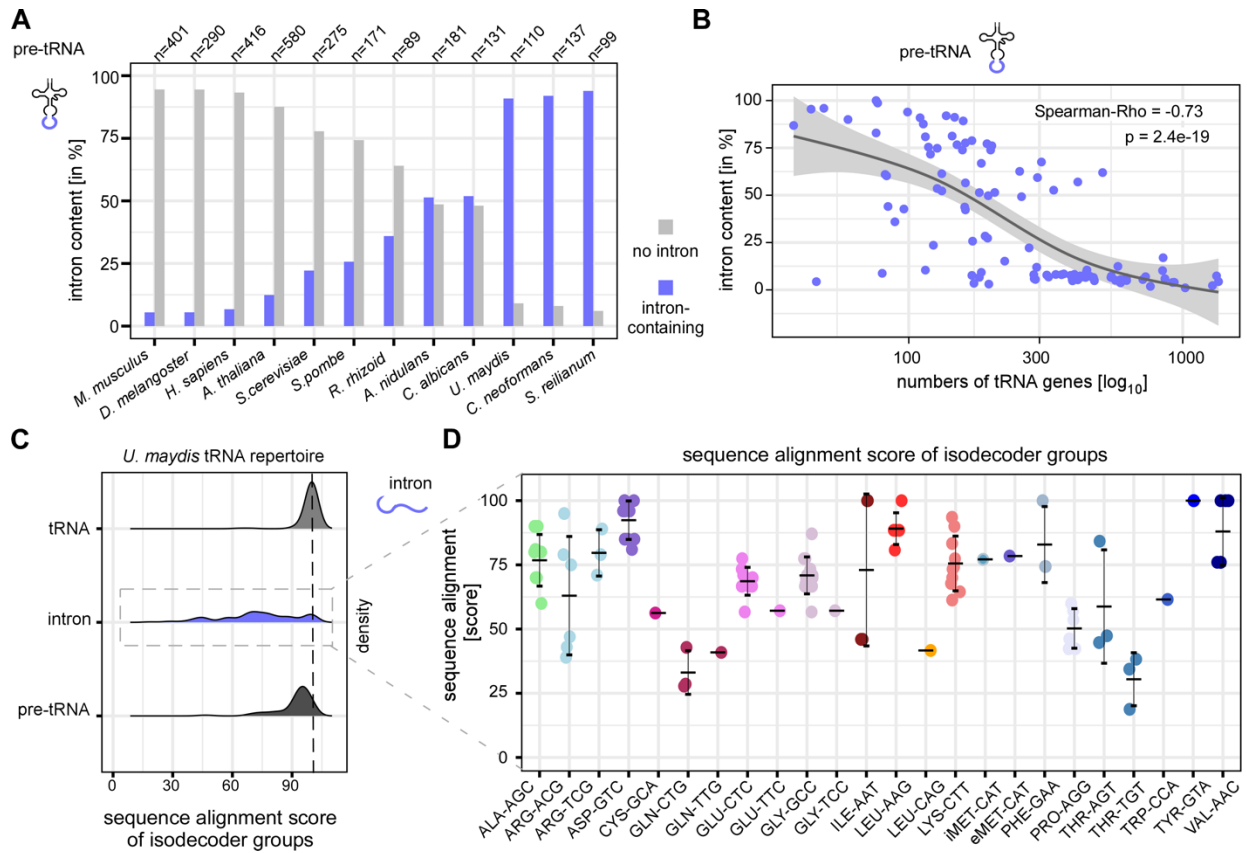


Fig. 1 The *U. maydis* (pre-)tRNA inventory reveals a high abundance of intron-containing tRNA genes

(A) Bar diagram visualizing the percentage of intron-containing tRNAs across diverse organisms and kingdoms. Data were obtained from the database GtRNAdb (25). (B) Spearman correlation analysis comparing the number of tRNA genes with the percentage of intron-containing tRNAs across selected eukaryotic organisms (n>100, SI-Table S6). (C) Sequence alignment scores of tRNAs, comparing isodecoders and separating matured, intron-region, and unspliced pre-tRNA sequences. (D) Intron sequence alignment scores from isodecoder families.

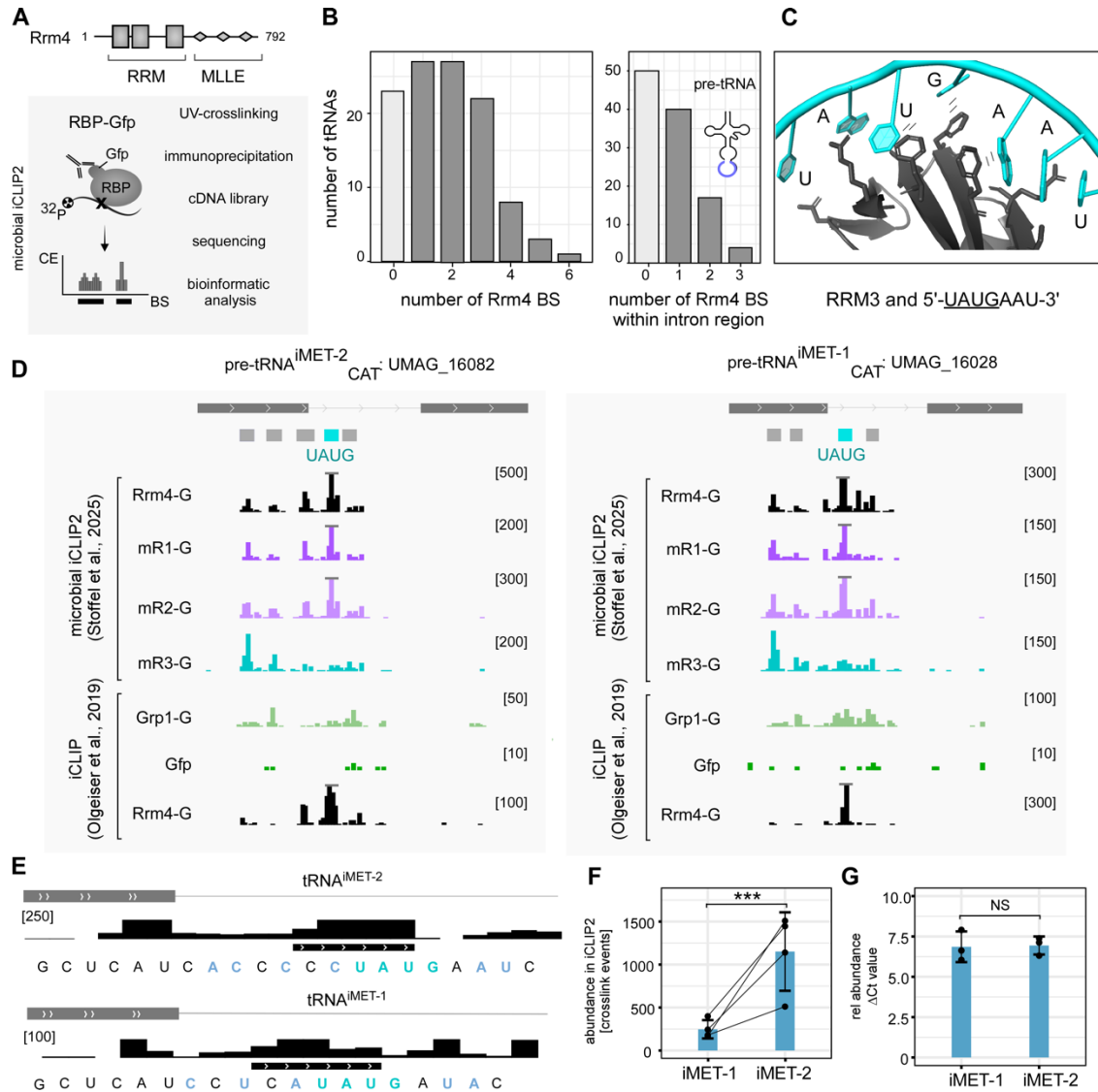


Fig. 2 Rrm4 binds intronic regions of pre-tRNA^{iMET} in a sequence-specific manner

(A) Schematic representation of the domain architecture of Rrm4, containing three RRM (rectangle) for RNA binding and MLLE domains (diamond) for endosomal attachment. (B) Bar graphs illustrating the distribution of Rrm4-binding sites across tRNAs in percentage. (C) AlphaFold3 prediction of the interaction between RRM3 and the intronic region of pre-tRNA^{iMET2} (UMAG_16082, ACCCCCUAUGAAUC, turquoise), highlighting RRM3-UAUG interaction. (D) Genome browser view of both initiator methionine tRNA isodecoder (UMAG_16082, UMAG_16028), highlighting a prominent UAUG-binding peak (turquoise) within the intron region, as detected by Rrm4 iCLIP2 (40), alongside previously published Rrm4 iCLIP datasets (38). The binding landscape is shown for Rrm4-G, RRM mutants (mR1-G, mR2-G, mR3-G), the small glycine-rich RBP Grp1, and Gfp control. Turquoise bar marks UAUG-harboring binding site and signal that extends beyond the visualized range are indicated by a grey line. (E) Intron sequences of both pre-tRNA^{iMET} isodecoders, with nucleotide differences (blue) and the RRM3-specific UAUG binding motif (petrol) highlighted. Genome browser view showing Rrm4 iCLIP2 crosslink events overlapping with isodecoder-specific sequences. (F) Bar diagrams comparing Rrm4 iCLIP2 crosslink events between pre-tRNA isodecoders (upper panel, n=5). (G) Bar diagram comparing the relative pre-tRNA^{iMET} isodecoder abundances measured by RT-qPCR (n=3, individual data points are indicated). For statistical analysis, paired Student's *t*-tests were applied.

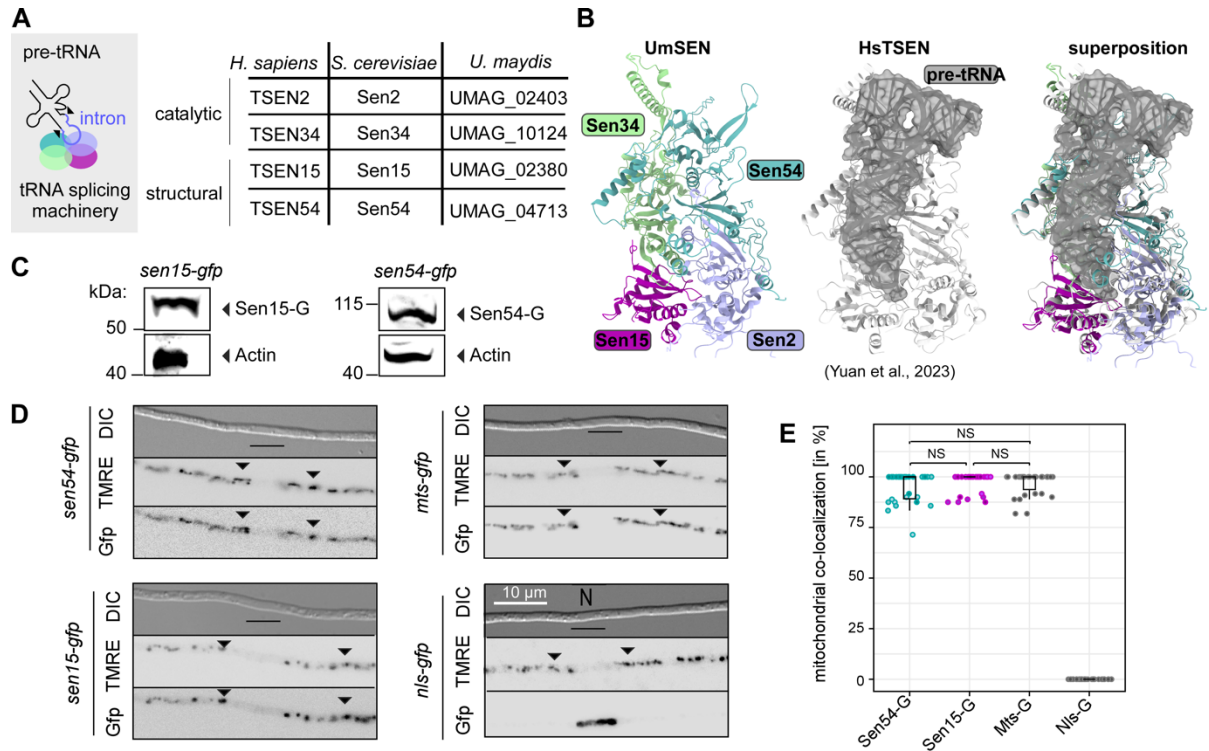


Fig. 3 The SEN complex colocalizes with mitochondria in polarized hyphae

(A) Table of protein homologs of the individual subunits of the tRNA splicing machinery (TSEN/SEN, SI-Table S4). (B) Structural superposition of the human TSEN complex cryo-EM structure (26), colored in gray, with the predicted *U. maydis* SEN model generated by AlphaFold3 (61), including Sen2 (purple), Sen15 (magenta), Sen34 (green), and Sen54 (cadet blue). Executive RMSD 1.157, 885 to 885 atoms. (C) Western blot analysis of C-terminal Gfp-tagged structural Sen subunits (Sen54-G and Sen15-G) under native promoter conditions. (D) DIC and fluorescence micrographs (scale bar: 10 μ m, nucleus underlined in black) showing colocalization of Sen subunits (Sen54-G and Sen15-G) with TMRE-stained mitochondria. Mts-Gfp (mitochondrial-targeting signal) as a mitochondrial marker, and Nls-G (nuclear localization signal), as a negative control. (E) Scatter plot quantifying colocalization events (three biological replicates, N>20 hyphae, t-test, two-tailed, unpaired).

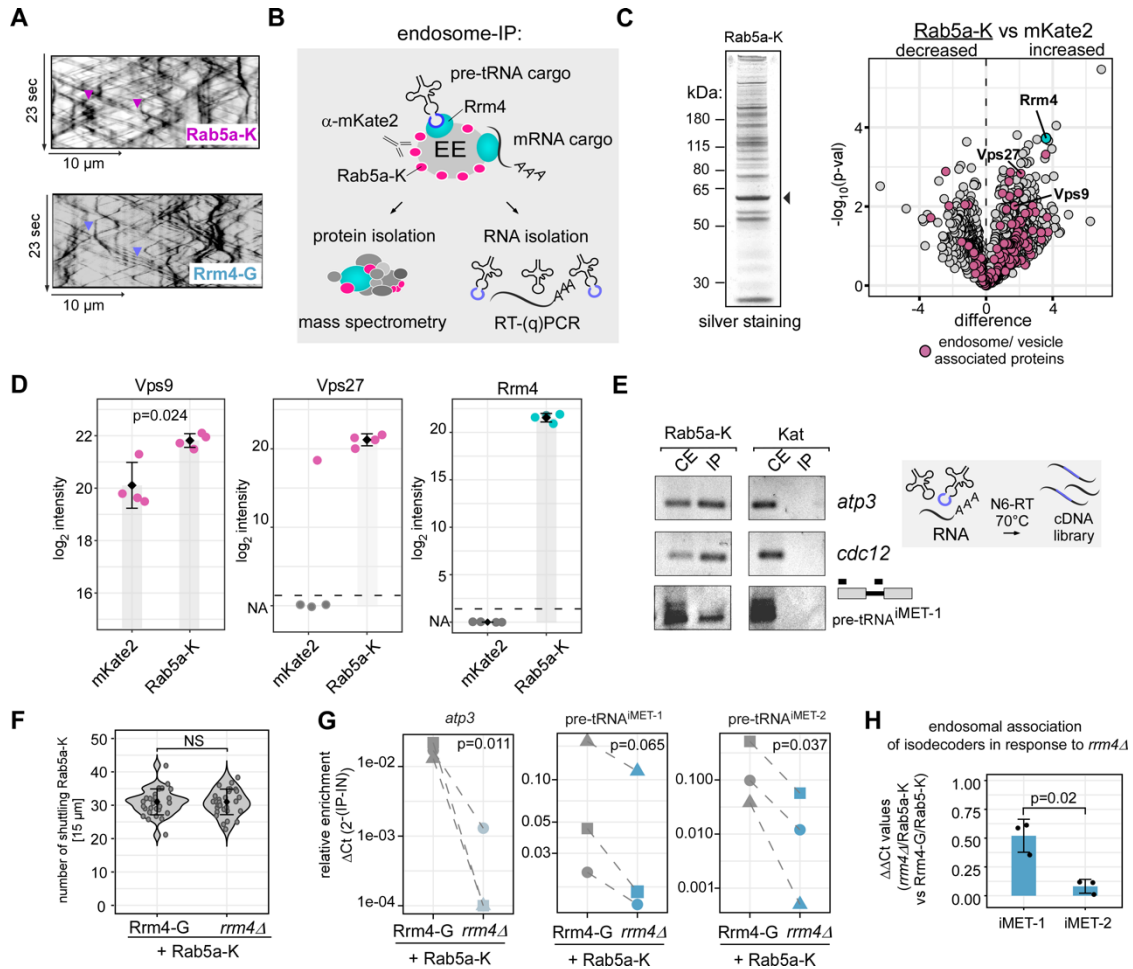


Fig. 4 Pre-tRNA^{iMET} isodecoders are associated with endosomes in an Rrm4-dependent manner

(A) Kymographs of shuttling particles of the early endosomal marker Rab5a-mKate2 (Rab5a-K) and the RBP Rrm4-G. (B) Model of the early endosomal mRNP model and the respective Rab5a-K-based immunoprecipitation approach (endo-IP) to purify endosomes as well as their cargo proteins or RNAs. (C) Silver-stained gel of immunoprecipitated Rab5a-K fractions (Rab5a-K indicated by arrowhead). Volcano plot of the comparative endo-IP (Rab5a-K versus free mKate2 control), i.e., an immunoprecipitation to capture mKate2-tagged complexes, followed by MS-based proteomics analysis (n=4), showing the enrichment of endosomal/vesicle marker proteins. The average $\log_2$ intensities between Rab5-K and mKate2 samples were used to measure protein abundance changes, defined as the difference of means (see Materials and Methods section). (D) Scatter plot of $\log_2$ protein intensities measured by MS-based proteomics for the targets Vps9, Vps27, and Rrm4 in mKate2 control IP and endo-IP. NA indicates proteins not detected in that measurement, marked in grey. (E) Semi-quantitative PCR on cDNA libraries (RT-PCR) from endosomal (Rab5a-K) or mKate2 co-purified RNAs. RNA isolated from the respective cell extracts (CE) serves as a positive control. The mRNAs encoding for the nuclear-encoded ATPase3 (*atp3*) and septin Cdc12 (*cdc12*) serve as positive controls. (F) Violin plots displaying Rab5a-K particle dynamics over 15 μ m in the presence or absence of Rrm4 (N>20). (G) Quantitative enrichment of pre-tRNA^{iMET} isoforms and *atp3* in endosomal fractions (endo-IP RT-qPCR) in strains expressing Rrm4 versus lacking *rrm4 Δ* . Respective cell extracts were used for normalization. Statistical significance was assessed using a paired two-tailed t-test on log-transformed ratios (n=3). (H) Scatter plot showing the direct comparison of the endosomal enrichment in dependence of Rrm4 expression of $\Delta\Delta$ Ct values (*rrm4 Δ* /Rrm4-G) obtained from Fig. 4F.

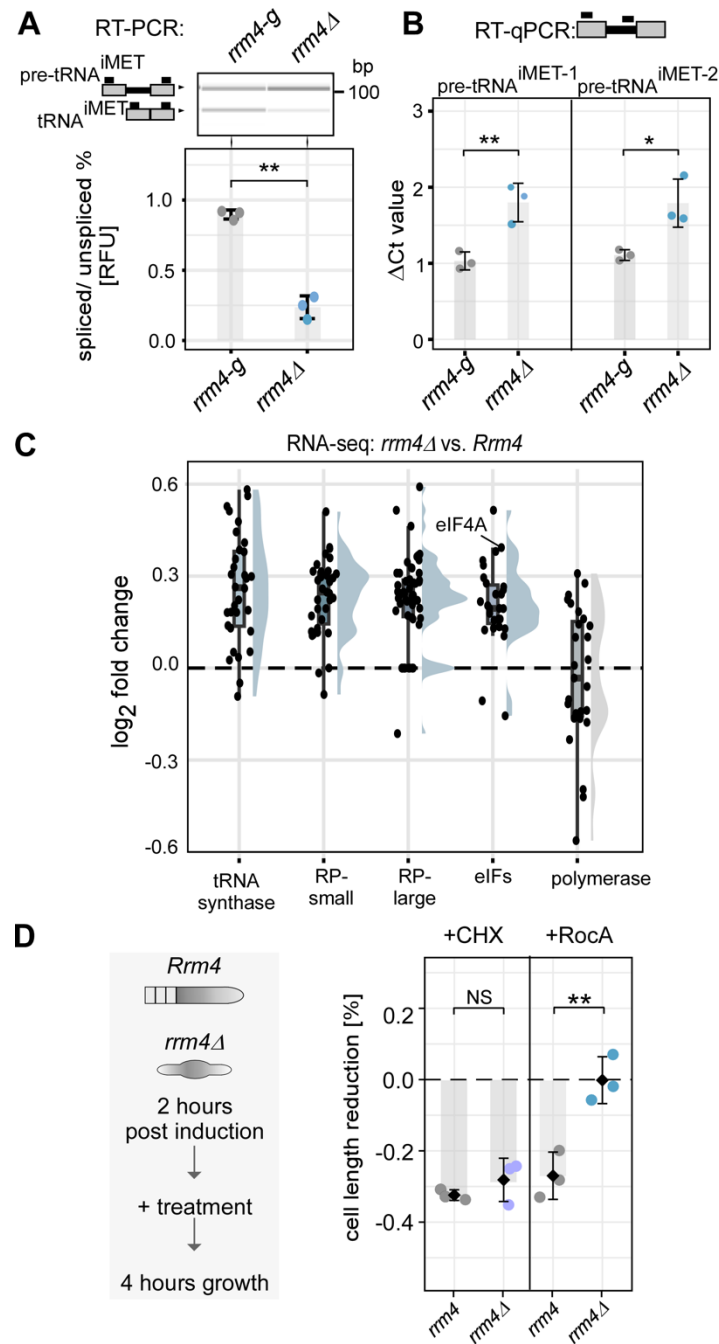


Fig. 5 Rrm4-mediated transport influences pre-tRNA^{iMET} maturation and impacts translational regulation

(A) Digital electrophoresis of pre-tRNA^{iMET} in hyphae expressing Rrm4-G and *rrm4Δ* mutants. Bar plot quantifying the percentage of spliced to unspliced pre-tRNA^{iMET} (n=3, two-tailed, t-test, unpaired). (B) Bar plot showing the quantitative determination of pre-tRNA^{iMET} isodecoders (Student's t-test, n=3, unpaired). (C) Transcriptomic analysis comparing *rrm4Δ* versus wild-type, highlighting differential expression of mRNAs encoding aminoacyl-tRNA synthetases, small and large ribosomal proteins, eukaryotic initiation factors (eIFs), and DNA polymerases. (D) Bar plot depicting *U. maydis* hyphal cell length (6 h.p.i.) under untreated conditions and after treatment with the translation initiation inhibitor rocaglamide A (RocA) or the translation elongation inhibitor cycloheximide (CHX) in wild-type and *rrm4Δ* strains.

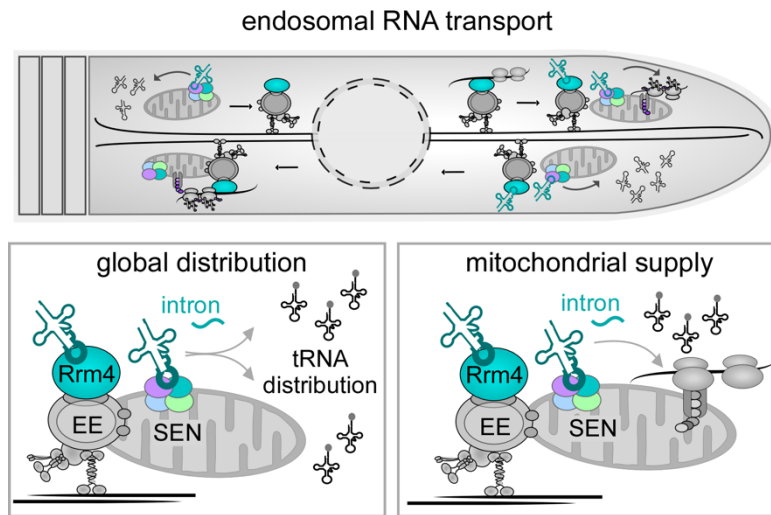


Fig. 6 Rrm4-mediated pre-tRNA transport in highly polarized cells

Schematic model of Rrm4-mediated endosomal transport of mRNAs and pre-tRNAs (turquoise) along microtubules in polarized hyphae. Pre-tRNAs are delivered to the mitochondrial surface, where splicing is mediated by the SEN complex. Mature tRNAs are then distributed to support local translation at mitochondria and/or cytoplasmic sites.

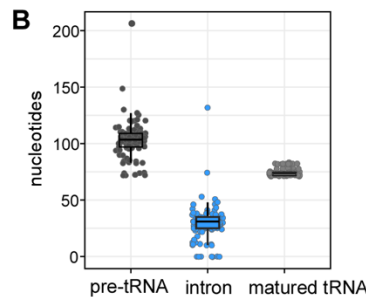
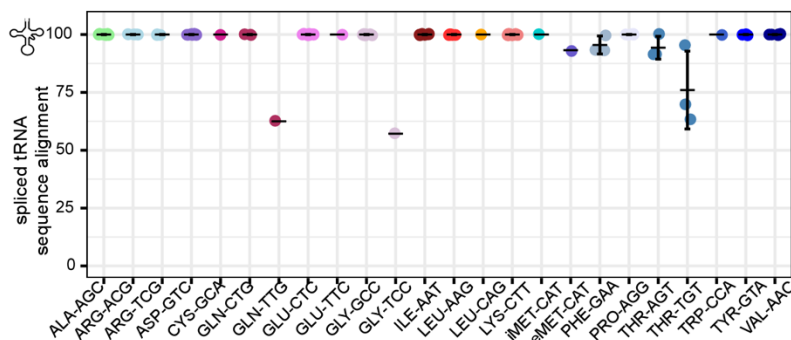
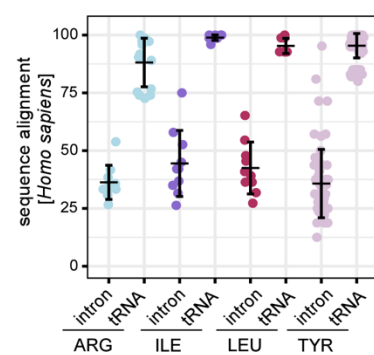
A

Intron-containing tRNAs in <i>U. maydis</i>							
ALA-AGC 5	ALA-CGC 1	ALA-TGC 1	GLY-GCC 6	GLY-CCC 1	GLY-TCC 2	PRO-AGG 4	PRO-CGG 1
PRO-TGG 1	THR-CGT 1	THR-TGT 1	VAL-AAC 4	VAL-CAC 1	VAL-TAC 1	SER-AGA 3	SER-CGA 3
SER-TGA 1	SER-GCT 2	ARG-ACG 4	ARG-TCG 3	ARG-CCT 1	ARG-TCT 1	LEU-AAG 4	LEU-CAG 2
LEU-TAG 1	PHE-GAA 3	ASN-GTT 1	LYS-CTT 5	LYS-TTT 1	ASP-GTC 5	GLU-CTC 4	GLU-TTC 2
HIS-GTG 1	GLN-CTG 3	GLN-TTG 2	ILE-AAT 4	ILE-TAT 1	MET-CAT 4	TYR-GTA 3	SUPR-TCA 2
CYS-GCA 2	TRP-CCA 2						

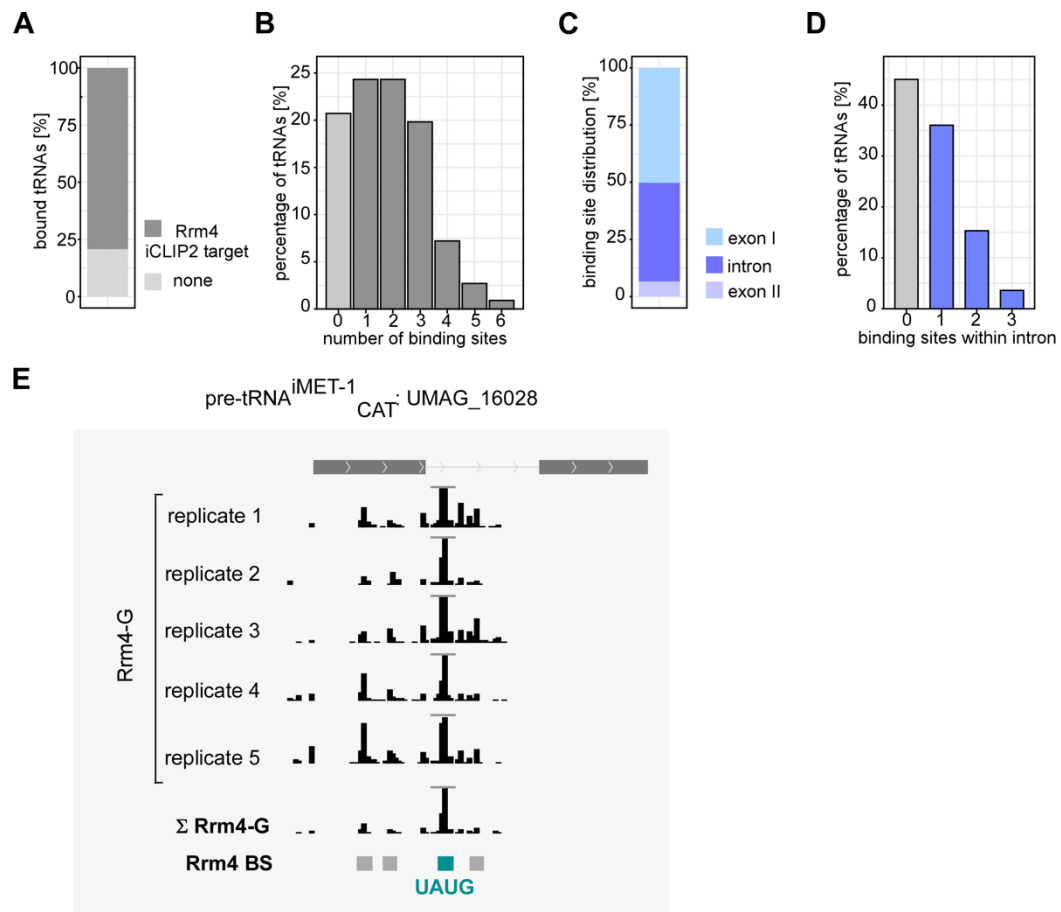
n = 100

Intron-less tRNAs in <i>U. maydis</i>			
ASN-GTT 3	GLU-CTC 2	LEU-TAG 1	THR-AGT 4

n = 10

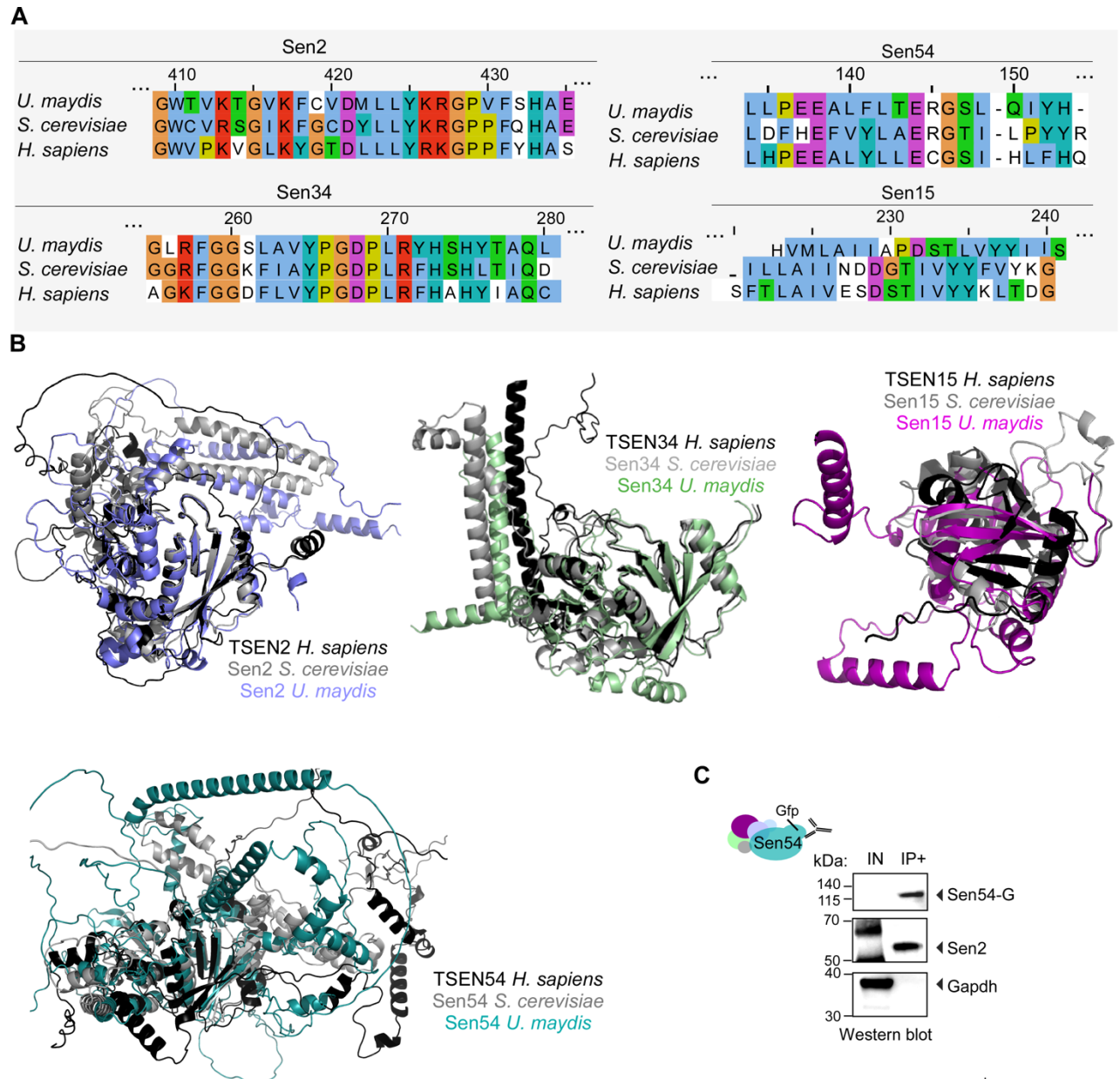
**C****D****SI-Fig. 1 *U. maydis* tRNA inventory**

(A) Table displaying the *U. maydis* tRNA inventory, categorized into intron-containing and intron-free tRNAs with anticodon resolution. (B) Scatter plot depicting nucleotide length across the *U. maydis* tRNA repertoire. (C) Scatter plot of sequence alignment scores for *U. maydis* spliced tRNAs of isodecoder groups ($n \geq 2$). Each dot represents the sequence alignment score between two isodecoders. (D) Sequence alignment score comparison between intronic (intron) and mature tRNA isodecoders (tRNA) in humans.



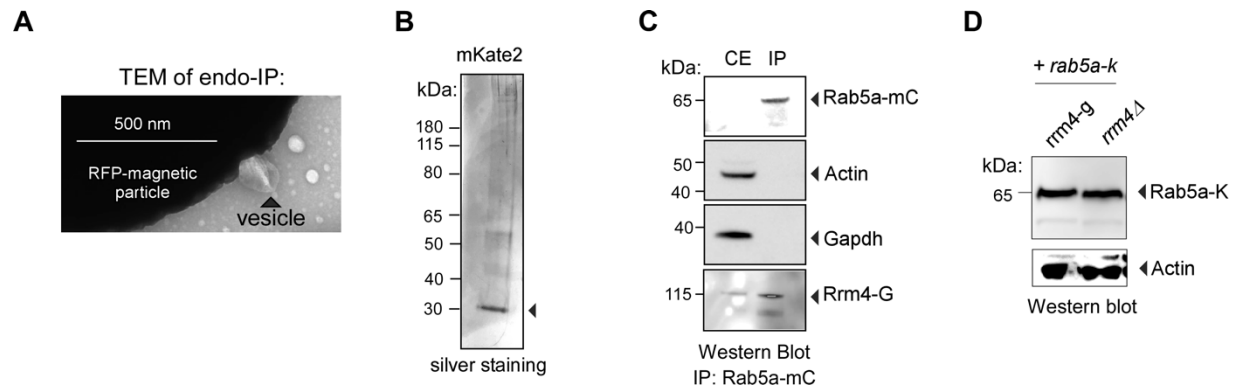
SI-Fig. 2 Rrm4 binds pre-tRNA^{iMET}

(A) Stacked bar graph showing the percentage of Rrm4-bound tRNAs. (B) Bar graph displaying the percentage of binding sites (BS) located within (pre-)tRNAs bound by Rrm4. (C) Stacked bar graph showing the distribution of Rrm4 binding sites across different regions of (pre-)tRNAs. (D) Bar graph illustrating the percentage of Rrm4 binding sites located within intron regions of bound (pre-)tRNAs. (E) Genome browser view pre-tRNA^{iMET-1} (UMAG_16028), showing a prominent UAUG-binding site (petrol) within the intron region. Grey bar marks signal that extend beyond the visualized range.



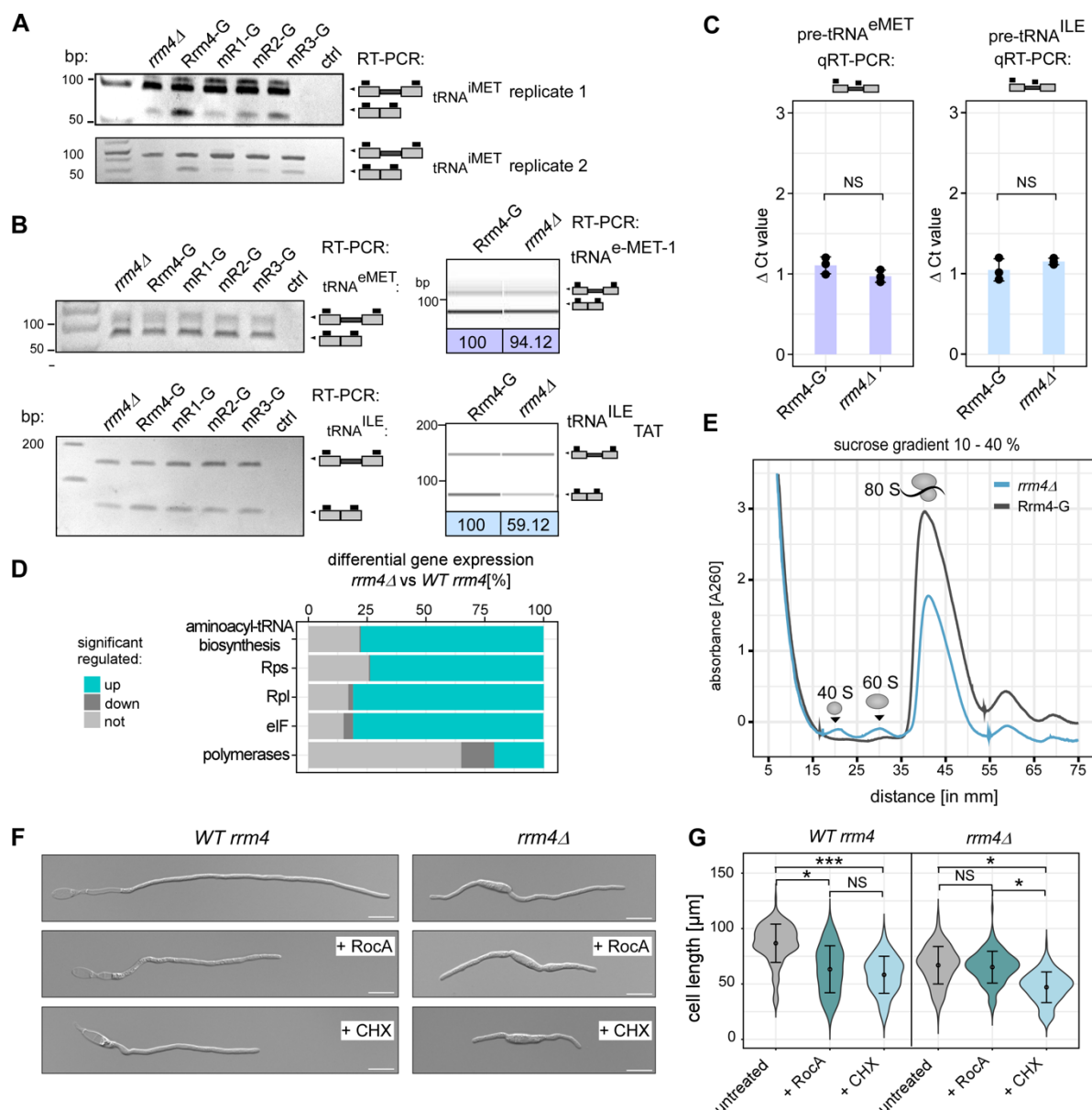
SI-Figure 3. *U. maydis* tRNA splicing machinery

(A) Multiple-sequence alignment of the SEN complex subunits Sen2, Sen34, Sen54, and Sen15 using Multiple Sequence Comparison by Log-Expectation, highlighting conserved regions across species (see Material and Methods). Accession numbers for protein sequences used are included in SI-Table S4). (B) Superposition of AlphaFold3 structural models of *U. maydis*, *S. cerevisiae*, and human (T)SEN subunits, illustrating structural conservation of the tRNA splicing machinery. (C) Western blot analysis of co-immunoprecipitated proteins using Sen54-G as bait.



SI-Figure 4. Establishment of the endosome-immunoprecipitation approach

(A) Transmission electron microscopy (TEM) image of an endo-IP approach showing a vesicle coupled to a magnetic particle. Cell extracts were diluted (1:20) before usage. (B) Silver-stained SDS-PAGE of mKate2 pulldown. (C) Western blot analysis of total cell extract and endosome-immunoprecipitation samples via Rab5a-mCherry, demonstrating enrichment of Rab5a-mC and Rrm4-Gfp in endosomal fractions. Actin and GAPDH serve as cytoplasmic controls and are, as expected, depleted in the endosomal fractions. (D) Western blot analysis showing Rab5a-K protein expression in Rrm4-G and *rrm4Δ* mutants. Actin serves as a loading control.



SI-Figure 5. tRNA maturation and transcriptomic changes

(A) Semi-quantitative PCR analysis of reverse-transcribed cDNA libraries optimized for tRNAs, showing spliced and unspliced pre-tRNA^{MET} in *Rrm4-G*, mutated versions (*mR1-G*, *mR2-G*, *mR3-G*), and deletion (*rrm4Δ*) background strains. (B) Semiquantitative and digital electrophoresis visualizing spliced and unspliced pre-tRNA^{MET} isoforms in various *Rrm4* background strains. Quantifying pre-tRNA^{MET} abundance (qPCR, n=3, two-tailed t-test, unpaired) in *Rrm4-G* and *rrm4Δ* background strains. The target *pab1* serves as a constitutive control. (C) Semiquantitative and digital electrophoresis visualizing spliced and unspliced pre-tRNA^{ILE} isoforms in various *Rrm4* background strains. Quantifying pre-tRNA^{ILE} abundance (qPCR, n=3, two-tailed t-test, unpaired) in *Rrm4-G* and *rrm4Δ* background strains. The target *pab1* serves as a constitutive control. (D) Bar graph showing the percentage of significantly up- and downregulated mRNA transcripts from differential RNA-seq analysis of *rrm4Δ* versus wild-type. (E) Sucrose gradient analysis of CHX-treated hyphae (6 h.p.i.) in *Rrm4-G* versus *rrm4Δ* strains, focusing on monosomes (80S) and free ribosomal subunits (40S and 60S). (F) DIC images of wild-type *rrm4* and *rrm4Δ* *U. maydis* hyphae 6 hours post-induction (6 h.p.i.), either untreated or treated with 50 μ M rocaglamide A (RocA) or 50 μ M cycloheximide (CHX). (G) Violin plot visualizing hyphal cell length (μ m) under the respective conditions (three biological replicates, N=150). For statistical analysis, the mean of each biological replicate was used for an unpaired two-tailed t-test.

Supporting information:

Endosomal pre-tRNA trafficking promotes subcellular splicing at mitochondria

Nina Kim Stoffel¹, Melissa Vázquez-Carrada¹, Thomas Lenz^{2,3}, Kira Müntjes¹, Johannes Postma¹, Srimeenakshi Sankaranarayanan^{1,4,5}, Kai Stühler^{2,3}, Kathi Zarnack^{4,5}, Julian König^{5,6}, Michael Feldbrügge¹

¹Institute of Microbiology, Heinrich Heine University Düsseldorf, 40204 Düsseldorf, Germany

²Molecular Proteomics Laboratory, Biological Medical Research Center, Heinrich-Heine-University Düsseldorf, 40225 Düsseldorf, Germany.

³Institute for Molecular Medicine, Proteome Research, University Hospital and Medical Faculty, Heinrich-Heine-University Düsseldorf, 40225 Düsseldorf, Germany.

⁴Buchmann Institute for Molecular Life Sciences (BMLS) & Institute of Molecular Biosciences, Goethe University Frankfurt, Max-von-Laue-Str. 15, 60438 Frankfurt, Germany

⁵Theodor Boveri Institute for Biosciences, Biocenter, Julius Maximilians University Würzburg, Am Hubland, 97074 Würzburg, Germany

⁶Institute of Molecular Biology gGmbH, Ackermannweg 4, 55128 Mainz, Germany

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Additional files:

Supplementary Table S5: Rrm4-BS within tRNA regions given as separate Microsoft Excel file

Supplementary Table S6: pre-tRNA-intron content across organisms given as separate Microsoft Excel file

Supplementary Table S7: Endo-IP proteomics given as separate Microsoft Excel file

Table S1. Description of *U. maydis* strains used in this study

Strain name with code	Locus	Progenitor strain	Short description
AB33 (UMa133)	<i>b</i>	FB2	promoter-nar:bw2bE1, expression of active b heterodimer under control of the nar1 promoter, filamentous growth can be induced upon nitrogen source switch (Brachmann et al., 2001)
AB33_rrm4Δ (Uma273)	<i>rrm4</i>	AB33 (UMa133)	carrying a deletion of <i>rrm4</i> (Becht et al., 2005)
AB33_rrm4-egfp (UMa274)	<i>rrm4</i>	AB33_rrm4Δ (UMa273)	expressing Rrm4 C-terminally fused to eGfp, endogenous promoter (Becht et al., 2005)
AB33_mR1-egfp (UMa311)	<i>rrm4</i>	AB33_rrm4Δ (UMa273)	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM1: TVEF (116-119 aa) to AAAA, endogenous promoter (Becht et al., 2006)
AB33_mR2-egfp (UMa312)	<i>rrm4</i>	AB33_rrm4Δ (UMa273)	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM2: VLEF (201-205 aa) to AAAA, endogenous promoter (Stoffel, Sankaranarayanan et al., 2025)
AB33_mR3-egfp (UMa313)	<i>rrm4</i>	AB33_rrm4Δ (UMa273)	expressing a mutated version Rrm4 C-terminally fused to eGfp mutation in RRM3: FVSF (362-368 aa) to AAAA, endogenous promoter (Becht et al., 2006)
AB33_sen54-egfp (UL0262)	<i>sen54</i>	AB33 (UMa133)	expressing Sen54 C-terminally fused to eGfp, endogenous promoter (this study, plasmid: pUL0367)
AB33_sen15-egfp (UL0250)	<i>sen15</i>	AB33 (UMa133)	expressing Sen15 C-terminally fused to eGfp, endogenous promoter (this study, plasmid: pUL0410)
AB33_mkate2 (UMa1987)	<i>cbx</i>	AB33 (UMa133)	expressing mKate2, overexpression promoter pOtef (Müntjes et al., 2019)
AB33_rab5a-mkate2/rrm4-egfp (UL0174)	<i>cbx</i> <i>rrm4</i>	AB33_rrm4-egfp (Uma274)	expressing Rrm4 C-terminally fused to eGfp and Rab5a N-terminally fused to mKate2 under the overexpression promoter pOtef (this study, plasmid: pUMa4237)
AB33_rab5a-mkate2/rrm4Δ (UL0175)	<i>cbx</i> <i>rrm4</i>	AB33_rrm4Δ (Uma273)	rrm4 deletion background strain expressing Rab5a N-terminally fused to mKate2 under the overexpression promoter pOtef (this study, plasmid: pUMa4237)

Table S2. Description of plasmids generated for *U. maydis* strain generation

Plasmid	Plasmid ID	Resistance cassette	Short description
potef:Rab5-mKate2_Cbx	pUMa4237	Cbx (carboxin resistance)	Plasmid vector for generating a N-terminal mKate2 tagged version of Rab5a under the constitutive overexpression promoter pOtef (plasmid designed by Muntjes)
pSen54:Sen54-eGfp_Hyg	pUL0367	Hyg (hygromycin resistance)	Plasmid vector for generating a C-terminal eGfp tagged version of Sen54 under the native promoter pSen54 (plasmid designed by Vázquez-Carrada)
pSen15:Sen15-eGfp_Hyg	pUL0410	Hyg (hygromycin resistance)	Plasmid vector for generating a C-terminal eGfp tagged version of Sen15 under the native promoter pSen54 (plasmid designed by Vázquez-Carrada)

Table S3: DNA oligonucleotides used in this study

Designation	Nucleotide sequence (5'→ 3')	Experiment/ Validation of:
UF_Sen54_fwd (oLA026)	GGTCTCGCCTGCAATATTGCACAACCAGCACCA	Generation of pUL0367
UF_Sen54_rev (oLA027)	GGTCTCCTGGCCAGCGGCGTGTGATGGCTCTGCGTCTG	Generation of pUL0367
DF_Sen54_fwd (oLA028)	GGTCTCCGGCCTTGTCAGTATGACATGGGCA	Generation of pUL0367
DF_Sen54_rev (oLA029)	GGTCTCGCTGCAATATTTTCGAGAGTCGCCGCAT	Generation of pUL0367
UF_Sen15_fwd (oLA141)	GGTCTCGCCTGCTATTCGGCACCATGTGTCTGTGAC	Generation of pUL0410
UF_Sen15_rev (oLA142)	GGTCTCCTGGCACAGTTGATCGGCTTGACCATAC	Generation of pUL0410
DF_Sen15_fwd (oLA143)	GGTCTCCGGCCTAGTTTAGTAATAGTCACAGAG	Generation of pUL0410
DF_Sen15_rev (oLA144)	GGTCTCGCTGCATCATATTATCAGCAACAAGCAGCTG	Generation of pUL0410
Atp3_fwd	CCTTGGTGACATCGTCTCTCC	RT-PCR
Atp3_rev	CTTTGCCCAAGAACCTTGCC	RT-PCR
Cdc12_fwd	ACGGATACGCTGCTTGAACA	RT-PCR
Cdc12_rev	CAAGAACAGCAACCCGAACG	RT-PCR
Umag_16082_tRNAmet2_Exon_P1_fwd	TCTTAGCGCAGTAGGTAGCG	RT-PCR
Umag_16082_tRNAmet2_Exon_P2_fwd	GTAGCGCGTTGGGCTCATA	RT-PCR
Umag_16082_tRNAmet2_Intron_P1_fwd	TTGGGCTCATAACCCCCTA	RT-PCR
Umag_16082_tRNAmet2_Exon_P1_rev	GGTTTCGATCCGACGACCTT	RT-PCR
Umag_16082_tRNAmet2_Exon_P2_rev	GCTTCTGTGCGTTTCGATCC	RT-PCR
Umag_16082_tRNAmet2_Intron_P1_rev:	ACGACCTTTGGGTTTCACCA	RT-PCR
Umag_16028_tRNAmet_Exon_P1_fwd	GGTTTCGATCCGACGACCTT	RT-PCR
Umag_16028_tRNAmet_Exon_P2_fwd	GCTTCTGTGCGTTTCGATCC	RT-PCR
Umag_16028_tRNAmet_Intron_P1_fwd	CGACCTTTGGGTTTCACCAC	RT-PCR
Umag_16028_tRNAmet_Exon_P1_rev	TTCTTAGCGCAGTAGGTAGCG	RT-PCR
Umag_16028_tRNAmet_Exon_P2_rev	GTAGCGCGTTGGGCTCATA	RT-PCR
Umag_16028_tRNAmet_Intron_P1_rev	GCGTTGGGCTCATACCTCAT	RT-PCR
Umag_16019_tRNAile_Exon_P1_fwd	GGACTCGAACCCACGATCTC	RT-PCR
Umag_16019_tRNAile_Intron_P1_fwd	CGATCTCGCGTTTCGTGTA	RT-PCR
Umag_16019_tRNAile_Intron_P2_fwd	GAAAAGCAACCTTTTCTGCGA	RT-PCR
Umag_16019_tRNAile_Exon_P1_rev	CCGGTGGCCAAGTTGGATTA	RT-PCR

Umag_16019_tRNAile_Exon_P2_rev	GTGGCCAAGTTGGATTAAGGC	RT-PCR
Umag_16019_tRNAile_Intron_P1_rev	AGTTCATGGGGTTTTAATCGCAG	RT-PCR
UMAG_16084_tRNA_eMET_Intron_fwd	CAGCTCGTAGGTTGATTCCTGT	RT-PCR
UMAG_16084_tRNA_eMET_ExonI_rev	CGTGGCCTAGTTGGTTAGGG	RT-PCR
UMAG_16084_tRNA_eMET_Exon2_fwd	GTCCTCGGCGAGAGTCGAAC	RT-PCR
UMAG_16084_tRNA_eMET_ExonI_rev	TCTTCGTGGCCTAGTTGGTT	RT-PCR
UMAG_16110_tRNA_prolin_Exon2_fwd	AATCGAACCGGGACCTCTG	RT-PCR
UMAG_16110_tRNA_prolin_ExonI_rev	GGGCCAATCGTATAGTGGCT	RT-PCR
Pab1_fwd	TACGTTGGTGAGCTTGACCC	RT-PCR
Pab1_rev	TCGTGGAGAGCCTTGTTGTC	RT-PCR
Actin_fwd	GGCCTTCTACGTCGCTATCC	RT-PCR
Actin_rev	TCGAGACGCAGAATCGAGTG	RT-PCR

Table S4. Accession numbers of SEN complex subunits

Organism name	Protein name	Uniprot ID	Length (aa)
<i>Ustilago maydis</i>	Sen2	A0A0D1C8Y2	527
<i>Ustilago maydis</i>	Sen54	A0A0D1BXJ9	760
<i>Ustilago maydis</i>	Sen34	A0A0D1E8E1	363
<i>Ustilago maydis</i>	Sen15	A0A0D1C8V1	246
<i>Saccharomyces cerevisiae</i>	Sen2	P16658	377
<i>Saccharomyces cerevisiae</i>	Sen54	Q02825	467
<i>Saccharomyces cerevisiae</i>	Sen34	P39707	275
<i>Saccharomyces cerevisiae</i>	Sen15	Q04675	128
<i>Homo sapiens</i>	TSEN2	Q8NCE0	465
<i>Homo sapiens</i>	TSEN54	Q7Z6J9	526
<i>Homo sapiens</i>	TSEN34	Q9BSV6	310
<i>Homo sapiens</i>	TSEN15	Q8WW01	171

8.1 Author contributions

Figure 1

(A–D) Data analysis and visualization were performed by Nina Kim Stoffel (NKS) and Prof. Dr. Michael Feldbrügge (MF).

Figure 2

(A–G) Data analysis and visualization were performed by NKS with input from MF.

Figure 3

(A) Data analysis and visualization were performed by NKS with input from MF. (B) Structural analysis was performed by Dr. Florian Altegoer with input from NKS and MF. (C) *Ustilago maydis* strains were prepared by Dr. Melissa Vázquez-Carrada1 (MVC); Western blot analysis and data visualization were performed by NKS. (D) *Ustilago maydis* strains were performed by MVC; microscopy analysis was performed by NKS and Dr. Kira Müntjes; visualization was performed by NKS. (E) Data analysis and visualization were performed by NKS.

Figure 4

(A–B) Data analysis and visualization were performed by NKS with input from MF. (C–D) Endo-IP mass spectrometry and data analysis were performed by NKS and Dr. Thomas Lenz (MS Facility); data visualization was performed by NKS with input from MF. (E–F) Data analysis and visualization were performed by NKS with input from MF.

Figure 5

(A–B) Data analysis and visualization were performed by NKS with input from MF. (C) RNA-seq analysis was performed by Srimeenakshi Sankaranarayanan (SM) and re-analysis was performed by NKS with input from MF. (D) Microscopy analysis and data analysis were performed by Johannes Postma (JP), MVC, and Kira Müntjes (KM).

Figure 6

Visualization was performed by NKS and MF.

SI-Figure 1

(A–D) Data analysis and visualization were performed by NKS with input from MF.

SI-Figure 2

(A–E) Data analysis and visualization were performed by NKS with input from MF.

SI-Figure 3

(A–C) Data analysis and visualization were performed by NKS with input from MF.

SI-Figure 4

(A) Electron microscopy data were performed by NKS and Dr. Miriam Bäumers. (B–D) Biochemical analysis and visualization were performed by NKS with input from MF.

SI-Figure 4

(A–C) Biochemical analysis and visualization were performed by NKS with input from

MF. (D) Differential RNA-seq analysis was analyzed by Srimeenakshi Sankaranarayanan (SM) and re-analysis was conducted by NKS under guidance of MF. (E) Sucrose gradient were performed by NKS. (F) Microscopy analysis and data analysis were performed by JP, MVC, and KM.

Supplementary Table:

SI-Tab. 1–3: Data analysis compiled by NKS with input from MF.

SI-Tab. 4: Protein homologs compiled by MVC with input from MF.

SI-Tab. 5–6: Data analysis conducted by NKS with input from MF.

SI-Tab. 7: Endosome IP-MS data compiled by NKS in collaboration with TL.

Due to the large amount of data, Supplementary Tables 5–7 are provided separately.

Literature research:

NKS and MF conducted the literature review.

Writing:

The manuscript was written by NKS and MF, with input from Drs. Florian Altegoer, Kerstin Schipper, Alexander Steffen, Heiner Schaal, Dierk Niessing, and all co-authors.

9 tRNA trafficking: Subcellular processing and local translation

Review article:

tRNA trafficking: Subcellular processing and local translation

Nina Kim Stoffel (unpublished)

tRNA trafficking: Subcellular processing and local translation

ABSTRACT

Transfer RNAs (tRNAs) are indispensable for protein synthesis, yet their processing, transport, and subcellular distribution remain surprisingly underexplored. Interestingly, the localization of the pre-tRNA splicing machinery varies widely across eukaryotes—from nuclear and cytoplasmic sites to the mitochondrial surface. Here, we examine how the mitochondrial surface localization of the tRNA endonuclease (SEN) complex may facilitate both cytoplasmic and mitochondrial translation, thereby supporting cellular homeostasis. Beyond its canonical role, we discuss potential moonlighting functions of the SEN complex, including the regulation of nuclear-encoded mitochondrial mRNA and rRNA metabolism, as well as the processing of free introns into small regulatory RNAs. Additionally, we highlight diverse (pre-)tRNA transport mechanisms—such as cytoskeletal-based movement and endosomal hitchhiking—that are conserved across species from fungi to neurons. Unraveling how pre-tRNA splicing, transport, and localization coordinate to fine-tune translation not only deepens our understanding of subcellular RNA dynamics but also opens new avenues for exploring regulatory pathways with implications for neurological disorders and antifungal therapies.

KEYWORDS: endosomal RNA transport, local translation, pre-tRNA splicing, tRNA localization, tRNA transport, TSEN complex

Highlights:

- The mitochondrial surface acts as a central hub for pre-tRNA splicing, linking processing to translational control.
- Endosomal hitchhiking enables long-distance pre-tRNA transport, mirroring conserved mRNA transport pathways in fungi and neurons.
- Spatially regulated tRNA trafficking may define translation hotspots, such as hyphal tips and neuronal growth cones.

INTRODUCTION

Over the past decade, the spatial regulation of mRNA transport and localized translation has become a central theme in RNA biology, with striking parallels observed from yeast to neurons [1, 2, 3]. Advances in biochemical profiling and live-cell imaging have revealed diverse strategies for mRNA localization, including mRNA stability control, active transport, and organelle hitchhiking [4, 5]. Furthermore, the discovery of translation hotspots has highlighted the intricate spatial regulation of protein synthesis [6, 7]. In parallel, the concept of specialized ribosomes has gained momentum, adding another layer of complexity to local translation control [8, 9].

While mRNA and ribosome localization are under intense investigation, the subcellular dynamics of tRNAs—fundamental components of translation—remain surprisingly underexplored. Yet, growing evidence links aberrant tRNA expression, splicing, aminoacylation, and altered nucleotide modifications to a wide array of

diseases, including cancer and neurological disorders [10]. These insights highlight an urgent need to unravel the spatial organization and regulatory functions of tRNAs in (localized) translation.

UNRAVELING THE ENIGMA

Mitochondrial association of the tRNA splicing machinery (SEN) and its potential implications

Similar to pre-mRNAs, intron-containing pre-tRNAs require splicing to achieve functional maturity [11, 12]. Introns within pre-tRNAs disrupt the anticodon stem-loop—a critical element for accurate mRNA decoding—making their removal indispensable for translation [13, 14, 15]. Unlike pre-mRNA introns, which are excised by the major spliceosome, pre-tRNA introns are removed exclusively by specialized, evolutionarily conserved protein complexes: the tRNA splicing endonuclease complex—termed TSEN in mammals and SEN in fungi [11, 14, 16,

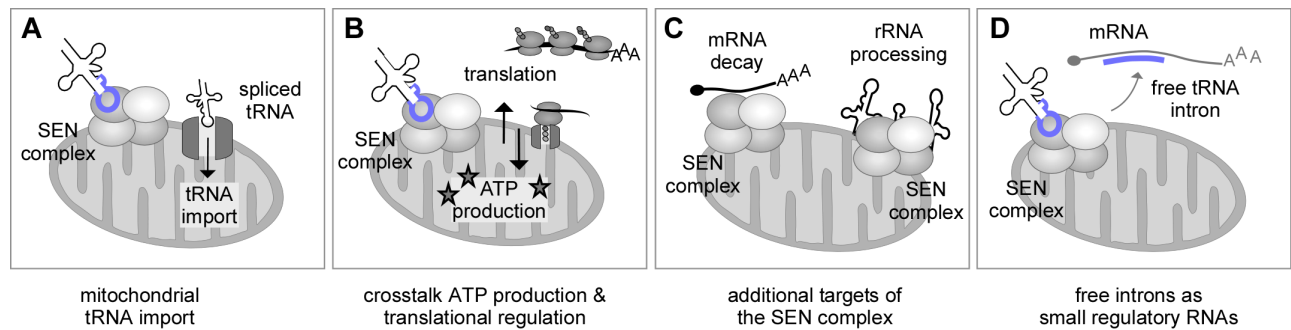


Fig. 1. Functional roles of the SEN complex

Potential explanations for localization of the SEN complex to the cytoplasmic mitochondrial surface. **(A)** Import of spliced tRNAs in mitochondria and their role in mitochondrial translation. **(B)** Crosstalk between the mitochondrial ATP production machinery and the pre-tRNA splicing machinery (SEN) as a regulator of translation. **(C)** Additional roles of the SEN complex in regulating mitochondrial and nuclear-encoded mitochondrial target mRNAs, as well as rRNA processing. **(D)** Pre-tRNAs as vehicles for free tRNA introns, acting as small regulatory RNAs that influence the fate of complementary mRNAs.

17].

Although pre-tRNA splicing is highly conserved, the number of tRNA gene copies and the frequency of introns vary greatly across species [18]. Fungal organisms such as *Ustilago maydis*, *Sporisorium reilianum*, and *Cryptococcus neoformans* possess a compact tRNA repertoire with a high proportion of intron-containing tRNAs ($\approx 90\%$), in stark contrast to budding yeast ($\approx 22\%$) and mammals ($\approx 5\text{--}7\%$) [13, 19]. The genetic and evolutionary drivers underlying these interspecies differences remain poorly understood. Notably, the subcellular localization of the tRNA splicing endonuclease (TSEN/SEN) complex also varies among eukaryotes [20, 13, 14]. In plants, the complex localizes to both the cytoplasm and the nucleus [21]. In human cells, the TSEN complex was traditionally considered nuclear [22]; however, recent findings challenge this view, revealing a cytoplasmic localization as well [23, 24]. In fungal systems—including budding and fission yeast [25, 26] and polarized hyphae of *U. maydis*—the SEN complex localizes to the cytosolic surface of mitochondria (Fig. 1, Stoffel et al., in preparation). This spatial association underscores subcellular localization as a critical determinant of the pre-tRNA lifecycle [20]. Whether mammalian pre-tRNA splicing similarly relies on such spatial coordination remains an open and compelling question [14, 23, 24].

The positioning of the SEN complex at the mitochondrial surface raises fundamental questions: How do pre-tRNAs reach their destinations? Why is this crucial splicing machinery localized to the mitochondrial cytoplasmic face? Despite extensive investigation, these questions remain unresolved [13, 14, 27].

Spatial optimization for tRNA splicing and import

One compelling explanation for the mitochondrial localization of the SEN complex is that it represents a spatial optimization strategy, functionally linking pre-tRNA splicing with subsequent mitochondrial import (Fig. 1A). Since most eukaryotic mitochondria lack a complete set of tRNA genes [28], nuclear-encoded tRNAs must be exported from the nucleus, processed at the mitochondrial surface, and, in some cases, imported into mitochondria to sustain organellar translation [29, 30]. Thus, the positioning of the SEN complex may provide a logistical advantage, streamlining the processing and import of tRNAs into mitochondria (Fig. 1A).

A nexus of two fundamental processes: ATP production and translation

An additional explanation involves the crosstalk between two major metabolic pathways: ATP production within mitochondria and cytoplasmic translation control (Fig. 1B) [31]. Mitochondria, as the primary energy producers, supply the ATP required for accurate translation. A decline in ATP production could regulate pre-tRNA splicing efficiency on the mitochondrial surface, potentially impairing translation and regulating cellular homeostasis. Given the essential role of intron removal and its impact in cytoplasmic translation, it is crucial to investigate whether mitochondrial inhibitors, such as oligomycin and rotenone targeting respiratory chain complexes I and V [32], respectively, affect pre-tRNA splicing efficiency and, in turn, influence cytoplasmic translation regulation.

Conversely, does pre-tRNA splicing influence mitochondrial function? Both local translation on the cytoplasmic surface of mitochondria [33] and tRNA import for intramitochondrial translation [30] are essential for

mitochondrial physiology. Thus, it is conceivable that pre-tRNA splicing at the mitochondrial surface directly contributes to mitochondrial function, for example by modulating the local supply of mature tRNAs or coordinating with mitochondrial protein synthesis (Fig. 1B). To dissect this potential interplay between tRNA processing and mitochondrial activity—particularly ATP production—multi-omics approaches, including RNA-seq, proteomics, and metabolomics, could reveal regulatory mechanisms and identify key molecular players governing this crosstalk (Fig. 1B).

Unraveling the multifaceted roles of the SEN complex in mRNA and rRNA regulation

Beyond its canonical role in pre-tRNA splicing, the mitochondrial localization of the SEN complex points to additional functions in RNA metabolism, including the regulation of mRNAs and rRNAs (Fig. 1C) [14, 34, 35]. In yeast, for example, the SEN complex is essential for the regulation of the nuclear-encoded mitochondrial target mRNA *CBP1*, underscoring a deeper interplay between SEN activity and mitochondrial physiology [34]. Moreover, beyond its role in mRNA regulation, the SEN complex also participates in pre-rRNA processing—a function that becomes particularly evident upon SEN mislocalization, which results in the accumulation of unprocessed pre-rRNAs [36].

Despite its central role, all known SEN-RNA targets have so far been identified through indirect means, underscoring the need for direct, high-resolution mapping of its interactome (e.g., iCLIP2) [35, 37, 38]. A promising approach could involve dual RNA interactome profiling, simultaneously targeting both endonuclease subunits, Sen2 and Sen34. This strategy could provide critical insights into (I) the precise RNA-binding landscape of SEN, (II) the spatial arrangement and subunit-specific contributions to RNA recognition, and (III) the mechanistic basis of substrate cleavage. Such data would not only refine our understanding of pre-tRNA splicing but also reveal potential mRNA and rRNA substrates of the SEN complex [34, 39].

The emerging roles of free tRNA introns

A recent study in yeast revealed that tRNA introns can function as small regulatory RNAs, modulating the fate of complementary mRNAs (Fig. 1D) [40]. This discovery raises the intriguing possibility that pre-tRNAs serve as delivery vehicles, transporting non-coding tRNA introns to the cytoplasm and even to mitochondria located far from the nucleus. Such a mechanism introduces a novel layer of post-transcriptional regulation [40], whereby free tRNA introns are locally released upon splicing at specific subcellular sites (Fig. 1D). Notably, the intronic sequences of isodecoder tRNAs often differ, raising the possibility

that the selective transport or localization of specific pre-tRNA isodecoders might mediate specialized regulatory functions (Stoffel et al., in preparation). This could occur through the release of their distinct introns, which may act as spatially restricted regulatory elements. Systematic identification of intron sequences with complementary mRNA targets across diverse species will be critical for uncovering conserved regulatory networks and defining distinct regulons governed by free tRNA introns [40]. The localization of pre-tRNAs and the SEN complex at the mitochondrial surface suggests regulatory roles that extend beyond processing. Yet, this observation prompts a fundamental question: How are pre-tRNAs efficiently directed to their mitochondrial destinations?

THE ROLE OF TRANSPORT AND LOCALIZATION

Spatial control of pre-tRNA processing

Nuclear-encoded mitochondrial target mRNAs reach distant mitochondria in neurons and fungi either through direct transport via motor proteins or by hitchhiking on vesicles [1, 3, 5, 33]. Does a similar mechanism may also govern pre-tRNA transport?

Indeed, in polarized hyphae of *U. maydis*, long-distance pre-tRNA transport is facilitated by endosomal hitchhiking along microtubules (Fig. 2A-B, Stoffel et al., in preparation). This process relies on the RNA-binding protein Rrm4 [41], which specifically recognizes the intron region of pre-tRNA^{iMET}. Loss of Rrm4 disrupts pre-tRNA splicing, elevates pre-tRNA levels and impairs translation initiation, uncovering a spatially coordinated network that links endosomal pre-tRNA transport, mitochondrial surface-mediated splicing, and translational regulation (Fig. 2A-B). Yet, do additional pre-tRNA trafficking routes exist?

In general, diverse RNA trafficking mechanisms have been described across a wide range of species [1, 4, 9]. For example, in polar-growing cells such as neurons and *U. maydis*, long-distance RNA transport is mediated by microtubules [5, 42, 43, 44], whereas in yeast, short-range, actin-dependent transport predominates, exemplified by the localization of *ASH1* mRNA [45, 46]. Notably, in yeast, mutations in cytoskeletal components—including actin, myosin V, and profilin—lead to elevated pre-tRNA levels, implicating actin-dependent pathways in the targeted delivery of pre-tRNAs to mitochondria [47]. Together with the discovery of endosomal pre-tRNA transport in polarized *U. maydis* hyphae, these findings reveal a conserved yet adaptable paradigm of pre-tRNA trafficking that may extend across eukaryotes.

Beyond fungi, the spatial organization of pre-tRNA splicing in mammals remains poorly understood [14]. However, emerging evidence indicates that pre-tRNAs are also localized in the cytoplasm [23, 24]. RNA-centric

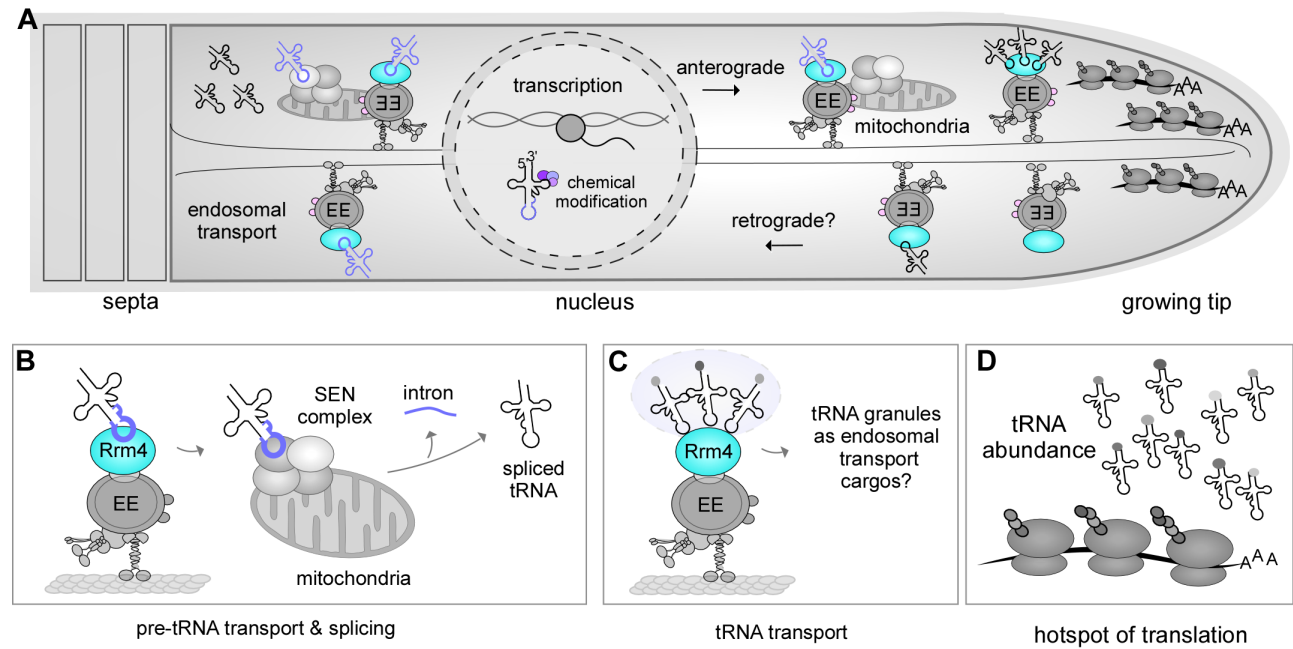


Fig. 2. Pre-tRNA transport and processing

(A) Schematic representation of bidirectional, microtubule-dependent long-distance endosomal transport along the highly polarized *Ustilago maydis* hypha. (B) Endosomal pre-tRNA transport mediated by the RNA-binding protein Rrm4, delivering pre-tRNA to mitochondria over long distances. (C) Potential Rrm4-mediated tRNA granule transport and its importance in regulating subcellular tRNA abundance and its implications in (D) hotspots of translation at the growing hyphal tip.

approaches, coupled with mass spectrometry [48], to identify pre-tRNA-associated proteins may offer critical insights into potential RNA transporters. Resolving this question will deepen our understanding of the evolutionary conservation of pre-tRNA transport systems across eukaryotes and reveal their broader functional implications.

Moreover, defects in cytoskeletal-mediated transport disrupt mRNA localization in diverse systems, leading to perinuclear accumulation of mRNAs—from mammalian muscle cells [49, 50] to *U. maydis*. This raises the intriguing possibility that pre-tRNAs similarly accumulate near the nucleus when transport is impaired, with broad implications for pre-tRNA processing, tRNA availability, and translational control.

Retrograde transport?

In yeast, following splicing at the mitochondrial surface, a subset of tRNAs is re-imported into the nucleus for further modifications prior to re-export and translation [20, 51, 52]. This retrograde trafficking introduces an additional regulatory layer, coupling tRNA transport with maturation processes beyond intron removal [52, 53, 54]. Although the potential involvement of endosomal transport in this pathway remains unresolved, these findings underscore the critical role of subcellular localization in orchestrating the tRNA lifecycle.

tRNA trafficking and its contribution to local translation sites

Although pre-tRNA transport mechanisms remain undefined in mammals, bidirectional movement of tRNA-containing granules—independent of translation—has been observed in neurons [55, 56]. Given the parallels between mRNA trafficking in neurons and fungi [1, 9, 42, 43, 50], it is plausible that fungi employ analogous mechanisms for tRNA granule trafficking. This raises the intriguing possibility that (Rrm4-mediated) endosomal transport extends beyond pre-tRNAs (Fig. 2C) to include mature tRNA granules, potentially supporting translational homeostasis across the hyphae or enabling spatially defined sites of localized translation (Fig. 2A,C,D).

Despite their abundance and potential role in translationally active endosomal transport [44, 57], mature tRNAs have been largely overlooked in recent Rrm4 iCLIP2 studies (Stoffel et al., in preparation). Expanding investigations to profile the endosome-associated tRNAome will therefore be crucial. However, current endosome purification strategies lack the spatial and temporal resolution necessary to monitor real-time dynamics. A key methodological advance would be the application of RNA-FISH or live-cell imaging approaches, enabling dynamic visualization of tRNA localization [58, 59].

To date, only a single study has applied tRNA-FISH in *U. maydis*, revealing tRNA^{GLY} accumulation at the hyphal tip [60]. The growing tip likely functions as a local translation hub (Fig. 2C-D), facilitating the synthesis of cell wall remodeling factors and secreted proteins [61, 62]. This mirrors neuronal growth cones, where localized translation maintains cellular polarity [2]. However, the mechanisms governing tRNA localization, composition, and transport to these subcellular sites remain largely unexplored. Deciphering these processes could unveil fundamental principles of spatially regulated protein synthesis conserved across fungi and neurons.

FUTURE PERSPECTIVES AND TECHNICAL CHALLENGES

- What determines the specificity and regulation of pre-tRNA transport to mitochondria and other subcellular locations?
- To what extent do tRNA processing and localization contribute to translational control under stress conditions or in disease states?
- Does endosomal hitchhiking play a broader role in tRNA localization?
- How does local tRNA abundance influence hotspots of translation?

To date, most studies have focused on specific pre-tRNA splicing events or their subcellular localization *in vivo*, leaving a comprehensive, tRNAome-wide resolution largely unaddressed. This gap stems from technical challenges—including the short length of tRNAs, their strong secondary structures, extensive chemical modifications, and high sequence similarity—which complicate both sequencing and data analysis. However, emerging technologies offer promising solutions. Quantitative tRNA nanopore sequencing enables tRNAome-wide quantification, distinguishing between spliced and unspliced tRNAs while simultaneously detecting modification sites [63]. Coupling this approach with subcellular purification strategies, such as endosome immunoprecipitation (Stoffel et al., in preparation) or physical separated neutrite-fraction [64], could provide unprecedented insights into (pre-)tRNA transport, localization, and regulatory functions. Moreover, integrating multi-omics with live-cell imaging will enable precise identification and compositional profiling of tRNA pools at translation hotspots, spanning from fungal hyphae to neurons.

Traditionally, ribosomes and tRNAs are assumed to localize wherever mRNAs are present. But what if, conversely, mRNAs migrate to pre-existing translation hotspots optimized for ribosome and tRNA availability?

COMPETING INTERESTS

No competing interest is declared.

AUTHOR CONTRIBUTIONS STATEMENT

NKS conducted the literature research, created graphical visualizations, and wrote the review article.

ACKNOWLEDGMENTS

I am particularly grateful to Dr. Kerstin Schipper for her critical proofreading of the manuscript.

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9.1 Author contributions

Figure 1:

Graphical visualization designed by NKS.

Figure 2:

Graphical visualization designed by NKS.

Literature Research:

NKS conducted the literature review.

Writing:

NKS wrote the manuscript. Proofreading was carried out by Dr. Kerstin Schipper.

10 Discussion

RBPs orchestrate the mRNA lifecycle, from synthesis to decay [3, 49]. Given their central roles, it is unsurprising that RBPs are implicated in a wide range of human diseases [4, 93, 94], plant climate adaptation [95, 96], as well as fungal growth and virulence [87, 97]. Despite their importance, key challenges in RNA biochemistry remain—particularly in understanding how RBPs select target transcripts, recognize specific RNA-binding sites, discriminate functional from accessory sites, and assemble regulatory mRNP complexes [1, 7, 13, 15, 21].

A hallmark of RBPs is their modular domain architecture, often comprising multiple RNA-binding domains, which collectively contribute to RNA-binding specificity and affinity [3, 8, 23]. Importantly, RBDs can mediate not only RNA binding but also direct protein–protein interactions, thereby broadening their regulatory scope [28, 48, 98]. Through this modularity, RBPs can fine-tune RNA recognition while dynamically shaping mRNP-protein composition via direct or RNA-mediated interactions [19, 23, 27, 48]. Notable examples of multi-RBD RBPs include ELAV-like proteins, such as the mammalian RNA transporter HuD, and the fungal endosome-associated RBP Rrm4 [30, 32, 74, 99]. While the presence of multi-RBDs is a common feature within the RBPworld [8, 28], how individual domains contribute to the RNA and protein interactomes—and influence downstream mRNP functions—remains poorly understood, especially *in vivo* and at a global scale [21, 22, 23]. This thesis combined multi-omics and molecular cell biology approaches to dissect the mechanistic framework of the ELAV-like RBP Rrm4. Specifically, it:

- (I) mapped the RNA-binding specificities of individual RRM domains and evaluated their functional contributions to target mRNA regulation.
- (II) defined the RRM-dependent endosomal cargo proteome.
- (III) elucidated how the RRM domains influence the protein interactome, integrate cooperative RBP actions, and coordinate shared mRNA regulons.

10.1 Optimized multi-omics workflows for high-resolution analysis of Rrm4

To enable high-resolution multi-omics analyses and resolve subtle transcriptome- and proteome-wide effects caused by mutations within individual RRM domains of Rrm4, key experimental workflows were systematically improved.

The microbial iCLIP2 protocol was refined through customized cell lysis procedures and tailored biochemical buffers [100]. These improvements enhanced RNA and protein stability, significantly increasing data quality and enabling transcriptome-wide,

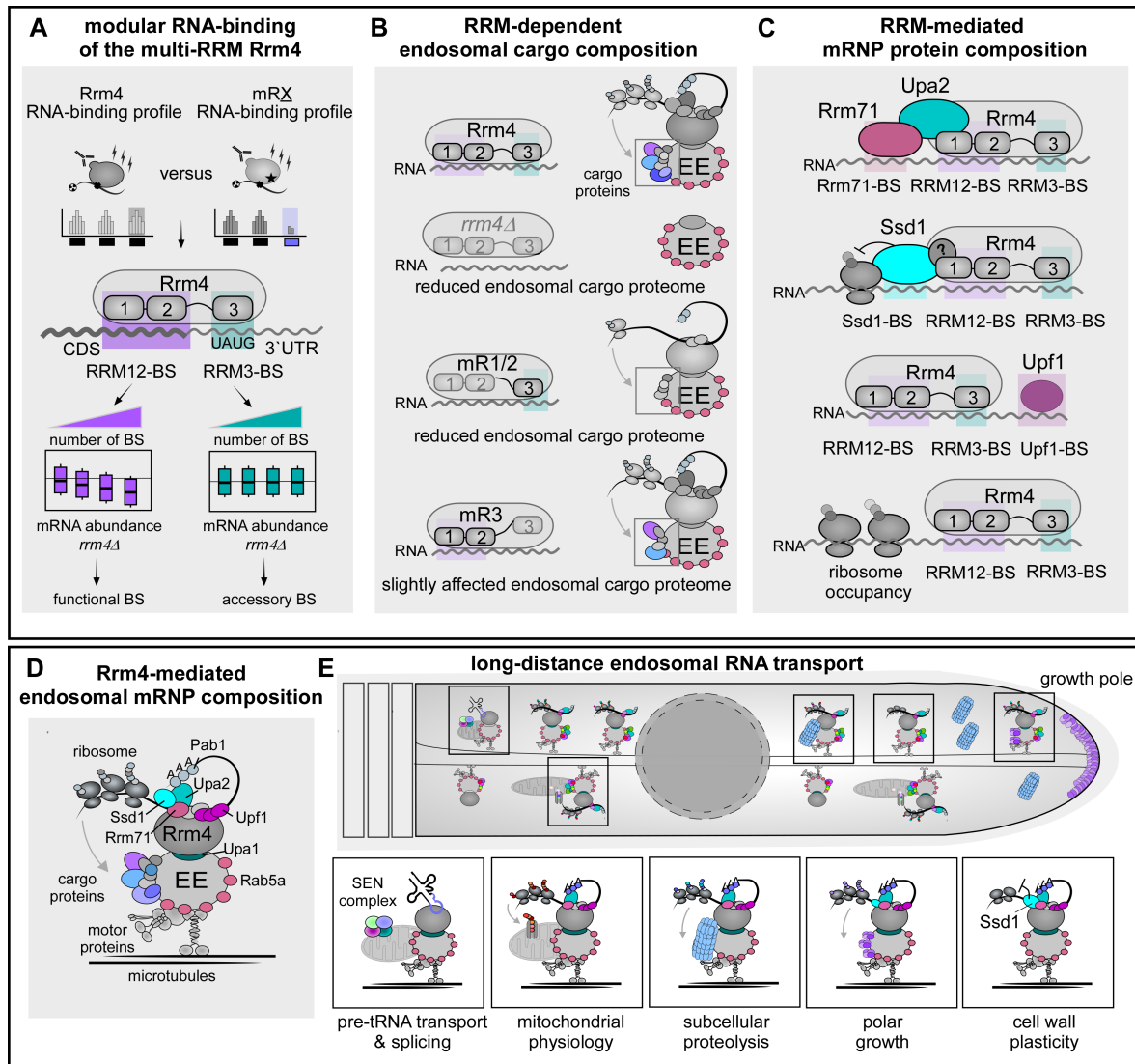


Figure 3: Fig. 3 Rrm4- / RRM-mediated endosomal RNA transport and its functional implications

(A) Schematic representation of comparative iCLIP2 analysis highlighting the modular RNA-binding mode of Rrm4. The functional relevance of RRM12-bound sites (purple) is shown in relation to mRNA abundance. (B) Schematic overview of the endosomal cargo proteome composition, dependent on Rrm4 and its RRMs. (C) Modular interactome and potential cooperative binding modes of Rrm4 with additional RBPs, including Rrm71, Ssd1, and Upf1. The schematic also illustrates the modular arrangements of the RRMs near translating ribosomes. (D) Updated model of endosomal mRNA transport incorporating newly identified RBPs (e.g., Rrm71, Upf1). While this model depicts translationally active transport, Ssd1-associated mRNPs likely represent translationally silent states (see E regarding cell wall plasticity). (E) Schematic of long-distance endosomal transport along microtubule bundles in *U. maydis* hyphae. Zoomed-in panels illustrate distinct RNA cargo regulons, including pre-tRNA transport, nuclear-encoded mitochondrial mRNAs translated on early endosomes near mitochondria, translationally active transport of proteasomal target mRNAs and polarity factors, and translationally repressed transport of mRNAs encoding cell wall remodeling enzymes.

high-sensitivity detection of Rrm4 BS *in vivo* (Fig. 3A) [82, 100]. Furthermore, the en-

hanced microbial iCLIP2 approach enabled high-resolution mapping of domain-specific binding sites for the RRM mutants (mRX, Fig. 3A), which exhibit up to 60% reduced RNA-binding capacity [80, 101].

Building on these advances, the cell disruption protocol was further refined to develop an endosome immunoprecipitation (endo-IP; Fig. 3B) method tailored to *U. maydis*. This enabled the selective isolation of intact endosomes, along with their associated cargo RNAs and proteins (see Section 6).

In parallel, the co-IP MS workflow was optimized by integrating mechanical disruption steps from the microbial iCLIP2 protocol and by adjusting co-IP buffer conditions (Fig. 3C, see Section 7). These enhancements stabilized Rrm4-containing mRNP complexes, preserved RNA integrity, and markedly reduced non-specific protein background, thereby surpassing the sensitivity and specificity of previous Rrm4 co-IP protocols [82, 89].

10.2 Rrm4’s modular control of RNA binding

A longstanding mystery in the field of RNA–protein interactions is how RNA-binding specificity is established and how functional binding sites are distinguished from accessory ones [15, 17]. Comparative RRM mutant-based iCLIP2 combined with differential RNA-seq analyses revealed two distinct Rrm4 RNA-binding modes: RRM12-BS and RRM3-BS. The tandem-RRMs (RRM1/2) primarily define subtle but functionally critical crosslink sites (RRM12-BS), while RRM3 provides additional support. Notably, RRM12-BS are enriched in critical cargo mRNAs that govern polar growth, cell wall plasticity, and mitochondrial function—processes intimately linked to the observed aberrant growth phenotype (Fig. 3A) [101]. In contrast, RRM3-specific binding sites (RRM3-BS) frequently overlap with the UAUG motif, forming sharp and prominent crosslink peaks—consistent with previous iCLIP results and yeast three-hybrid assays [82, 87]. However, while RRM3 contributes to RNA binding, it is dispensable for maintaining mRNA abundance and unipolar hyphal growth, in contrast to the essential tandem-RRMs (Fig. 3A) [31, 82]. Functional RRM12-BS are often subtle and easily overlooked in conventional CLIP analyses [13, 40, 82], whereas RRM3-BS tend to be more prominent. This discrepancy likely arises from UV crosslinking biases specific to amino acids–nucleotide interactions (π -stacking) [102, 103]. These findings underscore the importance of a comparative iCLIP2 approach to distinguish functionally relevant sites from accessory binding sites and highlight the need for high-depth iCLIP2 libraries to detect even minor binding changes [13, 41]. Given the established functional importance of individual binding sites (RRM12-BS vs RRM3-BS) and the potential π -stacking bias between the RRM, Rrm4 represents an ideal candidate to evaluate alternative zero-distance crosslinkers [104, 105]. Such comparative analyses could illuminate how

different crosslinkers reshape the RNA-binding landscape compared to conventional UV crosslinking, thereby revolutionizing the field of RNA-protein interaction studies [12, 13, 39, 40, 102, 105].

Overall, the coomparative iCLIP2 analysis using RRM-mutant strains revealed a defined modular binding mode of Rrm4 [82, 101]. Interestingly, a similar principle was observed *in vitro* in the human RNA transporter HuD, where tandem-RRMs drive target RNA recognition, while RRM3 enhances binding affinity [32]. This suggests a conserved ELAV-like modular binding mechanism, reinforcing that distinct RRM domains contribute differentially to RNA recognition and affinity [15, 23]. Thus, this comparative iCLIP2 approach provided a high-resolution, transcriptome-wide dissection of RBD-mediated RNA-binding specificity, offering a valuable blueprint for unraveling the functional roles of multi-domain RBPs *in vivo*.

For the field of RNA biochemistry, it would be valuable to deepen our understanding of the RNA recognition process in a time-resolved manner, shedding light on how binding is regulated by multi-RBD proteins [23]. Since iCLIP(2) captures only endpoint binding events [41, 45, 106], it cannot provide information on real-time binding dynamics. In this context, kinetic-iCLIP combined with RRM mutants represents a promising approach to further unravel this complex regulatory process over time [17, 107].

10.3 Rrm4-driven endosomal transport regulates mRNA localization and abundance

Rrm4 is an RNA transporter that localizes to the surfaces of motile endosomes, orchestrating the global distribution of bulk mRNA throughout the hyphae [74, 78, 82]. In this thesis, microscopy-based analyses revealed that mutations in individual RRMs cause pronounced perinuclear accumulation of bulk mRNA [101]. Mutations of all three RRMs—or loss of Rrm4—results in an additive effect, highlighting the collective contributions of all RRMs to RNA binding and subcellular mRNA positioning [82, 101]. This phenotype mirrors redistribution patterns observed in cardiovascular cells upon microtubule disruption, suggesting a conserved cellular response to impaired microtubule-dependent mRNA transport [74, 82, 108].

Beyond its role in global mRNA distribution, Rrm4 is essential for the subcellular localization of select transcripts, such as the septin-encoding mRNA *cdc3* [78]. Tip localization and endosomal shuttling of Cdc3 are abolished in *rrm4* Δ strains and upon mutation of RRM1, whereas RRM3 mutations have only a modest impact [78, 101]. This supports the notion that RRM12-BS are selective and functionally significant [101], highlighting the distinct roles of individual RRMs in RNA target recognition and regulation [23, 31]. Moreover, differential RNA-seq analyses of the RRM mutants and *rrm4* Δ strains revealed that mRNAs harboring RRM12-BS are specifically reduced

in strains carrying tandem-RRM mutations or lacking Rrm4. In contrast, transcripts containing RRM3-BS remain largely unaffected, highlighting functional differences among the RRMs *in vivo* [31, 101]. This establishes a link between endosomal RNA transport and steady-state mRNA levels [101]. The observed connection between accurate mRNA transport and mRNA abundance parallels findings in mammalian cells, where disrupted endosomal trafficking is associated with reduced levels of transcripts involved in mitochondrial and proteasomal function [69, 85]. Notably, in highly polarized neurons, mRNA stability is a key determinant of subcellular localization [60, 109, 110]. Collectively, these findings underscore a conserved relationship between spatial mRNA transport and transcript abundance, from fungi to neurons [60, 63, 69, 85].

10.4 The endosomal cargo proteome is shaped by the RNA-binding capacity of Rrm4

Endosomes as platforms for localized translation have emerged as a conserved feature in both fungi and mammals [63, 67, 71, 78, 84, 86]. In this thesis, an RRM-dependent endo-IP MS approach combined with comparative iCLIP2 both corroborates and significantly extends the current model of Rrm4-mediated cargo transport in *U. maydis* (Fig. 3B; Stoffel et al., unpublished; see Section 6). This integrated strategy reveals that specific mRNA regulons, along with associated ribosomes and translation products—including mitochondrial, proteasomal, and polar growth-associated proteins—are co-enriched with early endosomes in an Rrm4/ RRM-dependent manner (Fig. 3B). For example, the well-characterized cargo mRNA *cdc11*—an RRM12-BS target—exhibits endosomal localization of both its transcript and protein in an RRM-dependent manner, as confirmed by endo-IP RT-PCR and proteomics (see Section 6). These findings align with previous live-cell imaging studies demonstrating co-transport of septin mRNAs, their translation products, and ribosomal components on motile endosomes [78, 79, 81, 84, 87]. Together, these findings identify Rrm4 as a key determinant of endosomal proteome composition (Fig. 3B). Its tandem-RRM-guided RNA selectivity likely enables the spatiotemporal control of protein synthesis on early endosomes, thus linking mRNA transport, local translation, and subcellular proteome shaping in polarized cells [58, 62, 63]. However, analyzing local translation on endosomes in a time-resolved manner (e.g., SunTag) [111, 112] or in a transcriptome-wide manner (e.g., Ribo-Seq) [113, 114] would substantially deepen the mechanistic understanding, with potential implications for vesicle-based translocation systems in both mammalian and plant cells [63, 67, 68, 91, 92].

10.5 RRM1 of Rrm4 integrates RNA binding and protein interactions to shape mRNP composition

In addition to characterizing Rrm4's RNA-binding capacity, defining its protein interactome and its role in endosomal mRNA transport is crucial (Fig. 3C; see Section 7). A key question is whether—and by what mechanisms—individual RRM domains influence mRNP composition *in vivo* [18, 19, 47, 48, 98, 115, 116]. Comparative RRM-focused proteomic profiling revealed distinct interaction modes mediated by Rrm4's RNA-binding domains: (I) RNA-dependent associations with RBPs such as Upf1 and Rrm71, likely co-transported and involved in mRNA regulation, and (II) RNA-independent direct protein–protein interactions, exemplified by the scaffold protein Upa2 (Fig. 3C–D). Both live-cell imaging and *in vitro* binding assays demonstrate that endosomal movement and interaction rely on a direct contact between Upa2's GWW motif and Rrm4's RRM1 (Fig. 3C–D; see Section 7).

Beyond Upa2, the translational repressor Ssd1 [89, 117, 118] also exhibits an RNA-independent but RRM1-dependent association with Rrm4 (Fig. 3C–D, 7). This suggests that Rrm4 not only stabilizes the mRNP complex via protein–protein interactions via Upa2 [88] but also may influence the translational state of cargo mRNAs via Ssd1 (see below) [117, 119]. Similar complex mechanisms of translational regulation during transport have been described in other translocation systems, including yeast [62, 120, 121] and neurons [55, 59, 60, 65, 110]. Overall, these findings indicate that Rrm4's RRMs—particularly RRM1—not only mediate RNA binding but also influence higher-order mRNP assembly through both RNA-dependent and direct protein–protein interactions (see Section 7). This combination of RNA binding and protein–protein interactions likely acts synergistically, fine-tuning target recognition, translational regulation, and stabilizing transport mRNPs for efficient endosomal mRNA hitchhiking (Fig. 3C–D) [60, 63, 87]. For future studies, it will be highly valuable to dissect this synergistic effects more comprehensively using diverse *in vivo* and *in vitro* iCLIP2 approaches (e.g., Rrm4 iCLIP2 in Upa2/Ssd1 deletion backgrounds) [13, 40, 41, 122]. Such analyses could offer a transcriptome-wide perspective on the mechanisms defining cooperative RNA binding and its implications for RNA transport [15, 58].

10.6 Mechanisms and functional significance of Rrm4 in mRNA transport

The multi-omics perspective offers mechanistic insights into how RRMs influence the interactome of Rrm4 at both the RNA and protein level (Fig. 3A–C). Beyond these, novel RNA regulons were identified, directly linking (m)RNA transport to cellular and subcellular organization.

10.6.1 Polarity factors as key targets of Rrm4-driven mRNA transport

The connection between mRNA transport and cell polarity is well-established across diverse organisms, including budding yeast, plants, and neurons [55, 57, 59, 60, 62]. In the filamentous fungus *U. maydis*, this relationship is also evident: most polarity factors are encoded by mRNAs containing RRM12-BS, and endosomal mRNA trafficking contributes to the formation of polarity gradients within hyphae [78, 79, 87]. This functional relationship is further highlighted by the identification of Rrm4-dependent endosomal protein cargos—including the GTPases Cdc42, Rho1, and Rac1, which play central roles in cytoskeletal organization and polarity establishment (Fig. 3B,E) [123, 124, 125, 126]. Moreover, in tandem-RRM mutant strains, the endosomal association of these cargo proteins is significantly reduced (Fig. 3B,E). Interestingly, the endosomal association of mRNAs and proteins linked to polarized growth is not confined to *U. maydis* or Rrm4. Cytoskeleton-related mRNAs and proteins have also been detected in mammalian endosomal fractions and identified as direct targets of the human endosome-associated RNA-binding complex FERRY [69, 71, 127]. Collectively, these findings underscore a conserved role of endosomal mRNA transport in coordinating cell polarity, extending from fungal hyphae to neurons [58, 60, 62, 63, 87, 123, 128].

10.6.2 Endosomal mRNA transport and mitochondrial physiology

Beyond polarity factors, mRNAs encoding nuclear-encoded mitochondrial proteins are conserved targets of endosomal transport systems across diverse species [67, 69, 70, 73, 85, 86, 129]. In *U. maydis*, Rrm4 has been previously shown to bind nuclear-encoded mitochondrial mRNAs, particularly at the stop codons of transcripts encoding components of respiratory chain complex V [63, 82]. Using the improved microbial iCLIP2 protocol, this stop codon binding spectrum was extended to include transcripts encoding subunits of respiratory complexes I and III [100]. Notably, mitochondrial transcripts containing RRM12-BS represent a highly conserved class of endosomal mRNA cargos across fungi and humans [69, 101].

In neurons, mitochondrial transcripts are translated on endosomes positioned near mitochondria [67, 70, 73, 130], and a similar mechanism appears to operate in *U. maydis* [63, 100, 129]. Comparative endo-IP MS analyses revealed that mitochondrial proteins are enriched in endosomal fractions and are significantly depleted in *rrm4* Δ strains (Stoffel et al., unpublished, Fig. 3E).

Together, these findings support a model in which Rrm4 regulates the recruitment, transport, and possibly translation of nuclear-encoded mitochondrial mRNAs on endosomes (Fig. 3E) [63, 82, 100]. However, definitive evidence for translation on endosomes in *U. maydis* is currently limited by the lack of subcellular Ribo-Seq or SunTag data [111, 113, 114, 131].

The importance of this translocation system is underscored by mitochondrial dysfunction in mammals, which has been linked to neurological disorders such as Charcot-Marie-Tooth disease [67, 70, 73, 86, 129]. In *U. maydis*, strains lacking Rrm4 exhibit increased resistance to the respiratory chain V inhibitor oligomycin, suggesting an adaptive response to mitochondrial stress (Devan et al., in preparation) —likely due to insufficient supply caused by defective mRNA transport. A similar phenotype was observed in the RRM1 mutant but not in the RRM3 mutant strain (Stoffel, data not shown). This further highlights the modular RNA-binding mode of Rrm4 and the functional significance of the tandem-RRMs as key regulators of nuclear-encoded mitochondrial mRNA transport [63, 101]. For future research, it will be highly valuable to investigate the Rrm4-dependent endosomal mRNA supply from both transcriptomic and subcellular perspectives. Given the hypothesis that mitochondria distal to the nucleus are more strongly affected than those in close proximity, advanced techniques such as spatial transcriptomics or single-mitochondrion RNA-seq would be instrumental in resolving these differences [132, 133]. Overall, the data confirm and extend the link between endosomal mRNA transport, potential local translation, and mitochondrial physiology [61, 63, 67, 69, 82, 86, 129, 130, 134].

10.6.3 Rrm4-mediated endosomal transport of proteasomal mRNAs

Furthermore, the combination of comparative iCLIP2 (Fig. 3A), endo-IP MS (Fig. 3B), and comparisons to mammalian systems at both the transcriptome [69] and proteome-wide [127] levels indicates the existence of a novel cargo regulon—the proteasomal core complex (Fig. 3E). The majority of this mRNA regulon harbors RRM12-BS, underscoring their functional relevance [101]. Consistent with this, mutations within Rrm4’s RRM—particularly the tandem-RRMs—alter the endosomal association of the corresponding proteins (Fig. 3E).

Beyond *U. maydis*, studies in neurons have shown that defects in endosomal mRNA transport lead to reduced levels of transcripts encoding proteasomal subunits [85]. This supports the idea that proteasomal mRNAs are *bona fide* cargos of endosomes (see Section 6). Moreover, the combined iCLIP2 and endo-IP data suggest that mRNAs encoding proteasomal core components are transported in a translationally active state, with their corresponding proteins anchored on the endosomal surface (Fig. 3E), reminiscent of the transport mechanism described for septins [78, 79]. Aligning with this hypothesis, the association of proteasomal proteins with shuttling vesicles and their anchoring via ECM23 on endosomes has been independently demonstrated in mammalian systems [135, 136]. In neurons, the subcellular localization of the proteasome is tightly connected to spatially restricted proteolysis, which is essential for synaptic plasticity [137, 138, 139]. However, the mechanisms defining the subcellular localization of the proteasome remain poorly understood [137, 140]. It is therefore plausible that

endosomes serve as key delivery hubs, coordinating the continuum from mRNA supply to subcellular protein turnover [58, 137]. Given the established parallels between polarized hyphae and neurons [141, 142], spatially regulated proteolysis at the tip of *U. maydis* likely plays a critical role in polar growth and infection. Consequently, uncovering the Rrm4-dependent spatiotemporal regulation of protein turnover presents a compelling direction for future research. Advanced approaches such as proximity-dependent SILAC mass spectrometry and *in vivo* crosslinked proteasome purification [137, 143, 144] offer promising strategies to dissect these dynamics.

10.6.4 A cooperative binding mode of Rrm4-Ssd1 mediating translational repression of cell wall mRNAs during transport

Cooperative interplay by RBPs is a widespread mechanism across post-transcriptional processes including splicing, modification, and localization [15, 21, 47, 145, 146, 147], yet its role in defining transport specificity remains largely unexplored [58]. Proteomics and live-cell imaging revealed the translational repressor Ssd1 [117] as an RRM1-dependent, RNA-independent component of endosomal Rrm4-mRNP transport (Fig. 3C; see Section 7, Müntjes et al., in preparation) [89]. A comparable repression mechanism is mediated by the RBP Glorund through quasi-RRM2 interactions with the translational repressor dFMRP, regulating *nanos* translation elongation in *Drosophila melanogaster* [148], suggesting a conserved regulatory strategy.

The Rrm4-Ssd1 interaction may expand the RNA-binding interface (see Section 7, Müntjes et al., in preparation), thereby enhancing RNA target specificity and affinity [19, 47, 48]. Cross-species comparison of the RNA interactome of Ssd1 in *N. crassa* and Rrm4 binding targets in *U. maydis* revealed a conserved mRNA regulon comprising transcripts encoding cell wall remodeling proteins (Fig. 3; Müntjes et al., in preparation) [101, 117]. This observation is consistent with reports from other fungal systems showing that Ssd1 functions as a translational repressor of mRNAs involved in cell wall plasticity [90, 149, 150, 151]. Thus, Ssd1 likely acts as a translational gatekeeper during endosomal mRNA transport, particularly for transcripts implicated in cell wall plasticity (Fig. 3C,E). Supporting this, comparative proteomics show that Ssd1 predominantly associates with the large ribosomal subunit and exhibits reduced association with active elongation factors (see Section 7). These suggest that the transport complex accommodates both translationally active cargos—such as septin mRNAs [78, 79]—and a translationally regulated pool modulated by Ssd1, linked to defined cell wall plasticity (Fig. 3, see Section 7) [101, 117]. In hyphae of *Trichoderma reesei*, the β -glucan synthase Gcs1 co-shuttles with early endosomes and localizes to the hyphal tip [152], underscoring the link between endosomes and cell wall control [142, 153]. Future studies could focus on validating, via live-cell imaging in *U. maydis*, whether the abundance and dynamics of shuttling Gcs1 particles are affected by Ssd1 deletion or overexpression. Moreover, recent

reports suggest that the hyphal tip functions as a translation hotspot, analogous to the neuronal growth cone [56, 57]. At this site, mRNAs encoding secretion factors and cell wall enzymes are likely translated locally to support polar growth and morphogenesis [118, 154, 155]. It is therefore possible that upon release at the hyphal tip, Rrm4-Ssd1 cargo mRNAs like *gsc1* undergo local translation [118, 155]. This model is reminiscent to the translationally repressed transport of *ASH1* mRNA mediated by She2p-She3p in yeast and its local translation at the bud tip [55, 62, 120, 156]. Collectively, these findings highlight the mechanistic complexity of (endosomal) transport mRNPs and demonstrate that translational regulation during transit is executed in a regulon-specific manner (Fig. 3C–E). Notably, analogous regulatory principles have been described in neurons, where multi-omics analyses reveal finely tuned translational control of distinct mRNA regulons during intracellular transport [157].

10.6.5 Rrm4-mediated hitchhiking of pre-tRNAs on early endosomes

Moreover, this thesis unexpectedly identifies a novel class of endosomal cargo transcripts—pre-tRNAs—revealing that Rrm4 is not limited to mRNAs but also binds non-coding RNAs (Fig. 3E) [82, 100]. In fungal systems such as *U. maydis* (Stoffel et al., in preparation), as well as in budding [158] and fission yeast [159], the tRNA splicing machinery (SEN complex) localizes to the cytoplasmic surface of mitochondria [160]. Given the well-established link between endosomal RNA transport and mitochondria as a key destination [63, 70, 73, 129], it is plausible that pre-tRNAs hitchhike on endosomes to reach their mitochondrial-associated splicing site (Fig. 3E) [161, 162]. Rrm4 specifically binds both isodecoders of initiator methionine pre-tRNA^{iMET} within their intronic regions (Stoffel et al., in preparation). Notably, a prominent UAUG binding site—recognized by RRM3—dominates the crosslinking landscape of these pre-tRNAs, highlighting the specificity. This binding preference mirrors the well-established RRM3–UAUG interaction observed in Rrm4’s mRNA targets [82, 87, 101]. Loss of Rrm4 leads to reduced endosomal association of pre-tRNA^{iMET}, impaired splicing efficiency, elevated pre-tRNA^{iMET} levels, and abnormal translational profiles (Stoffel et al., in preparation). Moreover, all RRM mutants displayed reduced splicing efficiency, indicating that all three RRMs contribute to pre-tRNA^{iMET} recognition. Together, these findings support a model in which Rrm4 transports pre-tRNA^{iMET} to mitochondria for subcellular splicing, thereby maintaining tRNA homeostasis [160, 162]. Whether Rrm4 also transports mature tRNAs to supply localized translation remains an open question. Addressing this and defining the broader spectrum of (pre-)tRNAs associated with Rrm4 will benefit from subcellular quantitative tRNA nanopore sequencing in future studies [163].

10.7 Outlook and perspectives

Aberrant RBPs are implicated in a wide range of diseases, making them promising yet challenging therapeutic targets [10, 11, 93]. A prime example is the multi-RRM protein HuR, which is frequently overexpressed in aggressive brain tumors such as glioblastoma, where it stabilizes oncogenic mRNAs and promotes tumor progression [164, 165, 166]. Despite this promise, targeting RBPs remains difficult because many compounds lack specificity, binding to multiple RBPs and causing off-target effects [11]. To overcome these limitations and achieve highly specific therapies, it is crucial to build a detailed understanding of the target RBP—including its RNA and protein interaction networks *in vivo* [10, 93, 167].

By integrating iCLIP2, RNA-seq, co-IP MS, and endo-IP MS—with molecular cell biology, this thesis provides deeper insights into the functional regulation of Rrm4 during polar growth [63, 87]. Additionally, comparative analyses using RRM mutants enabled precise dissection of RRM-dependent RNA [31, 82, 101] and protein interaction modes (see Section 7).

Notably, the tandem-RRMs play a pivotal role in mediating RNA transport [31, 78, 82, 88, 101]. Moreover, RRM1 directly interacts with the mRNP scaffolding protein Upa2 [63, 88], underscoring its role as a central hub in higher-order mRNP assembly. Thus, targeting RRM1 presents a potential strategy to disrupt Rrm4 function. Selective inhibition of the RRM1–Upa2 interaction could destabilize mRNP transport, while blocking RNA binding might prevent the transport of RRM12-BS containing mRNA targets. Both strategies are likely to impair unipolar growth and virulence [31, 80]. In disease contexts, dual-targeted therapies that address both RNA and protein interactions could enhance therapeutic specificity and efficiency [10, 11]. By providing a comprehensive multi-omics dissection of the ELAV-like RBP Rrm4, this study establishes a conceptual and methodological framework for RRM-dependent mRNP profiling at both the proteome- and transcriptome-wide levels *in vivo* [31, 101] (Müntjes et al., in preparation; see Section 6-7). This integrative approach offers a scalable model for characterizing multi-RBDs proteins such as HuC/D/R [2, 32, 99, 168], and holds promise for guiding the development of targeted therapeutic strategies [10, 11, 93, 164, 169].

11 References

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12 Abbreviation

Abbreviation	Meaning
³² P	Radioactive isotope of phosphorus
AB	Antibody
BS	Binding sites
CDS	Coding sequence
CE	Crosslink events
°C	Degree Celsius
co-IP	Co-immunoprecipitation
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
DUF	Domain of unknown function
ELAV	Embryonic lethal abnormal vision
endo-IP	Endosome immunoprecipitation
Gfp	Green fluorescent protein
Grp1	Glycin-rich protein 1 (a RNA-binding protein)
h	Hour
Hz	Hertz
HuC	Human antigen C (a neuronal RNA-binding protein)
iCLIP2	Individual-nucleotide resolution crosslinking and immunoprecipitation (version 2)
IP	Immunoprecipitation
Kat	mKate2 (a red fluorescent protein)
KH	K homology (RNA-binding domain)
kDa	Kilodalton
L	Liter
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
mL	Milliliter
min	Minutes
MLLE	MLLE domain (also known as PAM2-interacting motif)
mM	Millimolar

Abbreviation	Meaning
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein complex
MS	Mass spectrometry
MW	Molecular weight
nM	Nanomolar
nm	Nanometer
OD	Optical density
PNK	Polynucleotide kinase
PPI	Protein-protein interaction
pre-tRNA	Precursor transfer RNA
PUF	Pumilio and FBF homology domain
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RBD	RNA-binding domain
RBP	RNA-binding protein
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RNP	Ribonucleoprotein
RRM	RNA recognition motif
rRNA	Ribosomal RNA
rpm	Revolutions per minute
SG200	Solopathogenic <i>Ustilago maydis</i> strain SG200
TRAP	Translating ribosome affinity purification
UPF1	Up-frameshift protein 1
UTR	Untranslated region
UV	Ultraviolet light
U	Enzyme unit
V/V	Volume/volume
W/V	Weight/volume
WT	Wild type

Abbreviation	Meaning
μCi	Microcurie
μL	Microliter
μM	Micromolar

13 Tools and Software

This thesis was written using both Microsoft Word and Overleaf (Digital Science). A manuscript template based on the *Nucleic Acids Research* format served as a reference but was heavily modified to generate customized manuscript templates for this work. Microsoft Excel and RStudio were employed for data analysis, summarization, and visualization. Figures and graphics were created using Canvas15 and Affinity Designer 2. Grammarly and DeepL were used to support grammar and spell-checking throughout the writing process.

14 Acknockegments

Prof. Dr. Michael Feldbrügge – danke für die finanzielle Unterstützung meiner Projekte, den wissenschaftlichen Input und vor allem für das, was ich aufrichtig gemeint - *MF's Magic Marketing Mornings* - nenne. Es war beeindruckend, wie du aus meinen nächtlichen Schreibeskapaden etwas Prägnantes geformt hast. Nur wenige Menschen können sich so tief in meine oft sprunghafte wissenschaftliche Denkweise hineinversetzen. Danke!

Prof. Dr. Heiner Schaal – danke, dass du immer da warst! Für deine Begleitung auf wissenschaftlicher wie persönlicher Ebene, für Korrekturen, Diskussionen und das Gefühl, in deinem Denken gespiegelt zu sein. Ich stimme mit jedem deiner Kommentare überein. Du bist kein Betreuer – du bist ein Mentor! Danke für alles!

Prof. Dr. Kathi Zarnack und **Prof. Dr. Julian König** – danke für euren Support und das wertvolle Feedback zu den Papern. Ohne euch hätte ich vieles nicht so umsetzen können! Vielen Dank!

Prof. Dr. Dierk Niessing Vielen Dank für das konstruktive Feedback, die Unterstützung meiner Projekte – und den Versuch mit dem U-Boot-Experiment! Auch für die zahlreichen spannenden Gruppentreffen und die inspirierende Arbeitsatmosphäre möchte ich mich bedanken.

Prof. Dr. Anne Ephrussi – I believe you were the one who believed in me the most. Your enthusiasm for tRNA transport was contagious — without it, I might have given up early on. The way you think and interact with people has truly become a role model for me. Thank you, Anne!

Prof. Dr. Dorothee Staiger – bei dir hat alles angefangen. Du hast mein Interesse für RNA entfacht und mir immer den Raum gegeben, meiner Neugier zu folgen. Danke für diesen wichtigen Anfang!

Dr. Srimeenakshi Sankaranarayanan und **Dr. Anke Busch** – danke für die bioinformatische Unterstützung bei iCLIP2 und RNA-seq. Ich habe viel von euch gelernt!

Dr. Florian Altegoer – du warst mein heimlicher Betreuer, auch wenn du es vielleicht nicht wusstest. Mit jeder neuen Idee bin ich zu dir gestapft. Danke, dass du meine Arbeit so gründlich gelesen hast (microbial iCLIP2, tRNAs, co-IP, RRM-Code, Ssd1 – wow!) und mir immer zur Seite standest.

Dr. Kerstin Schipper – danke für deine beeindruckend sorgfältigen Korrekturen (Einleitung, microbial iCLIP2, RRM-Code, endo-IP, tRNAs, Perspektive, Diskussion – sooo viel!). Ich weiß nicht, wie du jeden Fehler findest – aber du findest ihn. Es gibt einen Grund, warum es „Fehler“ gibt, aber keine „Fehlerinnen“ – du bist der Grund!

Dr. Thomas Lenz – im letzten Jahr warst du meine Arbeitsgruppe. Danke, dass du bei jeder Frage da warst, Wissen geteilt und meinen Horizont erweitert hast. Danke dir!

Dr. Alexander Steffen – für mich bist du immer mein Betreuer geblieben. Ich habe nie jemanden getroffen, der mit so viel Energie, Zeit und Leidenschaft unterrichtet und sich kümmert. Danke!

Ana und **Franzi** – meine *Hot Lab Girls* und Herzens-Labor! Danke für alles – und ja: „Franzi, du kannst nicht mitreden – du brauchst nicht mal einen Schirm!“ sagt alles. . .

Kira – nicht nur Kollegin, sondern Freundin. Danke, dass du mich unterstützt und beschützt hast – ich weiß das sehr zu schätzen.

Lilli – danke für deine Hilfe, Verständnis und viel gemeinsames Lachen! dieser Blumenkohl-Salat war *next level*!

Woogie – du bist für mich wissenschaftliche Kreativität in Reinform. Ich wünschte, du könntest sehen, was ich in dir sehe!

Uli – danke für alles! Es gab keinen Tag, an dem ich dich nicht vermisst habe. Auch wenn du mir häufiger mal „Defekt“ in die Haare geklebt hast und meinen Tisch mit dem Logo der Warn-App „*NINA*“ zugehangen hast – genau dieser Humor hat mir jeden Tag ein Lächeln ins Gesicht gezaubert. Danke auch für die Millionen Dinge, die du für mich oder wegen mir repariert hast!

Lea – meine Pasta-Freundin! Danke für alles, was du für mich getan hast. Du bist etwas ganz Besonderes!

Carola – du Wilde! Dein Mut und deine Art haben mich immer wieder inspiriert. Danke!

Joanna – danke für deine Unterstützung und unsere Spaziergänge als *mental break*. Du hast gut auf mich aufgepasst. Danke dir!

Kai, Magnus, Peter, Silke und **Carl** – danke für lustige Abende und Support!

Senthil, Summia, Sai, Ex-Rüdiger und **Trusha** – für die Chai-Zeremonien: danke euch!

Melissa – #SEN-Sister!

Ute – du bist die Lab-Mum schlechthin, die gute Seele, auf die sich alle verlassen. Ich habe beobachtet, was du alles für die Menschen um dich herum tust, wenn keiner hinsieht – danke dir von Herzen!

Tina – dein Karma ist einfach gut. Sonst hätte ich die Karten für dich nie gewonnen. Danke für alles!

Simone – danke für deine Unterstützung, besonders für die Stammherstellung am Wochenende, als ich unter Druck war und du mir beistandest. Und ja: *Feuerwehrmänner-Kalender*!

Sai – mein *Blotting-Brother*! Danke für deine Hilfe, unsere Kochsessions an Samstagen und die durchtanzten Labor-Nächte. Du warst der beste Kollege, den man sich wünschen kann. Grüße an Cynthia – the Spider! Ich werde immer bei dir sein!

Marten – du bist und bleibst der lustigste Mensch, den ich kenne. Danke für deinen wissenschaftlichen wie menschlichen Support. Und ja: *Lipid-RNA interactions – hot topic* #NaturePaperIncoming

Vika – danke, dass du mir Ruhe und Frieden gegeben hast – in der Uni und später auch zuhause (auch wenn unfreiwillig). Ich werde unsere Zeit nie vergessen – auch nicht, dass selbst deine Mutter uns verwechselt hat. #GoWithTheFlow #SoulSister

Meine Pilatinas – ihr habt mir die HHU von ihrer besten Seite gezeigt. Ich bin stolz auf euch – und könnte platzen, wenn ich eure Fortschritte beobachte. *#KsssschtKsssscht – doch, doch, das klappt. Ich glaube an euch!*

Meine YogaMatten-Freunde Kathrin und Rajpal – danke, für anstrengende Auszeiten und eure Aufrichtigkeit! *#Namaste!*

Martin – danke, dass du mich begleitet hast und immer für mich da warst!

Meiner Hometown-Crew – Tobi, Emmanuel, Daniel, Pawel, Felix – danke für euren Rückhalt und euer Verständnis. Ab jetzt bin ich wieder bei jeder Party dabei. *#NoExcuse #ImmerNachVorne*

Meinen Chicas – Elena, Daria, Julia, Deva und Caro – ihr habt mehr an mich geglaubt als ich selbst! Danke für Essen, Vino, Abholaktionen, Motzi, Konfetti, Mutmachen – und dafür, dass ihr mich daran erinnert habt, wie viel mehr in mir steckt. *Sei Pippi, nicht Annika!* Gemeinsam sind wir alles!

Und schließlich meiner Familie – besonders **meinen Eltern** – danke für eure emotionale und finanzielle Unterstützung und dafür, dass ihr immer für mich da seid.

– *Time to shine again* –

15 Erklärung

Die Untersuchung der vorliegenden Dissertation sowie die experimentiellen Arbeiten wurde im Zeitraum von April 2018 bis Juli 2025 am Institut für Mikrobiologie, der Heinrich-Heine-Universität, unter Betreuung von Prof. Dr. Michael Feldbrügge durchgeführt.

Ich, Nina Kim Stoffel, versichere an Eides statt, dass ich diese Dissertation selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ angefertigt habe. Die Dissertation wurde bisher in keiner Form an anderen Fakultäten eingereicht, noch habe ich erfolglose Promotionsversuche unternommen.

Düsseldorf, den _____

Nina Kim Stoffel