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Grainyhead- like 3 (GRHL3) - ein neuer zentraler Regulator der Endothelzellmigration

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Erklärung

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(Margarete Lukosz)
Düsseldorf, 02. Mai 2011

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1 Einleitung – Kardiovaskuläre Alterung

Die Bevölkerungsstruktur westlicher Nationen ist gekennzeichnet von einem stetigen Zuwachs des Anteils älterer Menschen an der Gesellschaft. Dabei wird das „gesunde“ Altern von vielen bekannten Umwelteinflüssen, wie z. B. Ernährung und physischer Aktivität, beeinflusst. Im Allgemeinen kann Altern definiert werden als die mit dem Lebensalter zunehmende Unfähigkeit des Organismus sich verändernden Umweltbedingungen anzupassen.

Statistische Untersuchungen der Weltgesundheitsorganisation (WHO, World Health Organization) zeigen, dass koronare Herzkrankheiten die Haupttodesursache in westlichen Industrieländern sind (www.who.int). Kardiovaskuläres Altern führt zu einer Abnahme der maximalen Leistungsfähigkeit des Herz-Kreislauf-Systems. Dieser Prozess ist in allen alten Menschen zu beobachten und unabhängig von jeder kardiovaskulären Erkrankung zu sehen. Durch den kardiovaskulären Alterungsprozess sinkt die Kompensationsfähigkeit des Myokards und der Gefäße, Risikofaktoren, wie ungesunder Ernährung, Übergewicht, Rauchen, der Konsumierung von Alkohol und physischer Inaktivität, entgegenzuwirken. Der Alterungsprozess korreliert mit endothelialer Dysfunktion. Diese Fehlfunktion des Endothels bedingt in den meisten Fällen das Auftreten kardiovaskulärer Erkrankungen im Alter. Aus diesem Grund ist es von großer Bedeutung, die molekularen Mechanismen, die endothelialer Dysfunktion zugrunde liegen, aufzuklären. Essentielle Prozesse, die hierbei gestört sind, sind der Schutz der Endothelzellen vor Apoptose und ihre verringerte Migrationsfähigkeit. Der bekannteste Faktor, der diese Prozesse beeinflusst, ist Stickstoffmonoxid (NO). Die Regulation dieser Endothelfunktionen stellte einen Schwerpunkt meiner Arbeit dar und wird daher in den folgenden Kapiteln eingeführt.

2 NO und das Endothel

Das Endothel bildet mit seiner Einzelzellschicht die innerste Auskleidung des gesamten Gefäßsystems und steht im direkten Kontakt mit dem zirkulierenden Blut. Es wird umgeben vom subendothelialen Bindegewebe und einer Schicht aus glatten Muskelzellen. Das Endothel ist für die Versorgung des umgebenden Gewebes mit Sauerstoff und Nährstoffen verantwortlich, aber auch für die kardiovaskuläre Homöostase und den Gefäßtonus. Dementsprechend beeinflusst eine endotheliale Dysfunktion das komplette Gefäßsystem (Collins und Tzima, 2010). Der Hauptgrund für das Entstehen einer endothelialen Dysfunktion ist die verringerte Bioverfügbarkeit von NO, die auch im Alter auftritt (**Abb.1**).

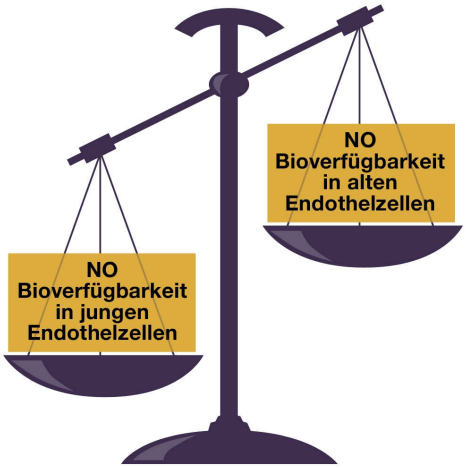


Abb. 1: Abnahme der NO Bioverfügbarkeit in alten Endothelzellen. Während des kardiovaskulären Alterungsprozesses kommt es durch eine Vielzahl von molekularen Veränderungen zu einer Abnahme des bioverfügbaren NO in Endothelzellen.

1998 erhielten Robert F. Furchgott, Louis J. Ignarro und Ferid Murad für ihre Untersuchungen zur Rolle von NO als Signalmolekül im kardiovaskulären System den Nobelpreis. Endotheliales NO besitzt eine gefäßerweiternde Wirkung, indem es die lösliche Isoform der Guanylylcyclase (sGC, soluble guanylyl cyclase) in den glatten Muskelzellen aktiviert und somit eine Muskelrelaxation hervorruft. Durch die Aktivierung der Guanylylcyclase kommt es zur Produktion von zyklischem Guanosin-monphosphat (cGMP, cyclic guanosine monophosphate) und der Aktivierung von cGMP-abhängigen zellulären Signalwegen (Zhao et al., 1999). NO ist ein freies Radikal und wird von drei verschiedenen Isoformen der NO-Synthase (NOS) im Körper produziert. Es handelt sich hierbei um Enzyme, die die Aminosäure L-Arginin in L-Zitrullin und NO umsetzen. Alle NOS-Isoformen sind homodimere Enzyme, die die gleichen Cosubstrate (molekularer Sauerstoff, NADPH) und Cofaktoren (FMN, FAD, Tetrahydrobiopterin, Eisen, Ca^{2+}/Calmodulin (CaM)) benötigen (Stuehr, 1999). Die verschiedenen Isoformen sind benannt nach dem Zelltyp, aus dem sie zuerst isoliert wurden: neuronale NOS, nNOS oder NOS1; induzierbare NOS, iNOS oder NOS2; endotheliale NOS, eNOS oder NOS3 (Bogdan, 2001). Die eNOS und die nNOS sind konstitutiv aktiv und produzieren moderate Mengen an NO (im picomolaren Bereich). Jedoch kann die NO-Produktion durch eine Erhöhung des intrazellulären Ca^{2+}-Levels oder durch Aktivierung der eNOS durch Phosphorylierung rapide ansteigen (Andrew und Mayer, 1999; Dimmeler et al., 1999; Fulton et al., 1999). Die induzierbare NOS hingegen ist ein Ca^{2+}-unabhängiges Enzym, das NO-Mengen im nanomolaren Bereich produziert. Die Regulierung der iNOS erfolgt durch transkriptionelle Aktivierung, z. B. als Antwort auf eine Entzündungsreaktion oder Infektion (Kroncke et al., 2000). Der Regulationsmechanismus der eNOS ist weitaus komplexer und erfolgt in mehreren Stufen. Hierbei ist anzunehmen, dass aufgrund der langen Halbwertszeit der eNOS-mRNA (10- 35 h) posttranslationale Regulierungen einen weitaus größeren Einfluss auf die eNOS-Aktivierung haben (Searles, 2006). Durch eine Ca^{2+}-Aktivierung ist die eNOS in der Lage, für eine vorübergehende Erhöhung der NO-Level zu sorgen. Die konstitutive eNOS-Aktivierung kann durch

Veränderungen des Phosphorylierungsgrades weiter vorangetrieben werden. Eine Erhöhung der intrazellulären Ca^{2+}-Level führt zur Bindung von CaM an eNOS und ermöglicht die gleichzeitige Dissoziation von Caveolin-1 (Cav-1) (Brouet et al., 2001). Die cytoplasmatische Oberfläche der Caveolae wird von dem Protein Cav-1 ausgekleidet (Anderson, 1998), von dem gezeigt werden konnte, dass es direkt mit der eNOS assoziiert (Smart et al., 1999). Diese Bindung wirkt sich hemmend auf die eNOS aus und die Freisetzung aus diesem Komplex stellt eine Voraussetzung für die NO-Produktion dar (Michel et al., 1997). Die durch CaM-vermittelte Aktivierung der eNOS resultiert in einer kurzfristigen Erhöhung der NO-Produktion. Die Interaktion von CaM und eNOS führt zur sofortigen Rekrutierung des Hitzeschockproteins 90 (HSP90) (Brouet et al., 2001). Es zeigte sich, dass die Co-Expression von HSP90 und eNOS die Aktivität dieses Enzyms erhöht (Garcia-Cardena et al., 1998). Man vermutet, dass entweder diese Bindung selbst sich aktivierend auf die Funktion der eNOS auswirkt oder die Bindung zu anderen aktivierenden Faktoren stabilisiert. Averna et al. konnten in diesem Zusammenhang nachweisen, dass durch Inhibition von HSP90 die NO-Produktion drastisch erniedrigt wird und eine vermehrte Degradation des eNOS-Proteins stattfindet. Die Assoziation von HSP90 und eNOS scheint somit eine schützende Funktion für die Synthase zu besitzen (Averna et al., 2008). Der CaM/eNOS/HSP90-Komplex sorgt für die Rekrutierung der Proteinkinase B (Akt) und der Phosphatase Calcineurin in die Nähe der eNOS (Brouet et al., 2001). Im Jahre 1999 konnte gezeigt werden, dass die Aktivierung der eNOS über die Phosphorylierung des Ser-1177 durch Akt vermittelt wird (Dimmeler et al., 1999; Fulton et al., 1999). Somit kann eine konstitutive und dadurch lang andauernde Aktivierung der eNOS gewährleistet werden. Das durch die eNOS auf diese Weise synthetisierte NO übernimmt im Endothel eine Vielzahl von Funktionen, auf die ich im nächsten Abschnitt genauer eingehen möchte.

3 NO und Endothelzellapoptose, -migration und –alterung

Neben seiner Aufgabe in der Regulierung des Blutdrucks wirkt das im Endothel produzierte NO dort antithrombotisch, antiapoptotisch und promigratorisch und übernimmt somit eine schützende Funktion für die Gefäße (**Abb.2**). NO ist in der Lage, die Proliferation der glatten Gefäßmuskulatur zu limitieren (Garg und Hassid, 1989), aber auch die Expression von Adhäsionsmolekülen für Lymphozyten und Monozyten (VCAM-1, vascular cell adhesion molecule-1) und neutrophile Granulozyten (ICAM-1, intercellular adhesion molecule-1, ICAM-1) auf Endothelzellen zu reduzieren (De Caterina et al., 1995).

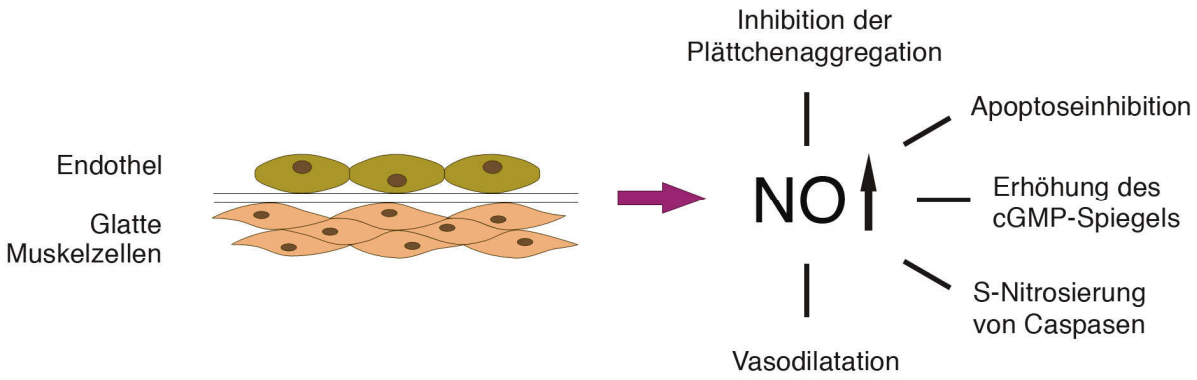


Abb.2: Funktionen von NO in der Gefäßwand. Das in den Endothelzellen produzierte NO wirkt sich über cGMP-abhängige Signalwege vasodilatierend auf die Gefäße aus. Darüber hinaus wirkt NO antiapoptotisch, da es für die S-Nitrosierung von Caspasen verantwortlich ist. Zusätzlich hat NO auch einen antithrombotischen Effekt, indem es die Plättchenaggregation inhibiert.

Apoptose von Endothelzellen stört die Integrität des endothelialen Monolayers (Dimmeler und Zeiher, 2000). Verschiedene proatherosklerotische Faktoren wie oxidiertes Low Density Lipoprotein (oxLDL) und proinflammatorische Zytokine sind in der Lage, die Apoptose von Endothelzellen zu induzieren (Dimmeler et al., 1997a; Dimmeler et al., 1997c). Wie in verschiedenen anderen Zelltypen sind auch in Endothelzellen spezifische Cysteinproteasen, die sogenannten Caspasen, entscheidende Effektormoleküle im apoptotischen Prozess (Dimmeler et al., 1997b; Nagata, 1997). Der „Caspase-Signalweg“, und dabei besonders die Aktivität der Caspase 3, spielen für die Endothelzellapoptose eine wesentliche Rolle. Die antiapoptotische Wirkung von NO lässt sich dadurch erklären, dass die S-Nitrosierung des Cys-163 in der katalytischen Untereinheit p17 der Caspase 3 die Bildung des aktiven Tetramers verhindert (Dimmeler et al., 1997b; Mannick et al., 1999). Während des kardiovaskulären Alterungsprozesses kann es zu ischämischen Situationen kommen, wie z. B. Gefäßverschlüssen im Rahmen von Herzinfarkten oder Schlaganfällen. Zur Kompensation solcher Ereignisse wird die Ausbildung neuer Blutgefäße, der Prozess der Angiogenese benötigt. Unter physiologischen Bedingungen ist die Gefäßneubildung z. B. an der Wundheilung beteiligt (Cave et al., 2006), kann allerdings unter pathophysiologischen Bedingungen zum Tumorwachstum oder deren Metastasierung beitragen (Benjamin und Keshet, 1997; Folkman, 1995). Für die Angiogenese sind Proliferation und Migration von Endothelzellen und somit die Kapillarbildung von großer Bedeutung (Ushio-Fukai und Urao, 2009).

Der Wachstumsfaktor „Vascular Endothelial Growth Factor“ (VEGF) ist ein wichtiger Stimulus für die Angiogenese und die Endothelzellmigration (Bernatchez et al., 1999). Nach VEGF Stimulation erfolgt die Signaltransduktion im Endothel hauptsächlich über den

Tyrosinkinase-Rezeptor VEGFR2 (Flk 1/KDR) (Feng et al., 1999; He et al., 1999; Kroll und Waltenberger, 1998; Thuringer et al., 2002). Hierdurch werden wichtige intrazelluläre Signalwege induziert, die die Aktivität der eNOS kontrollieren. Darunter der Phosphoinositid-3-Kinase (PI3K)/Akt Signalweg, der die Phosphorylierung von eNOS an Ser-1177 induziert (Dudzinski et al., 2006), und die durch die Phospholipase C (PLC) ausgelöste Signalkaskade, die zur Ausschüttung von Ca^{2+} aus intrazellulären Speichern, wie dem Endoplasmatischen Retikulum (ER), beiträgt, wodurch die Bindung der eNOS an CaM ermöglicht wird (Busse und Mulsch, 1990; Fujimoto et al., 1992). In einer Reihe von Untersuchungen konnte der Nachweis erbracht werden, dass die Aktivität der eNOS eine Voraussetzung darstellt für die stimulierende Wirkung von VEGF während der Angiogenese. So konnte durch den Einsatz eines eNOS Inhibitors die Endothelzellmigration unterdrückt werden (Murohara et al., 1999). Ebenfalls konnte demonstriert werden, dass die Akt-abhängige Aktivierung der eNOS notwendig ist für eine durch VEGF-induzierte Endothelzellmigration (Dimmeler et al., 2000). Zudem konnte in Untersuchungen von eNOS defizienten Mäusen gezeigt werden, dass sowohl die basale als auch die durch VEGF-induzierte Angiogenese im Vergleich zu Wildtyp-Mäusen signifikant reduziert ist (Fukumura et al., 2001). Die Expression von VEGF wird durch den Transkriptionsfaktor „Hypoxia inducible factor 1“ (HIF-1) reguliert (Levy et al., 1995; Rivard et al., 2000). Es konnte allerdings gezeigt werden, dass NO ebenfalls ein potenter Stimulus der VEGF-Neusynthese ist. Durch die Behandlung von Tumorzellen mit NO-Donoren konnte die HIF-1-DNA-Bindungsaktivität am VEGF-Promotor und damit die VEGF-Expression gesteigert werden (Kimura et al., 2000). Zusätzlich konnten Dulak et al. zeigen, dass die Überexpression von eNOS in vaskulären glatten Muskelzellen zu einer Heraufregulation der VEGF-Synthese führt (Dulak et al., 2000). Darüber hinaus ist NO allerdings in der Lage, unabhängig von VEGF die Migration von Endothelzellen zu induzieren. Dies konnte in Versuchen mit dem antiangiogenen Faktor Endostatin bestätigt werden. Die Phosphorylierung von eNOS an Ser-1177 wird durch Behandlung mit Endostatin erniedrigt und verhindert eine durch VEGF-induzierte Migration. Dieser durch Endostatin ausgelöste inhibitorische Effekt auf die Endothelzellmigration konnte durch Zugabe eines NO-Donors rückgängig gemacht werden. Zudem konnte durch Überexpression einer phosphomimetischen eNOS-Mutante kein inhibitorischer Effekt durch Endostatin auf die Endothelzellmigration nachgewiesen werden (Urbich et al., 2002). Diese Ergebnisse weisen deutlich auf, wie kritisch eNOS zum Einen für die durch VEGF-vermittelte Angiogenese ist, aber auch, dass NO unabhängig von VEGF promigratorisch wirkt.

Ein Zusammenhang zwischen Altern und Apoptose konnte bereits vor zehn Jahren in einer Primatenstudie aufgezeigt werden. In einem Vergleich von jungen und alten Affen (*Macaca fascicularis*) wiesen die Endothelzellen der alten Affen eine erhöhte Apoptoserate

auf. Diese Beobachtung konnte unabhängig von dem Auftreten von Atherosklerose und alternsassoziierter Krankheiten gemacht werden (Asai et al., 2000). Zusätzlich zeigten Untersuchungen von humanen Endothelzellen, dass Altern in Verbindung gebracht werden kann mit einer erhöhten Sensitivität gegenüber apoptotischen Stimuli und reduzierter eNOS-Level. Die gealterten humanen Endothelzellen wiesen nach einer Tumornekrosefaktor α (TNFα)- oder oxLDL-Behandlung im Vergleich zu jungen humanen Endothelzellen eine gesteigerte Apoptoserate bedingt durch eine erhöhte Aktivität der Caspase 3 auf (Hoffmann et al., 2001). Auf eine Verbindung zwischen Endothelzellmigration und Altern wurde durch die verringerten Proteinmengen der eNOS und der Akt, so wie einer reduzierten NO-Bioverfügbarkeit hingewiesen (Hoffmann et al. 2001). Diesen Zusammenhang haben wir nun im Labor experimentell bestätigt (**Lukosz***, Mlynek* et al., 2011, eingereicht).

Ein weiteres Enzym, das eine wesentliche Rolle in Alterungsprozessen spielt, ist die Telomerase, welche für die Aufrechterhaltung der Telomere verantwortlich ist. Das Holoenzym besteht aus der katalytischen Untereinheit Telomerase Reverse Transkriptase (TERT) und einer RNA Komponente (TERC), welche bei der Telomersynthese als Matrize dient (Greider und Blackburn, 1989; Nakamura und Cech, 1998). Während der Endothelzellalterung kommt es, zum einen bedingt durch die verringerte NO-Bioverfügbarkeit und zum anderen durch eine Erhöhung der intrazellulären reaktiven Sauerstoffspezies, zu einer Reduktion der TERT-Proteinmenge und damit zu einer verringerten Telomerase-Aktivität, was einhergeht mit dem Einsetzen von replikativer Seneszenz (Haendeler et al., 2004). Dementsprechend zeigte sich, dass eine Erhöhung der TERT-Level in humanen Endothelzellen und vaskulären glatten Muskelzellen zu einer verlängerten Lebensspanne führt (Chang und Harley, 1995; Minamino et al., 2001; Yang et al., 1999). Ebenfalls konnte gezeigt werden, dass eine Behandlung von Endothelzellen mit NO der Verringerung der Telomerase-Aktivität entgegenwirkt und somit den Beginn replikativer Seneszenz aufschiebt (Vasa et al., 2000). In unserer Arbeitsgruppe wurde gezeigt, dass es durch die im Alter verringerte NO-Bioverfügbarkeit und die Erhöhung der intrazellulären reaktiven Sauerstoffspezies zu einem Export von TERT aus dem Zellkern kommt (Haendeler et al., 2004). Vermittelt wird dieser Export durch die Kinasen der Src-Kinase Familie. Diese phosphorylieren TERT an Tyr-707 und sorgen somit für den Kernexport (Haendeler et al., 2003; Haendeler et al., 2004). Des Weiteren konnten wir die verantwortlichen Kinasen der Src-Kinase Familie auf Src und Yes eingrenzen (Jakob,…, **Lukosz** et al., 2008). Als Gegenspieler des TERT-Kernexports konnten wir die Phosphatase Shp-2 identifizieren (Jakob,…, **Lukosz** et al., 2008). Wir konnten zeigen, dass sich Shp-2 auch im Zellkern befindet, wo es mit TERT assoziiert vorliegt. Dort erhöht Shp-2 die Telomerase-Aktivität und verhindert unter physiologischen Bedingungen den Src-induzierten Kernexport von TERT. Dabei ist die katalytische Aktivität von Shp-2 essentiell, die entweder für eine

Dephosphorylierung von Tyr-707 in TERT verantwortlich ist oder durch Inaktivierung der Src-Kinasen Src und/oder Yes eine Phosphorylierung von Tyr-707 in TERT verhindert (Jakob,..., **Lukosz** et al., 2008). Vor wenigen Jahren konnte TERT auch in den Mitochondrien von Zelllinien nachgewiesen werden (Santos et al., 2004; Santos et al., 2006). Wir konnten erstmals zeigen, dass TERT in Mitochondrien von primären Endothelzellen lokalisiert ist und dort wichtige Aufgaben bei der Aufrechterhaltung der Mitochondrienfunktion und dem Schutz der mitochondriellen DNA (mtDNA) vor exogenen Noxen hat (Haendeler et al., 2009). Unsere Arbeitsgruppe konnte zudem zeigen, dass TERT in Mitochondrien durch die gleichen Modulatoren reguliert wird wie im Zellkern. So konnten wir in isolierten Mitochondrien aus primären humanen Endothelzellen die gleichen Proteine nachweisen, die an der TERT Regulation im Zellkern beteiligt sind, Akt und Src (Büchner,…, **Lukosz** et al., 2010). Dabei konnten wir beobachten, dass es unter oxidativem Stress, wie er auch im Alter auftritt, zu einer Inaktivierung der Kinase Akt und einer Aktivierung der Src-Kinase im Mitochondrium kommt. Dies führt zu einer Src-abhängigen Reduktion der mitochondriellen TERT-Proteinmenge. Dies konnten wir mit Hilfe einer TERT-Mutante, die nicht mehr durch Src am Tyr-707 phosphorylierbar ist, nachweisen.

Ein weiterer bedeutsamer Mechanismus in der Alterung ist eine Veränderung des Transkriptoms (Lee et al., 2002; Lee et al., 1999; Park et al., 2009; Park und Prolla, 2005). Bioverfügbares NO spielt für vielerlei Funktionen eine essentielle Rolle und möglicherweise auch in der Regulierung des Transkriptoms. Aus diesem Grund möchte ich im nächsten Kapitel genauer beschreiben, inwieweit NO zu veränderten Transkriptionsfaktoraktivitäten beiträgt.

4 NO und Transkriptionsfaktoren

Transkriptionsfaktoren haben pleiotrope Effekte, da sie das gesamte Genexpressionsprofil einer Zelle verändern können. Selber werden sie durch eine Vielzahl von Mechanismen in ihrer Funktion reguliert, wie z. B. über ihre Interaktion mit Cofaktoren, Oligomerisation oder aber auch über ihre Lokalisation innerhalb der Zelle. Auf diese Weise sind Zellen in der Lage, sich durch Modifikationen ihres Transkriptoms bestimmten physiologischen und pathophysiologischen Bedingungen anzupassen. In gealterten Endothelzellen wird erhöhter oxidativer Stress und eine verringerte NO-Bioverfügbarkeit beobachtet. Zudem ist bekannt, dass eine Vielzahl von Transkriptionsfaktoren redox-sensitiv und direkt durch NO regulierbar ist. Daher möchte ich im Folgenden beispielhaft einige Transkriptionsfaktoren und deren Modulatoren, die durch NO reguliert werden, vorstellen.

Einer der ersten Transkriptionsfaktoren, der im Säugetier identifiziert wurde**,** ist **Activator Protein-1 (AP-1)** (Lee et al., 1987). AP-1 besteht nicht aus einer einzelnen Polypeptidkette, sondern ist ein Überbegriff für Homo- und Heterodimere aus Mitgliedern der

bZIP-Familie (bZIP, basic region-leucine zipper). Diese Proteine können zu den Unterfamilien Jun, Fos, Maf und ATF der bZIP-Familie gehören. Die DNA-Bindeaktivität von AP-1 wird über den Redox-Zustand eines Cysteins, das innerhalb der DNA-Bindedomäne einer Untereinheit liegt, reguliert. Hier konnte gezeigt werden, dass sich Oxidationen des Cys-154 innerhalb des humanen Fos Proteins und Cys-269 innerhalb des humanen c-Jun Proteins *in vitro* inhibierend auf die DNA-Bindekapazität von AP-1 auswirken (Abate et al., 1990; **Lukosz** et al., 2010). NO kann sich auf die Funktion von AP-1 entweder aktivierend oder suppressiv auswirken. Hierbei reguliert NO zum einen Untereinheiten von AP-1 (Pilz et al., 1995), aber auch Mediatoren, wie JNK1 (c-Jun NH_2-terminal kinase 1) und JNK2 (c-Jun NH_2-terminal kinase 2), in der Signalkaskade oberhalb von AP-1 (Kim et al., 1997; Pilz et al., 1995; So et al., 1998). Es zeigte sich, dass NO-Donoren und cGMP-Analoga zu einer gesteigerten Expression von c-fos und junB in Rattenfibroblasten führen, was eine erhöhte AP-1-DNA-Bindung zur Folge hat (Pilz et al., 1995). Zudem können die Transaktivierungseigenschaften von AP-1 durch S-Nitrosierung kritischer Cysteine direkt, unabhängig von der Expression, gesteigert werden (Pilz et al., 1995). Auch konnte in nNOS-überexprimierenden Zellen die Aktivierung von AP-1 durch Einschalten des JNK-Signalweges nachgewiesen werden. Hierbei sorgt eine gesteigerte Aktivität von JNK1 für eine vermehrte Phosphorylierung seines Substrates, c-Jun, und dadurch für eine gesteigerte AP-1-Aktivität. Dieser Effekt konnte durch Zugabe von NO-Donoren verifiziert und durch NOS-Inhibitoren oder Antioxidantien verhindert werden (Kim et al., 1997). In einer Reihe von Untersuchungen konnte allerdings auch gezeigt werden, dass von NO ein suppressiver Effekt auf die Aktivität von AP-1 ausgeht. In Versuchen mit eNOS-überexprimierenden Mauskardiomyozyten konnte der Nachweis erbracht werden, dass durch S-Nitrosierung der AP-1 Untereinheit c-Jun die Expression des Tissue Inhibitor of Metalloproteinases 3 (TIMP-3) erniedrigt wird. Durch Behandlung dieser Zellen mit dem Reduktionsmittel Dithiothreitol konnte die S-Nitrosierung revertiert und der Effekt auf TIMP-3 aufgehoben werden (Hammoud et al., 2007). NO ist zudem in der Lage, oberhalb von AP-1 inhibitorisch in den Signalweg einzugreifen, indem es die direkte Bindung von JNK1 und seinem Substrat c-Jun verhindert und zu einer verminderten JNK1-Kinaseaktivität beiträgt (Park et al., 2006). Zusätzlich kann sich NO auch auf die Kinaseaktivität von JNK2 inhibierend auswirken. Die Behandlung mit NO-Donoren führt zur S-Nitrosierung von JNK2 und einer damit verbundenen Inhibition der Phosphotransferaseaktivität, was in einer verminderten c-Jun-Phosphorylierung resultiert (So et al., 1998). Da sowohl JNK1 als auch JNK2 in apoptotische Prozesse involviert sind, spielt NO möglicherweise im Regulationsmechanismus von Zelltod und Zellüberleben eine wichtige Rolle.

Nuclear Factor-kappa B (NF-κB) ist ein Sammelbegriff für induzierbare dimere Transkriptionsfaktoren, die alle der Rel-Familie angehören. Diese DNA-bindenden Proteine

erkennen ein gemeinsames spezifisches DNA-Element, das sogenannte kB Motiv. NF-κB ist verantwortlich für die Aktivierung von Genen als Antwort auf Stresssituationen, die eine schnelle Umprogrammierung des Genexpressionsprofils erfordert, wie Infektionen und Entzündungen. NF-κB repräsentiert einen Proteinkomplex, der aus Hetero- bzw. Homodimeren zusammengesetzt ist: NF-κB1 kodiert für p50 und p105; NF-κB2 für den p52 Vorläufer p100; RelA für p65; RelB und c-Rel. Die verschiedenen Dimer-Zusammensetzungen regulieren unterschiedliche Sets an Genen (**Lukosz** et al., 2010 ; Natoli und De Santa, 2006). In seiner inaktiven Form liegt NF-κB im Zytosol vor, wo es an inhibitorisch wirkende Proteine der IκB-Familie gebunden ist. Durch Phosphorylierung von IκB, anschließende Ubiquitinierung und proteosomale Degradation, wird es NF-κB ermöglicht, sich aus diesem Komplex zu lösen und in den Nukleus zu translozieren (Stancovski und Baltimore, 1997). Die Phosphorylierung von IκB erfolgt durch IκB-Kinasen (DiDonato et al., 1997). NO kann sich auf die Regulation von NF-κB sowohl aktivierend als auch hemmend auswirken. Entscheidend hierbei sind die NO-Konzentrationen. Durch die Behandlung von Endothelzellen mit niedrigen Konzentrationen eines NO-Donors konnte eine Zunahme der TNFα-induzierten NF-κB-DNA-Bindungsaktivität beobachtet werden, während höhere, nicht toxisch wirkende Konzentrationen diese drastisch erniedrigten (Umansky et al., 1998). Die Aktivierung von NF-κB durch Zytokine führt zur vermehrten iNOS-Transkription, was in einer Erhöhung der NO-Level resultiert. Ist eine kritische Konzentration an NO erreicht, wird die Zytokin-induzierte Aktivierung von NF-κB inhibiert (Umansky et al., 1998). Des Weiteren konnte von Matthews et al. in einem zellfreien System gezeigt werden, dass durch Zugabe von NO-Donoren eine S-Nitrosierung des Cys-62 innerhalb der p50 Untereinheit von NF-κB erfolgt, was eine verminderte DNA-Bindeaktivität von p50 zur Folge hat (Matthews et al., 1996). Diese Ergebnisse konnten von Grumbach et al. in Endothelzellen verifiziert werden. Sie postulierten, dass zwischen NO und NF-κB ein Zusammenhang der negativen Rückkopplung besteht. Durch Aktivierung von NF-κB kommt es zu einer vermehrten Expression der eNOS. Die dadurch erhöhte Menge an NO sorgt für S-Nitrosierung des Cys-62 innerhalb der p50 Untereinheit und einer damit verbundenen Inhibition, was eine fortwährende Aktivierung von NF-κB verhindern soll (Grumbach et al., 2005). Zusätzlich führt die Behandlung von Endothelzellen mit NO-Donoren zu einer erhöhten Expression von IκB und somit zu einer gesteigerten Retardation von NF-κB im Zytosol (Spiecker et al., 1997).

NF-E2-related factor 2 (Nrf-2) ist ein Transkriptionsfaktor, der involviert ist in die zelluläre Antwort auf oxidativen Stress. Nrf-2 ist ein Mitglied der sogenannten „cap´n´collar basic region leucine zipper“ (CNC-bZIP) Familie von Transkriptionsfaktoren. Es bildet Heterodimere mit anderen bZIP Transkriptionsfaktoren (Igarashi et al., 1994; Itoh et al., 1995; Moi et al., 1994 ; **Lukosz** et al., 2010). Nrf-2 wird durch die Bindung an Kelch-like

ECH-associated protein 1 (Keap1), das mit dem Aktinzytoskelett verankert ist, im Zytosol festgehalten und der proteasomalen Degradation zugänglich gemacht (Kang et al., 2004). Auf diese Weise wird eine Aufregulation von Nrf-2-regulierten Genen verhindert (Kobayashi et al., 2004; Zhang et al., 2004). Durch die Erkennung chemischer Signale, vermittelt über Kinasen oder direkt über oxidative und elektrophile Moleküle, erfolgt die Loslösung aus diesem Komplex und Nrf-2 kann sich der proteasomalen Degradation entziehen und in den Nukleus translozieren. Eine Freisetzung aus diesem Komplex erfolgt durch Modifizierungen in Form von kovalenter Bindung oder Oxidierung von Cys-257, Cys-273, Cys-288 und Cys-297 innerhalb des Keap1 Proteins (Dinkova-Kostova et al., 2002; Nguyen et al., 2003). Nrf-2 kann daraufhin im Kern die transkriptionelle Aktivierung einer Reihe von Genen, die für die zelluläre Redoxhomöostase und den oxidativen Schutz von großer Bedeutung sind, bewirken (Rangasamy et al., 2004; Thimmulappa et al., 2002). Die Behandlung von Endothelzellen mit NO-Donoren führt zu einer Steigerung der Nrf-2 Proteinmenge, zu einer verstärkten nukleären Translokation von Nrf-2 und einer dadurch ausgelösten transkriptionellen Aktivierung von Zielgenen (Buckley et al., 2003).

Ein wichtiger Modulator für die Funktion von Transkriptionsfaktoren ist **Thioredoxin-1 (TRX)**. Das Thioredoxin-1/Thioredoxin-Reduktase System (TRX/TR) zählt zu den wichtigsten antioxidativen Systemen von Endothelzellen. Die Funktion von TRX hängt von seiner subzellulären Lokalisation ab. So konnte gezeigt werden, dass zytosolisches TRX in der Lage ist über seine zwei redox-aktiven Cysteine (Cys 32 und Cys 35) oxidierte Proteine zu reduzieren (Holmgren, 2000; Saitoh et al., 1998; Yamanaka et al., 2000). Durch die Ausbildung von Disulfidbrücken interagiert TRX mit einer Vielzahl von Proteinen und verhindert auf diese Weise die Induktion von Apoptose. Diese protektive Funktion von TRX konnte durch seine Bindung an Apoptosis signal-regulating Kinase 1 (ASK1) beobachtet werden. Reduziertes TRX schützt durch seine inhibitorische Bindung an ASK1 vor Apoptose, während dieser Schutz durch Oxidation von TRX verloren geht (Saitoh et al., 1998). Des Weiteren konnte von Frau Haendelers Arbeitsgruppe gezeigt werden, dass TRX im Nukleus unter bestimmten Umständen akkumuliert. Hier ist TRX dann in der Lage, oxidierte Transkriptionsfaktoren zu reduzieren und ermöglicht somit ihre Bindung an DNA (Schroeder et al., 2007). Hierbei kann es sich zum einen um eine direkte Interaktion mit dem Transkriptionsfaktor handeln, wie es für z. B. NF-κB, AP-1 und Nrf-2 bereits bekannt ist, aber auch um eine indirekte Interaktion über die APEX Nuklease 1 (APEX1), wie wir 2010 bereits zeigen konnten (Harper et al., 2001; Hirota et al., 1997; **Lukosz** et al., 2010 ; Hansen et al., 2004). In diesem Fall bindet TRX an APEX1, das wiederum selbst den Transkriptionsfaktor reduziert (**Abb.3**).

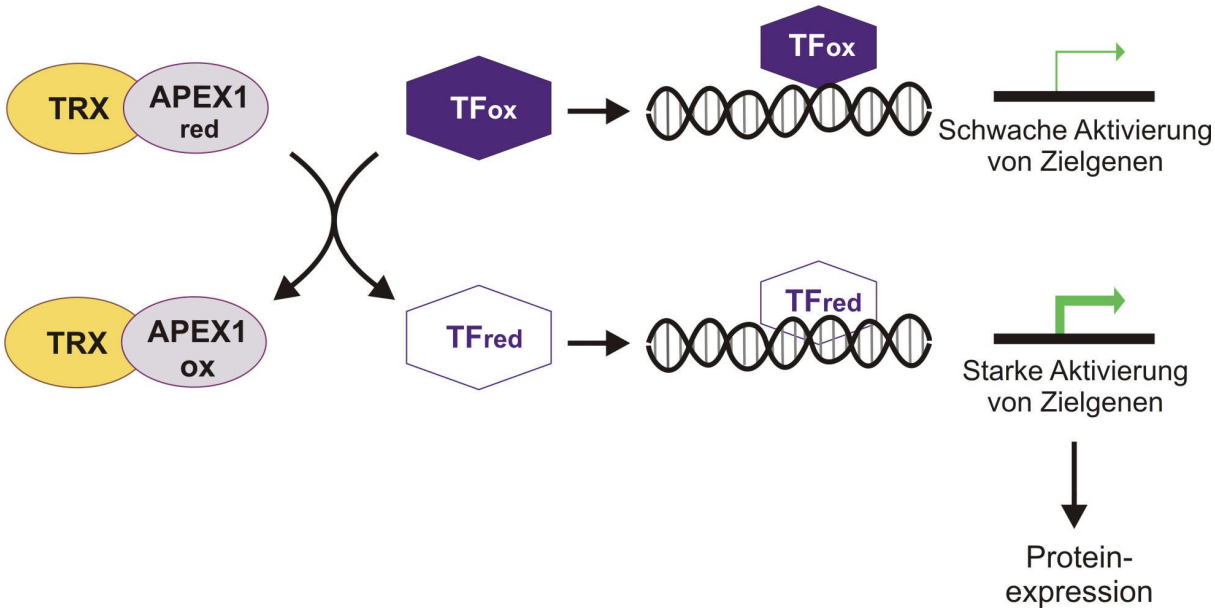


Abb.3: Indirekte Regulation von Transkriptionsfaktoren durch TRX . Durch die Bindung von TRX an APEX1 wird APEX1 reduziert und ist selbst in der Lage oxidierte Transkriptionsfaktoren (TF) zu reduzieren. Durch die Reduktion wird die DNA-Bindungsaktivität des TF erhöht (modifiziert nach **Lukosz** et al., 2010). (Abkürzungen: reduziert (red) und oxidiert (ox))

Eine von NO ausgehende direkte Regulation von TRX in Endothelzellen konnte durch S-Nitrosierung an Cys-69 nachgewiesen werden. Eine S-Nitrosierung an dieser Aminosäure erfolgt bereits unter basalen Bedingungen und stellt eine Voraussetzung für die redox-regulatorische Funktion von TRX dar. Gleichzeitig wird dadurch die enzymatische Aktivität von TRX erhöht (Haendeler et al., 2002). Zusätzlich konnte von Mitchell und Marletta demonstriert werden, dass TRX ebenfalls an Cys-73 S-nitrosiert werden kann. Durch eine Transnitrosierungsreaktion wird diese S-Nitrosierung von TRX auf das Cys-163 der Caspase 3 übertragen und führt zu einer Inhibition ihrer Aktivität und somit zu einem Schutz vor Apoptose (Mitchell und Marletta, 2005). Zusammenfassend zeigen diese Ergebnisse, dass die durch NO generierten Effekte auf TRX in einem Anstieg seiner antioxidativen und antiapoptotischen Kapazität resultieren.

Schließlich konnten wir in unserer Arbeitsgruppe einen Transkriptionsfaktor identifizieren, der durch NO reguliert wird und die Migration von Endothelzellen stark induziert – grainyhead-like 3 (GRHL3). Diesen Transkriptionsfaktor haben wir in einem Screen für neue antiapoptotsiche Gene, die durch TNFα induziert werden, identifiziert (Guardiola-Serrano,…, **Lukosz** et al., 2008). Im Folgenden werde ich nun GRHL3 näher vorstellen.

5 Die Familie der *Grainyhead* Transkriptionsfaktoren

GRHL3 ist ein Transkriptionsfaktor der zur *grainyhead*-like Gen-Familie gehört, die in der Evolution hoch konserviert ist. Entdeckt wurde das Gründungsmitglied, *grainyhead* (Grh), in der Taufliege *Drosophila*. Fliegen, die eine Nullmutation für das *grh* Gen tragen, sterben aufgrund von Anomalien ihrer Cuticula, ihrer Kopfstruktur und ihrer Tracheen (Bray and Kafatos, 1991; Ostrowski et al., 2002). Im Jahre 2002 sind zwei Grh-Homologe im Säuger entdeckt worden, Mammalian Grainyhead (MGR, GRHL1, grainyhead-like 1) und Brother-of-Mammalian Grainyhead (BOM, GRHL2, grainyhead-like 2) (Wilanowski et al., 2002). 2003 kam es zur Beschreibung eines weiteren Homologs Sister-of-Mammalian Grainyhead (SOM, GRHL3, grainyhead-like 3) (Ting et al., 2003). MGR, BOM und SOM weisen hohe Sequenzähnlichkeiten in ihrer DNA-Binde-, ihrer Protein-Dimerisierungs- und ihrer Aktivierungs-Domäne auf. Durch Nutzung unterschiedlicher erster Exons und alternatives Splicen existieren im Menschen drei distinkte Isoformen von GRHL3, die sich in ihren Funktionen unterscheiden (Ting et al., 2003).

6 GRHL3 in der Endothelzellmigration und –apoptose

Während der Transkriptionsfaktor *grainyhead* für die Cuticulaentwicklung von *Drosophila* unersetzlich ist, konnte gezeigt werden, dass GRHL3 in der Maus für die Ausbildung und Gewährleistung der epidermalen Schutzschicht verantwortlich ist. Mausembryonen, die defizient sind für GRHL3, weisen eine nicht intakte Barrierefunktion ihrer Haut und eine gestörte Wundheilung auf. Diese Mäuse sind zudem nicht lebensfähig und versterben kurz nach der Geburt aufgrund eines offenen Rückens, der auch als *spina bifida* bezeichnet wird. Der Grund für das Auftreten der *spina bifida* ist kein Proliferations-, sondern ein Migrationsdefekt (Ting et al., 2005). Zusätzlich konnte gezeigt werden, dass ein Knockdown von GRHL3 in der humanen Keratinozytenzelllinie HaCAT zu einer Beeinträchtigung ihrer Migrationsfähigkeit beiträgt (Caddy et al., 2010). Zusammenfassend implizieren diese Ergebnisse, dass GRHL3 eine wesentliche Rolle in der Migration von Zellen einnimmt. Da endotheliale Dysfunktion begleitet wird von einer verminderten Migrationskapazität von Endothelzellen, untersuchte ich die Bedeutung von GRHL3 für die Endothelzellmigration. Ich konnte zunächst zeigen, dass GRHL3 in humanen primären Endothelzellen (HUVEC, human umbilical vein endothelial cells) exprimiert ist (**Lukosz***, Mlynek* et al., 2011, eingereicht). Durch Überexpression von GRHL3 konnte ich nachweisen, dass der Transkriptionsfaktor eine starke promigratorische Wirkung auf diese Zellen hat. Dieser Effekt ist vergleichbar mit der starken Migrationsinduktion, die nach einer Behandlung mit VEGF zu beobachten ist (Guardiola-Serrano,…, **Lukosz** et al., 2008). Um nachzuweisen, ob GRHL3 eine entscheidende Rolle in der Migration spielt, untersuchte ich das migratorische Verhalten von Endothelzellen nach shRNA-vermittelter Herunterregulation

von GRHL3. In Vorversuchen testete ich fünf verschiedene shRNAs, die alle gegen GRHL3 gerichtet waren. Ziel dieses Versuches war es zu überprüfen inwieweit diese die Expression des Zielgens verringern können, um im Anschluss die physiologische Bedeutung dieser Genproduktreduktion (Knockdown) zu untersuchen. Mittels PCR war ich in der Lage einen erfolgreichen Knockdown von GRHL3 zu verifizieren. In anschließenden Migrationsstudien konnte ich zeigen, dass dieser Knockdown zu einer erniedrigten Migration von Endothelzellen führte, und das bereits unter basalen Bedingungen (**Lukosz***, Mlynek* et al., 2011, eingereicht). Da der Wachstumsfaktor VEGF ein wichtiger Stimulus für die Angiogenese und die Endothelzellmigration ist (Bernatchez et al., 1999), wollte ich herausfinden, ob der promigratorische Effekt von GRHL3 über VEGF vermittelt wird. Aus diesem Grund untersuchte ich, inwieweit eine Überexpression von GRHL3 zu einer erhöhten VEGF- Produktion in Endothelzellen führt. Ich konnte allerdings keine Veränderungen in der VEGF-Proteinmenge nach Überexpression von GRHL3 in Endothelzellen nachweisen. Auch konnte ich zeigen, dass GRHL3 keinen Einfluss auf den VEGF-mRNA Level nimmt. Umgekehrt zeigte sich auch, dass VEGF die Expression von GRHL3 nicht beeinflusst. So konnte ich demonstrieren, dass VEGF selbst GRHL3 nicht beeinflusst und somit nicht zu den von GRHL3 ausgelösten Effekten beiträgt (**Lukosz***, Mlynek* et al., 2011, eingereicht). Ein weiterer wichtiger Faktor für die Funktion des Endothels ist NO. Es konnte gezeigt werden, dass NO auch in der Lage ist unabhängig von VEGF Migration und Angiogenese zu induzieren (Urbich et al., 2002). Aus diesem Grund lag es nahe zu untersuchen, ob NO die GRHL3 Expresssion beeinflusst. So konnte ich zeigen, dass physiologische Konzentrationen an NO, von denen bekannt ist, dass sie promigratorisch und antiapoptotisch wirken (Dimmeler et al., 1999; Haendeler et al., 2002; Urbich et al., 2002), die Menge an GRHL3-mRNA erhöhen (**Lukosz***, Mlynek* et al., 2011, eingereicht). Durch einen Knockdown von GRHL3 in Endothelzellen lässt sich nur noch eine verminderte Migration dieser Zellen beobachten. Zudem konnte ich zeigen, dass eine Verringerung der GRHL3-Transkriptmenge die durch NO ausgelöste Migration außer Kraft setzt (**Lukosz***, Mlynek* et al., 2011, eingereicht). Demzufolge ist GRHL3 ein zentraler Mediator des durch NO hervorgerufenen promigratorischen Effektes. Im nächsten Schritt wollte ich untersuchen, ob GRHL3 die Produktion von NO in Endothelzellen beeinflussen kann. Daher habe ich den Effekt von GRHL3 Überexpression auf die Aktivierung von Akt und eNOS analysiert. Tatsächlich führte dies zu einer Phosphorylierung von Akt und eNOS. Zusätzlich konnte ich in einem modifizierten Griess Assay demonstrieren, dass durch Überexpression von GRHL3 die Menge an S-nitrosierten Proteinen zunimmt, was für eine Erhöhung der NO-Level spricht (**Lukosz***, Mlynek* et al., 2011, eingereicht). Insgesamt demonstrieren diese Untersuchungen, dass NO die Expression von GRHL3 erhöht, aber auch, dass umgekehrt GRHL3 die NO Produktion in Endothelzellen steigert. Zudem konnte ich zeigen, dass die

durch NO vermittelte Migration abhängig ist von der GRHL3 Expression (**Lukosz***, Mlynek* et al., 2011, eingereicht). Sowohl von Akt als auch eNOS ist bekannt, dass sie Schutz vor Apoptose in Endothelzellen vermitteln (Brazil et al., 2002). Aus diesem Grund könnte eine Aktivierung der Akt und eNOS durch GRHL3-Überexpression auch auf eine antiapoptotische Funktion von GRHL3 hindeuten, was durch die Isolation von GRHL3 in einem Screen für anti-apoptotische Gene untermauert wird (Guardiola-Serrano,…, **Lukosz** et al., 2008). Um dieser Hypothese nachzugehen, transfizierte ich Endothelzellen mit einem Expressionsvektor für GRHL3 und ermittelte die Apoptoserate dieser Zellen mittels Annexin V-Bindung an Phosphatidylserin. Bereits zu einem frühen Zeitpunkt des programmierten Zelltodes wird die asymmetrische Struktur der Phospholipidmembran zerstört, was zur Exposition von Phosphatidylserin auf der Zelloberfläche führt (Fadok et al., 1992; Hammill et al., 1999). Annexin V bindet mit hoher Affinität an Phosphatidylserin (Andree et al., 1990; Hammill et al., 1999). Diese Bindung von fluoreszenzmarkiertem Annexin V an exponiertes Phosphatidylserin apoptotischer Zellen kann mittels Durchflusszytometrie nachgewiesen werden und erlaubt somit eine Aussage über die Apoptoserate einer Zellpopulation (Koopman et al., 1994).

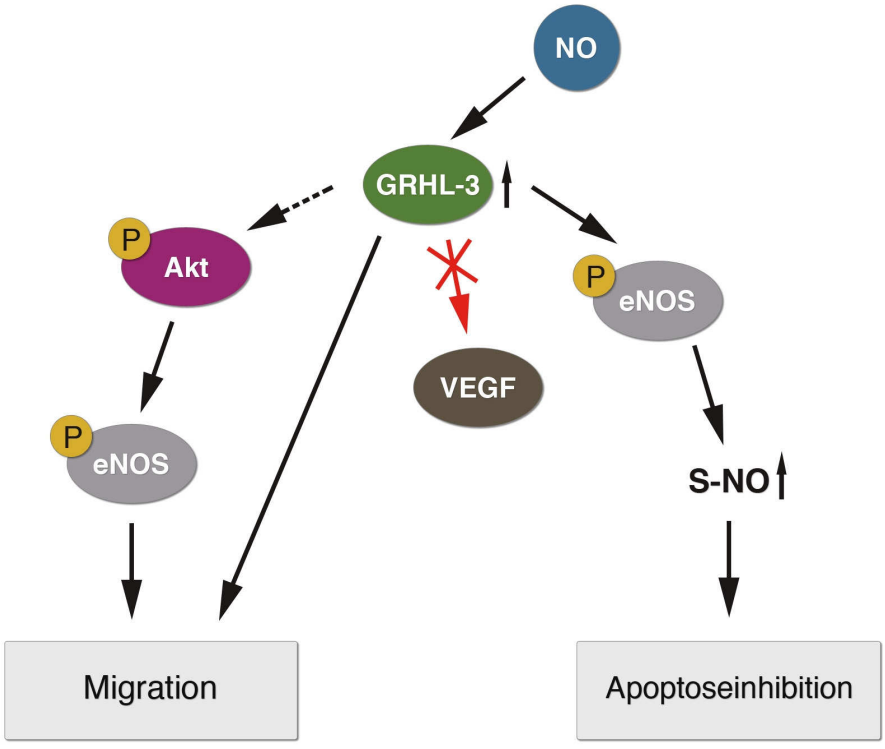


Abb. 4: Modell der von GRHL3 ausgehenden Effekte in Endothelzellen. GRHL3 wirkt promigratorisch auf Endothelzellen. Dieser Effekt ist unabhängig von VEGF. Gleichzeitig führt die Überexpression von GRHL3 zu einer erhöhten Phosphorylierung von Akt und eNOS. Dies geht einher mit einer Steigerung der eNOS Aktivität und einer vermehrten Menge an S-nitrosierten Proteinen, was sich inhibitorisch auf die Apoptose auswirkt. Auch NO selbst hat einen Einfluss auf GRHL3. Durch Behandlung mit NO-Donoren wird der GRHL3 mRNA-Level in Endothelzellen erhöht (**Lukosz***, Mlynek* et al., 2011, eingereicht).

In diesen Experimenten konnte ich nachweisen, dass eine Überexpression von GRHL3 antiapoptotisch wirkt (**Lukosz***, Mlynek* et al., 2011, eingereicht). Um herauszufinden, ob eine Aktivierung der eNOS für diesen protektiven Effekt benötigt wird, behandelte ich die Endothelzellen mit dem NOS-Inhibitor NG-Monomethyl-L-Arginin (L-NMMA). Die Apoptose-Messungen zeigten, dass der protektive Effekt von GRHL3 durch Inhibition der eNOS vollständig aufgehoben wird. Demzufolge ist der antiapoptotische Effekt von GRHL3 abhängig von einer aktiven eNOS (**Lukosz***, Mlynek* et al., 2011, eingereicht). Zusammenfassend ergibt sich aus diesen Daten das in **Abb. 4** vorgestellte Modell.

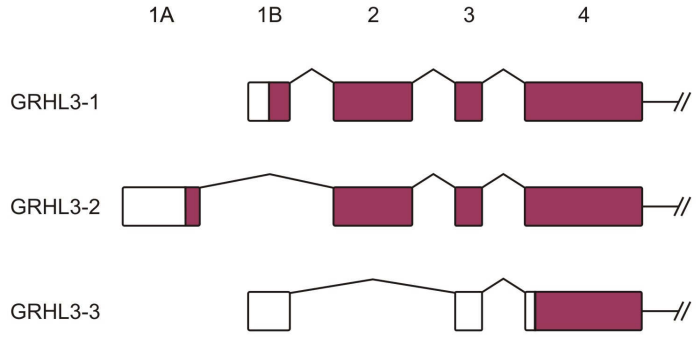
Die Src-Kinase spielt in Endothelzellen eine ambivalente Rolle. So konnte gezeigt werden, dass abhängig von der Aktivitätsdauer der Src-Kinase verschiedene Signalwege und Prozesse innerhalb der Zelle beeinflusst werden. Die Aktivierung über einen kurzen Zeitraum (bis zu 120 min) ist z.B. in VEGF-abhängige Signalwege involviert (Eliceiri et al., 1999; Tanimoto et al., 2002). Im Gegensatz dazu steht die Langzeit-Aktivierung (18 h bis 14 Tage) der Src-Kinase, die in Endothelzellen Apoptose und Seneszenz induziert (Chen et al., 2008; Haendeler et al., 2003; Haendeler et al., 2004). Vor diesem Hintergrund wollte ich überprüfen, inwieweit eine Blockade dieser Langzeit-Aktivierung Einfluss nehmen kann auf die Expression von GRHL3 in Endothelzellen. Durch eine 19-stündige Behandlung mit dem spezifischen Src-Kinasen Inhibitor PP2 (Igishi und Gutkind, 1998) konnte ich die Phosphorylierung der Src-Kinase an Tyr-418 reduzieren und somit die Aktivität der Kinase hemmen. In einer anschließenden quantitativen RT-PCR konnte ich zeigen, dass diese Langzeit-Suppression der Src-Aktivität die GRHL3-mRNA Menge erhöht (**Lukosz***, Mlynek* et al., 2011, eingereicht). Untersuchungen mit gealterten Endothelzellen zeigten, dass diese eine erhöhte Phosphorylierung des Tyr-418 der Src-Kinase aufweisen (Haendeler et al., 2004). Eine Phosphorylierung an dieser Aminosäure führt zu einer Erhöhung der Src-Aktivität (Porter et al., 2000). Da zudem die Migrationskapazität von Endothelzellen im Alter erniedrigt ist, wollte ich überprüfen, inwieweit ein Zusammenhang zwischen der Src-Kinase-Aktivität und dem migratorischen Verhalten von Endothelzellen besteht. Durch Behandlung von Endothelzellen mit dem spezifischen Src-Kinase Inhibitor PP2 (Igishi und Gutkind, 1998) konnte ich nachweisen, dass dies zu einer gesteigerten Migration der Endothelzellen führt (**Lukosz***, Mlynek* et al., 2011, eingereicht). Ebenfalls konnte von Frau Haendelers Arbeitsgruppe gezeigt werden, dass die Src-Kinase verantwortlich ist für den Kernexport von TERT und somit wesentlich zum Alterungsprozess beiträgt, da dadurch den Zellen die Fähigkeit zur Telomerverlängerung genommen wird (Haendeler et al., 2004). Ich konnte in Untersuchungen von gealterten Endothelzellen nachweisen, dass die Menge an GRHL3-mRNA in diesen erniedrigt ist (**Lukosz***, Mlynek* et al., 2011, eingereicht). Interessanterweise erhöht GRHL2 die Expression von TERT in Keratinozyten, was zu einer signifikant verlängerten Lebensdauer dieser Zellen führt (Chen et al., 2010). Da von den

Mitgliedern der *grainyhead*-like Familie bekannt ist, dass diese miteinander heterodimerisieren können (Ting et al., 2003), lassen diese Erkenntnisse die Hypothese zu, dass eine verminderte Expression von GRHL3 zu alternsassoziierten Veränderungen in Endothelzellen beitragen könnte.

7 GRHL3-Isoformen in humanen Endothelzellen

Im Gegensatz zur Maus, die nur ein GRHL3 Protein aufweist, konnten im Menschen für GRHL3 drei distinkte Isoformen identifiziert werden. GRHL3-1 und die zur Maus homologe Isoform GRHL3-2 sind fast identisch, abgesehen davon, dass die zweite Isoform ein alternatives erstes Exon nutzt. Die dritte Isoform (GRHL3-3) wird kodiert von einer Splicevariante, der das zweite Exon der Isoform 1 fehlt (Ting et al., 2005). Nach der Translation ergeben sich aus den unterschiedlichen RNAs drei Proteine, die sich in ihrer Größe (GRHL3-1: 607 Aminosäuren (NP_067003.2), GRHL3-2: 602 Aminosäuren (NP_937816.1) und GRHL3-3: 509 Aminosäuren (NP_937817.2)) und vermutlich ihrer Funktion unterscheiden. In **Abb.5 A** sind die 5`-Enden der verschiedenen Transkripte und in **Abb. 5 B** die N-Termini der zugehörigen Proteine dargestellt.

A



B

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hGRHL3-2    1  -----MSNELDFRSVRLLKNDPVNLQKFSYTSEDEAWKTYLENPLTAATKAMMRVNGDDDSVAALSFLYD  65
hGRHL3-3       ------------------------------------------------------------------
hGRHL3-1    1  MWMNSILPIFLFRSVRLLKNDPVNLQKFSYTSEDEAWKTYLENPLTAATKAMMRVNGDDDSVAALSFLYD  70

hGRHL3-2   66  YYMGPKEKRILSSSTGGRNDQGKRYYHGMEYETDLTPLESPTHLMKFLTENVSGTPEYPDLLKKNNLMSL  135
hGRHL3-3    1  ----------------------------MEYETDLTPLESPTHLMKFLTENVSGTPEYPDLLKKNNLMSL  42
hGRHL3-1   71  YYMGPKEKRILSSSTGGRNDQGKRYYHGMEYETDLTPLESPTHLMKFLTENVSGTPEYPDLLKKNNLMSL  140
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Abb. 5: Humane GRHL3 Isoformen. (A) Schematische Darstellung des 5'-Endes der drei GRHL3-Transkripte; Exons sind maßstabsgetreu als Boxen dargestellt, Introns nicht maßstabs-getreu, die translatierten Bereiche sind lila unterlegt. (B) Primärsequenz der N-Termini der GRHL3 Isoformen; der Unterschied zwischen Isoform 1 und 2 ist farblich gekennzeichnet.

Erste Untersuchungen zur transkriptionellen Aktivität der einzelnen Isoformen wurden mit einem "two-hybrid" System, in dem einzelne GRHL3-Domänen an die DNA-

Bindedomäne des Hefe-Transkriptionsfaktors Gal4 fusioniert wurden, durchgeführt. Aus diesen Untersuchungen ergab sich die Schlussfolgerung, dass GRHL3-1 ein aktiver Transkriptionsfaktor ist und GRHL3-3 aufgrund einer fehlenden Aktivierungsdomäne möglicherweise als Repressor fungiert oder in einem Heterodimer mit GRHL3-1 dominant negativ wirkt (Ting et al., 2003). Meine in Kooperation mit Arne Mlynek durchgeführten Untersuchungen zur Frage nach transkriptionellen Unterschieden der Isoformen zeigten jedoch, dass sich alle drei Isoformen aktivierend auf die Transkription auswirken (**Lukosz***, Mlynek* et al., 2011, eingereicht). Im Gegensatz zu Ting et al. verwendeten wir Vektoren zur Expression der kompletten GRHL3-Proteine und ausserdem ein Reportergen mit einer GRHL3-Konsensussequenz. Auf diese Weise wollten wir einer *in vivo* Situation möglichst nahe kommen und artifizielle Effekte vermeiden. Im Folgenden möchte ich nun auf die Ergebnisse zur Untersuchung der Rolle der einzelnen GRHL3-Isoformen im Endothel genauer eingehen, da sich die bislang diskutierten Ergebnisse auf die Isoform 2 bezogen. Durch Überexpression von GRHL3-1 und GRHL3-3 in humanen primären Endothelzellen konnte ich zeigen, dass sich die Isoform 1 promigratorisch verhält. Im Gegensatz wirkt sich GRHL3-3 repressiv auf das Migrationsverhalten von Endothelzellen aus (Mlynek*, **Lukosz*** et al., 2011, eingereicht). In weiteren Untersuchungen wollte ich klären, ob auch diese Isoformen Einfluss nehmen können auf die NO-Produktion. Ich konnte durch Überexpression von GRHL3-1 und -3 in Endothelzellen zeigen, dass Isoform 1 sowohl die Phosphorylierung von Akt als auch der eNOS erhöht. Die Überexpression der dritten Isoform hingegen führte zu keiner gesteigerten Phosphorylierung von Akt und eNOS (Mlynek*, **Lukosz*** et al., 2011, eingereicht). Als nächstes untersuchte ich den Effekt von GRHL3-1 und -3 auf Endothelzellapoptose. Durch Transfektion von Endothelzellen mit Expressionsvektoren für GRHL3-1 oder GRHL3-3 und anschließende Messung der Apoptoserate dieser Zellen mittels Annexin V konnte ich zeigen, dass von GRHL3-3 keine protektive Funktion für Endothelzellen ausgeht. Die Überexpression von GRHL3-1 zeigte einen antiapoptotischen Effekt (Mlynek*, **Lukosz*** et al., 2011, eingereicht). Die gegenläufigen Effekte von GRHL3-1 und GRHL3-3 sind in **Abb. 6** zusammengefasst.

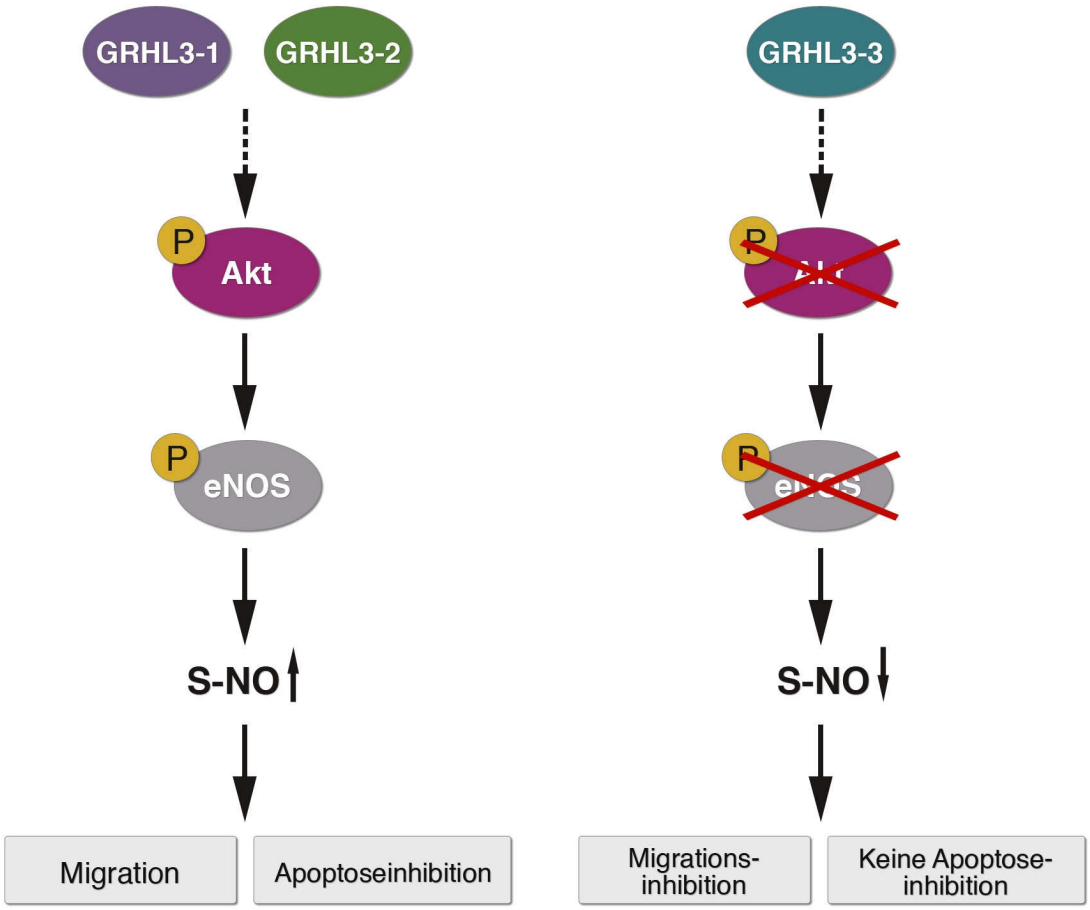

Abb. 6: GRHL3-Isoform-spezfische Effekte in Endothelzellen. (Mlynek*, **Lukosz***, et al., 2011, eingereicht).

In Kooperation mit Christoph Winkler und Martin Graf an der National University of Singapore waren wir in der Lage entwicklungsbiologische Untersuchungen im Zebrafisch (*Danio rerio*) durchzuführen. Durch Injektion von mRNA der einzelnen Isoformen wenige Stunden nach Fertilisation (34 hpf, hours post fertilization) war es möglich, die morphologischen Auswirkungen dieser Überexpression während der Fischentwicklung zu beobachten, da die Embryonen des Zebrafisches durchsichtig sind. Hierbei wurde festgestellt, dass die Überexpression von GRHL3-3 zu schwerwiegenden Missbildungen und einer erhöhten Letalität führte. Nach Überexpression von GRHL3-1 hingegen entwickelten sich die Fische normal.

Zusammenfassend zeigen unsere Untersuchungen der drei GRHL3-Isoformen, dass sich zwar alle drei Isoformen transkriptionsaktivierend in Reportergenanalysen verhalten, sich ihre Überexpression in Endothelzellen allerdings unterschiedlich auf die Prozesse der Migration und Apoptose auswirkt (**Lukosz***, Mlynek* et al., 2011, eingereicht) (Mlynek*, **Lukosz*** et al., 2011, eingereicht). Unsere Ergebnisse weisen eindeutig darauf hin, dass die Isoformen unterschiedliche Zielgene aktivieren (**Lukosz***, Mlynek* et al., 2011, eingereicht).

8 Ausblick

Wie zuvor angedeutet, weisen die erhobenen Daten darauf hin, dass die drei Isoformen von GRHL3 als Transkriptionsfaktoren unterschiedliche Zielgene aktivieren, was auf Interaktionen mit anderen Transkriptionsfaktoren beruhen könnte (**Abb. 7**). Zur Identifikation von Isoform-spezifischen GRHL3-Zielgenen werden wir Microarray-Analysen durchführen. Hierbei soll durch lentivirale Überexpression der drei Isoformen in Endothelzellen geklärt werden, inwieweit sich diese auf unterschiedliche Gene der Bereiche Angiogenese, Apoptose und Migration auswirken (**Abb. 7**).

Eine weitere Möglichkeit, wie die GRHL3-Isoformen ihre unterschiedlichen Funktionen in Endothelzellen ausüben könnten, wären Interaktionen mit verschiedenen Proteinen. Interessanterweise konnte ich in Vorversuchen nach Überexpression von GRHL3-2 in Endothelzellen nachweisen, dass GRHL3-2 nicht nur im Nukleus lokalisiert ist, sondern auch im Zytosol und an der Plasmamembran. Daher könnten unterschiedliche Interaktionspartner der GRHL3-Isoformen auch außerhalb des Nukleus mitverantwortlich sein für die unterschiedlichen Funktionen dieser Isoformen in Endothelzellen (**Abb. 7**). In ersten Untersuchungen zur Identifikation von GRHL3-Interaktionspartnern konnte ich in Zusammenarbeit mit Frau Sabine Metzger vom BMFZ zeigen, dass HSP90 mit GRHL3 co-immunpräzipitiert (unpublizierte Ergebnisse). Ich konnte ebenfalls erste Hinweise darauf gewinnen, dass eNOS mit an diesen Komplex gebunden ist (unpublizierte Ergebnisse). In weiterführenden Experimenten möchte ich versuchen, die Frage zu klären, ob eventuell diese Bindung für die gesteigerte eNOS-Aktivität in Form einer verstärkten Phosphorylierung des Ser-1177 verantwortlich ist oder dadurch nur weitere regulatorische Proteine, wie z. B. Kinasen rekrutiert werden. Hierbei stellt die Kinase Akt einen interessanten Kandidaten dar, da ich bereits zeigen konnte, dass eine Überexpression von GRHL3 in Endothelzellen zu einer erhöhten Phosphorylierung der Akt an Ser-473 führt (**Lukosz***, Mlynek* et al., 2011, eingereicht). Zudem lässt sich auch vermuten, dass die Interaktion der Isoformen GRHL3-1 und GRHL3-2 mit HSP90 zu einer verstärkten Aktivierung der eNOS führt und, dass GRHL3-3 möglicherweise nicht an HSP90 binden kann oder durch seine Bindung die eNOS Aktivierung verhindert. Für HSP90 wird vermutet, dass es die Bindung von eNOS zu anderen aktivierenden Faktoren stabilisiert, möglicherweise könnte GRHL3 eines dieser Proteine sein. Die Bindung des Proteins Caveolin-1 (Cav-1) an eNOS wirkt sich hingegen hemmend aus. Cav-1 ist ein Bestandteil der Caveolae, den Einstülpungen der Plasmamembran. Da die Freisetzung aus dieser hemmenden Bindung eine Voraussetzung für die NO-Produktion ist (Michel et al., 1997), könnten GRHL3-1 und GRHL3-2 auch hier aktivatorisch eingreifen (**Abb. 7**). Daher möchte ich die zytosolische Komplexbildung von GRHL3-1, GRHL3-2 und GRHL3-3 zukünftig untersuchen.

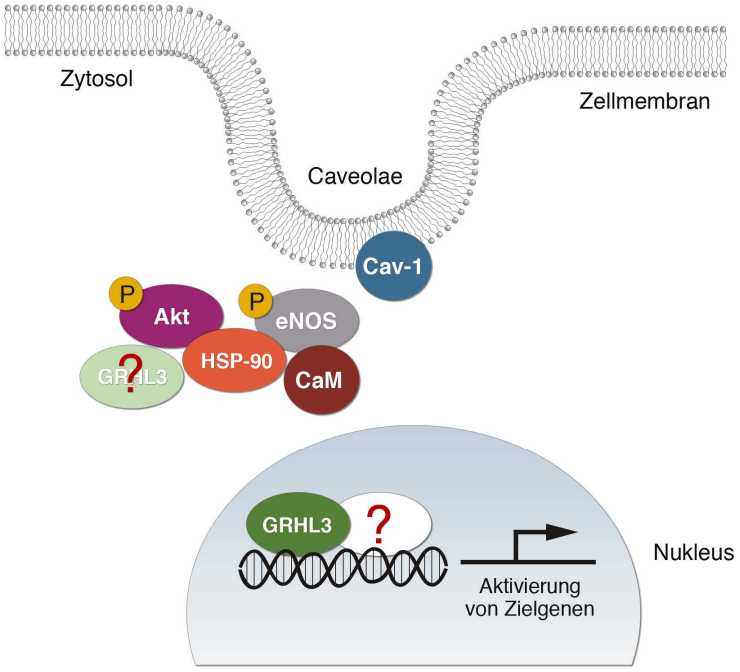

Abb. 7: GRHL3-Interaktionspartner. Durch die Bindung von Calmodulin (CaM) an eNOS im Zytosol erfolgt die Loslösung der eNOS aus dem inhibitorischen Komplex mit Cav-1. Diese Interaktion sorgt für die sofortige Rekrutierung von HSP90 und Bildung des CaM/eNOS/HSP90-Komplexes. Der CaM/eNOS/HSP90-Komplex rekrutiert die Proteinkinase B (Akt), die eNOS an Ser-1179 phosphoryliert. Inwieweit GRHL3 an diesem Komplex beteiligt ist, ist bislang nicht bekannt. Im Zellkern kann es durch differentielle Interaktionen der einzelnen GRHL3 Isoformen mit anderen Transkriptionsfaktoren zur Aktivierung unterschiedlicher Zielgene kommen.

Zudem konnte ich nachweisen, dass GRHL3-2 essentiell für die Endothelzellmgration ist und die Expression von GRHL3-2 in seneszenten Endothelzellen reduziert ist. Dies spricht dafür, dass GRHL3-2 wichtig für die Endothelzellfunktion ist. Daher wäre eine Aktivitätssteigerung oder auch schon die Konservierung der Aktivität/Expression von GRHL3-2 ein möglicher, neuer therapeutischer Ansatz, um die Integrität des Endothels zu erhalten.

9 Zusammenfassung

Physiologische Alterungsprozesse und auch kardiovaskuläre Erkrankungen gehen mit endothelialer Dysfunktion einher. Diese ist gekennzeichnet durch eine verringerte Verfügbarkeit an Stickstoffmonoxid (NO), eine erhöhtes Auftreten von apototischen Endothelzellen und eine verringerte Migrationsfähigkeit dieser Zellen. Kürzlich konnten wir in unserer Arbeitsgruppe den Transkriptionsfaktor Grainyhead-like 3 (GRHL3) in Endothelzellen nachweisen. Jedoch war über die Rolle von GRHL3 in Endothelzellen nichts bekannt. Daher war es Ziel dieser Arbeit zu untersuchen, ob GRHL3 Apoptose und Migration von Endothelzellen beeinflusst und wie GRHL3 bei der Alterung von Endothelzellen reguliert ist. Es zeigte sich, dass physiologische Konzentrationen an NO, aber auch die Inhibition der Aktivität von Src Kinasen, die Menge an GRHL3-mRNA erhöhen. Gleichzeitig wirken diese Behandlungen promigratorisch. Die Überexpression von GRHL3 führt zu einer vermehrten Phosphorylierung und damit Aktivierung der Protein Kinase B/Akt und der endothelialen NO-Synthase. Zudem führt die Überexpression von GRHL3 zu verminderter Apoptose in Endothelzellen. Dieser protektive Effekt ist NO abhängig, da er durch Behandlung mit dem NOS-Inhibitor L-NMMA aufgehoben werden kann. Im Gegensatz dazu unterdrückt die Ablation von GRHL3 die basale und die durch NO induzierte Migration von Endothelzellen. Während der Endothelzellalterung kommt es zu einer gesteigerten Aktivität der Src Kinasen und zu einem Verlust an GRHL3 Expression. Demzufolge wirkt GRHL3 promigratorisch und antiapoptotisch in Endothelzellen und ist während des Alterungsprozesses herunterreguliert. Im Gegensatz zur Maus gibt es im Menschen 3 Isoformen von GRHL3. Diese sind Translationsprodukte zweier Primärtranskripte mit unterschiedlichen ersten Exons von denen eines zudem zwei Splicevarianten aufweist. Untersuchungen zu den Funktionen dieser Isoformen (GRHL3-1, GRHL3-2, GRHL3-3) sind bis heute nicht durchgeführt worden. In Endothelzellen sind alle 3 Isoformen exprimiert und transkriptionsaktivierend. Jedoch induziert eine Überexpression von GRHL3-1 oder -2 Migration und schützt vor Apoptose in Endothelzellen, wohingegen GRHL3-3 repressiv auf das Migrationsverhalten wirkt und keinen Schutz vor Apoptose gewährt. Im Zebrafisch zeigt sich zudem, dass nur die Überexpression von GRHL3-3, nicht aber von GRHL3-1, zu schwerwiegenden Missbildungen und zu einer geringeren Anzahl normal entwickelter Embryonen führt. Diese Ergebnisse weisen darauf hin, dass die Isoform-spezifischen Effekte durch die Aktivierung unterschiedlicher Zielgene ausgelöst werden.

Zusammenfassend ist in dieser Doktorarbeit die funktionelle Relevanz von GRHL3 in Endothelzellen aufgeklärt worden. Eine Konservierung der Expression von GRHL3-1 oder -2 bzw. eine Verhinderung der Expression von GRHL3-3 könnte zur Aufrechterhaltung der Endothelzellintegrität beitragen.

10 Summary

Endothelial dysfunction is involved in physiological aging processes but also cardiovascular diseases. The dysfunction is characterized by reduced bioavailability of nitric oxide (NO), increased apoptosis sensitivity and reduced migratory capacity. Recently, we discovered the transcription factor Grainyhead-like 3 (GRHL3) in endothelial cells. However, the functions of GRHL3 in endothelial cells are completely unknown. Therefore, the aim of my PhD thesis was to investigate whether GRHL3 influences apoptosis and migration of endothelial cells and if GRHL3 is regulated during endothelial cell aging. Physiological concentrations of NO, but also inhibition of the activity of Src kinases, increased the expression of GRHL3, enhanced migration and inhibited apoptosis. Overexpression of GRHL3 increased phosphorylation and thereby activation of the Protein kinase B/Akt and of the endothelial NO-synthase and inhibited endothelial cell apoptosis. This process was dependent on NO, since inhibition of the endothelial NO-synthase with a NOS-inhibitor completely abrogated the protective effect. On the other hand, ablation of GRHL3 reduced basal and NO-induced migration of endothelial cells. During the process of endothelial cell aging activation of the Src kinases is increased and the expression of GRHL3 is decreased. Thus, GRHL3 acts promigratory and antiapoptotic in endothelial cells and is reduced during vascular aging processes. In contrast to mice humans express 3 isoforms of GRHL3. These isoforms are translation products of two primary transcripts with different first exons, one of which is alternatively spliced. Functional analyses of these three isoforms (GRHL3-1, GRHL3-2, GRHL3-3) were not performed so far. All of these isoforms are expressed and active transcription factors in human endothelial cells. Overexpression of GRHL3-1 or -2 induced migration and inhibited apoptosis of endothelial cells, whereas GRHL3-3 decreased migration and did not protect from apoptosis. Moreover, overexpression of GRHL3-3, but not GRHL3-1, induced severe malformations and reduced the number of normally developed zebrafish embryos. These results suggest that activation of different target genes accounts for the isoform-specific effects.

Taken together, the functional relevance of GRHL3 for endothelial cells has been elucidated in this PhD thesis. Maintenance of GRHL3-1 or -2 expression or alternatively inhibition of GRHL3-3 expression may result in prolonged endothelial monolayer integrity.

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12 Eigene Veröffentlichungen

1. **Gene trapping identifies a putative tumor suppressor and a new inducer of cell migration**
   F. Guardiola-Serrano*, J. Haendeler*, **M. Lukosz**, K. Sturrm, H. Von Melchner, J. Altschmied
   BBRC *376*, 748-752, 2008 (* Autoren zu gleichen Teilen am Manuskript beteiligt)

2. **Nuclear protein tyrosine phosphatase Shp-2 is one important negative regulator of nuclear export of telomerase reverse transcriptase**
   S. Jakob, P. Schroeder, **M. Lukosz**, N. Büchner, I. Spyridopoulos, J. Altschmied, J. Haendeler
   J Biol Chem *283*, 33155-33161, 2008

3. **Nuclear redox signaling**
   M. Lukosz*, S. Jakob*, N. Buechner, T.C. Zschauer, J. Altschmied, J. Haendeler
   Antioxid. Redox Signal. *12*, 713-742, 2010 (* Autoren zu gleichen Teilen am Manuskript beteiligt)

4. **Downregulation of mitochondrial telomerase reverse transcriptase induced by H_2O_2 is Src kinase dependent**
   N. Büchner, TC. Zschauer, **M. Lukosz**, J. Altschmied, J. Haendeler
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5. **The transcription factor Grainyhead like 3 (GRHL3) a new player in endothelial cell senescence, apoptosis and migration**
   M. Lukosz*, A. Mlynek*, P. Czypiorski, J. Altschmied, J. Haendeler
   Aging Cell, eingereicht, 2011 (* Autoren zu gleichen Teilen am Manuskript beteiligt)

6. **Two isoforms of the transcription factor Sister of Mammalian Grainyhead derived from an alternatively spliced transcript have opposing functions in cells and in vivo**
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Gene trapping identifies a putative tumor suppressor and a new inducer of cell migration

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Gene trapping identifies a putative tumor suppressor and a new inducer of cell migration

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ABSTRACT

Tumor necrosis factor alpha (TNFα) is a pleiotropic cytokine involved in apoptotic cell death, cellular proliferation, differentiation, inflammation, and tumorigenesis. In tumors it is secreted by tumor associated macrophages and can have both pro- and anti-tumorigenic effects. To identify genes regulated by TNFα, we performed a gene trap screen in the mammary carcinoma cell line MCF-7 and recovered 64 unique, TNFα-induced gene trap integration sites. Among these were the genes coding for the zinc finger protein ZC3H10 and for the transcription factor grainyhead-like 3 (GRHL3). In line with the dual effects of TNFα on tumorigenesis, we found that ZC3H10 inhibits anchorage independent growth in soft agar suggesting a tumor suppressor function, whereas GRHL3 strongly stimulated the migration of endothelial cells which is consistent with an angiogenic, pro-tumorigenic function.

Tumor necrosis factor alpha (TNFα) is a pluripotent cytokine involved in the control of inflammation, cell proliferation, apoptosis, and endothelial cell migration [1,2] and the cellular responses vary according to specific physiological contexts.

Tumors are continuously infiltrated by monocytes, which after differentiating into tumor-associated macrophages (TAMs) secrete a vast array of cytokines, including TNFα [3]. In tumors, TNFα has conflicting roles, as both a pro- and anti-tumorigenic factor. Local administration can inhibit tumor growth and even lead to tumor regression, like for example in the treatment of soft tissue sarcomas [4] or hepatic metastases of ocular melanomas [5]. The tumor promoting function of TNFα has been documented in numerous experimental models. For example, it has been demonstrated that TNFα directly promotes skin cancer in mice [6] and metastases formation in experimentally induced tumors [7]. Along the same lines, Helicobacter pylori, which is a bacterial carcinogen for stomach cancer, induces TNFα in the stomach [8].

To identify pro- and anti-tumorigenic genes induced by TNFα in MCF-7 breast cancer cells, we used a gene trap approach that enables the recovery of transiently expressed genes [9,10]. Among several known TNFα-responsive genes, we identified the zinc finger protein ZC3H10 and the transcription factor grainyhead-like 3 (GRHL3) as novel TNFα-regulated anti- and pro-tumorigenic genes, respectively. Here we show that ZC3H10 inhibits the anchorage independent growth of tumor cells, which is consistent with a tumor suppressor function. In contrast, GRHL3 strongly stimulated primary endothelial cell migration, suggesting that it is a putative tumor-angiogenesis factor. Identification of these two genes and their opposing functions supports TNFα's dual role as a tumor promoter and suppressor.

Materials and methods

Cell culture. MCF-7 cells were cultured in DMEM without phenol red, 4.5 mg/ml glucose, 1 mM sodium pyruvate, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. HUVEC were cultured as described [11].

Plasmids. Full length cDNAs for ZC3H10 and GRHL3 were obtained from RZPD and cloned into pcDNA™6/V5-His (Invitrogen). In the vectors for stable transfections the blasticidin deaminase gene was replaced with a neomycin-phosphotransferase selection marker.

Retrovirus production and transduction. Production of retroviral particles was performed as described (http://www.stanford.edu/

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group/nolan/protocols/pro_helper_dep.html) by transfecting 293 T cells with a plasmid encoding the retroviral RNA and the packaging vectors pVPACK-GP (Stratagene) and pMD2.G [12] using FuGene6 (Roche) according to manufacturer's specifications. Target cells were transduced by incubation with retroviral suspensions over night.

Inverse PCR. Retroviral integration sites were mapped by inverse PCR [13] on NspI or PstI digested genomic DNA; for details see Supplemental Information.

Transfection. MCF-7 cells were transfected with TransPass D1 (New England Biolabs) according to manufacturer's specifications. To obtain stable clones, transfected cells were selected for 14 days in medium containing 1 mg/ml G418. HUVEC were transfected as described [11].

Northern blotting. Total cellular RNAs were prepared using the RNeasy mini kit (Qiagen) according to manufacturer's specifications, separated on formaldehyde–agarose gels and transferred to Hybond N+ membranes (Amersham) with standard methods. Blots were hybridized with ^{32}P-labelled single stranded cDNA fragments obtained from asymmetric PCR in ULTRAhyb™ (Ambion) according to manufacturer's specifications. Quantitation was performed with a Phosphoimager (Molecular Dynamics).

Immunoblotting and immunofluorescence. Immunoblotting [11] and immunofluorescence [14] were performed as described, for details see Supplemental Information.

Soft agar colony formation. Soft agar colony formation assays were performed essentially as described [15]; 500 cells were seeded into 35 mm tissue culture plates; 3 weeks later colonies were stained with 0.005% crystal violet for counting.

Cell migration assays. Spheroid assays [16] and scratch wound assays [17] were performed as described previously.

Results

Gene trapping strategy in MCF-7 cells

To identify genes that are regulated by TNFα we used a strategy combining gene trap mutagenesis and site-specific recombination [9,10]. The approach is based on the transcriptional activation of an N-terminally truncated variant of the Fas associated death domain protein (dnFADD) [18] in MCF-7 mammary carcinoma cells, which blocks TNFα-triggered apoptosis [19]. Analogous to the previously described strategies designed to recover genes induced by a biological stimulus [10], we first developed an MCF-7 reporter cell line with a Cre-inducible molecular switch consisting of two selectable marker genes (Fig. 1A). After integration into the cellular genome, a floxed 5′ thymidine kinase-neomycin phosphotransferase fusion gene (tkneo) expressed from a CMV promoter confers resistance to G418. Due to a transcriptional termination (polyA) signal downstream of tkneo the downstream cassette encoding dnFADD is not expressed and therefore the G418 resistant MCF-7 cells are susceptible to TNFα-induced apoptosis. Cre-mediated excision of the polyA site along with tkneo moves the dnFADD cassette next to the CMV promoter initiating its expression and thereby rendering the MCF-7 cells resistant to TNFα. Out of 44 reporter cell lines tested, we selected the clone MCF-7/103 for all further experiments, because it consistently survived in TNFα after Cre mediated recombination (Supplemental Fig. 1). MCF-7/103 cells were infected with a retroviral gene trap vector (U3Cre, [10]) to randomly insert a promoterless Cre recombinase gene throughout the genome. By using 5×10^6 target cells and a multiplicity of infection of 0.5, we obtained a gene trap integration library consisting of approximately 2×10^6 unique proviral integrations. Cells with gene trap insertion in TNFα-regulated genes were recovered in a two-step protocol. To eliminate cells with Cre insertions in constitutively active genes, in which Cre excises tkneo, the integration library was first selected in G418. The surviving cells now enriched for insertions into previously "silent" genes were exposed to TNFα. Since MCF-7 cells are killed by TNFα, only cells expressing dnFADD as result of Cre expression from genes induced by TNFα (Fig. 1B) survived and were selected for further analysis.

Identification of gene trap integration sites by inverse PCR

Genomic sequences adjacent to the gene trap integration sites (gene trap sequence tags; GTSTs) were isolated by inverse PCR. From a total of 78 cell clones, we obtained 64 unique integration sites, of which 24 were in protein coding genes, 5 in ESTs and 35 in intergenic regions. Of these 35 intergenic integrations 16 were less than 3 kb upstream of transcription start sites of known genes or ESTs. Interestingly, 49% of the integrations were in antisense transcriptional orientation on the non-coding DNA strand. Similar results with a high number of antisense integrations were obtained in a larger scale, enhanced gene trap screen [20], suggesting that highly sensitive gene trap protocols such as this, where only a few Cre molecules are required to activate the molecular switch, may pick up previously uncharacterized transcripts.

TNFα regulation of trapped genes

To confirm that the gene trap strategy recovered TNFα-regulated genes, we analyzed the expression of several trapped genes in MCF-7 wildtype cells. Towards this end, MCF-7 cells were exposed for various time intervals to TNFα and analyzed by Northern blotting using gene specific probes. Fig. 2 shows TNFα-dependent gene expression for three different genes, including JUNB, a well-known TNFα target gene [21].

For further functional characterization, we selected two genes based on their novelty as TNFα-regulated genes and on their putative involvement in tumorigenesis. One encodes the zinc finger protein ZC3H10 (GenBank Accession No. NM_032786), whose expression inversely correlates with breast cancer progression, hence suggesting a tumor suppressor function [22]. The second gene, GRHL3 (GenBank Accession No. NM_198173), codes for a transcription factor of the mammalian grainyhead-like family [23], whose inactivation in the mouse results in spina bifida and wound closure defects [24,25], suggesting a role in cell migration, which is the core of angiogenesis and metastasis formation.

Functional analyses of candidate genes

For functional analysis, the ZC3H10 and GRHL3 coding regions were cloned into an expression vector in frame with a C-terminal V5-tag. Protein expression and subcellular localization were verified by transiently transfecting the expression plasmids into MCF-7 cells and staining with an anti-V5 antibody. As expected from their gene ontology annotations, both ZC3H10 and GRHL3 were mainly detected in the nucleus (Supplemental Fig. 2).

Based on ZC3H10's downregulation in highly malignant breast cancer cells [22], we investigated whether ZC3H10 has any effect on cellular transformation. For this, three MCF-7 clones stably transfected with the ZC3H10 expression vector were tested for anchorage independent growth in soft agar. Fig. 3 shows that ZC3H10 significantly inhibited colony formation in a dose dependent manner.

Guided by the phenotypes observed in GRHL3 deficient mice suggesting a role for this protein in cell migration, which is crucial for tumor angiogenesis and metastasis formation, we transiently transfected human umbilical vein endothelial cells (HUVEC) with the GRHL3 expression vector and subjected these to two different types of cell migration assays. As shown in Fig. 4, expression of GRHL3 expression in HUVEC strongly induced cell migration in the scratch wound assay to a level comparable to vascular endothelial growth factor (VEGF), the most potent pro-angiogenic factor

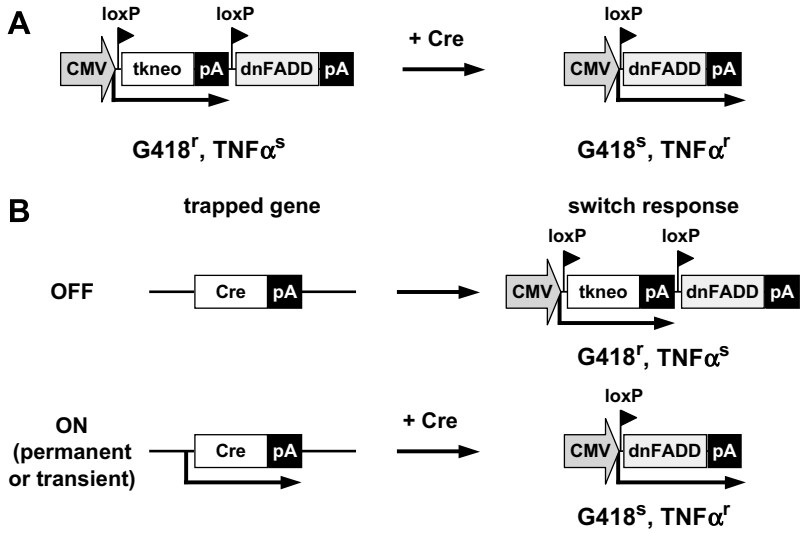

Fig. 1. (A) Anatomy of the Cre-dependent gene switch. In the starting configuration the tkneo marker gene is expressed under the control of the CMV-promoter. Cre-induced deletion of the floxed tkneo-cassette results in expression of dominant negative FADD. (B) Activation of the gene switch by the Cre gene trap. Integration of the Cre gene trap into a non-transcribed region of the genome leaves the gene switch unaffected; insertion into an active gene induces recombination in the gene switch. CMV, Cytomegalovirus promoter; tkneo, thymidine kinase-neomycin phosphotransferase fusion gene; pA, polyadenylation signals; dnFADD, dominant negative FADD; $G418^{r/s}$, G418 resistance/sensitivity; $TNF\alpha^{r/s}$, TNFα resistance/sensitivity.

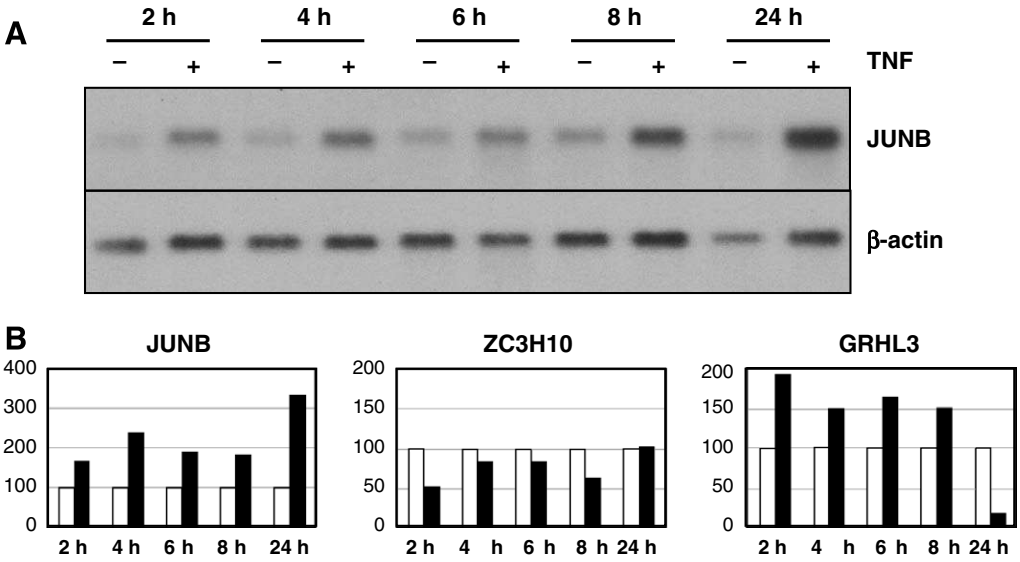

Fig. 2. Regulation of trapped genes by TNFα. Cells were treated for the indicated times with 25 ng/ml TNFα. Total cellular RNAs (20 μg) were analyzed in Northern blots. (A) Exemplary autoradiograph of a blot hybridized with a JUNB specific probe and β-actin for normalization. (B) Quantitative analyses of Northern blots probed for JUNB, ZC3H10 and GRHL3. Expression was normalized for β-actin and is shown relative to the RNA levels in untreated cells (white bars, set to 100%; black bars, TNFα treated cells).

discovered thus far. Expression of GRHL3 also induced sprout formation in a spheroid assay, a three-dimensional angiogenesis model, suggesting a potential role in tumor angiogenesis.

Discussion

By using a one-way gene expression switch that uncouples the expression of a marker gene from a trapped cellular promoter, we recovered 41 U3Cre gene trap insertions in TNFα-induced genes. This number is low compared to the number of TNFα-regulated genes recovered by expression profiling [21,26] and simply reflects the high stringency of selection inherent to the gene trap approach. As expected, TNFα induction kinetics were highly variable with some genes being induced almost instantaneously others requiring up to 24 h to respond (data not shown).

Like the enhanced gene trap vectors recently described [20] nearly one third of the U3Cre gene trap insertions were in intergenic regions of which a significant proportion are likely to correspond to novel genes. Thus, trapping with U3Cre should greatly assist future genome annotation [27]. Moreover, a large proportion of the gene trap integrations were in antisense orientation on the non-coding strand of genes or ESTs. One explanation could be an effective trapping of naturally occurring antisense transcripts. Indeed, four U3Cre insertions mapped to known overlapping sense/antisense transcript pairs (data not shown). While the gene trap activation mechanism from the remaining antisense insertions remains to be established by future experiments, high throughput trapping using highly sensitive gene trap vectors such as U3Cre may aid the functional annotation of the large number of antisense transcripts expressed in the human genome [28,29]. Although the

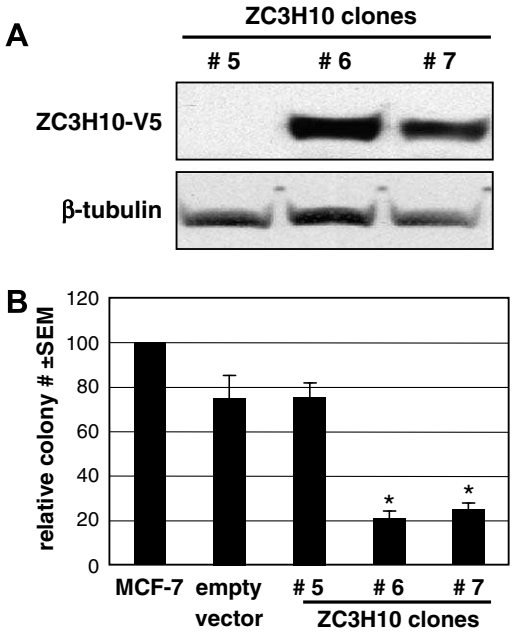

Fig. 3. Inhibition of soft agar colony formation by ZC3H10. Three different MCF-7 clones stably transfected with an expression vector for ZC3H10-V5 were analyzed for colony formation in soft agar. (A) Western blot for ZC3H10-V5 and β-tubulin as loading control. (B) Relative colony numbers ± SEM. The numbers were normalized to the colony numbers obtained with empty vector transfected cells ($n = 3$, $^{*}p < 0.01$ vs. empty vector transfected cells).

functions of antisense RNAs are still largely unknown, some have been already shown to be involved in fundamental aspects of gene regulation at transcriptional and posttranscriptional levels including epigenetic silencing of gene expression [30].

The functional analysis of the two TNFα-regulated genes recovered in this screen revealed that both are linked to tumorigenesis. The inhibition of anchorage independent growth by ZC3H10 fits well with this gene's downregulation in advanced stages of primary breast cancers. The observations suggest that ZC3H10 is a putative tumor suppressor, which is inactivated during the late stages of tumor development. Tumor suppressors often slow down proliferation of cancer cells and thereby promote differentiation. Accordingly, ZC3H10 is strongly expressed in grade 1 breast tumors consisting of still well differentiated cells. Expression decreases in the less differentiated grade 2 tumors and is hardly detectable in the poorly differentiated and highly proliferating cells of grade 3 tumors [22]. Moreover, breast cancers with lymphocytic infiltrates exhibit lower ZC3H10 expression than tumors without such infiltrates [22], suggesting that the increased levels of TNFα secreted by these cells downregulate ZC3H10 in the tumor cells. However, little is known about the molecular mechanisms responsible for the tumor suppressive effect. Proteomic studies revealed that ZC3H10 interacts with the two RNA binding proteins ATXN1 and RBPMS [31,32], and may thus be involved in RNA processing.

We also identified GRHL3 as a TNFα-induced endothelial cell migration factor with a promigratory activity as high as VEGF. Interestingly, the GRHL3 related protein LBP-1a is essential for extraembryonic angiogenesis during mouse development [33], suggesting that the grainyhead family of proteins has a more general role in blood vessel formation. GRHL3 was originally identified in a search for human homologs of the Drosophila transcription factor grainyhead and is closely related to the other mammalian homologs of grainyhead, GRHL1 and GRHL2. The three proteins share a high degree of sequence identity and can both homo- and heterodimerize [23]. Thus, the lack of obvious embryonic angiogenesis defects in GRHL3-deficient mice is probably due to some

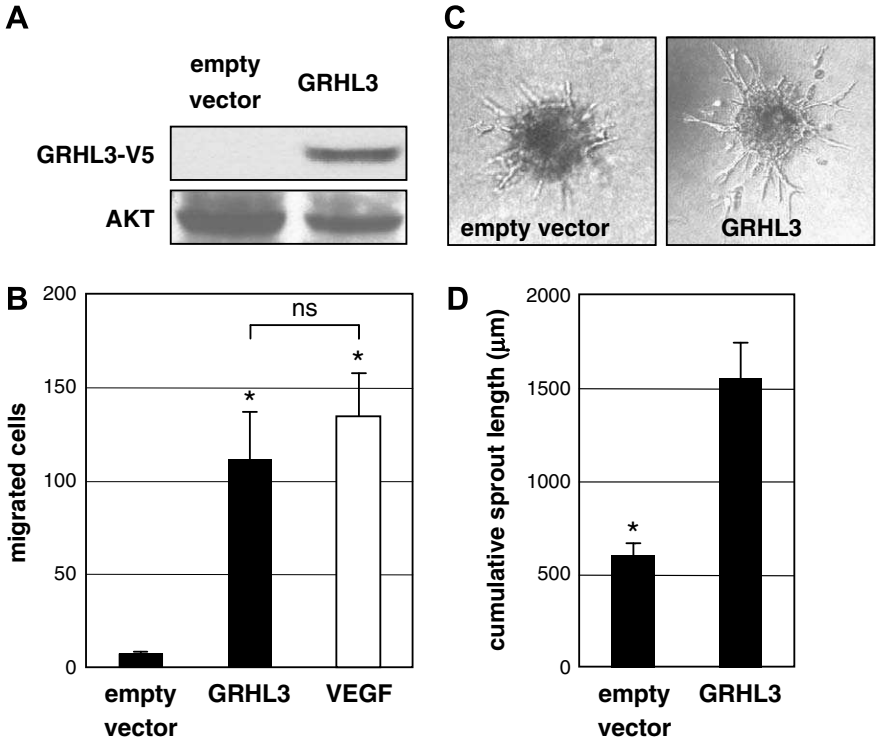

Fig. 4. Induction of endothelial cell migration by GRHL3. Human umbilical vein endothelial cells were transiently transfected with an expression vector for GRHL3-V5 or an empty vector and assayed for migration induction. (A) Expression of GRHL3-V5 in transfected cell populations determined by Western blotting; blots were reprobed for AKT as loading control. (B) Scratch wound assay; shown is the absolute number of migrated cells ± SEM, for comparison cell migration upon treatment with 50 ng/ml VEGF is shown ($n = 4$, $^{*}p < 0.015$ vs. empty vector, ns = non significant). (C)+(D) Spheroid assay. (C) Exemplary pictures of spheroids. (D) Quantitation of sprout formation; shown is the cumulative sprout length of single spheroids ± SEM ($n = 7$ for empty vector, $n = 5$ for GRHL3, $^{*}p < 0.0002$).

functional redundancy within this gene family. In this context, it will be interesting to determine whether GRHL1 and 2 also stimulate endothelial cell migration. In addition to characterizing the grainyhead related proteins, it will be of major importance to identify their downstream effectors in endothelial cells. Up to now only a few genes regulated by GRHL3 have been identified in the context of epidermal differentiation and skin barrier development [34]. Since GRHL3 is induced by TNFα in the mammary carcinoma cell line MCF-7 it is likely to play a role in tumor cell migration and metastasis which is presently under investigation. It is interesting to note that along with GRHL3 another prometastatic gene was recovered, namely BLC9-2, which has been to shown strongly stimulate epithelial-mesenchymal transition (EMT) [35].

In summary, by using a gene trap approach in MCF-7 breast cancer cells, we have identified two novel TNFα target genes with previously unknown functions, which are likely to have an impact on tumor progression by controlling growth, vascularization and metastasis formation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.070.

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Nuclear protein tyrosine phosphatase Shp-2 is one important negative regulator of nuclear export of telomerase reverse transcriptase

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Nuclear Protein Tyrosine Phosphatase Shp-2 Is One Important Negative Regulator of Nuclear Export of Telomerase Reverse Transcriptase*[S]

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Aging is one major risk factor for numerous diseases. The enzyme telomerase reverse transcriptase (TERT) plays an important role for aging and apoptosis. Previously, we demonstrated that inhibition of oxidative stress-induced Src kinase family-dependent nuclear export of TERT results in delayed replicative senescence and reduced apoptosis sensitivity. Therefore, the aim of this study was to investigate mechanisms inhibiting nuclear export of TERT. First, we demonstrated that H_2O_2-induced nuclear export of TERT was abolished in Src, Fyn, and Yes-deficient embryonic fibroblasts. Next, we wanted to identify one potential negative regulator of this export process. One candidate is the protein tyrosine phosphatase Shp-2 (Shp-2), which can counteract activities of the Src kinase family. Indeed, Shp-2 was evenly distributed between the nucleus and cytosol. Nuclear Shp-2 associates with TERT in endothelial cells and dissociates from TERT prior to its nuclear export. Overexpression of Shp-2 wt inhibited H_2O_2-induced export of TERT. Overexpression of the catalytically inactive, dominant negative Shp-2 mutant (Shp-2(C459S)) reduced endogenous as well as overexpressed nuclear TERT protein and telomerase activity, whereas it had no influence on TERT(Y707F). Binding of TERT(Y707F) to Shp-2 is reduced compared with TERTwt. Ablation of Shp-2 expression led only to an increased tyrosine phosphorylation of TERTwt, but not of TERT(Y707F). Moreover, reduced Shp-2 expression decreased nuclear telomerase activity, whereas nuclear telomerase activity was increased in Shp-2-overexpressing endothelial cells. In conclusion, Shp-2 retains TERT in the nucleus by regulating tyrosine 707 phosphorylation.

Telomeres are the physical ends of the chromosomes. They maintain chromosome stability, genetic integrity and cell viability in a variety of different species (1, 2). Telomeres can also function as a mitotic clock, because telomeres are progressively shortened during each cell division. The enzyme telomerase, with its catalytic subunit telomerase reverse transcriptase (TERT),[5] counteracts the shortening of telomeres. Introduction of TERT into human cells extended both their life-span and their telomeres to lengths typical of young cells (3–5). In addition to this well known function of TERT, functions independent of telomere shortening have been described. TERT has been shown to inhibit apoptosis (6, 7). Recently, it has been demonstrated that TERT is also localized in the mitochondria, but its function there is discussed controversially (8–10). TERT is regulated by transcriptional and post-translational mechanisms. Phosphorylation, binding proteins, and cellular localization have been described for post-translational modifications of TERT (11–15). TERT can be phosphorylated and its activity is regulated by kinases like c-Abl, PKC, ERK1/2, and Akt (16–20). We demonstrated that TERT is tyrosine-phosphorylated by the Src kinase family under conditions of oxidative stress (21, 22). Functionally, this results in nuclear export of TERT disrupting the anti-apoptotic and telomere-extending potential of the enzyme, which subsequently leads to enhanced apoptosis sensitivity and accelerated senescence of cells (21, 22).

One important regulator of the Src kinase family activity is the protein tyrosine phosphatase Shp-2 (Shp-2). The importance of Shp-2 for survival has been documented by the Shp-2 knockout mice which are embryonic lethal (23). All the described mechanisms for the regulatory mechanisms of Shp-2 are associated with its ability to dephosphorylate target molecules such as Src kinase family members, which are bound to growth factor receptors at the membrane to inhibit the permanent activation of the receptors by kinase phosphorylation (for review see Ref. 24). Recently, it has been demonstrated that Shp-2 is also localized in the nucleus, where it binds to the transcription factor STAT5a and thereby regulates its function (25). Because the regulation, which leads to nuclear export of TERT has to occur in the nucleus, we hypothesized that Shp-2 might counteract the nuclear export of TERT.

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[5] The abbreviations used are: TERT, telomerase reverse transcriptase; Shp-2, protein tyrosine phosphatase Shp-2; EC, human umbilical venous endothelial cells; HEK, human embryonic kidney cells; MEF, mouse embryonic fibroblasts; wt, wild type; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Our data demonstrate that nuclear export of TERT is triggered by Src and/or Yes. One negative regulator of this export is Shp-2. Shp-2 is localized in the nucleus, is associated with TERT and dissociates from TERT prior to its export. The catalytic activity of Shp-2 is crucial for retaining TERT in the nucleus.

EXPERIMENTAL PROCEDURES

Cell Culture—Endothelial cells (EC) were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamycin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10% fetal calf serum. After detachment with trypsin, cells were grown for at least 18 h (26, 27). Human embryonic kidney cells (HEK) were cultured in DMEM basal medium with 10% heat-inactivated fetal calf serum. Mouse embryonic fibroblasts deficient of Src, Fyn, and Yes and their wild-type counterparts were cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum and gentamycin.

Plasmids—Human Shp-2 was cloned out of endothelial cell-derived cDNA incorporating EcoRI and BamHI restriction sites. The amplified PCR product was subcloned into pcDNA3.1 (−) vector containing a Myc tag (Invitrogen) or into pGFP vector creating a Shp-2 GFP fusion. The catalytically inactive mutant of Shp-2 (Shp-2(C459S)) was generated by site-directed mutagenesis (Stratagene) out of Shp-2 wt. The hTERT construct was kindly donated by Dr. Weinberg (28). TERT was subcloned into pcDNA3.1(−)Myc-His vector (TERTwt) containing the Myc tag at the C terminus. TERT(Y707F) was generated by site-directed mutagenesis. ShRNA vectors were obtained from Sigma (Mission shRNA series).

Transfection—HEK were transiently transfected with Lipofectamine/Plus according to the manufacturer's protocol (Invitrogen) as previously described (29) with a transfection efficiency of 90 ± 4%. EC were transiently transfected with Superfect (Qiagen) as described previously. Mouse embryonic fibroblasts were transiently transfected with Lipofectamine/Plus according to the manufacturer's protocol (Invitrogen). Transfection of siRNA was performed by using INTERFERRin™ according to the manufacturer's protocol (POLYPLUS transfection). Sequence of scrambled siRNA: 5′-AACUUGAGAAUCGCCUGAA-3′, sequence of Shp-2 siRNA: 5′-GAAGCACAGUACCGAUUUA-3′.

Telomerase Enzyme Activity Measurement—Telomerase enzyme activity was measured using a commercially available PCR-based assay according to the manufacturer's protocol (Roche Applied Science) as previously described (30). In brief, after PCR amplification, PCR products were either used for detection of telomerase enzyme activity by 1) ELISA or by 2) telomerase-mediated DNA laddering. 1) For ELISA, PCR products are immobilized via the biotin-labeled TS primers (provided with the assay) to a streptavidin-coated microtiter plate. The linearity of the assay was assured by the positive controls provided by the company, and as negative controls heat (95 °C, 2 min) and RNase-treated samples were used in the presence of the biotinylated primers (*inset*, Fig. 5*b*).

Separation of Nuclear and Cytosolic Extracts—Nuclear and cytosolic extracts were separated using a commercially available kit according to the manufacturer's protocol (Pierce). In brief, cells were scraped off the dish and centrifuged at 800 × *g* for 5 min at 4 °C. The resulting pellet was resolved in cytosolic extraction reagent I (CERI buffer) and incubated for 10 min at 4 °C. After adding cytosolic extraction reagent II (CERII buffer) and further incubation for 1 min at 4 °C, samples were centrifuged at 16,000 × *g* for 5 min at 4 °C. The resulting supernatant contained the cytosolic fraction. The resulting pellet was washed with phosphate-buffered saline and resuspended in nuclear extraction reagent (NER buffer) and incubated for 60 min at 4 °C. After centrifugation for 15 min at 16,000 × *g* at 4 °C, the resulting supernatant was obtained as nuclear fraction. Purity of the nuclear and cytosolic extracts was always assured by immunoblotting with topoisomerase 1 (nuclear) and HSP70 (cytosolic).

Immunoprecipitation and Immunoblotting—Lysates (250 μg) were immunoprecipitated with 2.5 μg of Shp-2 antibody or 2.5 μg of Myc antibody overnight at 4 °C. After incubation with A- and G-Sepharose (Amersham Biosciences) for 2 h at 4 °C, resulting beads were washed, subjected to SDS-PAGE sample buffer, and resolved on a 10% SDS-PAGE.

Immunoblotting was performed with antibodies directed against TERT (1:200, overnight, 4 °C, Calbiochem or 1:500, overnight, 4 °C, Rockland), actin (1:8000, overnight 4 °C, Sigma) and Shp-2, Myc, Hsp70, Ref-1, topoisomerase I (2 h, 1:250, all Santa Cruz). Antibodies were detected by the enhanced chemiluminescence system (Amersham Biosciences). Semi-quantitative analyses were performed on scanned immunoblots using Scion Image 1.6 (Scion Corp.).

Immunostaining—Cells were fixed in 4% paraformaldehyde and permeabilized using 0.3% Triton X-100 and 3% bovine serum albumin in phosphate-buffered saline. For immunostaining, cells were incubated with an antibody against TERT and stained with an anti-rabbit Texas-Red-conjugated Fab fragment. Nuclei were counterstained with Sytox-Blue or DAPI (1:1000, 5 min, Molecular Probes). Cells were visualized with confocal microscopy (Zeiss, LSM 510 META, magnification 1:40 oil).

Statistics—Statistical analysis was performed with Student's *t* test or Wilcoxon, Mann-Whitney test using XLSTAT 2008.

RESULTS

Nuclear Export of TERT Depends on Src and Yes—Recently, we demonstrated that short term exposure to oxidative stress as well as aging-induced reactive oxygen species formation led to a Src kinase family-dependent nuclear export of TERT. Loss of nuclear TERT resulted in increased apoptosis sensitivity and accelerated senescence (21, 22). To reduce the number of candidates of the Src kinase family, which are responsible for oxidative stress-induced nuclear export of TERT, we used embryonic mouse fibroblasts deficient for Src, Fyn, and Yes and overexpressed TERTwt in these cells. Treatment with 500 μM H_2O_2 for 6 h resulted in a reduction of nuclear TERT protein and an increase in cytosolic TERT protein in wildtype fibroblasts. In contrast, in fibroblasts deficient for Src, Fyn, and Yes, nuclear export of

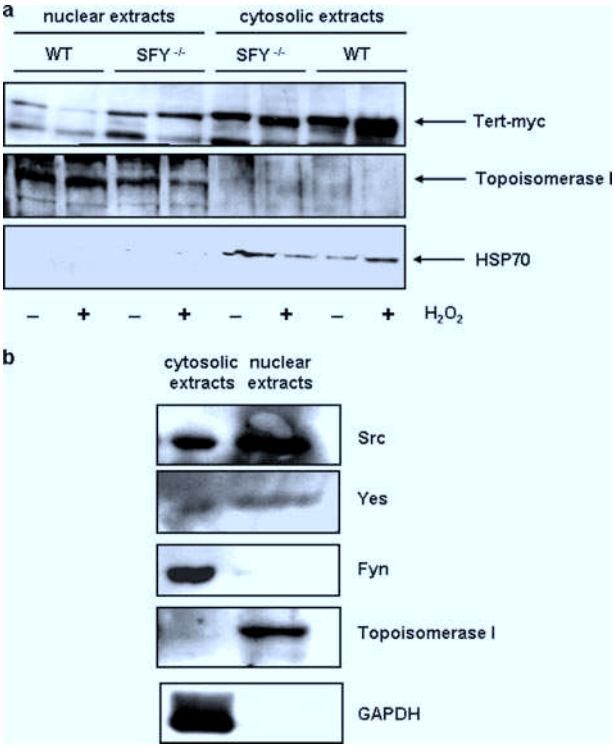

FIGURE 1. **Absence of Src, Fyn, and Yes completely abrogates nuclear export of TERT.** *a*, Src, Fyn, Yes-dependent nuclear export of TERT. Embryonic fibroblasts from mice deficient for Src, Fyn, and Yes ($SFY^{-/-}$) and from their wild-type counterparts (*WT*) were transfected with TERT-myc and incubated with 500 μM H_2O_2 for 6 h. Nuclear and cytosolic extracts were prepared as described under "Experimental Procedures" and used for immunoblotting; shown is a representative blot of three independent experiments. *Upper panel*, detection of TERT-myc with an anti-Myc antibody. The *middle* and *lower panel* show the purity of the nuclear and cytosolic extracts with the nuclear marker topoisomerase I and the cytosolic protein Hsp70. *b*, nuclear localization of Src and Yes. Nuclear and cytosolic extracts were prepared from EC and used for immunoblotting. The following proteins were detected with specific antibodies (from *top to bottom*): Src, Yes, Fyn, topoisomerase I (nuclear marker), and GAPDH (cytosolic marker).

TERT was completely abolished (Fig. 1*a*) demonstrating that these three kinases play a role in this process.

The functional relevance of these kinases in EC is substantiated by the fact that Src and Yes could be detected in nuclear extracts of them (Fig. 1*b*). Having demonstrated that Src and/or Yes are responsible for oxidative stress induced nuclear export of TERT, we next wanted to identify one of the counter players, which inhibits nuclear export of TERT. One known inhibitor of Src kinase family functions is Shp-2. However, to inhibit nuclear export of TERT, Shp-2, like Src and Yes must be localized in the nucleus. Therefore, we first investigated the cellular localization of Shp-2 in EC. We found that Shp-2 is evenly distributed between the nuclear and cytosolic extracts (Fig. 2*a*). Thus, we hypothesized that Shp-2 could be indeed the counter player for oxidative stress-induced nuclear export of TERT. Similar to endogenous Shp-2, overexpressed, myc- or GFP-tagged Shp-2 were also localized in the nucleus (Fig. 2*a* and supplemental Fig. S1).

If Shp-2 is involved in TERT nuclear export by oxidative stress, we hypothesized that Shp-2 itself must be regulated by H_2O_2 in EC. Indeed, incubation with 200 μM H_2O_2, which led to a nuclear export of TERT, resulted in a minor, but significant decrease of total Shp-2 protein and activity (Fig. 2, *b* and *c*).

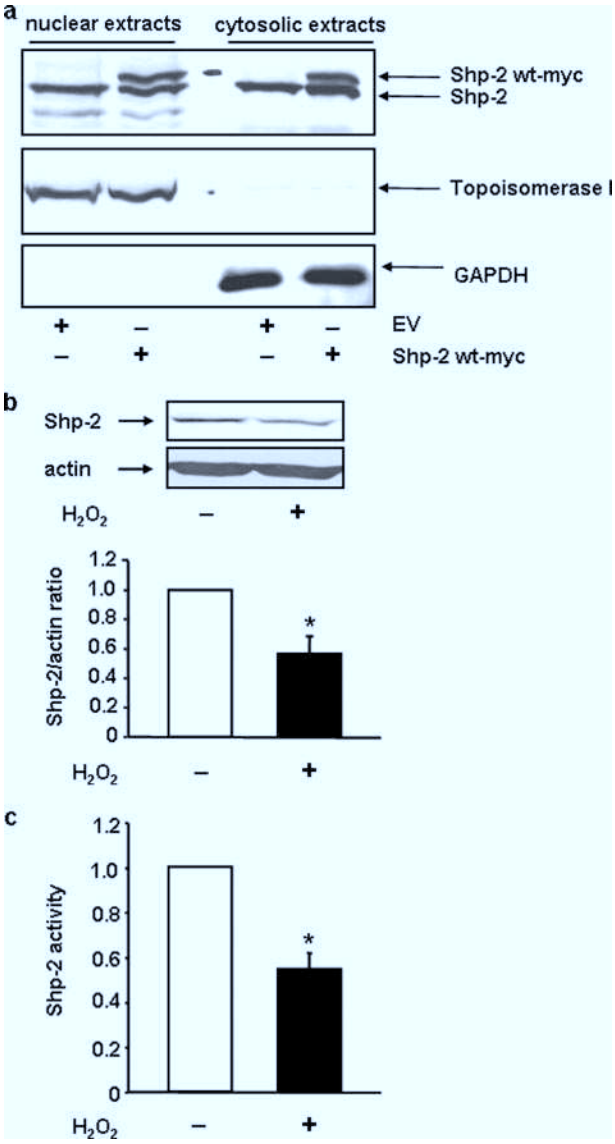

FIGURE 2. **Subcellular localization of Shp-2 and regulation by H_2O_2.** *a*, endogenous and overexpressed Shp-2 are evenly distributed between nucleus and cytoplasm in EC. Shp-2 wt-myc was overexpressed in EC. Nuclear and cytosolic extracts were prepared as described under "Experimental Procedures" and used for immunoblots. *Upper panel* shows an immunoblot with an anti-Shp-2 antibody, *middle* and *lower panels* demonstrate the purity of the extracts using antibodies against the nuclear marker topoisomerase I and the cytosolic marker GAPDH. One representative immunoblot is shown ($n = 6$). *EV*, empty vector; *b*, H_2O_2 reduces Shp-2 protein levels. *Upper panel*, EC were incubated with 200 μM H_2O_2 for 6 h and immunoblots were performed with an anti-Shp-2 antibody and anti-actin for normalization. *Lower panel*, semi-quantitative analysis of four independent experiments. Shown are the Shp-2 levels relative to actin, data are means ± S.E. (relative levels in untreated cells set to 1; *, $p < 0.05$). *c*, H_2O_2 reduces Shp-2 activity. EC were incubated with 200 μM H_2O_2 for 6 h, lysed, and Shp-2 activity was measured in the lysates. Data are means ± S.E. ($n = 6$) and are shown relative to Shp-2 activity in untreated cells (*, $p < 0.05$).

Shp-2 Associates with TERT and Retains It in the Nucleus—To get first insights whether Shp-2 could be a direct player in the regulation of TERT, we next determined whether TERT and Shp-2 associate with each other in the nucleus. Therefore, we co-immunoprecipitated endogenous Shp-2 and TERT-myc from nuclear extracts of TERT-myc-transfected cells. Indeed, the majority of TERT is associated with Shp-2 in the nucleus (Fig. 3). This association seems to be specific as we did not find

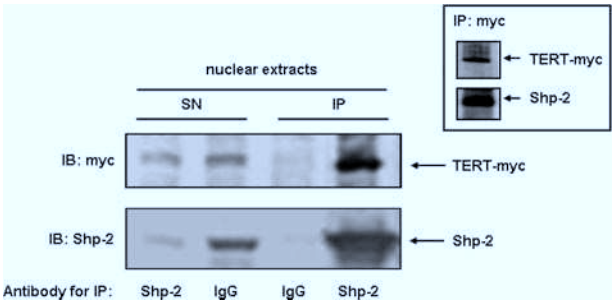

FIGURE 3. **Shp-2 associates with TERT.** HEK cells were transfected with TERT-myc and endogenous Shp-2 was immunoprecipitated from 250 μg of nuclear protein. Immunoblots with the precipitate (*IP*) and 25 μg of total protein from the supernatant (*SN*) were performed with an anti-Myc antibody (TERT-myc, *upper panel*) and anti-Shp-2 (*lower panel*). IgG served as negative control. The *inset* shows the reciprocal immunoprecipitation with an anti-Myc antibody.

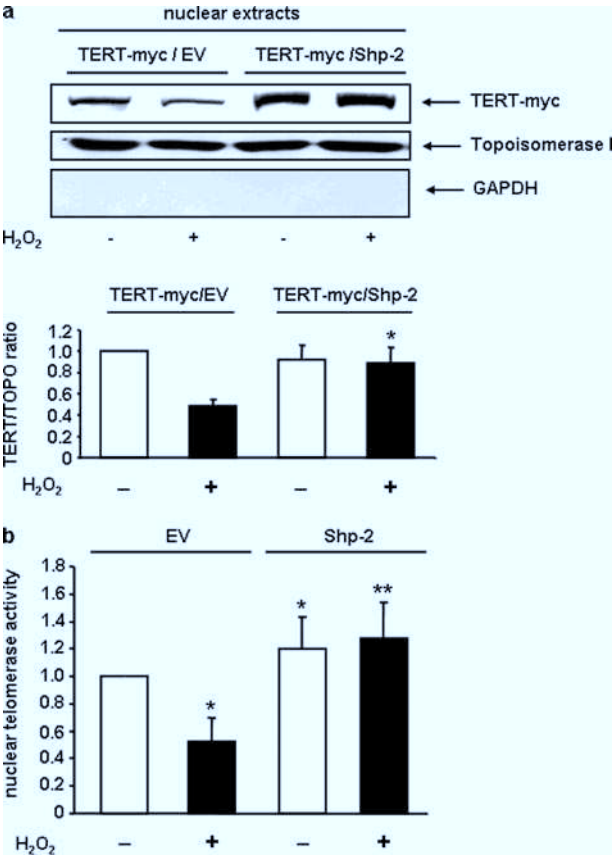

FIGURE 4. **Shp-2 wt rescues H_2O_2-induced loss of nuclear TERT protein and telomerase activity.** *a*, EC were transfected with either empty vector (*EV*) and TERT-myc or with Shp-2 and TERT-myc. After incubation with 200 μM H_2O_2, immunoblot against TERT-myc and topoisomerase I was performed in nuclear extracts. *a*, *upper panel* shows a representative immunoblot with an Myc antibody, the *upper middle panel* shows purity of nuclear fraction with an anti-topoisomerase I antibody and *upper lower panel* shows purity of nuclear fraction with an anti-GAPDH antibody ($n = 4$). *Lower panel* shows the semi-quantitative analysis of four independent experiments. TERT levels were normalized to topoisomerase I and are shown relative to the levels of cells transfected with EV/TERT-myc not treated with H_2O_2. Data are means ± S.E. (*, $p < 0.05$ *versus* TERT-myc/EV + H_2O_2). *b*, overexpression of Shp-2 rescued H_2O_2-induced reduction of nuclear telomerase activity. EC were transfected and treated as in *a*. Nuclear telomerase activity was measured as described under "Experimental Procedures" and is shown relative to the level in EV-transfected cells not treated with H_2O_2. Data are means ± S.E. ($n = 6$; *, $p < 0.05$ *versus* EV-H_2O_2. **, $p < 0.05$ *versus* EV + H_2O_2).

complexes between TERT-myc, Shp-2, and the transcription factor Ref-1 (supplemental Fig. S2). Having demonstrated that nuclear Shp-2 interacts with TERT, we next examined whether

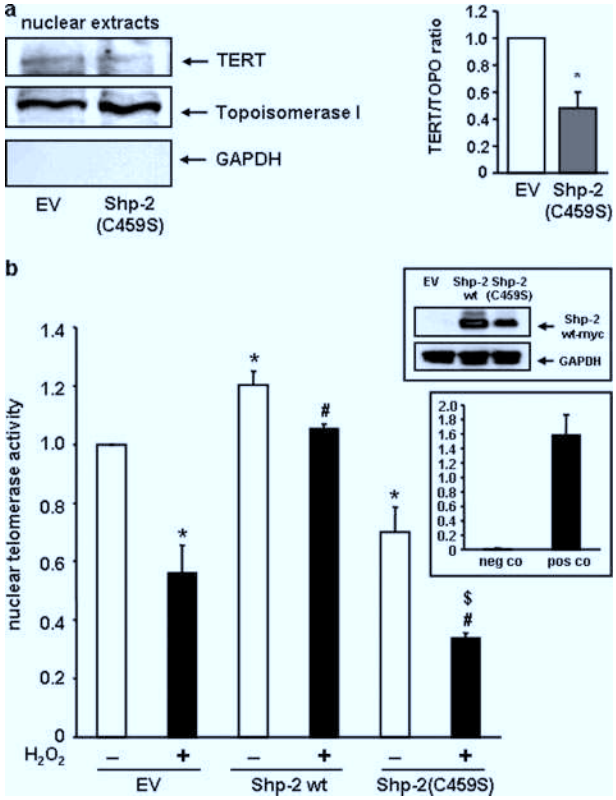

FIGURE 5. **Nuclear retention of TERT requires catalytically active Shp-2.** *a*, EC were transfected with empty vector (*EV*) or Shp-2(C459S). Immunoblot was performed with an anti-TERT antibody (*left panel*, *top*), an anti-topoisomerase I antibody (*left panel*, *middle*) and an anti-GAPDH antibody (*left panel*, *bottom*). TERT levels were determined by semi-quantitative analysis and normalized to topoisomerase I (*right panel*). Data are means ± S.E. of four independent experiments, the relative TERT levels in EV-transfected cells are set to 1 (*, $p < 0.05$). *b*, EC were transfected with empty vector (*EV*), Myc-tagged Shp-2 wt or Shp-2(C459S) and incubated with 200 μM H_2O_2 for 6 h. Nuclear telomerase activity was measured and is shown relative to the level in EV-transfected cells not treated with H_2O_2. Data are means ± S.E. ($n = 3$; *, $p < 0.05$ *versus* EV; #. $p < 0.05$ *versus* EV + H_2O_2; $, $p < 0.05$ *versus* Shp2(C459S)). *Lower inset* shows absolute values (A_{450}) of negative and positive controls; the measured absorbances of all samples were within the range of these controls. To assess the levels of both Shp-2 wt and Shp-2(C459S) immunoblot of whole cell lysates was performed with an anti-Myc antibody (*upper inset*, *top panel*), equal loading was confirmed using GAPDH (*upper inset*, *bottom panel*).

Shp-2 has an inhibitory effect on oxidative stress-induced nuclear export of TERT.

We overexpressed Shp-2 and TERT-myc in EC and incubated them for 6 h with 200 μM H_2O_2 to induce nuclear export of TERT. As shown in Fig. 4*a*, overexpression of Shp-2 completely abolished nuclear export of TERT. In line with this finding, overexpression of Shp-2 inhibited H_2O_2-induced reduction of endogenous, nuclear telomerase activity (Fig. 4*b*).

Nuclear Retention of TERT Requires Catalytically Active Shp-2—To determine whether the catalytic activity of Shp-2 is required for retaining TERT in the nucleus, we investigated the effects of the catalytically inactive, dominant negative Shp-2 mutant, Shp-2(C459S). First we verified that this mutant like the wild-type protein is evenly distributed between nucleus and cytoplasm (supplemental Fig. S3).

Overexpression of Shp-2(C459S) induced already under basal conditions reduction of nuclear TERT protein (Fig. 5*a*). In addition, we transfected EC with Shp-2 wt or Shp-2(C459S), incubated them with H_2O_2 and measured telomerase activity in

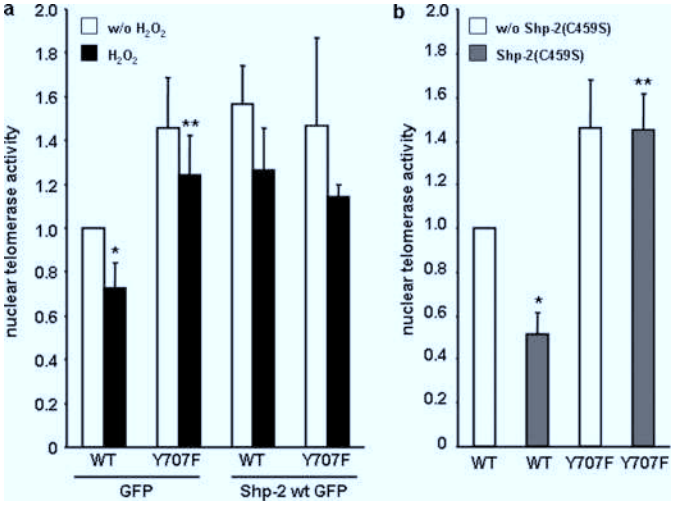

FIGURE 6. **Tyrosine 707 is a target for Shp-2.** *a*, EC were transfected with Myc-tagged TERT-wt (*WT*) or TERT(Y707F) (*Y707F*) together with GFP or Shp-2 wt GFP. After incubation with H_2O_2 nuclear extracts were prepared and nuclear telomerase activity was measured. Data are means ± S.E. ($n = 4$) relative to the values obtained with TERT-wt/GFP in untreated cells (*, $p < 0.05$ *versus* TERT wt/GFP w/o H_2O_2; **, $p < 0.05$ *versus* TERT wt/GFP with H_2O_2. *b*, EC were transfected with Myc-tagged TERT-wt (*WT*) or TERT Y707F (*Y707F*) together with GFP or Shp-2(C459S)-GFP. 24 h after transfection, nuclear extracts were prepared, and nuclear telomerase activity was measured. Data are means ± S.E. ($n = 4$) relative to the values obtained with TERT-wt/GFP (*, $p < 0.05$ *versus* TERT-wt/GFP; **, $p < 0.05$ *versus* TERT-wt/Shp2(C459S)-GFP).

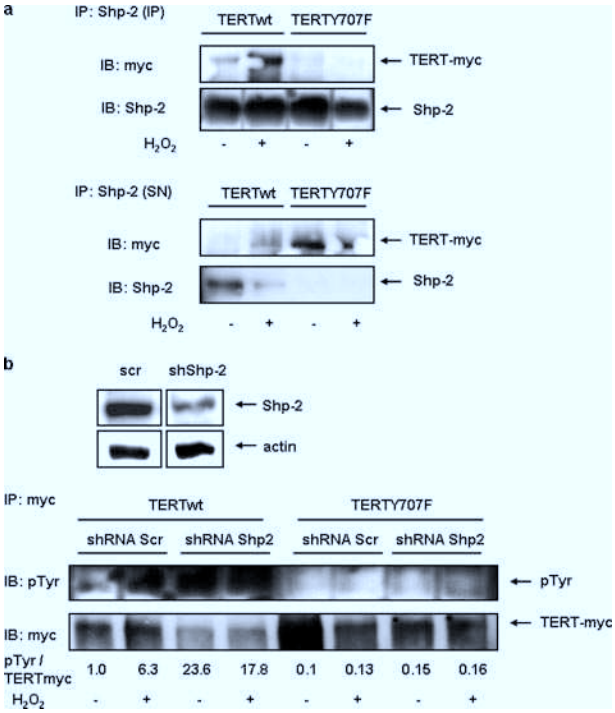

FIGURE 7. **Tyrosine phosphorylation of TERT depends on Shp-2.** *a*, HEK cells were transfected with Myc-tagged TERT-wt (*WT*) or TERT(Y707F) (*Y707F*), incubated with H_2O_2 for 1 h as indicated and immunoprecipitated with an anti-Shp-2-antibody from nuclear extracts. Immunoblots with the precipitates (*IP*) and the supernatants of the IP (*SN*) were performed with an anti-Myc antibody (*upper panel*) or an anti-Shp-2 (*lower panel*). *b*, HEK cells were first transfected with a shRNA vector targeting Shp-2 (shShp-2) or a scrambled control (*scr*) and 24 h later with TERT-wt (*WT*) or TERT(Y707F) (*Y707F*). Another 24 h later, cells were treated with H_2O_2 for 1 h, and nuclear lysates used for immunoprecipitation (*IP*) with an anti-Myc antibody. Immunoblots with the precipitates were performed with an anti-phosphotyrosine antibody (*upper panel*) or an anti-Myc antibody (*lower panel*).

nuclear extracts. Shp-2 wt increased, whereas Shp-2(C459S) reduced endogenous, nuclear telomerase activity under basal conditions. This effect seems to be specific for Shp-2, because overexpression of another phosphatase, namely the protein phosphatase 2a (PP2A) did not alter nuclear TERT protein and telomerase activity (supplemental Fig. S4). Shp-2 wt completely abrogated the effect of H_2O_2 on endogenous, nuclear telomerase activity, which is in accordance with our data presented in Fig. 4*a*. Interestingly Shp-2(C459S)-overexpressing cells showed a stronger effect on endogenous, nuclear telomerase activity compared with empty vector transfected cells under H_2O_2 treatment (Fig. 5*b*). These data clearly demonstrate that nuclear retention of TERT depends on the presence of Shp-2 protein and its activity. This is in accordance with the findings shown in Fig. 2 that H_2O_2 treatment reduces total Shp-2 protein and activity, providing one possible explanation for the H_2O_2-induced nuclear export of TERT.

We previously reported that TERT(Y707F) is retained in the nucleus under H_2O_2 treatment, suggesting that this tyrosine is crucial for nuclear export (21). The involvement of Shp-2 in this process suggests that phosphorylation/dephosphorylation regulates subcellular TERT localization. As expected, Shp-2 wt did not alter nuclear telomerase activity in TERT(Y707F)-overexpressing cells even after H_2O_2 treatment because this mutant cannot be phosphorylated at position 707 and thus does not require dephosphorylation for nuclear retention (Fig. 6*a* and Ref. 21). Moreover, under basal conditions, overexpression of dominant negative Shp-2(C459S) reduced nuclear telomerase activity in TERT-wt transfected cells, whereas it had no influence on TERT(Y707F) (Fig. 6*b*), suggesting that tyrosine 707 in TERT is the target for Shp-2 phosphatase activity. Next, we investigated the influence of tyrosine 707 on the TERT Shp-2 association in the nucleus in the presence and absence of H_2O_2. Association of TERT(Y707F) to Shp-2 is markedly reduced compared with TERT-wt. This is also reflected in the supernatants of the immunoprecipitations where more unbound TERT(Y707F) is found (Fig. 7*a*). Finally, ablation of Shp-2 increased tyrosine phosphorylation of nuclear TERT-wt, whereas TERT(Y707F) was unaffected (Fig. 7*b*). Similarly, reduced Shp-2 expression decreased nuclear telomerase activity and TERT protein already under basal conditions (supplemental Fig. S5 and Fig. 7*b*).

DISCUSSION

The present study demonstrates that nuclear Shp-2 is associated with nuclear TERT and dissociates from TERT prior to its export, that Shp-2 inhibits nuclear export of TERT and that tyrosine 707 in TERT is a target for Shp-2.

Shp-2 was identified as a cytosolic SH2 domain containing tyrosine phosphatase, which is ubiquitously expressed. The wide distribution of the enzyme indicates that it might regulate various physiological functions. Homozygous Shp-2 knockout mice are embryonic lethal underscoring the importance of the enzyme. It is clear that Shp-2 plays a critical role in regulating signal transduction; however, a profound biochemical basis for the different functions of this phosphatase remains to be elucidated. Our data now add another important issue to the list of

unaddressed questions: What is the function for nuclear Shp-2? Chughtai *et al.* (25) reported that Shp-2 exists in a complex with the transcription factor Stat5a. The authors speculate that active Shp-2 and a tyrosine-phosphorylated Stat5a translocate to the nucleus and potentially function in the nucleus to regulate transcription as both Shp-2 and Stat5a as a complex bind DNA. Our data now provide evidence that Shp-2 is localized in the nucleus already under basal conditions, where it is associated with TERT. Thus, it is tempting to speculate that some of the physiological functions of Shp-2 have to be addressed to its nuclear localization. The question remains how Shp-2 is imported into the nucleus, because it lacks a nuclear localization sequence. Therefore, a binding protein for Shp-2 could be required to induce its nuclear import. A possible candidate is Gab1, which has a nuclear import sequence (31) and has been shown to bind to Shp-2 (32). Preliminary data from our laboratory indicate that overexpression of a nuclear localization deficient Gab1 mutant prevents nuclear import of Shp-2.[6]

Under oxidative stress, TERT is tyrosine-phosphorylated and thereby exported from the nucleus in a Src kinase family/CRM1-dependent manner (21). In this study we demonstrate that Src and Yes are also localized in the nucleus of EC and thereby could be responsible for tyrosine phosphorylation of TERT. Moreover, the Src kinase family has been implicated in aging processes. This is mainly due to the fact that reactive oxygen species, which accumulate with age and induce lipid peroxidation, protein modification, DNA strand breaks, and cause oxidative damage, enhance the activity of the Src kinase family, which results in a progressive loss of cell function, a hallmark for aging processes (22, 33–35). We provide functional evidence that Src, Fyn, and Yes could play a role in aging processes, because cells deficient in these kinases can retain TERT in the nucleus under oxidative stress.

It has to be assumed that the export of nuclear TERT is regulated. Here, we demonstrate that Shp-2 is associated with TERT in the nucleus under basal conditions. More importantly, endogenous Shp-2 dissociates from nuclear TERT prior to its export and down-regulation of endogenous Shp-2 expression reduced nuclear telomerase activity. Thus, one may speculate that Shp-2 protects TERT from nuclear export by complex formation with TERT. Our data also suggest that tyrosine 707 in TERT is a target for the phosphatase activity of Shp-2. Therefore, Shp-2 might indeed be the unknown counterplayer for oxidative stress-induced nuclear export of TERT. However, we still do not know whether it acts solely via dephosphorylation. However, our data presented here strongly suggested that tyrosine 707 in TERT is a target of Shp-2 because the phosphorylation status of TERT(Y707F) is unaffected by Shp-2 and more importantly tyrosine phosphorylation of TERT-wt is negatively regulated by Shp-2. As a functional consequence nuclear Shp-2 could protect the cell either against accelerated senescence or increased apoptosis sensitivity or even both.

To our knowledge Shp-2, or more precise nuclear Shp-2, has not been implicated in aging processes until now. It has only been suggested that Shp-1 is responsible for aging-related attenuation of EGF receptor signaling in dermal fibroblasts (36), which suggests that Shp-1 negatively contributes to aging processes. This is in line with findings that Shp-1 plays a negative role in transducing signals for cellular responses (24). Previous biochemical evidence has shown that the enzymatic activity of Shp-2 is required for its function in signal transduction (37, 38). Replacing cysteine 459 with serine completely abolished its enzymatic activity. Binding of this mutant to other proteins via its SH2 domains remained unaltered. However, introduction of this mutant markedly inhibited the activation of MAP kinases in response to EGF and insulin (37, 39). This is in line with our data presented here that Shp-2(C459S) has also a nuclear localization (data not shown), but reduced nuclear telomerase activity and TERT protein already under basal conditions.

In summary, our study demonstrates a new important function for nuclear Shp-2 in retaining TERT in the nucleus. In addition, tyrosine 707 is a target for Shp-2. It counteracts the Src and Yes effects on TERT protein and telomerase activity in the nucleus under conditions of oxidative stress, most likely by regulating the net phosphorylation status of tyrosine 707 in TERT. This suggests that keeping Shp-2 in the nucleus delays aging processes and inhibits apoptosis.

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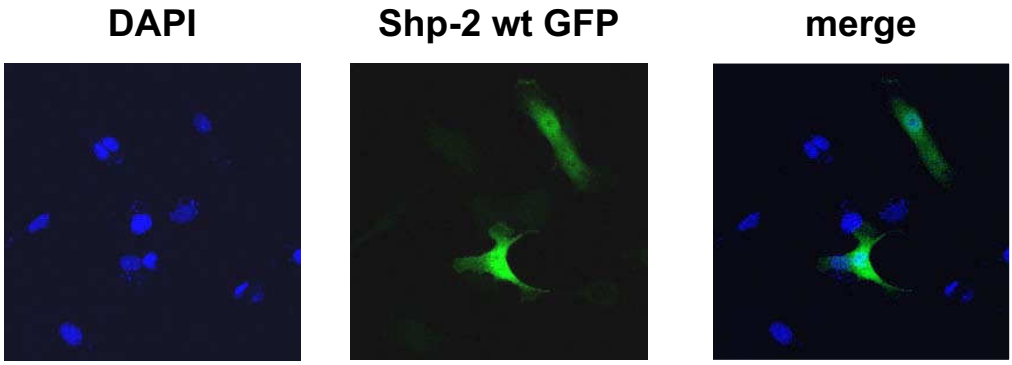
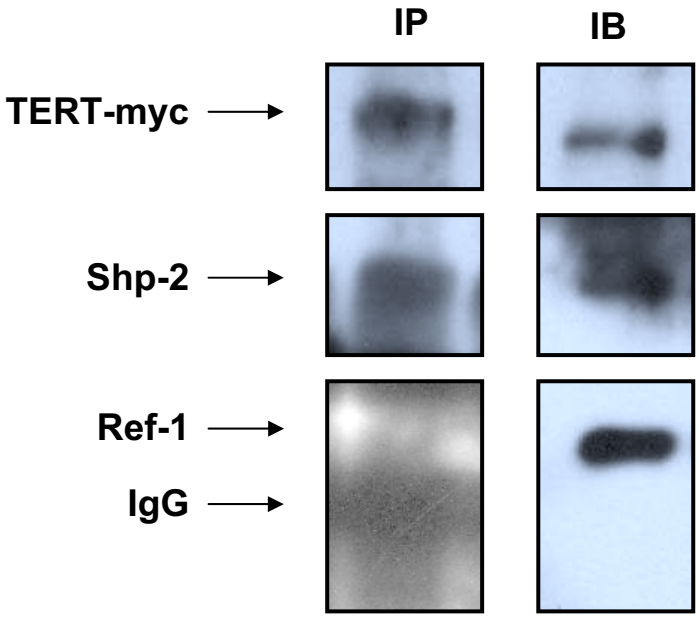
supplementary figure 1: Jakob et al.



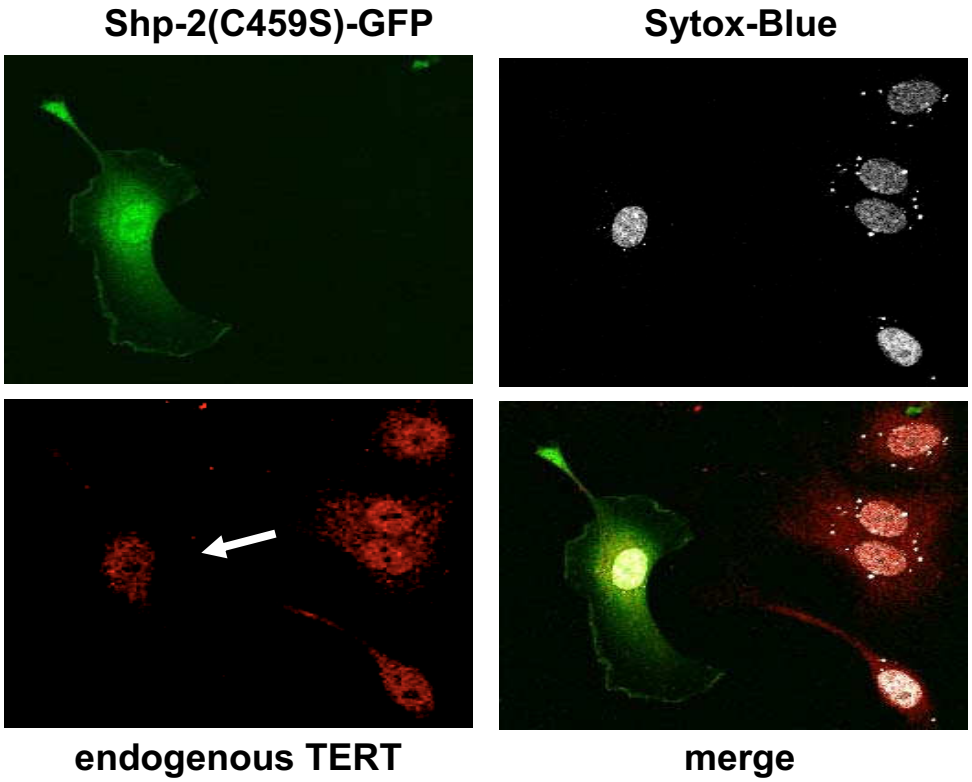
Shp-2 localization in endothelial cells. Shp-2 wt-GFP was overexpressed in EC and detected by fluorescence microscopy. Left panel: nuclear staining with DAPI, middle panel: Shp-2 wt GFP, right panel: merge. Transfected cells show an even distribution of Shp-2 wt-GFP between nucleus and cytoplasm.

supplementary figure 2: Jakob et al.



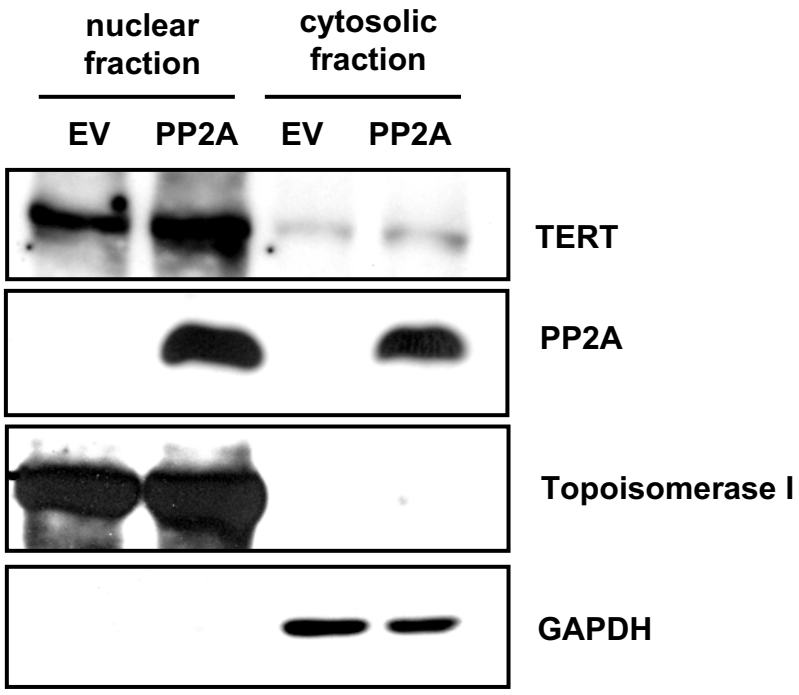
The nuclear transcription factor Ref-1 does not associate with TERT-myc and endogenous Shp-2. HEK cells were transfected with TERT-myc, lysed and the lysates used for immunoprecipitations (IP) with an anti myc-antibody. Immunoblots with the precipitates (left panels) and 25 µg of total protein (IB) (right panels) were performed with an anti-myc antibody (TERT-myc, upper panels), anti-Shp-2 (middle panels) and anti-Ref-1 (lower panels). IgG denotes the antibody used for immunoprecipitation.

supplementary figure 3: Jakob et al.



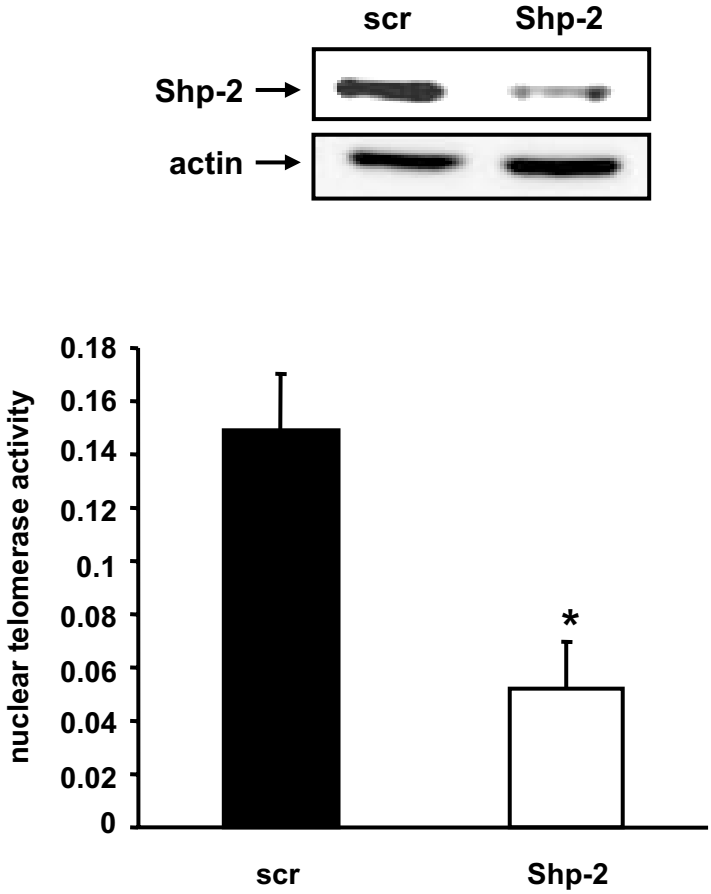
Dominant negative Shp-2 reduces nuclear TERT protein. Endothelial cells were transfected with Shp-2(C459S)-GFP and analyzed by fluorescence microscopy. Endogenous TERT was detected by immunostaining using a Texas Red coupled secondary antibody. Top left panel: Shp-2(C459S)-GFP, top right panel: nuclear staining with Sytox Blue, bottom left panel: endogenous TERT, bottom right panel: merge. In Shp-2(C459S)-GFP transfected cells (white arrow) the nuclear staining of TERT is reduced in comparison to non-transfected cells.

supplementary figure 4: Jakob et al.



PP2A did not alter endogenous nuclear TERT protein. HEK cells were transfected with empty vector (EV) or PP2A. Immunoblots of lysates from nuclear and cytosolic extracts were performed with an anti-TERT antibody (upper panel) and an anti-PP2A antibody (lower upper panel). Anti-topoisomerase I antibody (upper lower panel) was used as nuclear marker and anti-GAPDH antibody as cytosolic marker (lower panel).

supplementary figure 5: Jakob et al.



Downregulation of Shp-2 reduces nuclear telomerase activity. HUVEC were transfected with Shp-2 siRNA (Shp-2) and scrambled siRNA (scr). Upper panel verifies downregulation of Shp-2. Middle panel shows equal loading using an anit-actin antibody. Nuclear telomerase activity was measured as described under experimental procedures. Data are means +/- SEM (n=3, *p< 0.05 vs scr, n=3) (lower panel).

Nuclear redox signaling

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COMPREHENSIVE INVITED REVIEW

Nuclear Redox Signaling

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Abstract

Reactive oxygen species have been described to modulate proteins within the cell, a process called redox regulation. However, the importance of compartment-specific redox regulation has been neglected for a long time. In the early 1980s and 1990s, many *in vitro* studies introduced the possibility that nuclear redox signaling exists. However, the functional relevance for that has been greatly disregarded. Recently, it has become evident that nuclear redox signaling is indeed one important signaling mechanism regulating a variety of cellular functions. Transcription factors, and even kinases and phosphatases, have been described to be redox regulated in the nucleus. This review describes several of these proteins in closer detail and explains their functions resulting from nuclear localization and redox regulation. Moreover, the redox state of the nucleus and several important nuclear redox regulators [Thioredoxin-1 (Trx-1), Glutaredoxins (Grxs), Peroxiredoxins (Prxs), and APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1)] are introduced more precisely, and their necessity for regulation of transcription factors is emphasized. *Antioxid. Redox Signal.* 12, 713–742.

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I. Introduction

Oxygen is one of the most relevant molecules for all aerobic organisms. For many years, it has been clear that aerobic organisms produce reactive oxygen species (ROS) from oxygen. The molecules, which belong to the ROS, are superoxide anion $O_2^{-\bullet}$, hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2). Even under physiologic conditions, all of these molecules are produced within cells.

Several oxidative systems exist that generate $O_2^{-\bullet}$ from oxygen. Potential sources of this ROS production are, for example, the NADPH oxidases, the respiratory chain within the mitochondria, and the xanthine/xanthine oxidase system (Fig. 1A). Conversely, a pool of several antioxidative enzymes scavenge, reduce, or inactivate ROS (Fig. 1A). Specifically, $O_2^{-\bullet}$ is reduced by superoxide dismutases (SODs) to H_2O_2. H_2O_2 is then metabolized to H_2O by several enzymes, mainly by catalase, but also by glutathione peroxidase (GPx), which in turn results in oxidation of glutathione. The glutathione disulfide (GSSG) itself is then reduced to glutathione by the glutathione oxidoreductase (GR) to feed back into this cycle (Fig. 2). Another important antioxidative system, the thioredoxin/thioredoxin-reductase (Trx/TR) also has been described to metabolize H_2O_2 directly. However, this seems to be a rather rare event in cells, and the Trx/TR system mainly reduces oxidized proteins. In the course of this process, reduced Trx itself is oxidized. Reduced Trx is then regenerated by TR by using the cofactor NADPH to be further available for the reduction of oxidized proteins (Fig. 3).

Therefore, a controlled redox balance exists in cells. Perturbation of this balance either by increased production of ROS or by reduced antioxidative capacity will result in so-called oxidative stress (Fig. 1B). Increased ROS lead to modifications of biological molecules, including proteins, DNA, and lipids. In line with this, under conditions of oxidative stress, $O_2^{-\bullet}$ can directly react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), which readily crosses cell membranes. Although possessing a short half-life, $ONOO^-$ interacts with target molecules even in neighboring cells. Peroxynitrite is involved in protein nitration by nitrating tyrosine residues irreversibly to form 3-nitrotyrosine (95). The interplay of $ONOO^-$ with ROS to damage cells is then termed nitrosative stress. $ONOO^-$ itself is extremely toxic to cells because it is readily converted to two other radical species, $OH^{\bullet}$ and $NO_2^{\bullet}$, by hemolytic decomposition (92). Therefore, these species are often referred to as ROS/RNS (reactive nitrogen species). Oxidative and nitrosative stress–induced modifications of biological molecules have been implicated in a variety of diseases, such as cardiovascular diseases, neurologic disorders, and cancer. Over a long period, it was believed that the production of ROS is, in principle, bad for cells, and therefore, the term "redox signaling" was underestimated. However, several lines of evidence have established that redox signaling exists and is required for organisms to survive. Moreover, ROS have a wide range of action because of their ability to interact with almost all biological molecules. The mode of action of ROS is also dependent on the localization of their target molecules. Thus, the modifications induced by ROS can be separated depending on the cellular compartments they affect. Several studies investigated whether mitochondrial and nuclear redox signaling exist, in addition to the well-described cytosolic (previously termed cellular) redox-dependent events. Under physiologic conditions, ROS can induce changes in gene expression (203), whereas under conditions of oxidative and nitrosative stress, ROS and RNS can directly damage DNA in the nucleus, which can result in apoptosis or malignant transformation (140, 233).

This review focuses on nuclear redox signaling and oxidative stress–induced nuclear translocation of proteins and their resultant nuclear modes of actions. The antioxidative enzymes, Trx-1 and the APEX nuclease (multifunctional DNA repair enzyme) 1 (APEX1, also known as Ape/Ref-1), and their important role in nuclear redox signaling are highlighted. A number of proteins, which belong to the classes of transcription factors, chromatin-modifying enzymes, kinases, and phosphatases, are discussed in detail. All of the mentioned proteins have in common that they have a nuclear localization and function and are redox regulated or regulated by oxidative stress in higher eukaryotes. Although this review tries to be as comprehensive as possible, we are aware that we might not have discussed all of them.

II. Nuclear Redox Regulators

A. Redox state of the nucleus

The term redox state describes the balance between the oxidized and reduced forms of biologically relevant redox pairs, including $NADH/NAD^+$, $NADPH/NADP^+$, and GSH/GSSG. It is reflected in the balance of several sets of

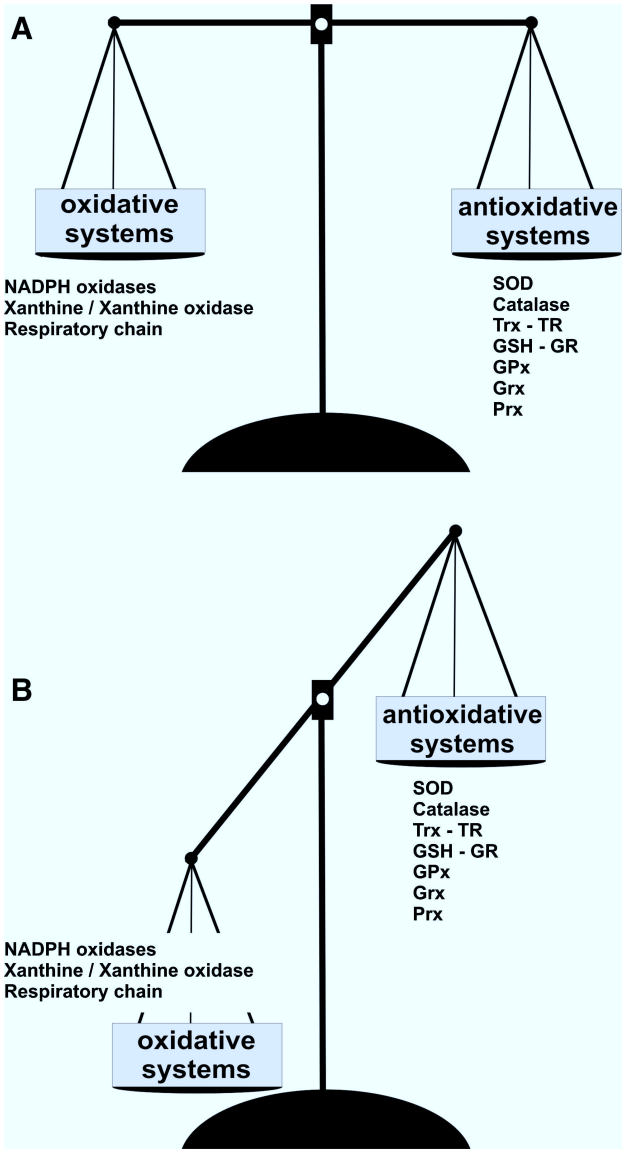

FIG. 1. Balance between oxidative and antioxidative systems. (**A**) The generation of ROS by oxidative systems (*e.g.*, NADPH oxidase, xanthine/xanthine oxidase, and the respiratory chain) is controlled by the activity of antioxidant systems, like superoxide dismutase (SOD), catalase, thioredoxin/thioredoxin reductase (Trx/TR), glutathione/glutathione reductase (GSH/GR), glutathione peroxidase (GPx), glutaredoxin (Grx), and peroxiredoxin (Prx). They scavenge ROS or reduce their levels and thus maintain the redox balance in healthy cells and tissues. (**B**) A state of oxidative stress occurs either by increased activity of the oxidative systems or by reduced antioxidative capacity of the cells.

metabolites (*e.g.*, lactate and pyruvate) whose interconversion is dependent on these ratios. An abnormal redox state can develop in a variety of deleterious situations, such as hypoxia, shock, and sepsis. Determination of the relative concentrations of the components of these redox pairs showed that the GSH/GSSG and NADPH/$NADP^+$ ratios are >1 (234, 236), whereas the NADH/NAD^+ ratio is <1 (221, 261). Generally, the nucleus provides a reductive environment.

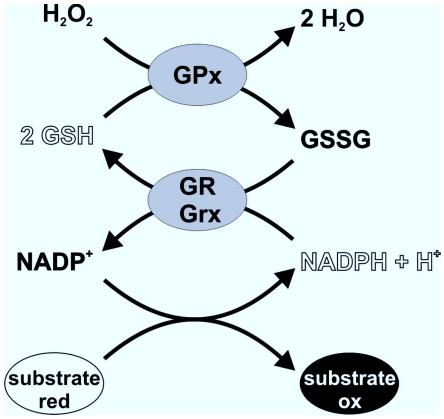

FIG. 2. The GSH/GR/GPx system: a general model of the GSH/GR/GPx system. Glutathione peroxidase (GPx) reduces H_2O_2 to H_2O and thereby oxidizes GSH to GSSG. GSSG itself is reduced by glutathione reductase (GR) to regenerate GSH. The reductant in this reaction is NADPH + H^+, created by metabolic substrate oxidation.

NAD^+/NADH is required as a coenzyme for metabolic processes. The high NAD^+/NADH ratio allows this coenzyme to act as both an oxidizing and a reducing agent. In contrast, the main function of $NADP^+$ is as a reducing agent in anabolism. Since NADPH is needed to drive redox reactions as a strong reducing agent, the NADPH/$NADP^+$ ratio is kept high. Because no barrier exists to diffusion of these dinucleotide coenzymes across the nuclear membrane (160, 261), it is assumed that the NAD^+/NADH and $NADP^+$/NADPH ratios are the same in the cytoplasm and the nucleus. The majority of these coenzymes are protein bound, and the concentration of free NADH in the nucleus has been calculated to be 130 n*M* (261). It is assumed that the NADPH/NADH ratio is ~4 (210); therefore, the concentration of free NADPH in the nucleus must be ~500 n*M*.

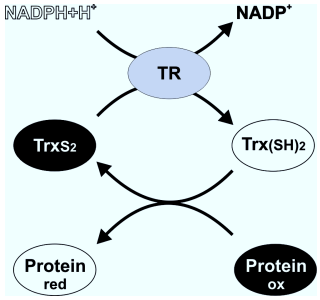

FIG. 3. The Trx/TR system: simplified model of the Trx/TR system and Trx function. The Trx/TR system consists of two oxidoreductase enzymes, thioredoxin (Trx) and thioredoxin reductase (TR). Reduced Trx ($Trx_{(SH)2}$) directly interacts with oxidized proteins by forming disulfide bridges. This involves formation of a mixed-disulfide intermediate in the thiol-disulfide exchange reaction (124). As a consequence of this two-step reaction, the protein is reduced, and Trx itself is oxidized (Trx_{S2}). The regeneration of reduced Trx from its oxidized form is catalyzed by TR by using NADPH + H^+.

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a ubiquitous thiol tripeptide and is the most abundant thiol present inside the cell. GSH has multiple direct and indirect functions in many critical cellular processes like synthesis of proteins and DNA, amino acid transport, enzyme activity, and metabolism (149). GSH also serves as a reductant to destroy free radicals, hydrogen peroxide, and other peroxides and as a storage form of cysteine. The redox state of the GSH/GSSG couple is often used as an indicator of the overall redox environment of the cell (200). GSH is found in a fairly high concentration of ~10 m*M* within cells. As it can freely diffuse (160, 172), the cytoplasmic and nuclear concentrations are similar (215, 236). Interestingly, the nuclear levels change during the cell cycle, with the highest levels found in the S and G_2/M phases (137). In line with these findings, depletion of GSH leads to reduced proliferation and apoptosis (138, 151). Taken together, one would hypothesize that during the G_2/M phase, a more reductive nuclear environment is required for cell proliferation to proceed. This was corroborated by a combined *in vitro*/bioinformatic investigation, which showed that 69 proteins containing redox-sensitive motifs have functions in central cell cycle processes like transcription, nucleotide metabolism, (de)phosphorylation, and (de)ubiquitinylation. The majority of these oxidant-sensitive proteins function during the G_2/M phase, indicating that oxidant-sensitive proteins may be temporally regulated by oscillation of the intracellular redox environment (39).

B. Antioxidative enzymes in the nucleus

As mentioned earlier, this review focuses on nuclear redox signaling. Therefore, it is important to introduce the antioxidative systems that have been described to be localized in the nucleus. Already in the late 1980s, Cu/Zn SOD and catalase were detected in the nuclear fractions of the developing rat cerebral cortex (43). In contrast, in adult Langerhans cells in catalase-overexpressing mice, catalase could not be detected in the nucleus (34). These findings point out that the presence of catalase in the nucleus seems to be dependent on cell type and perhaps also on age.

Several studies investigated the nuclear localization and activity of enzymes of the glutathione system, including GR, GPx, and glutathione *S*-transferases (GSTs). In rat liver cells, all proteins were found to be localized in the nucleus. Moreover, enzymatic activity could be measured in nuclear extracts (191). Recently, the exact localization of GSTα-a was determined to be at the nuclear membrane. Thus, the authors speculated that GSTα-a probably has a role as a defense barrier at the nuclear envelope (220). Several lines of evidence demonstrated that Trx-1 is localized in the nucleus, and its major nuclear function seems to be the binding to and reduction of transcription factors, thereby modulating their activities. Other thiol reductases, namely nucleoredoxin and glutaredoxin (Grx), which have similar, but also distinct functions from those of Trx-1, have been reported to be localized in the nucleus (80). Another important enzyme this context is APEX1. It is known that the DNA base excision-repair pathway is responsible for the repair of alkylation and oxidative DNA damage. A crucial step in the base excision-repair pathway involves the cleavage of an apurinic/apyrimidinic (AP) site in DNA by AP endonucleases (248). The major AP endonuclease in mammalian cells is APEX1, a multifunctional enzyme that acts not only as an AP endonuclease but also as a redox-modifying factor for a variety of transcription factors. Moreover, several studies demonstrated that APEX1 and Trx-1 act in concert in regulating transcription factors, which is discussed in more detail later in this review.

The variety of antioxidative enzymes reported to be localized in the nucleus and to be present in their active forms underscores the existence of a nuclear redox signaling network. Several nuclear proteins will now be introduced whose activity and functions depend on the redox balance and on nuclear redox signaling.

C. Thioredoxin-1 (Trx-1)

One major redox regulator in cells besides the glutathione system is the thioredoxin system. Thioredoxin was first discovered by Peter Reichard and co-workers (118) in 1964 as an electron donor for ribonucleotide reductase from *Escherichia coli*. In 1968 Holmgren and co-workers (85) determined the amino acid sequence of thioredoxin from *E. coli* after isolation of the pure protein and showed the classic active site -Cys-Gly-Pro-Cys-. The crystal structure of *E. coli* Trx in its oxidized form resulted in the definition of the thioredoxin fold: a central β-sheet surrounded by α-helices with the active site at the end of a β-strand and in the beginning of an α-helix (88). This structure defines a large superfamily of proteins (124). It is adopted by bacterial glutaredoxins (Grx) and appears in the other members of the family as a substructure or domain (9, 50, 139). Besides Trxs and Grxs, protein disulfide isomerases, GSTs, GPxs, peroxiredoxins, and chloride intracellular channels (CLICs) are members of the Trx superfamily (12, 139, 202). Mammalian cells contain two Trxs, Trx-1 and Trx-2 (exclusively localized in mitochondria) and three TRs: cytosolic TR1, mitochondrial TR2, and the testis-specific thioredoxin glutathione reductase (TGR). In 1985, Holmgren (87) introduced Trx-1 as a small, ubiquitous protein with two redox-active cysteine residues in an exposed active center, having the same amino acid sequence as *E. coli* Trx -Cys-Gly-Pro-Cys- (Cys 32 and Cys 35 within Trx-1), which is essential for its redox-regulatory function. The thioredoxin-1 system consists of Trx-1 and TR1. The regeneration of reduced Trx-1 from its oxidized form is catalyzed by TR1 by using NADPH (162). One important function of Trx-1 is the reduction of oxidized proteins, which depends on cysteine 32 and cysteine 35. The functions of Trx-1 are dependent on its cellular localization. Cytosolic Trx-1 interacts with its active-site cysteines with several proteins by forming disulfide bridges and thereby inhibits apoptosis induction. Trx-1 associates with ASK1 and the vitamin D_3–upregulated protein 1 (Txnip, also named VDUP-1) (194, 258). Thereby, reduced Trx-1 protects cells from apoptosis through an inhibitory binding to ASK1, whereas this binding is lost when Trx-1 is oxidized (194). Similarly, binding of Trx-1 to Txnip completely abrogates the antiproliferative function of Txnip (204). It has been demonstrated that, under certain conditions, Trx-1 accumulates in the nucleus and directly or indirectly interacts with different transcription factors (203). In the case of direct association, Trx-1 reduces oxidized transcription factors and thereby allows them to interact with DNA. The capacity of Trx-1 to interact with several transcription factors and thereby alter their functions is discussed in more detail later in this review. In case of an indirect action on transcription factors,

Trx-1 binds to APEX1, and APEX1 itself reduces the oxidized transcription factors. APEX1 is a complex protein with several functions, including endonuclease activity, redox factor, and redox chaperone properties. Because of its importance in nuclear redox signaling, APEX1 is introduced in more detail later in this review.

D. Glutaredoxins (Grxs) and peroxiredoxins (Prxs)

Glutaredoxins (Grxs) belong to the Trx superfamily of proteins. Grxs were first described as glutathione-dependent reductases three decades ago (86). They play an important role in cellular redox-dependent processes, mainly through deglutathionylation of proteins. In addition to the early-discovered dithiol Grxs, another group of Grxs has been identified. These monothiol Grxs lack the C-terminal active-site cysteine but contain all structural and functional elements to bind and use GSH. Therefore, two distinct mechanisms are known, the dithiol reaction in which the two cysteines in the active center take part in reducing both low-molecular-weight and protein disulfides and the monothiol mechanism in which only one cysteine in the active center is present (for review, see ref. 123). In humans, four Grx isoforms have been identified: the dithiol isoforms Grx1 and Grx2 and the monothiol isoforms Grx3 (PICOT/TXNL-2) and Grx5 (94, 131, 249, 250). Grx1 is considered mainly to be a cytosolic protein, although some studies show a nuclear localization (133, 219) and a presence in the intermembrane space of mitochondria (171). Several studies have shown that Grx1 is able to deglutathionylate a variety of proteins, like actin, protein tyrosine phosphatase-1B, glyceraldehyde-3-phosphate dehydrogenase, Ras, and caspase 3 (3, 19, 126, 173, 240) and thereby influences several important cellular functions, including actin polymerization, hypertrophy, and apoptosis (3, 173, 240, 242). Furthermore, Grx1 regulates transcription factor activity directly as well as indirectly, which is discussed later in this review in the respective transcription factor sections. Grx2 is known to exist in at least three different splicing variants, Grx2a, Grx2b, and the newly discovered Grx2c. Grx2a is considered a mitochondrial protein, and Grx2b was detected in nuclear fractions (62, 131). A recent screening of diverse tissues showed a ubiquitous expression of Grx2a, whereas Grx2b and Grx2c were found exclusively in testis and some cancer cell lines (129). Grx3, also termed PICOT (protein kinase C–interacting cousin of thioredoxin), was first identified in 2000 as an interaction partner of protein kinase C θ (PKCθ) and described to exist in the cytosol (250). Grx5 is a monothiol enzyme with a mitochondrial localization signal and therefore resides within the mitochondria (190). Thus, only Grx1 and Grx2b are localized in the nucleus, and regulation of transcription factor activity in the nucleus has been demonstrated only for Grx1.

Peroxiredoxins (Prxs) are members of a superfamily of Se-independent peroxidases. Six members have been identified and characterized in mammals. Prxs execute enzymatic degradation of H_2O_2 and organic hydroperoxides by using electrons donated by Trx-1 (Prx1 to Prx5), cyclophilin A (Prx1 to Prx4), or glutathione (Prx6). Prxs are divided into three classes: typical 2-Cys Prxs; atypical 2-Cys Prxs; and 1-Cys Prxs (for review, see refs. 102 and 187). These enzymes share the same basic catalytic mechanism, in which the single, redox-active cysteine is oxidized to a sulfenic acid by the peroxide substrate (38). The recycling of the sulfenic acid back to a thiol distinguishes the three enzyme classes: 2-Cys Prxs are reduced by thiols, particularly thioredoxin (188), whereas the 1-Cys enzymes are reduced by glutathione (136) and ascorbic acid (152). By using crystal structures, a detailed catalytic cycle has been derived for typical 2-Cys Prxs, including a model for the redox-regulated oligomeric state proposed to control enzyme activity (251). Prxs have a wide tissue distribution and specific subcellular localization. Nuclear localization has been demonstrated for Prx1, Prx2, Prx4, Prx5, and Prx6, at least in tumor cells (109). However, only for Prx5 has a nuclear function been demonstrated. Nuclear Prx5 significantly reduced nuclear DNA damage induced by H_2O_2 (15).

E. APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1)

APEX1 is a multifunctional protein. Its first abbreviation, human apurinic/apyrimidinic (AP) endonuclease, or Ape1 (also called HAP1 or APEX) derived from one of its functions as an essential enzyme in the base-excision repair (BER) pathway. This pathway is responsible for repair of apurinic/apyrimidinic (AP) sites in DNA, which are a major end product of ROS damage. Unrepaired AP sites can halt mRNA and DNA synthesis or act as noncoding lesions resulting in the increased generation of DNA mutations (248). To initiate repair, APEX1 cleaves 5′ to the baseless site, which leads to generation of a 3′-hydroxyl group and an abasic deoxyribose-5-phosphate. Subsequently, DNA polymerase β and DNA ligase I are recruited to the abasic site to complete the repair process (223).

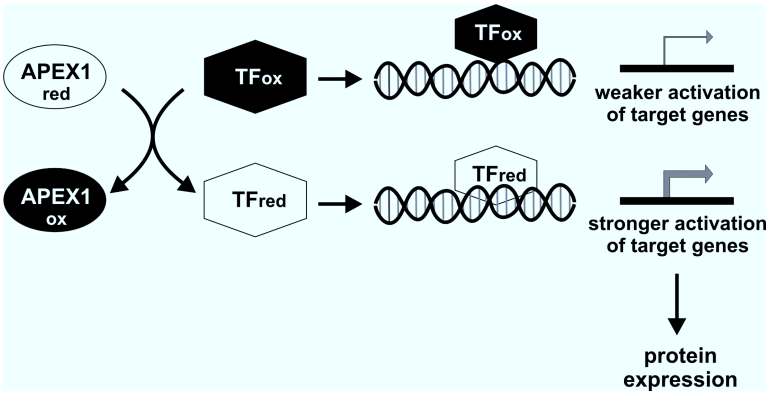

FIG. 4. APEX1. General model of APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1) redox regulator function. APEX1 converts a transcription factor (TF) from an oxidized, less DNA binding competent state to its reduced state that can bind more avidly to the regulatory regions of a variety of genes. This leads to activation of these downstream targets.

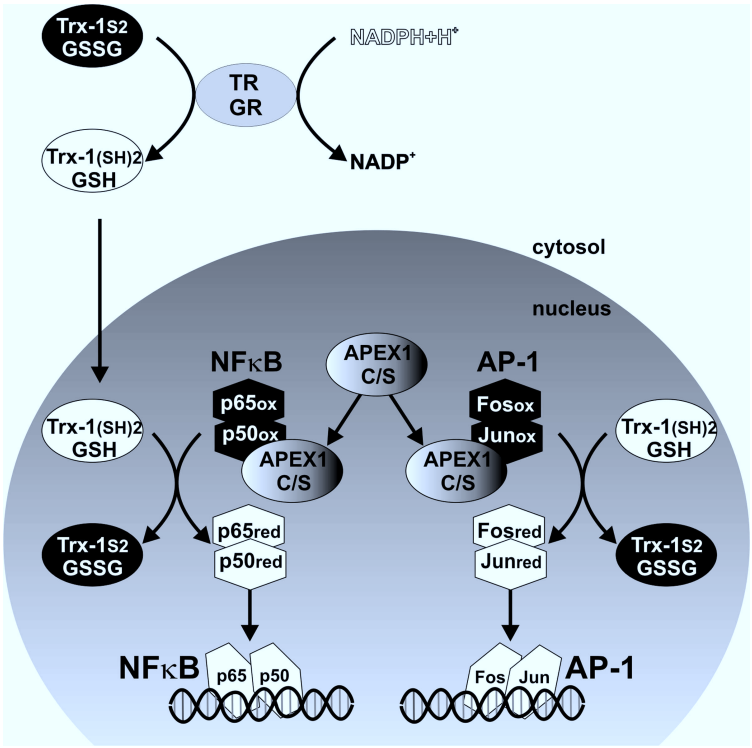

FIG. 5. APEX1 as a redox chaperone: model for the redox chaperone function of APEX1. A mutant of APEX nuclease (multifunctional DNA-repair enzyme) 1, in which all seven cysteine residues are replaced with serine (APEX C/S), can bind to the p50 and Jun subunits of the transcription factor complexes nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1), respectively. In this complex, APEX C/S can recruit thioredoxin-1 (Trx-1) or glutathione (GSH), which subsequently reduce both subunits of the heterodimeric transcription factors. Reduction enables them to bind their cognate response elements and activate transcription of their target genes.

The second abbreviation, Ref-1, stands for redox effector factor-1 and reflects its function as a redox regulator of transcription factors (Fig. 4). Through its redox function, APEX1 maintains transcription factors in an active, reduced state required for DNA binding and transcriptional activation. The two functions of APEX1, repair and redox regulation, are independent and located in separate domains of the protein, which was demonstrated by deletion analysis. The N-terminal portion of APEX1 that is not present in functionally related proteins from other organisms is required for the redox activity, whereas the DNA-repair activity requires conserved C-terminal sequences. Chemical alkylation or oxidation of cysteines inhibits the redox activity of APEX1 without affecting its DNA-repair activity (255). In addition, mutation of cysteine residue 65 (Cys 65), which is unique to mammalian APEX1, abrogates the redox function of the human protein. The wild-type zebrafish APEX has a threonine residue in the corresponding position (Thr 58) and is redox inactive, but can be converted to a redox enzyme by conversion of Thr 58 to cysteine (59). Conversely, when Cys 65 in human APEX1 is converted to alanine, the resulting protein is redox deficient (132).

Two reports uncovered a third function of APEX1, the stimulation of transcription factor reduction independent of its intrinsic redox activity. *In vivo* analysis of mouse APEX1 mutated at the cysteine previously identified as the redox catalytic site revealed a surprising result. Unlike APEX1-null mice, which die very early in embryonic development, homozygous APEX1(C64A) mice were viable with no overt phenotype. Although APEX1 is the major redox regulator of activator protein 1 (AP-1) in murine cells, AP-1 DNA binding activity and reduction of Fos and Jun were unaltered compared with wild-type mice, demonstrating that the redox activity of APEX1 is not required for redox regulation of AP-1 (169). A more-robust proof was obtained by the analysis of human APEX1, in which all seven cysteine residues were substituted to serine (APEX1 C/S; Fig. 5). It was demonstrated that APEX1 can directly reduce *in vitro* oxidized recombinant p50, a subunit of nuclear factor-kappa B (NF-κB), at relatively high concentrations (*i.e.*, at concentrations >50-fold higher than that of p50). However, when Trx-1 or GSH was included in the reaction, a much lower concentration of APEX1 was sufficient to stimulate p50 DNA binding activity, indicating that APEX1 might facilitate reduction of transcription factors by other reducing molecules such as Trx-1. Surprisingly, unlike the direct reduction of p50, this effect was not dependent on the redox activity of APEX1 because APEX1 C/S increased p50 DNA binding as efficiently as APEX1 wt in the presence of GSH or Trx-1. This activity of APEX1 was not restricted to the NF-κB transcription factor complex, as also the DNA binding activity of AP-1 was stimulated by APEX1 C/S. Interestingly, a physical interaction of APEX1 and the C/S mutant occurs only with one subunit of these heterodimeric transcription factors (with p50, p52, c-Rel, and c-Jun, but not with p65 and c-Fos) (8).

Thus, APEX1 binding to certain subunits of heteromeric transcription factors leads to reduction of the transcription factor subunits by other reductive systems. Therefore, APEX1 can function as a redox chaperone. This activity may be important for the modulation of the activity of a subset of transcription factors.

F. Trx-1/APEX1 interactions

A direct interaction between overexpressed APEX1 and Trx-1 has been demonstrated in the nucleus. Detailed analysis

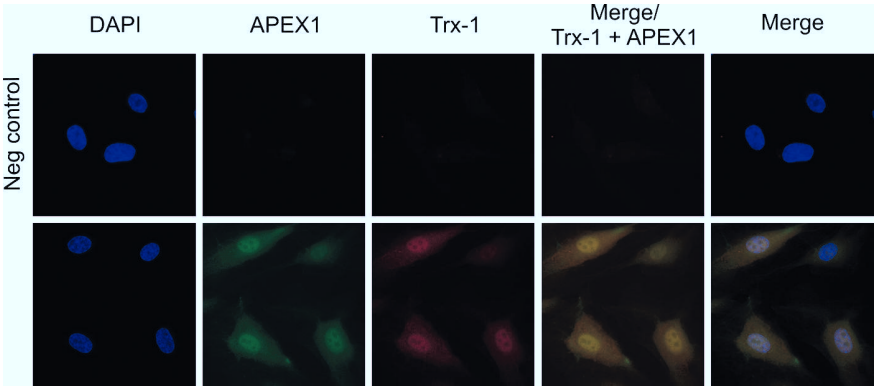

FIG. 6. Interaction of endogenous APEX1 and Trx-1 in human endothelial cells. Nuclear association of endogenous thioredoxin-1 (Trx-1) and APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1). Representative immunostainings of human umbilical vein endothelial cells are shown. Nuclear staining with DAPI is shown in blue, APEX1 staining in green, and Trx-1 staining in red. The second panel from the right shows the merging of APEX1 and Trx-1 staining; the rightmost panel, the merging of DAPI, APEX1, and Trx-1. Cells were fixed and permeabilized. For coimmunostaining, cells were first incubated with an antibody against human Trx-1 (mouse, 1:50, overnight, 4°C; BD Pharmingen); as a secondary antibody, anti-mouse rhodamine RedX (1:100, 30 min, 37°C; Invitrogen) was used. Because both the Trx-1 and the APEX1 antibodies are from the same host species, a blocking step with an excess of mouse IgG was performed. After that, the cells were incubated with an antibody against human APEX1 (mouse, 1:200, overnight, 4°C; Novus Biologicals) followed by a secondary anti-mouse Alexa 488 (1:200, 30 min, 37°C; Invitrogen). Nuclei were stained with DAPI (0.2 μg/ml). Cells were visualized with fluorescence microscopy (Zeiss Axiovert 100, magnification 1:40, oil). The top row represents the negative control without primary antibodies.

of the cysteines in Trx-1 in a yeast two-hybrid system revealed that cysteines 32 and 35 are required for direct interaction with APEX1 (79). Conversely, cysteines in APEX1 were not mapped for Trx-1 interaction. We found that colocalization of the two endogenous proteins, APEX1 and Trx-1, is detectable mainly in the nucleus in human endothelial cells (Fig. 6). Mapping of the interaction domain within APEX1 is under further investigation. It must be noted that, under certain conditions, Trx-1 and APEX1 act in concert to regulate transcriptional activation (8, 244), whereas in other cases, APEX1 or Trx-1 alone is sufficient to control DNA binding of transcription factors. This is discussed in closer detail for the redox-regulated transcription factors in the following sections of this review.

Table 1. Redox-regulated Transcription Factors

Transcription factor	*Critical amino acid*	*Affected function*	*Compartment of modification*
AP-1	Cys 269 (c-Fos) Cys 154 (c-Jun)	DNA binding (1)	Nucleus (79)
BPV E2	Cys 340	DNA binding (146)	n.d.
CBP/PEBP2	Cys 115, Cys 124	DNA binding (5)	n.d.
c-Myb	Cys 130	DNA binding (67, 155)	Nucleus (67)
CREB	Cys 300, Cys 310	DNA binding (64)	n.d.
Egr-1	n.d	DNA binding (91)	Nucleus (91)
Estrogen receptor	n.d. (DBD)	DNA binding (122)	n.d.
Glucocorticoid receptor	n.d. (DBD) Cys 481	DNA binding (93, 229) Nuclear import (167)	Nucleus (135) Cytoplasm (167)
HIF-1α	Cys 800	CBP interaction (51)	n.d.
HLF	Cys 28 Cys 844	DNA binding (116) CBP interaction (51)	n.d.
HoxB5	Cys-232	Cooperative DNA binding (58)	n.d.
MyoD	Cys 135	DNA binding (218)	n.d.
NFI/CTF	Cys 3 Cys 427	DNA binding (13) Transcriptional activation (154)	n.d.
NF-κB	Cys 62 (p50) Tyr 66, Tyr 152 (p65)	DNA binding (81, 142) Stability, nuclear retention (176)	Nucleus n.d.
NF-Y	Cys 85, Cys 89	DNA binding (156)	n.d
Nrf-2	Cys 506	DNA binding (26)	Nucleus (76)
p53	Cys 173, Cys 235, Cys 239	DNA binding (73, 185)	n.d.
Pax-5	n.d.	DNA binding (226, 227)	Nucleus (226, 227)
Pax-8	n.d.	DNA binding (103, 226)	Nucleus (226)
Sp1	n.d.	DNA binding (6, 7)	Nucleus (36)
TTF-1	Cys 87	DNA binding (11, 103, 225)	n.d.

III. Redox-Regulated Transcription Factors and Chromatin Modifiers

Transcription factors regulate cellular functions through altering the gene expression profile. A number of transcription factors have been shown to be redox regulated through modulation of their DNA binding capacity. Additional layers of regulation are on the level of transcriptional activation by changing cofactor interactions, oligomerization, or subcellular localization. Thereby cells can modulate their transcriptome to adjust to physiologic and pathophysiologic changes in ROS levels and exogenous noxae. Table 1 provides an overview over the mammalian transcriptional regulatory proteins for which a redox regulation has been shown. In the following sections, we review the molecules for which a more-detailed knowledge of their redox regulation is available.

A. Activator protein-1 (AP-1)

Activator protein-1 (AP-1) is one of the first mammalian transcription factors that were identified (119). AP-1 is not a single protein, but represents various homo- or heterodimers formed between the proteins of the basic region-leucine zipper (bZIP) family. They belong to the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, and Fra-2), Maf (c-Maf, MafB, MafA, MafG/F/K, and Nrl), and ATF (ATF2, ATF3/LRF1, B-ATF, JDP1, JDP2) subfamilies of the bZIP proteins. Their complexes bind to a canonical AP-1 site, originally described as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response element, or variants thereof. Recruitment of other transcription factors, coactivators, and chromatin-remodeling proteins generates a plethora of regulatory complexes with cell- and stimulus-specific transcriptional activities (for review, see ref. 35). Therefore, AP-1 is involved in a wide range of physiologic functions, including proliferation and survival, differentiation, growth, apoptosis, cell migration, transformation, and carcinogenesis. AP-1 itself is target of a variety of upstream kinases like c-Jun NH_2-terminal kinases (JNKs), extracellular regulated kinases (ERKs), and p38 mitogen-activated protein kinases (MAPKs) through phosphorylation of Jun and Fos proteins.

DNA binding of AP-1 is regulated by the redox state of a cysteine residue within the DNA binding domains of both proteins. Oxidation of Cys 154 in human Fos and Cys 269 in human c-Jun inhibits DNA binding of AP-1 *in vitro* (1). This was ascribed to an intermolecular disulfide bridge formed between the two cysteines. Oxidation of these residues could be achieved enzymatically, by using GPx, and DNA binding protected them from oxidation *in vitro* (16). In the viral homologue of *c-jun*, the transforming oncogene *v-jun*, the corresponding cysteine residue is replaced by a serine, which results in an insensitivity to oxidation, leading to constitutive DNA binding and aberrant transcriptional regulation. Similarly, Fos can escape regulation and is converted to a transforming protein when the critical cysteine is mutated (168). Shortly after uncovering the redox sensitivity of AP-1 DNA binding, a nuclear protein was identified that copurified with AP-1 and stimulated DNA binding of oxidized Fos-Jun heterodimers, Jun-Jun homodimers, and AP-1 complexes purified from HeLa cells (253, 254). This protein turned out to be APEX1. Besides a direct redox function, APEX1 can act as redox chaperone for AP-1, as discussed earlier.

B. Cyclic AMP response element–binding protein (CREB)

The transcription factor CREB binds the cAMP response element (CRE) and functions in glucose homeostasis, growth factor–dependent cell survival, and has been implicated in learning and memory. CREB is activated in response to, among other signals, cAMP. The accumulation of cAMP triggered by extracellular signals induces most cellular responses through protein kinase A (PKA). An increase in cellular cAMP levels liberates the catalytic from the regulatory subunits of PKA, which then translocate into the nucleus and induce cellular gene expression by phosphorylating CREB at serine residue 133. Together with the related activating transcription factor 1 (ATF1) and the CRE modulator (CREM), CREB comprises another family of bZIP transcription factors (for review of the CREB/ATF family, see refs. 145, 175, and 197). Originally, the Fos/Jun and ATF/CREB protein families were regarded as distinct sets of transcription factors that recognize closely related, but different DNA binding sites and form intrafamily dimers. However, it was shown that members of these two families can form selective cross-family heterodimers. These display DNA binding specificities distinguishable from each other and from their parental homodimers (21, 72). Analogously to AP-1, DNA binding of CREB is regulated by its oxidation status. The reduction of two cysteine residues (Cys 300 and Cys 310) located in the DNA binding domain, enhances the binding efficiency of CREB to DNA and regulates CRE-mediated gene expression. Substitution of these residues to serine renders CREB insensitive to reduction. These substitutions, which do not alter the secondary structure of the protein (189), enhance the binding of CREB to its cognate DNA sites under oxidative conditions and CREB dependent gene expression during normoxia (64). Interestingly, this redox regulation is bypassed by the Tax protein of the human T-cell leukemia virus type 1 (HTLV1), which recruits CREB independent of phosphorylation and redox status to the HTLV1 promoter (61, 64) to promote viral transcription.

C. Specificity protein 1 (Sp1)

Specificity protein 1 (Sp1) is one of the best-characterized zinc-finger transcription factors. It is a member of an extended family of DNA binding proteins, harboring three Cys_2-His_2 zinc-finger motifs, which bind to GC-rich DNA recognition elements. By regulating the expression of a large number of genes that have GC-rich promoters, Sp1-like transcription factors are involved in the regulation of many cellular processes, including proliferation, apoptosis, differentiation, and neoplastic transformation. Individual members of the Sp1 family can function as activators or repressors, depending on which promoter they bind and the co-regulators with which they interact (for review of the Sp1 family, see refs. 100 and 193).

A first hint that DNA binding by Sp1 is regulated by the cellular redox status came from the analysis of nuclear extracts from 30-month-old rat tissues, in which the DNA binding efficiency of Sp1 was greatly decreased, although the protein was present in levels comparable to those in younger tissues (6). This was attributed to increased levels of reactive oxygen intermediates in the aged animals because high concentrations of DTT, added to the aged tissue extracts, fully restored Sp1 DNA binding. Conversely, H_2O_2 treatment of extracts from young tissues strongly decreased the Sp1 DNA

binding activity, which again could be restored with DTT. The same results were obtained with purified Sp1, clearly demonstrating that oxidation directly affected Sp1 and not a cofactor (7). A similar phenomenon was observed during the transition of thymocytes from the resting to the proliferating state, in which production of ROS upon priming with phorbol 12-myristate 13-acetate (PMA) is nearly abolished. In the proliferating state, Sp1 DNA binding activity increased and could be compromised by the addition of H_2O_2 to extracts from these cells, whereas the binding activity in nuclear extracts from resting cells could be fully restored with DTT (199). This susceptibility to oxidation is conferred by thiol groups, depends on zinc coordination, and is prevented by DNA binding, suggesting that the DNA binding domain is the target for oxidation and is protected when in contact with DNA. This was corroborated *in vivo* by findings that arsenic treatment of promyelocytic leukemia cells prevented DNA binding of Sp1 to specific promoters. Of note, the nuclear levels of Sp1 did not change with arsenic treatment, suggesting that the oxidation occurs in the nucleus (36). In addition, Sp1 DNA binding was inversely correlated with the GSSG/GSH ratio added to nuclear extracts *in vitro* (110). Moreover, Trx-1 alone or in conjunction with the full thioredoxin system (Trx-1/TR and NADPH) was able to increase the DNA binding activity of recombinant Sp1 produced in *Escherichia coli* and of the protein from a mammalian cell line (27). Interestingly, not only DNA binding of Sp1 might be affected by oxidation, but also its transactivation properties; however, this phenomenon has not been investigated in detail (153).

D. Nuclear receptors

Nuclear receptors constitute to a large superfamily of ligand-activated transcription factors (for review, see ref. 147). These intracellular receptors are activated by lipophilic ligands and play crucial roles in development, differentiation, metabolic homeostasis, and reproduction. The prototypic glucocorticoid and estrogen receptor (ER) consist of an N-terminal transactivation domain (TAD), a central DNA binding domain (DBD) containing zinc-finger motifs, and a C-terminal ligand-binding (LBD) domain. In the cytosol, they are complexed with various chaperones of the heat-shock protein family. After steroid binding, glucocorticoid and estrogen receptors are released from these cytosolic complexes and translocate to the nucleus, where they interact as homodimers with their cognate DNA binding sites, the glucocorticoid response element (GRE), and estrogen response element (ERE), respectively, from where they activate transcription.

Early studies with biochemically purified glucocorticoid receptor showed that it changes its conformation under oxidizing conditions, when it seemingly can form intra- and intermolecular disulfide bonds. This conformational change was completely reversible with DTT, and only the reduced form of the receptor was capable of binding DNA (93, 212, 229), which was later confirmed in intact cells (52). A role for Trx-1 in this process was first suggested by experiments that showed that suppression of Trx-1 expression decreases glucocorticoid-inducible gene expression (134). Mammalian two-hybrid and pull-down assays finally demonstrated a direct interaction between the two proteins involving the DNA binding domain of the glucocorticoid receptor. Analysis of the subcellular localization demonstrated that this interaction most likely takes place in the nucleus under oxidative conditions (135). Besides DNA binding, the nuclear import of the glucocorticoid receptor also is under redox control, which affects a cysteine residue in its nuclear localization signal (167). Similar observations were made for the estrogen receptor, whose DNA binding activity is also sensitive to oxidation, which alters the conformation of the DNA binding domain (122). As for the glucocorticoid receptor, the transcription of endogenous and transfected synthetic ER target genes was shown to depend on Trx-1 when cells were placed under oxidative stress (78).

E. Nuclear factor-kappa B (NF-κB)

NF-κB is a collective name for inducible dimeric transcription factors composed of members of the Rel family of DNA binding proteins that recognize a common sequence motif, the κB site. NF-κB is found in essentially all cell types and is involved in activation of an exceptionally large number of genes in response to infections, inflammation, and other stressful situations requiring rapid reprogramming of gene expression. NF-κB was originally identified as a nuclear protein binding to the immunoglobulin kappa light-chain enhancer (208). Shortly thereafter, it was demonstrated by the same investigators that its DNA binding activity in pre-B cells can be induced by bacterial lipopolysaccharide (LPS) with a superinduction upon cycloheximide treatment (207), which then provided the first evidence that the activity of transcription factors can be regulated posttranslationally. Later it was shown that NF-κB represents a protein complex composed of hetero- or homodimeric combinations of five different members of the NF-κB/Rel family: NF-κB1 encoding p50 and p105, NF-κB2 encoding the p52 precursor p100, RelA or p65, RelB, and c-Rel. All subunits contain a conserved Rel-homology domain important for nuclear localization, dimerization, and DNA binding (for review see ref. 161). However, only the three Rel proteins contain transactivation domains required for the transcriptional activation of target genes. Therefore, and because each subunit has distinct biologic activities, different dimer combinations regulate specific sets of genes (for review, see ref. 157). This combinatorial mode of action results in diverse effects on cell fate and function.

In unstimulated cells, NF-κB is sequestered in an inactive form in the cytosol. It can be released from these cytosolic pools by two main pathways (for review, see ref. 201), resulting in nuclear translocation of NF-κB complexes. The canonic pathway, which is triggered by several proinflammatory cytokines, pathogen-associated molecules, and antigen receptors, depends on phosphorylation of the inhibitor of NF-κB (IκB) by an IκB kinase (IKK) complex consisting of the catalytic subunits IKKα, IKKβ, and a regulatory IKKγ subunit. Phosphorylated IκB is then subject to proteasomal degradation. This pathway leads primarily to the activation of p50/RelA and p50/c-Rel dimers. The noncanonic pathway engaged by various members of the tumor necrosis factor (TNF)-receptor family selectively requires IKKα activated by the upstream kinase NF-κB–inducing kinase (NIK). IKKα in turn phosphorylates p100, leading to proteasomal degradation of its C-terminus to generate p52, which then forms heterodimers predominantly with RelB. Besides these two major pathways, other mechanisms can release cytosolically

sequestered NF-κB components [*e.g.*, the phosphorylation of p105 by TPL-2, accelerating proteasome-mediated removal of the carboxy terminus yielding p50 (20)].

The observation that increased levels of the oxidoreductase Trx-1 are found in lymphocytes under conditions in which the transcription factor NF-κB is active [*e.g.*, after lymphocyte activation or in EBV- or HTLV-1–infected cells (238)], led to the suspicion that NF-κB might be under redox control. It could be shown that *in vitro* DNA binding activity of NF-κB is inhibited by agents modifying free sulfhydryls (141, 142, 230). Mutagenesis of conserved cysteine residues in the p50 subunit revealed that Cys 62 is critical for high-affinity binding to the κB motif. DNA binding activity of wild-type p50, but not a mutant, in which Cys 62 was exchanged to serine (C62S), was stimulated by Trx-1. Detection of disulfide cross-linked dimers in p50 wild type but not C62S suggested that Trx-1 stimulates DNA binding by reduction of a disulfide bond involving Cys 62 of the NF-κB subunit p50. Cotransfection of a plasmid expressing human Trx-1 and an NF-κB–dependent reporter construct demonstrated that Trx-1 also can regulate DNA binding and transcriptional activation by NF-κB in living cells (142). Interestingly, depending on its subcellular localization, Trx-1 can have opposing effects on NF-κB. In the cytosol, it interferes with signals to IKKs and thereby blocks the degradation of IκB, resulting in cytosolic retention of NF-κB, which prevents the activation of target genes. In contrast, nuclear Trx-1 has a positive effect on NF-κB transcriptional activity by enhancing its ability to bind to DNA (81). Molecular-modeling studies finally provided a clue to this dual role of Trx-1. Oxidized, disulfide-bridged NF-κB is more compact than the reduced form, which might facilitate its nuclear translocation. However, the inter-subunit disulfide blocks DNA from entering the active site of the oxidized dimer, explaining why reduction in the nucleus is essential for DNA binding and transcriptional activation to occur (32). Similar to Trx-1, Prx1 has the same dual role in the regulation of NF-κB activity (75). Besides disulfide cross-linked dimer formation, another oxidation product of NF-κB subunits has been detected. Both p50 and p65 can be glutathionylated, which leads to reduced NF-κB DNA binding and transactivation (181, 182). In the case of p65, glutathionylation was detected under hypoxic conditions, when the intracellular GSH levels were increased. The modification of p65 required Grx1, and the authors speculated that p65-SSG formation takes place in the cytosol and that modified p65 is still transported to the nucleus (182). However, as Grx1 can be detected in the nucleus, it also is possible that glutathionylation of this transcription factor subunit takes place there.

In addition to cysteine oxidation, the modification of specific tyrosine residues in p65 has been reported. Peroxynitrite inhibited NF-κB activity through nitration of p65 at Tyr 66 and Tyr 152, leading to p65 destabilization and nuclear export (176).

An interesting observation was made concerning the role of NF-κB redox regulation in the action of thalidomide, which causes severe malformations, especially of the extremities, in children when taken by the mothers during pregnancy. Thalidomide increases the production of free radicals and elicits oxidative stress. Oxidative stress, as marked by GSH depletion, occurs preferentially in limbs of thalidomide-sensitive rabbits. Activation of an NF-κB–dependent reporter gene is attenuated in limb bud cells of treated rabbits and can be restored by addition of *N*-acetylcysteine and a free radical spin-trapping agent (74). NF-κB is a key factor in limb development, because it regulates expression of *twist* and fibroblast growth factor 10 (*fgf10*), two genes necessary for proper limb outgrowth. Therefore, its oxidative inactivation triggered by thalidomide might explain the malformations occurring in children.

F. p53

The protein p53 is one of the best-characterized tumor suppressors and is constitutively expressed in nearly all cells and tissues. It functions as a tetrameric transcription factor found at very low levels in normal cells. Several genome-wide surveys have been undertaken to map genomic binding sites of p53 and thus to identify its target genes (83, 104, 213), yielding a plethora of genes potentially regulated by p53. Various kinds of cellular stress that alter normal cell cycle progression or induce mutations in the genome lead to stabilization and thus accumulation of the protein. Depending on the cell type and tissue and the extent of damage, p53 now either leads to cell cycle arrest to repair the lesions or forces the cell into apoptosis. Therefore, p53 has been termed "guardian of the genome" (117). Somatic mutations in the *p53* gene are critical events in a wide variety of malignancies, and *p53* is the gene most frequently mutated in human cancers. The majority of the mutations are missense mutations, and a hotspot is the region of the gene coding for the DNA binding domain (for review of *p53* and its mutations, see refs. 28, 84, 217, and 239).

Because of its prominent role in human cancers, the regulation of *p53* has been at the focus of intensive studies. With respect to redox regulation, it has to be noted that all 10 cysteine residues in p53 are within its DNA binding domain. This prompted very early studies on redox regulation. It was shown that oxidation of *in vitro* translated and recombinant, baculovirus-produced p53 disrupted its DNA binding, which was attributed to a change in conformation that could be reversed by reduction (44, 73). Interestingly, the redox state of p53 seemingly regulates only sequence-specific DNA binding and activation of p53-dependent genes. In contrast, no difference is found in the binding of oxidized p53 and reduced p53 to double-stranded nonspecific DNA (177).

That APEX1 can modulate the redox-dependent properties of p53 was demonstrated by the stimulation of sequence-specific DNA binding of oxidized p53 *in vitro* (97). The expression of reporter genes driven by p53-responsive promoters and endogenous p53 target genes, like *p21* and cyclin G, was equally stimulated on overexpression of APEX1 (57, 97). Importantly, downregulation of APEX1 caused a marked reduction in p53-dependent induction of p21 and diminished the transcriptional activation of *p21* and *Bax* by p53. In addition, the same authors demonstrated a physical interaction between APEX1 and p53, although only a small portion of both proteins was present in the same complex (57). Interestingly, APEX1 modulates p53 DNA binding, not only as a redox regulator, but also to facilitate formation of p53 tetramers, the most active form in terms of DNA binding (245), independent of its redox activity (77). This is substantiated by the fact that the cysteine residues, which are exclusively found in the DNA binding domain of p53 and are the targets for oxidation/reduction, have no influence on tetramerization

(185). Collectively, these data suggest that APEX1 stimulates p53 by both redox-dependent and -independent means and imply a key role for it in p53 regulation.

G. Nuclear factor I/CAAT transcription factor (NFI/CTF)

NFI/CTF was originally described as being required for the replication of adenovirus DNA (for review, see ref. 42). Later it was shown that NFI can regulate the transcription of a large number of cellular and viral genes. NFI represents a family of four genes in vertebrates (*NFI-A*, *NFI-B*, *NFI-C*, and *NFI-X*), which are expressed in overlapping patterns. The transcripts of all four genes can be spliced differentially, yielding distinct proteins. NFI proteins have been associated with changes in the growth state of cells and a number of malignancies (for review of NFI proteins, see ref. 65).

As for other transcription factors, it has been shown for NFI that its DNA binding activity is redox sensitive (164). A single cysteine residue (Cys 3) in the DNA binding domain of the NFI-family proteins, which is conserved from *Caenorrhabditis elegans* to humans is the target for this regulation, as was shown by site-directed mutagenesis (13). Oxidized, inactive NFI can be reduced to a DNA binding form by Grx1 *in vitro*. This requires the GSH/GR system to regenerate reduced Grx1. The *in vivo* relevance has been shown by treatment of HeLa cells with buthionine sulfoximine, an agent that inhibits its GSH synthesis. This GSH depletion potentiated the inactivation of NFI by the oxidizing agent diamide. Similarly, a stronger restoration of NFI activity after oxidation with diamide was observed, when the cells were treated with *N*-acetylcysteine, an agent that can replenish intracellular GSH (14).

In the case of NFI, not only DNA binding is subject to redox control. By using fusions with a heterologous DNA binding domain from the Gal4 protein, it was demonstrated that transcriptional activation by NFI also is regulated through oxidation of a cysteine residue (Cys 427) in the transcription-activation domain (TAD) (153) (Fig. 7). These findings were corroborated in living cells subjected to various stress conditions that induce cellular ROS formation, including inflammatory cytokine treatment, GSH depletion, heat and osmotic shocks, and chemical stress. In all cases, suppression of a reporter gene was specific for the NFI TAD, as no effects were observed with the transactivation domains of activator protein 2 (AP-2) and octamer transcription factor 2 (Oct-2). A common target for all these stressors was again Cys 427, leading to the suggestion that the NFI TAD might be a negative sensor of cellular stress (154). Based on the concentrations of exogenously applied H_2O_2 required to block transcriptional activation and DNA binding by NFI, it was calculated that a 100-fold difference in sensitivity to oxidation exists between the DNA binding domain of NFI and its TAD (153). During this investigation, TADs from other transcription factors (Sp1 and Oct-2) were analyzed to show that redox regulation of TADs is not a general effect. Whereas the Oct-2 TAD was not sensitive to oxidative stress, the analogous domain of Sp1 showed some responsiveness, indicating that other transcription factors could be subject to similar control mechanisms, which might have been overlooked during the characterization of the redox dependency of their DNA binding capacity.

It is not clear whether the thiol moiety of Cys 427 of NFI undergoes an oxidation with a gain of oxygen atoms or if it forms an intra- or intermolecular disulfide bridge, although mutations of a cysteine in the neighborhood (Cys 405) indicate that the formation of an intramolecular disulfide bridge within the TAD is not the mechanism most likely to trigger the effect. The oxidation of Cys 427 could affect the conformation of the TAD, which is the interface for interactions with the TATA-box–binding protein TBP, the coactivator CBP/p300, and histones H1 and H3.

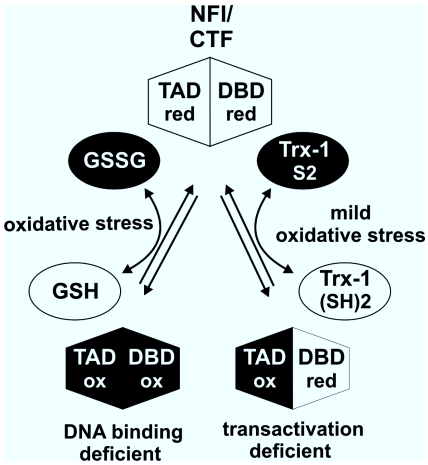

FIG. 7. Redox regulation of NFI/CTF: dual regulation of NFI/CTF transcriptional activity. Nuclear factor I/CAAT transcription factor (NFI/CTF) must be reduced to activate its target genes. Oxidative stress leads to oxidation of its transactivation domain (TAD) and DNA binding domain (DBD), which prevents DNA binding. Much lower concentrations of ROS only oxidize the TAD in a thioredoxin-1 (Trx-1)-dependent manner. This partially oxidized molecule can still bind to DNA, but is incapable of activating transcription.

H. Hypoxia-inducible factor 1 (HIF-1)

HIF-1 is the most prominent regulator of genes induced by hypoxia. It is a transcription factor that binds to the hypoxia-responsive element (HRE) in the promoters and enhancers of various hypoxia-inducible genes. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β, which is identical to the aryl hydrocarbon-receptor nuclear translocator (ARNT). Both proteins contain a basic helix–loop–helix (bHLH) and a PAS domain at their N-terminus, the latter being an acronym for Per, ARNT, and Sim, the first three members of the protein family characterized. Whereas the basic domain is essential for DNA binding, the HLH domain and the N-terminal half of the PAS domain are required for heterodimerization and DNA binding. HIF-1β contains a single C-terminal transactivation domain, whereas in HIF-1α, two such domains are found, termed NAD and CAD, according to their location closer to the N- or C-terminus. The NAD is embedded in a region controlling protein stability, the oxygen-dependent degradation domain (ODD). Both subunits of HIF-1 are expressed constitutively, but the α-subunit has an extremely short half-life (<5 min) under normoxic conditions, because of continuous proteolysis by the ubiquitin-proteasome pathway targeting the ODD. As both subunits are required for the activation of HIF-1 target

genes, these are not or only weakly expressed at normal oxygen tension. During hypoxia, HIF-1α is instantaneously stabilized, resulting in a functional heterodimer and expression of hypoxia-inducible genes. HIF-1α is hydroxylated at proline 564 (Pro 564) in the ODD in an oxygen-dependent fashion. This hydroxylated HIF-1α is bound by the von Hippel–Lindau (VHL) protein, which acts as an E3-ubiquitin ligase, tagging HIF-1α for proteasomal degradation. The critical molecules for the rapid turnover of HIF-1α under normoxia are members of a family of prolyl-4-hydroxylases (PHs). These enzymes require oxygen and 2-oxoglutarate as cosubstrates and contain iron liganded by two histidine and one aspartic acid residues. Oxygen binding requires the vitamin C–dependent maintenance of iron in its ferrous state. When oxygen is low, Pro 564 in HIF-1α remains unmodified, resulting in stabilization of the protein, which is no longer bound by VHL. Similarly, iron chelation or replacement by transition metal ions (Co^{2+}, Ni^{2+}, Mn^{2+}) can mimic hypoxia-inducible stabilization, explaining the apparent "upregulation" of HIF-1α by these metals (for review of HIF-1, see refs. 205, 206, 246, and 247).

In addition to HIF-1α, two other family members are known, HIF-2α/HLF (HIF-1α–like factor) and HIF-3α. They show a more-restricted expression pattern, but contain domains similar to HIF-1α and have comparable biochemical properties.

In 1996, it was shown that pretreatment of cells with hydrogen peroxide has an inhibitory effect on transcriptional activation by HIF-1, which suggested an additional redox control mechanism. Sulfhydryl alkylation or oxidation, the latter being reversible, indicated an involvement of cysteine residues in this process. These findings were supported by the fact that purified, oxidized Trx-1 in combination with DTT could stimulate HIF-1 DNA binding in hypoxic extracts. In addition, overexpression of Trx-1 or APEX1 enhanced the hypoxic induction of a HIF-1–dependent reporter gene (90). Interestingly, DNA binding of HLF-, but not HIF-1α–containing complexes was redox dependent. This is explained by the fact that the critical cysteine residue in the basic domain of HLF (Cys 28), which seems to be a target for APEX1, is replaced by a serine residue at the corresponding position (Ser 25) in HIF-1α. However, APEX1 also is important for transcriptional activation by HIF-1α (116). The last finding is in agreement with a previous report that the CADs of HIF-1α and HLF interact with the transcriptional coactivator CBP/p300 and that APEX1 and Trx-1 further enhanced the activity of a luciferase reporter activated by a fusion protein between the Gal4 DNA binding domain and CAD under hypoxic conditions. This potentiation required the catalytic activity of Trx-1, suggesting that a redox reaction is involved. Interestingly, a single cysteine residue is conserved in the 49-amino-acid CAD between HLF (Cys 844) and HIF-1α (Cys 800). Mutation of this cysteine abolished the hypoxia-inducible transcriptional activation by Gal4-CAD and interaction with CBP. The importance of this cysteine residue was confirmed by mutation in the context of full-length HIF-1α and HLF, which markedly reduced the transcription-enhancing activity (51).

I. Nuclear factor erythroid 2–related factor 2/ NF-E2 related factor 2 (Nrf-2)

The NF-E2–related factor 2 (Nrf-2), not to be confused with the GA-binding protein nuclear respiratory factor 2, which is also called Nrf-2, is a transcription factor implicated in the cellular responses to oxidative stress and to chemical compounds that are metabolically transformed to reactive or electrophilic intermediates. *Nrf-2*–deficient mice show a lower expression of xenobiotic enzymes and are predisposed to tumors induced by carcinogens (186). Nrf-2 is a member of the so-called cap 'n' collar basic region leucine zipper (CNC-bZIP) family of transcription factors, the bZIP region of which is distinct from other bZIP families, such as the Jun/Fos family. Nrf-2 heterodimerizes with other bZIP transcription factors, including the small Maf (sMaf) proteins. These heterodimers bind to antioxidant-response elements (AREs) and thereby upregulate numerous genes coding for detoxification enzymes, antioxidants, and the enzymes required for *de novo* GSH synthesis (148). However, Nrf-2 controls not only inducible, but also low-level gene expression under nonstressed conditions, suggesting that its activity is tightly controlled. It has been shown that Nrf-2 is tethered by the Kelch-like ECH-associated protein 1 (Keap1), and in this complex, is not available as a transcriptional activator. Keap1 serves not only simply to sequester Nrf-2, but also functions as an adaptor for a Cul3-dependent E3 ubiquitin ligase modifying Nrf-2 for proteasomal degradation, which explains the short half-life of Nrf-2 (~15 min). It is still a matter of debate whether Keap1 transiently enters the nucleus and targets Nrf-2 for ubiquitinylation there or is capable of engaging in a nucleocytosolic shuttling of Nrf-2 dependent on CRM-1 (235). Nevertheless, the generally accepted scenario involves a cytosolic retention and degradation of Nrf-2 by Keap1.

Keap1 contains an N-terminal BTB/POZ domain (for broad-complex, Tramtrack and Bric-a-brac; also known as a Poxvirus and zinc finger domain) potentially serving as an interface for homomeric or heteromeric interactions. The C-terminus comprises six Kelch repeats, each of which forms a four-stranded β-sheet resulting in a propeller-like structure binding Nrf-2. Keap1 contains 25 cysteine residues, the most reactive of which are found in the intervening region between the BTB/POZ and Kelch repeat domains (47). Inducers of ARE-dependent genes disrupt the Keap1/Nrf-2 interactions by modifying two of these residues (Cys 273 and Cys 288). Transfection of *Keap*1- and *Nrf*-2–deficient mouse embryonic fibroblasts with constructs expressing cysteine-to-alanine mutants of these two amino acids in Keap1 demonstrated that release of Nrf-2 is the consequence of the formation of an intermolecular, disulfide-linked Keap1 dimer. In this dimer, the disulfide bridges most likely are formed crosswise between Cys 273 and Cys 288 (237). After release from Keap1, Nrf-2 escapes degradation and can bind to AREs in a heteromeric complex with an sMaf to activate gene expression. Several kinases have been shown to phosphorylate Nrf-2, but the molecular consequences of these phosphorylation events have not been elaborated (for review of Nrf-2 and its regulation by Keap1, see refs. 107, 158, and 159).

Besides the cytosolic retention of Nrf-2 by Keap1 and its release upon formation of an intermolecular Keap1 dimer, a second layer of Nrf-2 activity regulation exists. Like other transcription factors, Nrf-2 must be in a reduced state for efficient DNA binding. The critical residue is Cys 506, whose oxidation reduced its affinity for the ARE, leading to decreased expression and antioxidant induction of NAD(P)H/quinine oxidoreductase 1 (*NQO1*). However, mutation of this residue to serine did not affect the retention of Nrf-2 by Keap1

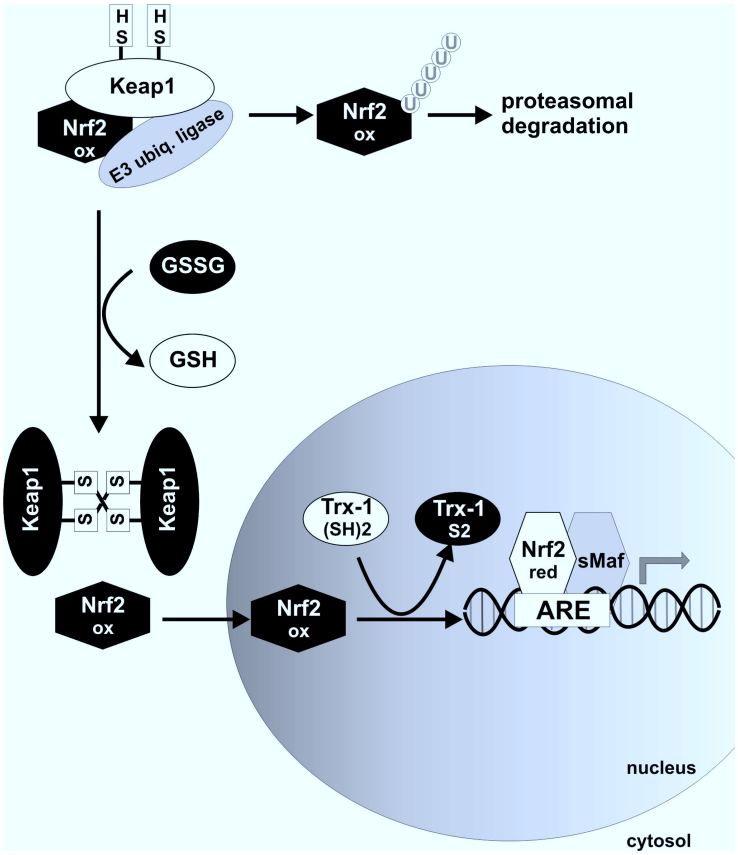

FIG. 8. Activation of Nrf-2: general model of gene induction by the Keap1/Nrf-2 pathway. Nuclear factor erythroid 2–related factor 2/NF-E2–related factor 2 (Nrf-2) is sequestered in the cytosol by Kelch-like ECH associated protein 1 (Keap1). In addition to binding Nrf-2, Keap1 functions as an adaptor for an E3 ubiquitin ligase, which ubiquitinates Nrf-2, thereby tagging it for proteasomal degradation. After induction, Keap1 is oxidized to an intermolecular, disulfide-linked dimer involving reciprocal cysteine residues of both monomers. This oxidation requires GSSG. Released Nrf-2 translocates to the nucleus, where it is reduced by thioredoxin-1 (Trx-1). Nrf-2 must be in a reduced state for efficient DNA binding as a heterodimer with a small Maf protein (sMaf). These heterodimers induce transcription of genes, whose promoters contain antioxidant responsive elements (AREs).

in the cytosol or its release in response to antioxidants (26). It has been shown that this two-layered, compartmentalized regulation of Nrf-2–dependent gene expression involves two cellular redox systems. Whereas cytosolic retention is controlled by GSH/GSSG ratios, the Nrf-2/DNA interactions depend on nuclear Trx-1 (76) (Fig. 8).

J. Homeobox B5 (HoxB5)

To our knowledge, only one case exists in which oxidation of a cysteine residue in a transcription factor can enhance DNA binding, homeobox B5 (HoxB5). It is a homeodomain (HD) protein of the antennapedia family and functions as a sequence-specific transcription factor that is involved in lung and gut development. In the cardiovascular system, HoxB5 is an upstream transcriptional switch for differentiation of the vascular endothelium from precursor cells (252). It was shown *in vitro* that the cooperative stabilization of HoxB5 DNA binding, but not sequence-specific DNA binding, is under redox regulation. Cooperative binding and redox regulation were found to require the presence of a cysteine residue (Cys 232) in the turn between homeodomain helices 2 and 3 and that oxidation of this cysteine is necessary for cooperative binding of the protein to tandem binding sites. This was shown by DTT treatment of purified, recombinant HoxB5, which resulted in loss of cooperativity (58). Multiple clustered HD protein-binding sites are found in the promoters of *Drosophila* and vertebrate genes whose expression is regulated by antennapedia-type HD proteins. Thus, cooperative interactions could have a large influence on the DNA binding of HD proteins to these sites.

K. Other redox-regulated transcription factors

Besides the transcription factors discussed in detail, DNA binding of several others is inhibited by oxidation: CBP/PEBP2 (5, 101), c-Myb (67, 155), Egr-1 (91), MyoD (218), NF-Y (156), Pax-5 (226, 227), Pax-8 (103, 224), TTF-1 (11, 103, 225), and bovine papilloma virus E2 (BPV E2) (146). In some of these cases, oxidation sensitivity has been mapped to the DNA binding domain; in others, it has been shown that Trx-1 or APEX1 can restore the DNA binding capacity after oxidation.

L. Histone deacetylase 2 (HDAC2)

Interestingly, not only transcription factors as *bona fide* DNA binding proteins are affected by modification of cysteine residues. Recently, it was shown that the chromatin modifier histone deacetylase 2 (HDAC 2) becomes nitrosylated after treatment of rat cortical neurons with neurotrophins. This modification occurs on two cysteine residues (Cys 262 and Cys 274) and is dependent on neuronal nitric oxide synthase (nNOS), as shown in neurons from nNOS-deficient mice and nonneuronal cells expressing nNOS. Intriguingly, nitrosylation of the two cysteines did not change the enzymatic activity of HDAC 2 but rather induced its release from chromatin. This dissociation of HDAC 2 leads to acetylation of histones H3 and H4, activation of BDNF target genes, and dendritic

Table 2. Nuclear Kinases and Phosphatases

	Critical amino acid	*Molecular consequence*	*Cellular consequence*	*Compartment of modification*	*Ref.*
Nuclear kinase					
PKCδ	Tyr 512	Kinase activation	Apoptosis	Cytosol	46, 222
PKA	n.d	Kinase activation	Cell survival	Nucleus	17
JNKs	n.d	Kinase activation	Apoptosis	Nucleus	24
Akt	Ser 473	Kinase activation	Apoptosis inhibition, delayed	Nucleus	68, 70
ERK2	Thr 183, Tyr 185	Kinase activation	*De novo* GSH synthesis	Nucleus	108, 263
Src	Tyr 416	Kinase activation	Senescence, apoptosis	Cytosol	68, 69
	Cys 277	Kinase inactivation	n.d.	Cytosol	106
Yes	Tyr 426	Kinase activation	Senescence, apoptosis	Cytosol	68, 69, 96
	n.d. (Cys)	Kinase inactivation	n.d.	Cytosol	106
Nuclear phosphatase					
Shp-2	Cys 459	Reduced phosphatase activity	ROS induction, apoptosis	n.d.	37, 96
	Cys 331, Cys 367	Backdoor cysteines, protection of Cys 459	n.d.	n.d.	33
TC-PTP	n.d.	Dephosphorylation of transcription factors	n.d.	Nucleus	228, 257
Cdc25C	Cys 330	Reduced phosphatase activity	Cell cycle progression	Nucleus	179, 198
	Cys 377	Backdoor cysteine, protection of Cys 330	Cell cycle progression		

growth (163). This mechanistic study could explain why HDAC 2 can negatively regulate memory formation and synaptic plasticity (66).

IV. Redox-Regulated Nuclear Kinases and Phosphatases

A. Nuclear-localized kinases

Protein kinases are involved in many different cellular signaling pathways. Therefore, a strict regulation of these kinases is necessary for the survival of the cell. The most important regulatory mechanism is their activation by phosphorylation (for review, see refs. 98 and 99). Kinases are phosphorylated under physiologic and pathophysiologic conditions. Recently a direct oxidation of Src and the fibroblast growth factor type 1 was demonstrated, leading to their inactivation. This mechanism seemingly only works for kinases containing a cysteine in the Gly loop capable of forming disulfide homodimers and therefore applies only to a small number of human protein tyrosine kinases (106).

In the context of this review, we restrict the detailed discussion to kinases, for which a nuclear localization has been demonstrated, even if the redox regulation takes place outside the nucleus. Nuclear localization has been shown for protein kinase C δ (PKCδ), PKA, JNKs, Akt, ERKs, and some members of the Src kinase family. The following paragraphs discuss the kinases mentioned earlier in more detail; a brief overview is given in Table 2.

1. Protein kinase Cδ (PKCδ). The protein kinase C (PKC) family of serine/threonine kinases is involved in many cellular signaling pathways such as growth, proliferation, and cell death (for review, see ref. 259). This family consists of three groups: (a) the conventional PKCs (cPKCs: α, βI, βII γ); (b) the novel PKCs (nPKCs: $\delta, \varepsilon, \theta, \mu$); and (c) the atypical PKCs (aPKCs: ζ, λ). PKCδ belongs to the novel PKCs and is redox regulated in several cell types. One well-described role for PKCδ is in mitochondria-dependent apoptosis induction. Overexpression of PKCδ in keratinocytes leads to translocation of PKCδ to mitochondria, alterations in mitochondrial functions, and induction of cell death (121). However, other studies also show translocation of PKCδ into the nucleus in various cell types. In response to cellular stresses, like oxidative stress, PKCδ is activated by tyrosine phosphorylation, and nuclear translocation occurs. PKCδ possesses its own nuclear localization sequence (NLS) (46) and has several tyrosine phosphorylation sites, which regulate its kinase activity. One of these sites, tyrosine 512, is phosphorylated by the proapoptotic tyrosine kinase c-Abl in response to oxidative stress. Phosphorylation results in activation of PKCδ followed by its nuclear translocation (222, 260). After nuclear translocation, activated PKCδ initiates a sequence of events that activates caspase 3, which in turn cleaves PKCδ (25, 46). This cleavage results in a 40 kDa catalytically active fragment and a 38 kDa regulatory fragment of PKCδ. The catalytically active fragment induces apoptosis by phosphorylation of the apoptosis-related protein DNA-dependent protein kinase (DNA-PK) (22, 60). DNA-PK is essential for repair of DNA double-strand breaks (214). Phosphorylation by PKCδ and also its catalytically active fragment induces the dissociation of DNA-PK from DNA, resulting in an inhibition of DNA repair and enhanced DNA fragmentation (22) DeVries *et al.* (46) showed that caspase 3–dependent cleavage of PKCδ increases the rate of nuclear translocation of the 40 kDa PKCδ cleavage fragment, which results in an amplification of the apoptotic signal (46) (Fig. 9).

PKCδ also interacts with and activates IKKα in response to oxidative stress. Active IKKα translocates into the nucleus

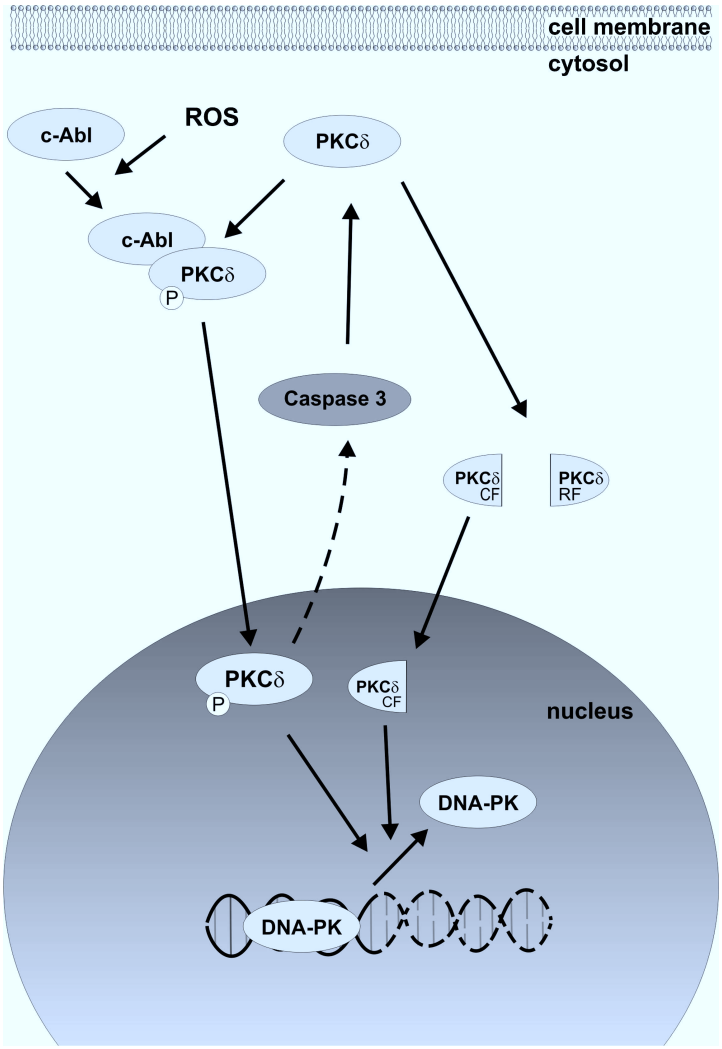

FIG. 9. Role of nuclear PKCδ. ROS induce association of protein kinase C δ (PKCδ) and c-Abl. This leads to phosphorylation, activation, and nuclear import of PKCδ. In a direct or indirect way, phosphorylated PKCδ activates caspase 3, which in turn leads to cleavage of PKCδ into a catalytically active (PKCδ CF) and a regulatory fragment (PKCδ RF). PKCδ CF translocates into the nucleus and, like PKCδ, induces the dissociation of DNA protein kinase (DNA-PK) from the DNA, which leads to fragmentation of DNA.

and regulates the transcriptional activity of the tumor-suppressor p53 by phosphorylation at serine 20 (256). This is a recently described mechanism for ROS-induced p53 activation.

2. Protein kinase A (PKA). Cyclic AMP formed by adenylyl cyclases binds the regulatory subunits (R) of the tetrameric PKA holoenzyme and promotes dissociation of the catalytic subunits (C-PKA). A fraction of C-PKA translocates to the nucleus and stimulates cAMP-dependent gene expression in different cell types (174). The best-characterized target of nuclear C-PKA is CREB, which is phosphorylated at serine 133 by C-PKA (71). H_2O_2 has been shown to initiate an increase in CREB phosphorylation at serine 133 in a non-transformed murine alveolar type II epithelial cell line (18), leading to transcriptional activation (145). The increased phosphorylation of CREB was due to an H_2O_2-induced increase in nuclear accumulation of C-PKA (17). This was confirmed by pharmacologic inhibition of PKA with H89, which reduced H_2O_2-mediated phosphorylation of CREB. Because the downregulation of CREB by siRNA increased the sensitivity of cells to H_2O_2-induced apoptosis and reduced transcription of the antiapoptotic gene B-cell lymphoma protein 2 (*Bcl*-2), CREB seems to play a role in cell survival in response to oxidative stress (17).

3. c-Jun NH_2-terminal kinases (JNKs). JNKs are involved in the regulation of cell proliferation and apoptosis. The activation of these pathways is dependent on the actual stress stimulus and cell type (125, 128). Sustained activation of JNKs leads to apoptosis, whereas the acute and transient activation induces survival pathways and cell proliferation (196). The sustained stress-induced activation of JNKs by phosphorylation through upstream MAP-kinases, ASK1, and MAP kinase kinase (MKK) 4/7 leads to an induction of apoptosis (for review, see ref. 127). JNKs translocate into the nucleus and phosphorylate c-Jun and activating transcription factor 2 (ATF2) (for review, see ref. 41), leading to the formation of an AP-1 complex and to the transcription of genes coding for proapoptotic proteins [*e.g.*, tumor necrosis factor α (TNF-α), Fas-L, and Bak] (53). However, JNKs also regulate physiologic and homeostatic processes. One attractive explanation for these differences is the existence of three isoforms of JNK and the subcellular pools of the JNKs. Only combined siRNA knockdown of all JNKs 1, 2, and 3 provides substantial protection from cell death. In contrast, knockdown or knockout

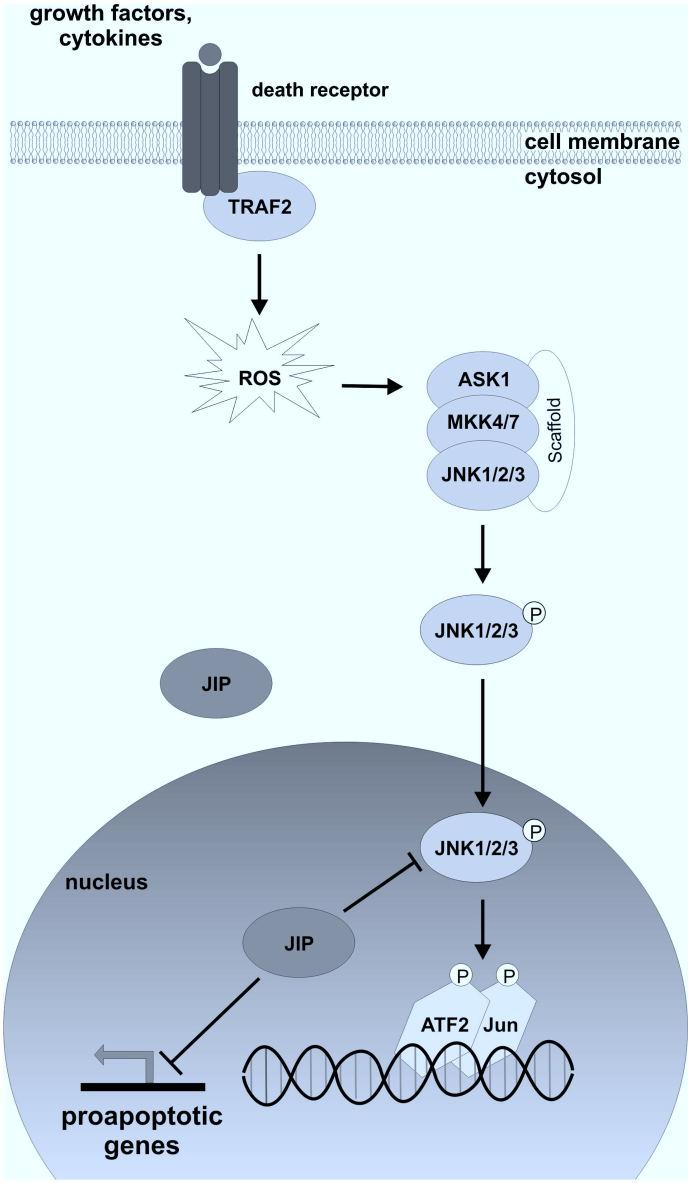

FIG. 10. Nuclear targeting and signaling of JNKs in the regulation of apoptosis. Growth factors and cytokines elevate ROS levels mediated by death receptor–coupled Traf2. This induces the activation of c-Jun NH_2-terminal kinases (JNKs) through upstream kinases apoptosis-signaling kinase 1 (ASK1) and MAP kinase kinase (MKK) 4/7. Activated JNK1, 2, and 3 translocate into the nucleus and phosphorylate c-Jun and activating transcription factor 2 (ATF2), which leads to formation of an activator protein 1 (AP-1) complex and to transcription of proapoptotic genes. Inhibition of nuclear active JNK1, 2, and 3 by JNK inhibitor protein (JIP) results in complete apoptosis inhibition.

of individual JNKs or two JNKs together does not protect. Moreover, to determine whether cytosolic or nuclear JNKs are responsible for JNK-dependent cell death, compartment-specific inhibitors for JNKs were generated. Therefore, a nuclear-exclusion sequence (NES) from MKK1 or three NLSs from SV40 large-T antigen were fused in tandem upstream of the JNK inhibitor protein JIP. These targeted proteins localized to cytosolic and nuclear compartments, respectively (23). Overexpression of these compartment-specific JNK inhibitors revealed that cell death requires nuclear but not cytosolic JNK activity, as the nuclear dominant-negative inhibitor of JNK protected against apoptosis, whereas the cytosolic form only blocked physiologic JNK function (24). Thus, any one of the three JNKs is capable of mediating apoptosis, and this is dependent on their nuclear localization (Fig. 10).

4. Protein kinase B (Akt). The activity of the serine/threonine kinase Akt is redox regulated by Grx. By keeping Akt in its reduced form, Grx enables the activation and stimulation of the endothelial NO-synthase/NO signaling pathway (241). However, it is unclear whether this has to be attributed only to cytosolic Akt or also to the nuclear Akt.

We previously reported that nuclear Akt is required for the maintenance of telomerase activity and inhibition of apoptosis in human umbilical vein endothelial cells (70). Mechanistically, after the first assembly of the telomerase holoenzyme, which requires Hsp90 and p23 (89), Akt is recruited into this complex in the nucleus. Upon its recruitment, Akt phosphorylates TERT at serine 823, which increases nuclear telomerase activity (29, 70). Furthermore, the complex formation of TERT with HSP90 and Akt protects Akt from being dephosphorylated by the protein phosphatase 2A and thereby keeps Akt and TERT in their active forms (70, 105). Independent of its telomere-elongation function, nuclear TERT also has antiapoptotic effects. Inhibition of telomerase activity or overexpression of nuclear TERT induces or inhibits

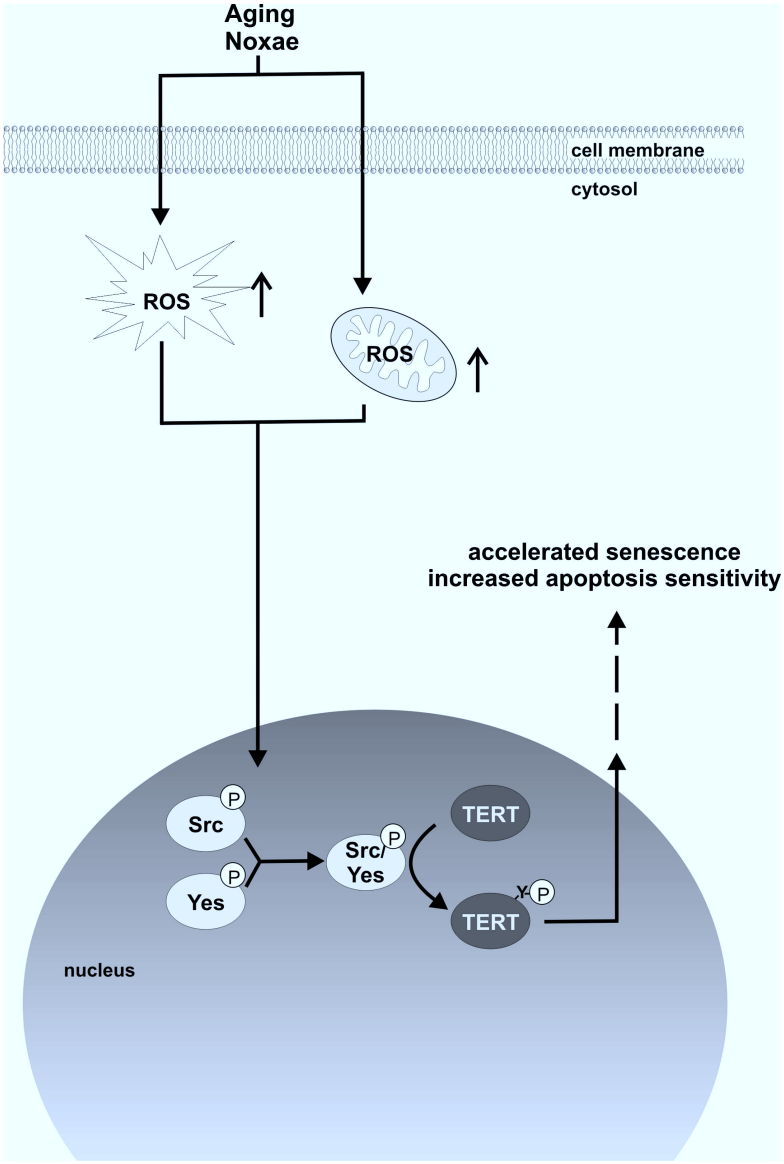

FIG. 11. Nuclear Src and Yes induce TERT export under conditions of oxidative stress. Cytosolic and mitochondrial ROS induce increased activity of nuclear Src and Yes. This leads to tyrosine phosphorylation of telomerase reverse transcriptase (TERT) and its nuclear export. The functional consequences are increased apoptosis sensitivity and accelerated senescence.

apoptosis, respectively (68, 166, 192, 262). Thus, maintaining TERT in its active state in the nucleus by Akt protects cells against apoptosis.

It has been demonstrated that, upon its activation, myocardial Akt accumulates in the nucleus (30). Therefore, important studies investigated the specific function of nuclear Akt in cardiomyocytes by using an adenovirus expressing Akt with nuclear localization signals. Nuclear-targeted Akt inhibited cardiomyocyte hypertrophy and apoptosis (211, 232). Moreover, transgenic mice with cardiac-specific expression of nuclear-targeted Akt were protected from ischemia/reperfusion injury (211). Given the fact that constitutive active Akt causes hypertrophy in cardiomyocytes, specifically, nuclear-targeted Akt provides a new opportunity for therapeutic applications.

5. Extracellular regulated kinases 1/2 (ERK1/2). ERK1/2s are a subfamily of the MAP kinases and are involved in many important cellular processes like cell proliferation, survival, apoptosis, and metabolism (165). Their stimulation is triggered by growth factors and different environmental and oxidative stresses. The cytokine epidermal growth factor (EGF) is one of the activators of ERK1/2 through phosphorylation of the EGF receptor (EGFR) (31), whereas H_2O_2 activates ERK1/2 by EGFR-dependent and -independent pathways (63, 243). The phosphorylation state of ERK2 is more important for its nuclear retention than is the activity of ERK2. This was confirmed with catalytically inactive ERK2, in which lysine 52 was mutated to arginine [ERK2(K52R)]. Like the wild-type protein, microinjected ERK2(K52R) transiently translocated to the nucleus after stimulation. To reduce the susceptibility to dephosphorylation, the two activating phosphorylation sites were thiophosphorylated *in vitro*. The stably phosphorylated wt and K52R mutant directly moved into and remained in the nucleus, demonstrating that ERK2 activity is not necessary for nuclear accumulation (108).

Besides phosphorylation of ERK2, homodimerization is necessary for its nuclear accumulation (108). The dimerization

of ERK2 induces a change of conformation and exposes a binding site for a NLS-containing protein. One of the potential candidates for such a NLS protein is growth factor–receptor bound protein 2-associated protein 1 (Gab1), which associates with ERK2. It was suggested that binding of ERK2 and Gab1 regulates the nuclear import of phosphorylated ERK2 (170). Another important role for ERK1 and ERK2 is their involvement in the nuclear localization of Nrf-2, which, among others, activates genes coding for enzymes required for *de novo* GSH synthesis (263). Thus, ERK1/2 or mainly ERK2 plays a role in ensuring an appropriate GSH supply for the cell.

6. Src kinase family. The Src kinase family consist of at least nine members (178). The most prominent members are the kinases Src, Fyn, and Yes. In the nucleus, only the kinases Src and Yes can be found, but not Fyn (96). In response to oxidative stress or growth factors, Src is phosphorylated at tyrosine 416, and Yes at tyrosine 426, which increases their enzymatic activity. Under conditions of oxidative stress or during the process of aging, these nuclear kinases are involved in tyrosine phosphorylation of TERT, which then leads to nuclear export of TERT. This has been demonstrated by mutation of tyrosine 707 within TERT, resulting in nuclear retention of the enzyme and by pharmacologic inhibition of Src kinase family activation, which inhibits tyrosine phosphorylation of TERT and its subsequent export from the nucleus (70, 96) (Fig. 11). The underlying export mechanism is mediated by a complex formation between TERT, the well-described export receptor CRM-1, and the nuclear GTPase Ran (70). Functional consequences of this Src kinase family–dependent nuclear tyrosine phosphorylation of TERT are increased apoptosis sensitivity and accelerated senescence (68, 69). This is in agreement with other studies, demonstrating that nuclear TERT acts as an inhibitor of apoptosis in several cell types (56, 144, 166). Therefore, an imbalance in the redox status seems to enhance active nuclear Src and Yes, which importantly contribute to apoptosis and accelerated senescence.

Recently a new mechanism for inactivation of the Src kinase was discovered. Src is active only in its reduced form, and oxidation results in complete inactivation. This inactivation is caused by a specific, reversible oxidation of Cys 277, located in the catalytic domain of Src, which results in homodimerization of Src linked by a disulfide bridge. This cysteine residue is conserved only in three of the Src family members, Src, Yes, and Egr, and could therefore be a specific mechanism for regulating a specific subset of Src kinases (106). Interestingly, only Src and Yes could be found in the nucleus, leading to the speculation that the formation of disulfide homodimers might be a prerequisite for nuclear import.

B. Redox-regulated phosphatases

Protein phosphatases can be divided into serine/threonine phosphatases and tyrosine phosphatases (PTPs). Both are important regulators in the activation and inactivation of cell-signaling pathways. Serine/threonine phosphatases are generally regulated by association with regulatory subunits and their own phosphorylation (49, 209). In the last few years, the role of oxidative stress in the regulation of PTPs has received more attention. PTPs have been shown to be redox sensitive and to be inhibited reversibly or irreversibly, depending on the degree and mechanism of oxidation (120, 150, 180, 231). Oxidation of the essential cysteine in the active site by H_2O_2 inactivates phosphatase activity (45). The reversible oxidation of this cysteine residue to sulfenic acid (Cys-SO^-) has been identified as a key mechanism for the regulation of many pathways. Higher oxidation to sulfinic (Cys-SO_2^-) or sulfonic (Cys-SO_3^-) acid leads to an irreversible inactivation of the phosphatase (Fig. 12A). Many PTPs prevent a higher oxidation by formation of intracellular disulfide bonds between the active-site cysteine and nearby so-called backdoor cysteines [for example, Cdc25 phosphatases (216) or the phosphatase and tensin homologue (PTEN) (113)]. These intramolecular disulfides can then rapidly and effectively be reduced by

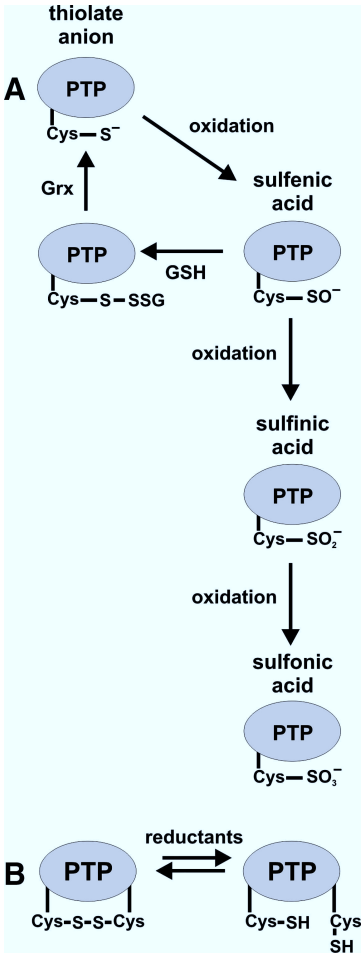

FIG. 12. Model for the regulation of PTP activity. (**A**) Under physiologic conditions, the catalytic cysteine of active protein tyrosine phosphatases (PTPs) is in the thiolate anion form. Oxidation leads to reversible sulfenic acid formation. Further oxidation is irreversible and leads to sulfinic and sulfonic acid formation. To prevent this further oxidation, GSH can form a mixed disulfide with the sulfenic acid, which is then reduced by glutaredoxin (Grx). (**B**) To prevent PTPs from being irreversibly oxidized, the reversible inactive state is stabilized by formation of an intramolecular disulfide bond between the cysteine in the catalytic center and a backdoor cysteine. This intramolecular disulfide bond can be rapidly and effectively reduced by several reductants.

reductants like dithiothreitol (DTT), GSH, Trx-1, or a combination of these (113, 216) (Fig. 12B). The oxidation of PTPs is important for the regulation of many cellular signaling pathways in response to oxidative stress.

C. Nuclear-localized phosphatases

Recently a new concept emerged, which emphasizes an important role for nuclear tyrosine phosphatases and their signaling pathways in response to oxidative stress. We concentrate on the detailed discussion of PTPs for which a nuclear localization has been shown, even if the redox regulation takes place outside the nucleus: Shp-2, T-cell protein tyrosine phosphatase (TC-PTP), and Cdc25C (an overview is given in Table 2).

1. Protein tyrosine phosphatase Shp-2. The Src homology 2 (SH2) domain containing protein tyrosine phosphatase Shp-2 is involved in many signal-transduction processes induced by cytokines and growth factors (2, 4, 54, 55). Further findings indicated a regulation of Shp-2 by ROS (150). Stimulation of Rat-1 cells with platelet-derived growth factor (PDGF) induced production of intracellular ROS, which leads to oxidation and inactivation of Shp-2. This oxidation requires complex formation between Shp-2 and the activated PDGF receptor (PDGFR). In its basal state, the active site of Shp-2 is covered by the N-terminal SH2 domain (N-SH2). The binding of a specific phosphotyrosyl ligand opens the active site of Shp-2 (82). Meng *et al.* (150) speculated that binding of PDGFR to Shp-2 promotes an open, active conformation of Shp-2, and that not only substrates but also ROS can interact with this site.

Like other PTPs, Shp-2 is oxidized by ROS. Recently, two backdoor cysteines were found to be involved in the redox regulation of Shp-2. Instead of forming a disulfide bond between the active-site cysteine and a backdoor cysteine to prevent the phosphatase from further oxidation, the reduced catalytic cysteine in Shp-2 (and also in Shp-1) is shielded by a disulfide bond between two backdoor cysteines (33).

In 2002, Chughtai *et al.* (37) reported a nuclear localization of Shp-2 in connection with an association of the signal transducer and activator of transcription 5 (STAT5) and Shp-2. This nuclear translocation of Shp-2 in a complex with Stat5 is induced by the stimulation with prolactin in mammary cells. Formation of this complex requires the carboxy-terminal SH2 domain and the catalytic activity of Shp-2 and correlates with the tyrosine phosphorylation of STAT5 by Janus kinase 2 (JAK2) on the tyrosine residue 694 (Fig. 13). The authors speculated that the nuclear Shp-2/STAT5 complex binds to DNA and regulates transcription of milk-protein genes (37). In endothelial cells, Shp-2 is localized in nuclear and cytosolic fractions under basal conditions (96). Our findings identified nuclear Shp-2 acting as a counterplayer for the nuclear export of TERT. Under conditions of oxidative stress, the nuclear export of TERT is blocked by Shp-2 overexpression. This is dependent on the activity of Shp-2 because the dominant-negative Shp-2(C459S) reduces nuclear TERT protein and telomerase activity. Ablation of endogenous Shp-2 leads to an increased tyrosine phosphorylation of TERT. Tyrosine 707 within TERT (which has previously been shown, once phosphorylated, to be essential for nuclear export of TERT) seems to be the target tyrosine for Shp-2. Thus, Shp-2 inhibits the nuclear export of TERT by regulating the tyrosine 707 phosphorylation (Fig. 14). This implies a new role for nuclear Shp-2 in protecting nuclear TERT, and therefore, nuclear Shp-2 may delay cellular senescence.

2. T-cell protein tyrosine phosphatase (TC-PTP). T-cell protein tyrosine phosphatase (TC-PTP) is a ubiquitously expressed PTP. As a result of alternative splicing, two isoforms of TC-PTP are generated, a 45 kDa isoform located in the nucleus and a 48 kDa isoform targeted to the endoplasmic reticulum (130). Specific cellular stresses cause a reversible cytosolic accumulation of the 45 kDa isoform of TC-PTP (114). For example, oxidative stress or hyperosmolarity induces the nuclear export of TC-PTP, whereas other stresses like heat shock have no effect on the localization of TC-PTP. This change in

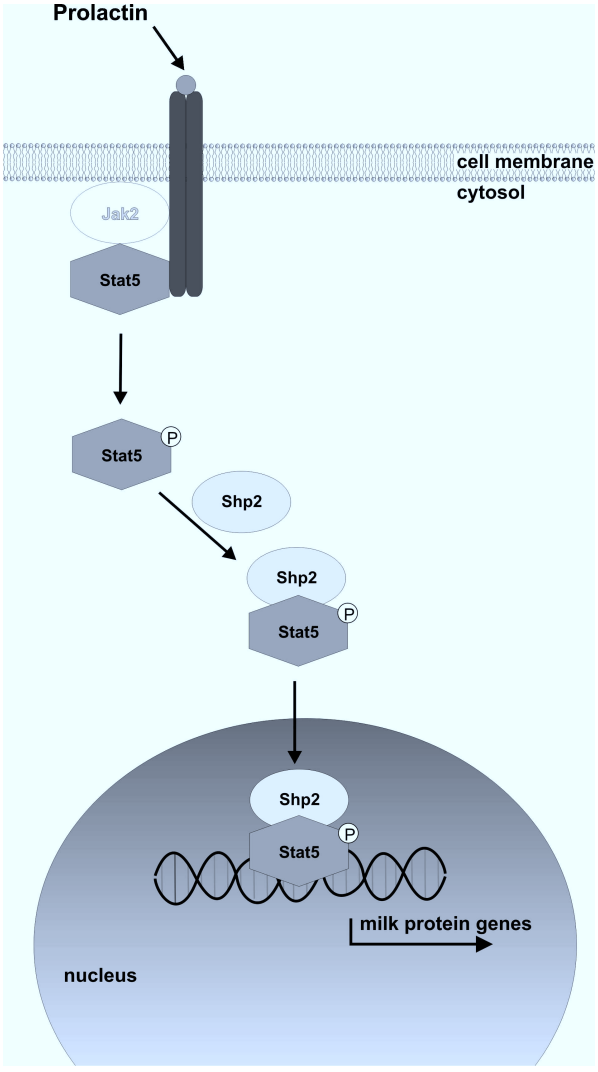

FIG. 13. Nuclear translocation of Shp-2 induces STAT5-dependent gene transcription. Prolactin induces tyrosine phosphorylation of STAT5 at tyrosine 694 by the Janus kinase 2 (JAK2), which leads to complex formation of Shp-2 and STAT5. This complex translocates into the nucleus, binds to DNA *via* STAT5, and induces milk-protein gene transcription.

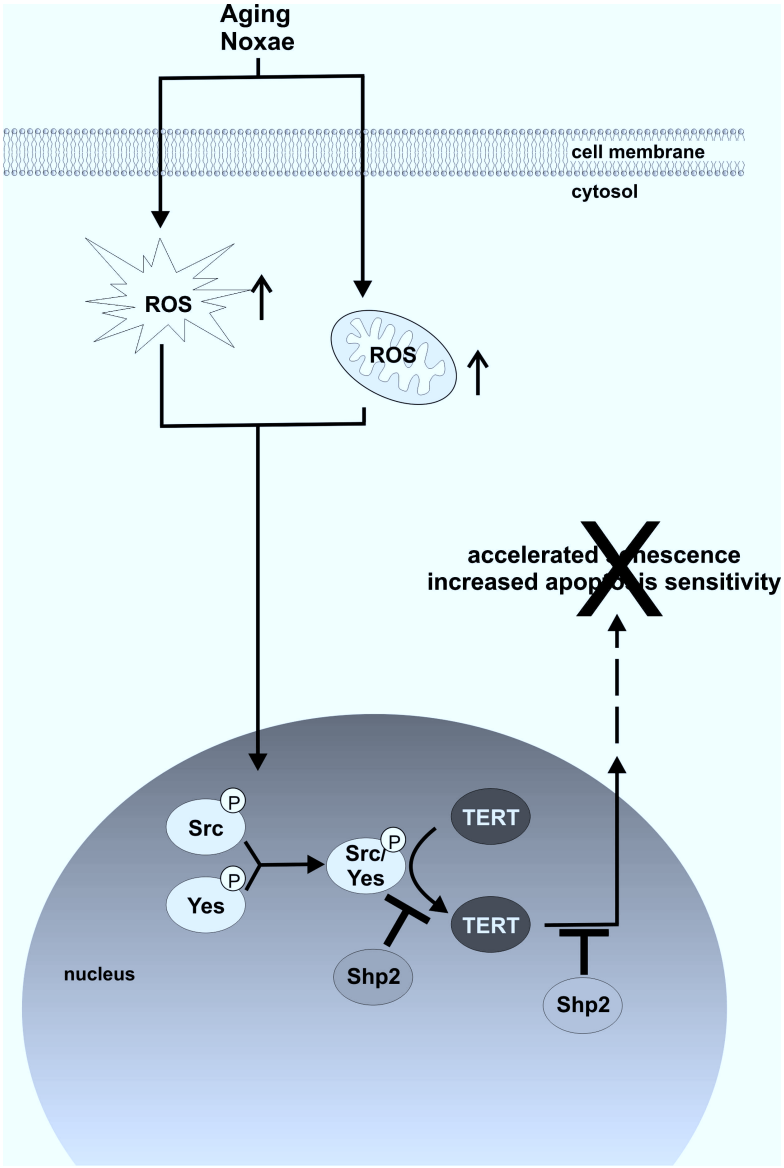

FIG. 14. Shp-2 inhibits TERT nuclear export induced by oxidative stress. ROS induce increased activity of nuclear Src and Yes. This leads to tyrosine phosphorylation of telomerase reverse transcriptase (TERT) and to its nuclear export. Nuclear Shp-2 prevents this export.

localization could be observed in different cell types. Nonnuclear TC-PTP is involved in the regulation of cell growth and cell differentiation in endothelial cells (143). There, TC-PTP binds to vascular endothelial growth factor receptor 2 (VEGFR2) and dephosphorylates specific tyrosine residues. Dephosphorylation of tyrosines 1045 and 1059 delays VEGF-induced VEGFR2 internalization and thus reduces sprouting angiogenesis.

Another target tyrosine of TC-PTP is tyrosine 1214, which has been implicated in VEGF-induced actin remodeling and thereby plays a role in endothelial cell migration (115). Together, these data suggest an important role for cytosolic TC-PTP in endothelial cell growth and differentiation.

Several other groups have investigated the functions of nuclear TC-PTP. The 45 kDa form of TC-PTP was first identified as a nuclear STAT1 tyrosine phosphatase. Upon stimulation, STAT1 becomes tyrosine phosphorylated by the family of JAKs and translocates into the nucleus. There it binds to DNA and activates transcription (40). To terminate this transcription, STAT1 must be dephosphorylated to dissociate from DNA. Ten Hoeve *et al.* (228) were the first to identify the 45 kDa form of TC-PTP to be the tyrosine phosphatase of STAT1. They also investigated the dephosphorylation of other STATs (STAT3, STAT5, and STAT6) and found that, in *TC-PTP*–deficient mouse embryonic fibroblasts, only the dephosphorylation of STAT1 and STAT3 is affected on interferon stimulation, but not that of STAT5 and STAT6 (228). This is in agreement with findings of Yamamoto *et al.* (257), who demonstrated dephosphorylated STAT3 after interleukin 6 treatment and showed a direct interaction between STAT3 and nuclear TC-PTP. Similarly, Aoki and Matsuda (10) found that in epithelial cells, stably expressing mouse *TC-PTP* STAT5a and STAT5b are dephosphorylated after prolactin stimulation (10). TC-PTP has different regulatory roles in diverse pathways, dependent on its localization. Because nuclear export of TC-PTP occurs under specific stresses, one

can imagine stress-specific functions of TC-PTP. This opens up an interesting field for further studies to understand why specific stress inducers like heat shock do not induce the nuclear export of TC-PTP.

3. Cdc25C. In mammalian cells, cell cycle progression is tightly regulated by the cyclin-dependent protein kinases (CDKs). CDK1 (also named Cdc2) is the key component of the checkpoint pathway, which delays mitotic entry after DNA damage or stalled replication. CDK1 forms complexes with cyclin B1 that, in their phosphorylated, inactive form, are retained in the cytosol. During prophase, CDK1/cyclin B1 complexes accumulate in the nucleus and are activated through the phosphatase Cdc25C by dephosphorylation of threonine 14 and tyrosine 15 of CDK1 (48, 112). As mentioned earlier, phosphatases can protect themselves from irreversible oxidation by forming a disulfide bond between their catalytic cysteine and a backdoor cysteine. This protection has also been demonstrated for Cdc25C. Its catalytic cysteine is cysteine 330, and the backdoor is cysteine 377. Mutation of cysteine 377 in Cdc25C leads to irreversible oxidation of cysteine 330, which results in degradation of Cdc25C (198). During interphase of the cell cycle, a formation of the disulfide bond between cysteine 330 and 377 in Cdc25C is induced, and Cdc25C is phosphorylated at serine 216. This phosphorylation leads to binding of Cdc25C to 14-3-3 proteins and results in the nuclear export of Cdc25C (179). Mutation of serine 216 in Cdc25C perturbs mitotic timing and allows cells to escape the G_2-checkpoint arrest (179, 195).

For the fate of the cytosolic Cdc25C, different hypotheses exist. One possible mechanism is the degradation of Cdc25C, according to the destruction hypothesis of Savitsky and Finkel (198). It has been demonstrated that vitamin C induces formation of ROS, which leads to decreased Cdc25C levels. Conversely, the formation of cytosolic Cdc25C/14-3-3 complexes occurs without reduction in the Cdc25C levels. Thus, one may speculate that, dependent on the levels of ROS, cells decide to induce cell death or G_2-checkpoint arrest. High ROS levels would lead to irreversible oxidation of Cdc25C, its degradation, and finally to cell death. Minor damage, conversely, could induce formation of the disulfide bond in Cdc25C, its phosphorylation, and cytosolic sequestration by 14-3-3 proteins. Thus, Cdc25C would be immediately available for cell cycle progression after damage repair.

V. Conclusions and Outlook

Redox regulation plays an important role in intracellular signal transduction. Numerous proteins have been described to be redox regulated. However, it must be noted that, for the nuclear import and export receptors, only initial studies in yeast have shown that these receptors change their localization from the nucleus to the cytosol and can be oxidized on oxidative stress, which could importantly contribute to gene regulation (111, 183, 184). Thus, further studies in higher eukaryotes are required to investigate the redox regulation of the nuclear import and export machinery. Furthermore, many studies do not distinguish between the intracellular compartments in which the redox modification of the protein of interest takes place. This is of special importance, because many antioxidative systems exist in different cellular compartments like the cytosol, the mitochondria, and the nucleus. However, not all antioxidative systems are equally distributed throughout the cell. The distribution strongly depends on the cell type and on the stimulus used. Therefore, it is noteworthy that cellular functions regulated by redox modifications of proteins are intimately associated with their cellular localization. Unfortunately, many previous investigations did not accurately discriminate between the compartments where redox regulation occurs and the corresponding cellular function. Without this differentiation, pharmacologic interventions may exhibit undesirable and unanticipated side effects because the compartment-specific effects of the proteins have been disregarded in the past. Therefore, compartment-specific investigations will help us to determine protein functions in more detail and to uncover misunderstood protein functions.

Finally, compartment-specific delivery of inhibitors and activators will open a new field of drug design to regulate protein actions more precisely and to reduce unwanted side effects.

Acknowledgments

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Abbreviations Used

AP-1 = activator protein 1
APEX1 = APEX nuclease (multifunctional DNA-repair enzyme) 1
ATF = activating transcription factor
bZIP = basic region-leucine zipper
cAMP = cyclic adenosine monophosphate
CDK = cyclin dependent kinase
CRE = cAMP response element
CREB = cAMP response element–binding protein
CREM = CRE modulator
DBD = DNA binding domain
DNA-PK = DNA protein kinase
DTT = dithiothreitol
Egr-1 = early growth-response factor 1
ER = estrogen receptor
ERE = estrogen response element
ERK = extracellular regulated kinase
GPx = glutathione peroxidase
GR = glutathione reductase
GRE = glucocorticoid response element
Grx = glutaredoxin
GSH = glutathione
GST = glutathione *S*-transferase
HIF = hypoxia-inducible factor
HLF = HIF-1α–like factor
HRE = hypoxia-response element
Hsp = heat-shock protein
HTLV = human T-lymphotropic virus
IκB = inhibitor of nuclear factor-κB
IKK = IκB kinase
JAK = Janus kinase
JIP = JNK-inhibitor protein
JNK = c-Jun NH_2-terminal kinase
Keap1 = Kelch-like ECH-associated protein 1
MAP kinase = mitogen-activated protein kinase
MKK = MAP kinase kinase
NFI/CTF = nuclear factor I/CAAT transcription factor
NF-κB = nuclear factor-κB
NIK = NF-κB–inducing kinase
NLS = nuclear localization signal
Nrf-2 = nuclear factor erythroid 2–related factor 2/NF-E2 related factor 2
ODD = oxygen-dependent degradation domain
PKA = protein kinase A
PKC = protein kinase C
Prx = peroxiredoxin
PTP = protein tyrosine phosphatase
ROS = reactive oxygen species
SOD = superoxide dismutase
Sp1 = specificity protein 1
STAT = signal transducer and activator of transcription
TAD = transcription-activation domain
TC-PTP = T-cell protein tyrosine phosphatase
TERT = telomerase transcriptase
TR1 = thioredoxin-1 reductase
Trx-1 = thioredoxin-1

Downregulation of mitochondrial telomerase reverse transcriptase induced by H_2O_2 is Src kinase dependent

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Downregulation of mitochondrial telomerase reverse transcriptase induced by H_2O_2 is Src kinase dependent

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ABSTRACT

Telomerase with its catalytic subunit telomerase reverse transcriptase (TERT) prevents telomere erosion in the nucleus. In addition, telomerase has also telomere-independent functions in protection from apoptosis. Unexpectedly, TERT was found in the mitochondria. However, its regulation in this organelle is completely unknown. Here, we demonstrate that mitochondrial TERT is downregulated by exposure to H_2O_2 in primary human endothelial cells. This depletion is dependent on the Src phosphorylation site within TERT, tyrosine 707. In accordance with this finding, we also detected Src in the mitochondria and demonstrated that Src is activated upon H_2O_2 treatment. This regulation of mitochondrial TERT is reminiscent of the situation in the nucleus from where TERT is exported under conditions of oxidative stress in a Src kinase dependent manner. In addition, Akt1 was also found in the mitochondria and H_2O_2 treatment led to reduced active Akt1 in these organelles, suggesting that similar regulatory mechanisms operate in mitochondria and the nucleus.

1. Introduction

The enzyme telomerase counteracts the shortening of telomeres, the physical ends of the chromosomes. Thereby it prevents the onset of replicative senescence and genetic instability (Blackburn, 2000; Buys, 2000; Collins, 2000; Hemann et al., 2001). Its catalytic subunit is the telomerase reverse transcriptase (TERT) (Collins, 2000). Several studies demonstrated that nuclear TERT increases cell survival and resistance against oxidative stress after short term incubation with different stimuli, which did not affect telomere length (Haendeler et al., 2003a,b; Rahman et al., 2005; Ramirez et al., 2003; Ren et al., 2001). Another unexpected finding was the observation that TERT is also localized in mitochondria (Ahmed et al., 2008; Haendeler et al., 2009; Santos et al., 2004, 2006). Since the circular mitochondrial DNA does not contain any telomeric repeats, mitochondrial TERT has to have also a telomere-independent role within mitochondria. However, the exact functions of TERT in mitochondria remain controversial. Santos et al. demonstrated in fibroblasts overexpressing TERT that it exacerbates oxidative injury (Santos et al., 2004, 2006). In contrast, Ahmed et al. showed in the same cells a protective role for mitochondrial TERT after oxidative stress (Ahmed et al., 2008). Furthermore, we recently revealed that endogenous, mitochondrial TERT protects mitochondrial DNA from damage and importantly contributes to the respiratory chain activity under basal conditions in endothelial cells as well as in human embryonic kidney cells (Haendeler et al., 2009). Besides this controversy, it is completely unknown how TERT is regulated in mitochondria. However, since changes in reactive oxygen species formation regulate nuclear TERT, one could speculate that also mitochondrial TERT is affected by oxidative stress. Moreover, there exists accumulating evidence that mitochondria are one compartment within the cell producing reactive oxygen species that contribute to aging processes, which has been demonstrated by several lines of evidence. Mice overexpressing mitochondrially targeted catalase show a significant increase in life span and a reduction in oxidative damage to DNA and consequently in apoptosis (Schriner et al., 2005). Moreover, overexpression of mitochondrially localized antioxidant enzymes lengthens lifespan of *Drosophila* (Orr and Sohal, 1994; Ruan et al., 2002) and deletion of manganese superoxide dismutase in mice results in the age-related decline of mitochondrial function, culminating in increased apoptosis (Kokoszka et al., 2001). Thus, aging and apoptotic processes are associated with increased reactive oxygen species formation within the mitochondria. Because also TERT is involved in aging and apoptotic processes, we elucidated for the first time the regulation of mitochondrial TERT by oxidative stress and the role of the Src kinase therein.

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2. Materials and methods

2.1. Cloning of expression vectors for mitochondrially targeted TERT

The N-terminal mitochondrial targeting sequence was amplified from pCMV/myc/mito (Invitrogen) using the primers mito-link for1 (5′-CAATTGCTAGCCACCATGTCCGTCCTGACGCCG-3′) and mito-link rev1 (5′-GCATGAATTCCAACGAATGGATCTTGGCGCG-3′) in a standard PCR reaction. The resulting amplification product was cut with NheI and EcoRI, inserted into hTERT expression vectors for TERT wild-type and TERT(Y707F), respectively (Haendeler et al., 2003a) and verified by sequencing.

2.2. Cell culture, transfection

Human embryonic kidney (HEK) cells were cultured in DMEM basal medium with 10% heat-inactivated fetal calf serum. Human umbilical vein endothelial cells (HUVEC) were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10% fetal calf serum. After detachment with trypsin, cells were grown for at least 18 h. HUVEC were transiently transfected with Superfect (QIAGEN) as described previously (Haendeler et al., 2002). The transfection efficiency in HUVEC was between 20% and 30%.

2.3. Cellular fractionation

Cellular fractionation and isolation of mitochondria was performed as described previously (Yang et al., 1997). To obtain total cell lysates, cells were lysed in RIPA-buffer as described previously (Haendeler et al., 2003a).

2.4. Immunoblotting

Immunoblotting was performed with antibodies directed against TERT (1:500, overnight, 4 °C, Rockland), total Src (1:500, Invitrogen), phospho-SrcY418 (1:500, Invitrogen), phospho-SrcY529 (1:1000, Abcam), phospho-Akt1 (1:1000, Cell Signaling Technol.), Akt1 (1:1000, Cell Signaling Technol.), tubulin (1:5000, Sigma), Thioredoxin-1 (1:500, BD Biosciences), and translocase of inner membrane 23 (TIM23, 1:500, BD Biosciences). Blots were incubated with primary antibodies overnight at 4 °C and with secondary HRP-coupled antibodies 2 h at room temperature. Antibodies were detected by the enhanced chemiluminescence system (GE Healthcare). Semi-quantitative analyses were performed on scanned immunoblots using Scion Image 1.6 (SCION Corporation) or ImageJ (Abramoff et al., 2004).

2.5. Statistics

Statistical analyses were performed with student's *T*-test using winLSTAT 2008.

3. Results

3.1. TERT and the Src kinase are localized in mitochondria

We recently demonstrated that TERT is localized in mitochondria, where it protects mitochondrial DNA from damage and preserves mitochondrial functions (Haendeler et al., 2009). However, the regulation of mitochondrial TERT is completely unknown. From our studies of nuclear TERT in endothelial cells as well as in human embryonic kidney cells, we know that TERT is exported from the nucleus under conditions of oxidative stress in a Src kinase dependent manner (Haendeler et al., 2003a, 2004). Moreover, it has recently been demonstrated that the Src kinase is localized in mitochondria in rat brain and that the complexes of the respiratory chain are substrates for Src, indicating that respiratory chain activity is partially dependent on tyrosine phosphorylation (Arachiche et al., 2008). Therefore, we first investigated whether the Src kinase is present in mitochondrial fractions from human embryonic kidney cells as well as endothelial cells. Indeed, the Src kinase is localized in the mitochondria of these cell types (Figs. 1A and 2).

3.2. Mitochondrial Src kinase is activated by treatment with H_2O_2

Next, we determined whether treatment with H_2O_2 activates the Src kinase in mitochondria. Therefore, human endothelial cells were incubated with 200 μM H_2O_2 and active Src kinase was measured by immunoblot analysis of mitochondrial lysates. For that purpose immunoblots were performed with an antibody against active Src, phosphorylated at tyrosine 418 and an antibody against inactive Src, phosphorylated at tyrosine 529. The ratio between

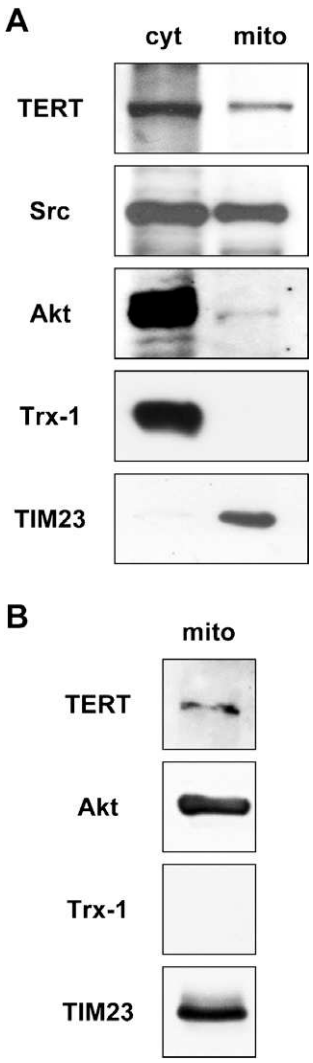

Fig. 1. Localization of TERT, Akt1 and Src kinase in mitochondria. (A) Mitochondrial and cytosolic fractions of HEK 293 cells were isolated as described in Section 2. Lysate proteins were resolved by SDS–PAGE, blotted onto PVDF membranes and the blots probed with antibodies against TERT, Src and Akt1. Antibodies against thioredoxin-1 (Trx-1) and translocase of inner membrane 23 (TIM23) were used to control for purity of the fractions. (B) Immunoblots with mitochondrial lysates from HUVEC were probed with antibodies against TERT, Akt1, Tim23 and Trx-1.

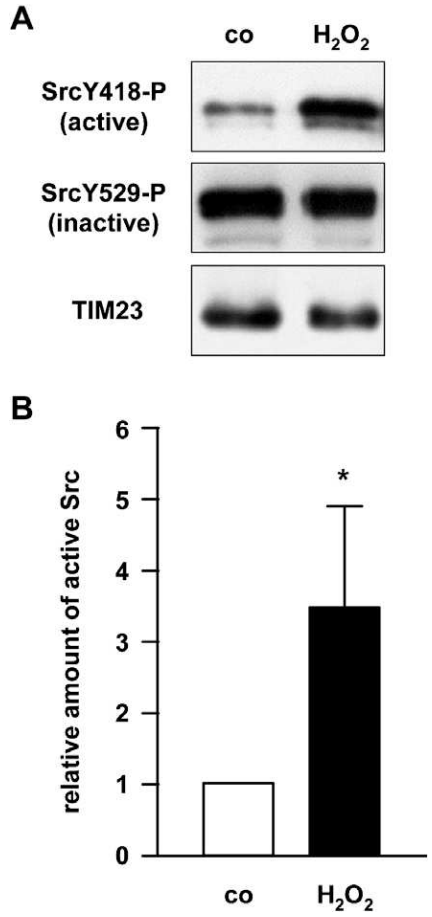

Fig. 2. Src kinase is activated by H_2O_2 in mitochondria. HUVEC were treated with 200 μM H_2O_2 for 3–4 h (H_2O_2) or left untreated (co) and mitochondrial lysates prepared as described in Section 2. The proteins were resolved by SDS–PAGE and blotted onto PVDF membranes. Blots were probed with antibodies against active Src phosphorylated on tyrosine 418 (SrcY418-P), inactive Src phosphorylated on tyrosine 529 (SrcY529-P) and translocase of inner membrane 23 (TIM23). (A) Representative blot. (B) Blots were quantitated densitometrically and the levels of SrcY418-P and SrcY529-P normalized to TIM23 levels. The graph shows the ratio of active to inactive Src. Data are mean +/− SEM of 4 independent experiments. * Significantly different to control ($p < 0.05$).

these two differently phosphorylated forms reflects the activity of Src in the mitochondria. Incubation with H_2O_2 increased the phosphorylation at tyrosine 418 within Src, whereas the phosphorylation at tyrosine 529 remained unaltered (Fig. 2A). Semi-quantitative analysis of the immunoblots revealed that exposure to H_2O_2 significantly increased the activity of the Src kinase in the mitochondria (Fig. 2B).

3.3. Mitochondrial Akt1 is deactivated by treatment with H_2O_2

We previously demonstrated that nuclear TERT is complexed with Akt1 and Heat Shock protein 90 (Haendeler et al., 2003b). Disruption of this complex resulted in inactivation of Akt1, which subsequently led to a reduction of nuclear TERT and to induction of apoptosis (Haendeler et al., 2003b). Therefore, we next determined whether Akt1 is also localized in the mitochondria. Indeed, Akt1 was identified in mitochondria of HEK293 cells as well as endothelial cells (Fig. 1A and B). Since Akt1 is negatively regulated by increased oxidative stress in endothelial cells (Hoffmann et al., 2001), we hypothesized that incubation with H_2O_2 could also reduce active Akt1 in the mitochondria. To determine active Akt1, we measured phosphorylation of Akt1 on serine 473 in mitochondria of endothelial cells. Incubation with H_2O_2 reduced active Akt1 (Fig. 3A and B). These data suggest that mitochondrial TERT and nuclear TERT are regulated similarly.

3.4. Downregulation of mitochondrial TERT by H_2O_2 depends on tyrosine 707 in TERT and thereby on the Src kinase

Having demonstrated that exposure to H_2O_2 increases mitochondrial Src kinase activity and decreases mitochondrial Akt1 activity, we next wanted to investigate whether mitochondrial TERT is regulated by H_2O_2. Taking into account that TERT is exported from the nucleus (Haendeler et al., 2003a) and that after nuclear export, the TERT level is increased in the cytosol and in the mitochondria (Ahmed et al., 2008; Haendeler et al., 2003a), we decided to use TERT exclusively targeted to the mitochondria (mitoTERT) containing a myc-tag to prevent detection of endogenous TERT, which may be newly imported into these organelles. After overexpression of myc-tagged mitoTERT in endothelial cells and treatment with 200 μM H_2O_2, we measured the levels of mitochondrial TERT by immunoblot. Interestingly, H_2O_2 induced a significant reduction of TERT in the mitochondria (Fig. 4A and B). From our previous studies, we knew that tyrosine 707 in TERT is a putative Src kinase phosphorylation site (Haendeler et al., 2003a; Jakob et al., 2008). Mutation of tyrosine 707 to phenylalanine resulted in complete inhibition of nuclear TERT export triggered by the Src kinase. Therefore, we inserted a mitochondrial targeting sequence into the TERT(Y707F) expression construct to

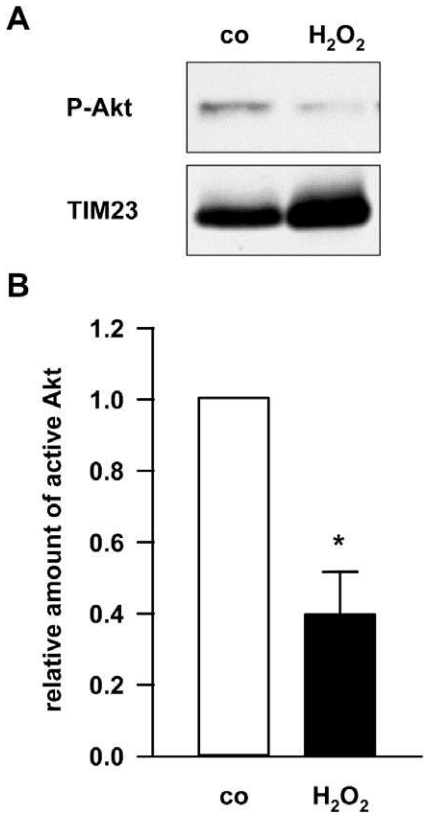

Fig. 3. Akt1 is deactivated by H_2O_2 in mitochondria. HUVEC were treated with 200 μM H_2O_2 for 3–4 h (H_2O_2) or left untreated (co) and mitochondrial lysates prepared as described in Section 2. The proteins were resolved by SDS–PAGE and blotted onto PVDF membranes. Blots were probed with antibodies against active Akt1 phosphorylated on Serine 473 (P-Akt) and translocase of inner membrane 23 (TIM23). (A) Representative blot. (B) Blots were quantitated densitometrically and the levels of active Akt1 (P-Akt) were normalized to TIM23 levels. Data are mean +/− SEM of 4 independent experiments. * Significantly different to control ($p < 0.05$).

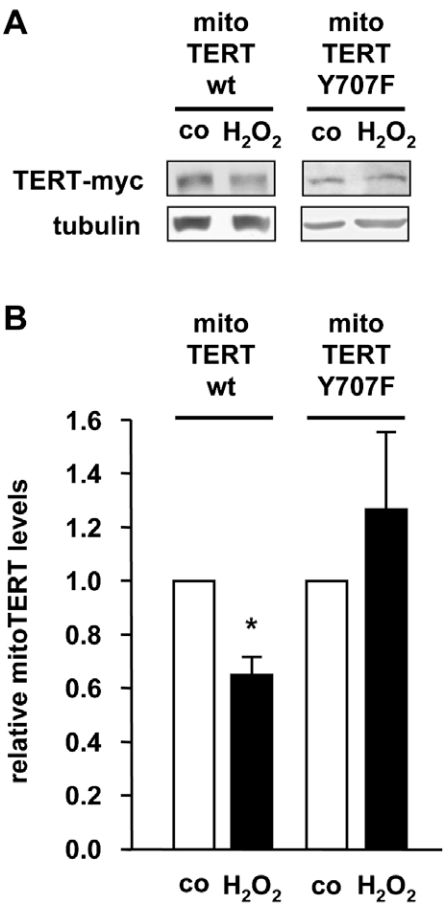

Fig. 4. Mitochondrial TERT wild-type, but not TERT(Y707F) is reduced by H_2O_2. HUVEC were transfected with expression vectors for mitochondrially targeted, myc-tagged human TERT wild-type (mitoTERT wt) or a mutant, in which tyrosine 707 was exchanged to phenylalanine (mitoTERT Y707F), treated with 200 μM H_2O_2 for 3–4 h (H_2O_2) or left untreated (co). Total lysates were prepared as described in Section 2, proteins resolved by SDS–PAGE and blotted onto PVDF membranes. Blots were probed with an anti-myc antibody (TERT-myc) and an antibody against tubulin. (A) Representative blot. (B) Blots were quantitated densitometrically and the levels of TERT normalized to tubulin. The graph shows the relative amount of TERT protein. Data are mean +/− SEM of 3–6 independent experiments. * Significantly different to untreated cells expressing mitochondrial TERT wild-type ($p < 0.05$).

force TERT(Y707F) into the mitochondria, to elucidate whether the downregulation of mitochondrial TERT levels is also dependent on the Src kinase. As shown in Fig. 4, downregulation of mitochondrial TERT by H_2O_2 was completely abrogated by mutation of this single tyrosine residue, demonstrating that the mitochondrial Src kinase is responsible for H_2O_2-induced downregulation of mitochondrial TERT.

4. Discussion

In our present study we investigated for the first time regulatory mechanisms of mitochondrial TERT. Here, we demonstrate that in primary human endothelial cells the Src kinase and Akt1 are localized in the mitochondria. The Src kinase is activated and Akt1 is deactivated upon stimulation with H_2O_2. H_2O_2 treatment results in downregulation of mitochondrial TERT levels, which is dependent on tyrosine 707 within TERT, indicating a phosphorylation-dependent mechanism.

The functions and role of nuclear TERT have extensively been studied and this years Nobel Prize in Physiology or Medicine was awarded for the discovery of how chromosomes are protected by telomeres and the enzyme telomerase, which underscores the importance of this enzyme. Therefore, it is important to understand the regulation of the catalytic subunit of telomerase, TERT. Over the last 10 years, it has become evident that nuclear TERT is not only regulated transcriptionally, but also post-translationally by binding proteins and phosphorylation. In human endothelial cells, we and others demonstrated that nuclear TERT requires protein kinase B (Akt) for its activation under basal conditions (Breitschopf et al., 2001; Haendeler et al., 2003b). In contrast, under conditions of oxidative stress, Src kinases are activated and TERT is exported from the nucleus, which results in the onset of replicative senescence and in increased apoptosis sensitivity (Haendeler et al., 2003a, 2004). Recent studies demonstrated a mitochondrial localization of TERT. However, the regulation of mitochondrial TERT is completely unknown. Several studies were undertaken to investigate, whether Akt1 and Src kinase are localized in mitochondria. Indeed, Akt1 and Src kinase were found to be localized in mitochondria of fibroblasts and rat brain, respectively (Antico Arciuch et al., 2009; Arachiche et al., 2008). Akt1 has been shown to cycle between the nucleus and the mitochondria dependent on the redox status of the cell (Antico Arciuch et al., 2009) and complexes of the respiratory chain have been demonstrated to be substrates of the Src kinase (Arachiche et al., 2008). Taking into account that mitochondrial TERT enhances respiratory chain activity and mitochondrial function (Haendeler et al., 2009) and the known regulation of TERT by the kinases Akt1 and Src in the nucleus as mentioned above, it is tempting to speculate that Akt1 and Src can also regulate mitochondrial TERT. Indeed, we also found the kinases Akt1 and Src in the mitochondria of primary human endothelial cells as well as human embryonic kidney cells (Figs. 1 and 2). Therefore, these kinases could also regulate mitochondrial TERT. Interestingly, H_2O_2 treatment led to a reduction in active Akt1, to an induction in active Src kinase and to a reduction in mitochondrial TERT levels. Thus, one may speculate that mitochondrial TERT is regulated in a similar fashion as nuclear TERT. This speculation is underscored by our finding that oxidative stress-induced downregulation of mitochondrial TERT (Fig. 4) like nuclear TERT (Haendeler et al., 2003a) depends on tyrosine 707. However, it has to be noted that export of nuclear TERT results in an increased amount of mitochondrial TERT (Ahmed et al., 2008). Taken these findings together, one has to suggest that oxidative stress initially leads to a reduction of TERT levels in the mitochondria, which is followed by a nuclear export of TERT and a concomitant mitochondrial import, but this is pure speculation and requires further experimental proof. However, the finding that an increased amount of TERT is found in the mitochondria after oxidative stress-induced nuclear export (Ahmed et al., 2008), suggests also that the mitochondrial Src kinase has to be inactivated at that time. Since we identified the tyrosine phosphatase Shp-2 as the counterplayer for Src dependent nuclear export of TERT (Jakob et al., 2008) and Arachiche et al. found that Shp-2 is also localized in the mitochondria of rat brain (Arachiche et al., 2008), it is tempting to speculate that Shp-2 inactivates the mitochondrial Src kinase.

In conclusion, our study demonstrates for the first time that the Src kinase and Akt1 are present also in the mitochondria of human endothelial cells and human embryonic kidney cells. Mitochondrial Src is activated, Akt1 is deactivated and mitochondrial TERT levels are downregulated by H_2O_2. This downregulation is dependent on a Src kinase dependent phosphorylation site, tyrosine 707, within TERT. Therefore, both mitochondrial and nuclear TERT might be regulated by the same mechanisms at least in primary human endothelial cells.

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The transcription factor Grainyhead like 3 (GRHL3) a new player in endothelial cell senescence, apoptosis and migration

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A. Mlynek: Auch Erstautor, führte zusammen mit Margarete Lukosz die Versuche und die Datenanalyse durch. Er war zudem am Entwurf des Manuskriptes beteiligt.

P. Czypiorski: Führte die Passagierungsversuche in Endothelzellen und die RNA-Isolation durch. Zudem gab er Feedback zum Manuskript.

J. Altschmied: Senior Autor, Identifizierte GRHL3 in Mammakarzinomzellen. Er klonierte alle Konstrukte und war an der Versuchsplanung der Luciferase-Assays beteiligt. Er gab Feedback zum Manuskript und war für die finale Abnahme der Abbildungen verantwortlich.

J. Haendeler: Senior Autorin, hatte die Idee zur Studie, war an der Versuchsplanung beteiligt und half Philip Czypiorski mit den Passagierungsversuchen. Sie schrieb das finale Manuskript.

Submission Confirmation

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The transcription factor Grainyhead-like 3 (GRHL3) a new player in endothelial cell senescence, apoptosis and migration

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Abstract

Migratory capacity and resistance to apoptosis are crucial for proper endothelial function. In a screen for anti-apoptotic genes in a breast cancer cell line, we identified Grainyhead like 3 (GRHL3). Therefore, the aim of our study was to investigate whether GRHL3 is expressed in endothelial cells (EC) and moreover, to determine its role in migration, apoptosis and senescence. GRHL3 is expressed in human EC. GRHL3 is required for endothelial cell migration. The underlying mechanism is independent of vascular endothelial growth factor. GRHL3 induces Akt and eNOS phosphorylation and its expression is increased by physiological concentrations of nitric oxide. Nitric oxide dependent migration is completely dependent on GRHL3 expression. Moreover, GRHL3 inhibits apoptosis of endothelial cells in an eNOS-dependent manner. Src kinases act as negative regulator of GRHL3. Inhibition of Src kinase activation by PP2 enhances GRHL3 expression and migration. Finally, activation of Src kinases is increased and GRHL3 expression is decreased in senescent endothelial cells, which results in decreased migratory capacity. Thus, loss of GRHL3 may result in endothelial dysfunction in vivo. One may consider new therapeutic strategies with the aim to conserve GRHL3 expression in the vasculature.

Introduction

Migratory capacity and resistance to apoptosis are crucial for proper endothelial function. One of the prerequisites for vascular aging as well as the development of atherosclerosis and other cardiovascular diseases is endothelial dysfunction. The early onset of vascular aging processes and atherosclerosis is accompanied by apoptosis and reduced migratory capacity of endothelial cells. Therefore, it is important to fully understand the molecular mechanisms involved in endothelial cell migration and apoptosis. Two important players in endothelial cell migration and apoptosis are the vascular endothelial growth factor (VEGF) and nitric oxide (NO). VEGF is required for developmental angiogenesis and tumor angiogenesis (Takeshita *et al.* 1994; Ferrara *et al.* 1996; Suri *et al.* 1996; Holash *et al.* 1999). NO has been shown to act as a vasodilator and it has anti-apoptotic, pro-migratory and anti-thrombotic properties (Haendeler *et al.* 1999; Berk *et al.* 2001; Dimmeler *et al.* 2002). Interestingly, a mammalian homolog of the Drosophila transcription factor grainyhead, grainyhead-like 3 (GRHL3), has been shown to be essential for cell migration. GRHL3 deficient mice die shortly after birth and show a pronounced spina bifida and defects in wound healing (Ting *et al.* 2005). These data together with knock down experiments in keratinocytes and analyses of keratinocytes isolated from GRHL3-deficient mice demonstrated a pivotal role for GRHL3 in epidermal cell migration (Hislop *et al.* 2008; Caddy *et al.* 2010). Furthermore, we identified GRHL3 in a screen for TNFα–induced survival genes in a breast cancer cell line (Guardiola-Serrano *et al.* 2008). Taken these findings together GRHL3 seems to be important for migration and protection against apoptosis. However, up to now, it is unknown whether GRHL3 is expressed in endothelial cells and whether it affects endothelial cell migration and apoptosis. Therefore, the aim of the present study was to investigate whether GRHL3 is expressed in human endothelial cells (EC), which

pathways are regulated by GRHL3 or regulate GRHL3 expression and which role GRHL3 has in migration and apoptosis of EC. Here, we identify GRHL3 as a new molecule expressed in EC. GRHL3 transcript levels are increased by NO. GRHL3 enhances activation of Akt and endothelial nitric oxide synthase (eNOS), but has no effect on VEGF production. Migration of EC depends on GRHL3 expression. GRHL3 acts anti-apoptotic in an eNOS dependent manner. GRHL3 expression is increased by long term blockade of Src kinase, a treatment known to inhibit stress-induced senescence in EC (Haendeler *et al.* 2004). In line with this finding, senescent EC show reduced GRHL3 expression and migratory capacity. Thus, we conclude that GRHL3 is a new, central mediator in migration and apoptosis of EC and is also regulated by senescence-related changes of in these cells.

Results

GRHL-3 is expressed in EC and essential for their migratory capacity

In a screen for survival genes in breast cancer cells, we identified GRHL3 (Guardiola-Serrano *et al.* 2008). GRHL3 is a transcription factor, which belongs to a large family of genes encoding developmental transcription factors. The founding member of the family is the Drosophila gene grainyhead. Mice lacking GRHL3 display defective skin barrier function and wound repair of the skin. They die shortly after birth due to a neural tube closure defect (Ting *et al.* 2005). GRHL3 knockdown in human keratinocytes strongly impairs their migration (Caddy *et al.* 2010). The cumulative evidence points to a role of GRHL3 in migratory capacity of cells. Endothelial dysfunction is accompanied by reduced migratory capacity of EC. Migration of EC contributes to the homeostasis of blood vessels. However, up to now it is unknown whether GRHL3 is expressed in EC and whether it plays a role in the maintenance of endothelial function. Therefore, we first determined whether GRHL3 is expressed in human EC. We could detect the transcript for the homolog of mouse GRHL3 in human EC and EaHy (Figure 1A, 3A and 5D). Unfortunately, up to now there is no antibody available, which recognizes endogenous human GRHL3. Thus, we had to perform all our experiments on endogenous GRHL3 on RNA. To test our hypothesis that this transcription factor is important for EC migration, we wanted to knock down GRHL3. Therefore, we first analyzed 5 different shRNAs against GRHL3. After verifying the knock down of GRHL3 by RT-PCR (figure 1A), we assessed the migratory capacity of EC, in which GRHL3 expression was reduced. As shown in figure 1B, basal migration of EC was significantly reduced. Together with our previous observation that overexpression of GRHL3 induces migration of EC (Guardiola-Serrano *et al.* 2008), these data demonstrate that GRHL3 plays a role in EC migration.

VEGF is not involved in GRHL3 dependent EC migration

Since GRHL3 is important for EC migration, we wanted to elucidate the underlying mechanisms. One of the most important contributors to EC migration and angiogenesis is vascular endothelial growth factor (VEGF). To determine whether overexpression of GRHL3 enhances VEGF production in EC, we transfected a GRHL3 expression vector into EC and measured VEGF in cell lysates. Although, overexpression of GRHL3 was confirmed (Figure 2A), we could not detect any change in VEGF in the same samples (Figure 2B). Thus, we believe that the strong migratory effect of GHRL3 is not due induction of VEGF. Therefore, we conclude that VEGF is not involved in GRHL3 dependent migration.

Role of NO on GRHL3 dependent EC migration

Another extremely important factor for endothelial function is NO. NO acts pro-migratory and anti-apoptotic on EC. It has been demonstrated that VEGF activates eNOS and thereby acts pro-angiogenic (Shizukuda *et al.* 1999; Dimmeler *et al.* 2000; Radisavljevic *et al.* 2000). On the other hand, NO can induce migration and angiogenesis independently of VEGF (Urbich *et al.* 2002). Therefore, we investigated the effect of physiological concentrations of NO on GRHL3 expression. Low doses of NO, which are known to induce migration of EC and act anti-apoptotic (Dimmeler *et al.* 1997; Haendeler *et al.* 2002; Urbich *et al.* 2002), increased GRHL3 expression (Figure 3A). Next, we analyzed the effects of GRHL3 knockdown (Figure 1B) on NO-induced migration of EC. Reduction of GRHL3 transcript levels completely abrogated NO-dependent migration (Figure 3B). Thus, GRHL3 is a central mediator of the pro-migratory effects of NO. To further investigate whether GRHL3 also influences NO production in EC, we overexpressed GRHL3 in EC and measured eNOS activation. Transfection of the GRHL3 expression vector into EC led to increased phosphorylation and thereby activation of eNOS on serine 1179 (Figure 3C). The

activation of eNOS on serine 1179 depends on Akt (Dimmeler *et al.* 1999; Fulton *et al.* 1999), thus, we also assessed the effect of GRHL3 on Akt phosphorylation. GRHL3 overexpression increased Akt phosphorylation on serine 473 in EC (figure 3D). Since enhanced eNOS activation leads to increased S-NO molecules, we determined the amount on S-NO molecules, which are important for proper endothelial function and reduced in EC senescence (Hoffmann *et al.* 2001). As expected, GRHL3 overexpression increased the S-NO content (1.97 fold +/- 0.31). These data demonstrate that NO enhances GRHL3 expression and conversely, GRHL3 increases NO production in EC. In addition, NO-induced migration depends on GRHL3 expression. However, GRHL3 is a transcription factor, which can not phosphorylate Akt and/or eNOS directly, suggesting that it modulates one or more upstream regulators of Akt on the transcriptional level. Indeed, we demonstrate that our GRHL3 expression construct codes for an active transcription factor, which can activate a luciferase reporter with a tandem GRHL3 binding site, but not a corresponding reporter, in which the binding sites are mutated (Figure 3E).

GRHL3 acts anti-apoptotic in an eNOS-dependent manner

Having demonstrated that overexpression of GRHL3 increases Akt and eNOS activation, which are both known to be required for apoptosis protection, we hypothesized that GRHL3 is not only pro-migratory but also anti-apoptotic. Therefore, we overexpressed GRHL3 in EC and measured apoptosis. GRHL3 inhibited apoptosis in EC (Figure 4). Protection from apoptosis by GRHL3 was dependent on eNOS activation, since blockade of the enzyme by L-NMMA completely abrogated the anti-apoptotic effect of GRHL3 (Figure 4). Thus, we identified with GRHL3 a new NO-dependent, pro-migratory and anti-apoptotic protein in EC.

Regulation of GRHL3 by Src kinases – role in migration and senescence of EC

It has been demonstrated that Src kinases play a double-edged role in EC. Short-term activation (up to 120 min) of Src kinases is involved in VEGF-dependent signalling (Eliceiri *et al.* 1999; Tanimoto *et al.* 2002). In contrast, long-term activation (18 h to 14 days) of Src kinases has been shown to induce apoptosis and cellular senescence of EC and tumor formation (Haendeler *et al.* 2003; Haendeler *et al.* 2004; Chen *et al.* 2008). Therefore, we next investigated the role of Src on GRHL3 expression by long-term blockade of its activation. Incubation with the Src kinase inhibitor PP2 for 19 h enhanced GRHL3 expression (Figure 5A), repressed phosphorylation of Src on tyrosine 418 (Figure 5B) and increased EC migration (Figure 5C). These data suggest that the pro-migratory and anti-apoptotic effects of GRHL3 depend on the activation of Akt and eNOS and on the inactivation of Src kinases. We have previously demonstrated that eNOS is reduced and Src kinase activity is increased in senescent EC (Hoffmann *et al.* 2001; Haendeler *et al.* 2004). Thus, we hypothesized that GRHL3 expression is downregulated in senescent (passaged) EC. As shown in figure 5D, GRHL3 expression is reduced in senescent EC. Since, we published previously that Src kinases are activated in senescent EC and that inhibition of Src kinases delayed EC senescence (Haendeler *et al.* 2004), we believe that the long term activation of Src kinases in senescent EC leads to reduction in GRHL3 expression (figure 5A). This is in line with our findings shown in figure 5A that inhibition of Src kinases leads to increased GRHL3 expression. Given the fact that GRHL3 is required for EC migration, we next determined whether the migratory capacity is reduced in senescent EC, which show reduced GRHL3 expression (figure 5D). Indeed, the migratory capacity of these cells is also diminished (figure 5E). We next wanted to investigate a causal relationship between Src kinases activation and GRHL3 expression by using knock down approaches of the Src kinases. However, to successfully reduce Src kinase activation in EC, a triple

knock down of the kinases Src, Fyn and Yes is needed, which we could not perform in EC as we published previously (Jakob *et al.* 2008). Therefore, we made use of mouse embryonic fibroblasts deficient for Src, Fyn and Yes, knowing that these fibroblasts are from mouse and not human origin and that fibroblasts and EC have different signalling pathways in senescence. Nevertheless, we found increased GRHL3 expression in Src, Fyn, Yes deficient fibroblasts (data not shown).

Taken these data together, we hypothesize that a loss of GRHL3 is dependent on increased Src kinases activation and contributes to the reduced migratory capacity and enhanced sensitivity towards apoptotic stimuli in senescent EC (Hoffmann *et al.* 2001; Murasawa *et al.* 2002; Thum *et al.* 2007).

Discussion

The present study for the first time identified the transcription factor GRHL3 in human EC and its requirement for migration and apoptosis protection. We have shown that GRHL3 enhances Akt and eNOS activation and that it is absolutely required for basal and in NO-induced EC migration. Moreover, GRHL3 acts anti-apoptotic in an eNOS dependent manner and is negatively regulated by long-term activation of the Src kinases.

GRHL3 belongs to a large family of genes encoding developmental transcription factors. We demonstrate here that GRHL3 is expressed in EC and required for migration. This is in line with findings in keratinocytes, in which a knockdown of GRHL3 induced defects in actin polymerization and wound healing, implicating GRHL3 as a central mediator in epidermal repair (Caddy *et al.* 2010). Furthermore, keratinocytes isolated from GRHL3 deficient mice fail to close artifical wounds set in a scratch wound assay (Hislop *et al.* 2008). Similarly, we here present data that knock down of GRHL3 reduces the migratory capacity of EC. It has to be noted that the migration of keratinocytes mainly relies on proliferation, whereas the migration of EC, which is dependent on NO, is nearly completely proliferation independent. Therefore, it is not astonishing that GRHL3 regulates different pathways in keratinocytes and EC. Whereas GRHL3 activates the extracellular regulated kinases 1 and 2 (ERK1/2), known inducers of proliferation, in keratinocytes, it enhances activation of Akt and eNOS, known pro-migratory and anti-apoptotic molecules, in EC. One interesting question is how a transcription factor like GRHL3 can induce phosphorylation of Akt and eNOS. Since our GRHL3 expression construct codes for an active transcription factor, one has to assume that GRHL3 modulates upstream regulators of Akt and/or eNOS on the transcriptional level. However, the number of known upstream regulators of Akt/eNOS is manifold and it is not clear whether all of

the regulators are already known by now. To answer this important question, we will perform microarray analyses in EC, in which either the expression of GRHL3 transcript levels is reduced or GRHL3 is overexpressed. From the data obtained in these experminents, we hope to reduce the number of potential candidates involved in activation of Akt and/or eNOS in the future.

Besides its pro-migratory function, GRHL-3 acts also anti-apoptotic. Interestingly, the anti-apoptotic effect of GRHL3 completely depends on eNOS activation. It has been demonstrated that Caspase-3 is one of the most important effector molecules for apoptosis induction in EC (Dimmeler *et al.* 1997). Since NO inhibits Caspase-3 by S-nitrosation and thereby blocks apoptosis in EC (Dimmeler *et al.* 1997), it is tempting to speculate that the anti-apoptotic effect of GRHL3 involves inhibition of Caspase-3 activation.

Another interesting aspect of the study presented here, is that GRHL3 does not increase VEGF levels in EC. GRHL3 has been demonstrated to be essential for epidermal wound repair (Ting *et al.* 2005; Hislop *et al.* 2008; Caddy *et al.* 2010), a process in which VEGF has also been implicated (Barrientos *et al.* 2008; Schiefelbein *et al.* 2008). However, none of the studies on GRHL3 and epidermal wound repair reported VEGF to be involved in GRHL3 dependent pathways (Ting *et al.* 2005; Hislop *et al.* 2008; Caddy *et al.* 2010). Thus, one could hypothesize that GRHL3 and VEGF activate two independent pathways in both organs leading to the same endpoints, epidermal wound repair and EC migration, respectively.

Furthermore, short term inhibition of Src kinases blocks VEGF signaling in EC (Tanimoto *et al.* 2002), whereas long term inhibition of Src kinases protects EC from apoptosis and stress induced senescence (Haendeler *et al.* 2003; Haendeler *et al.* 2004). Interestingly, short term inhibition of Src kinases (3 h) did not influence GRHL3 expression (unpublished observation), whereas long term inhibition

enhances GRHL3 expression and EC migration. These findings further support the notion that GRHL3 acts independently of VEGF and that GRHL3 dependent migration involves inhibition of the Src kinases. Long term activation of these kinases is important for the onset of age-related changes in EC like the known reduction in Telomerase Reverse Transcriptase (TERT) (Haendeler *et al.* 2004). Here, we show that GRHL3 expression and migratory function is reduced in senescent EC. Along the same lines GRHL3 expression is reduced in brains of 2 year old black 6 mice (data not shown). This is in line with findings that another member of the grainyhead family, GRHL2, enhances human TERT gene expression in keratinocytes (Chen *et al.* 2010). As GRHL2 and GRHL3 are known to interact with each other (Ting *et al.* 2003), increased GRHL3 expression may also lead to a reduction of age-related changes in EC.

Taken our data together, we present here for the first time that the transcription factor GRHL3 is expressed in EC. GRHL3 has pro-migratory and anti-apoptotic properties. NO and inhibition of Src kinases increased GRHL3 expression. In senescent EC, in which NO bioavailability is reduced and activity of Src kinases is increased, GRHL3 expression is diminished. Since, basal as well as NO-induced migration is fully dependent on GRHL3 expression, migration is also reduced in senescent EC. Therefore, GRHL3 seems to be a central mediator in the maintenance of endothelial function. Loss of GRHL3 may result in endothelial dysfunction in vivo. Thus, one may consider new therapeutic strategies with the aim to conserve GRHL3 expression in the vasculature.

Experimental Procedures

Cell culture

Human primary umbilical vein endothelial cells (EC) and the human EaHy endothelial cell line (EaHy) were cultured as previously described (Edgell *et al.* 1983; Haendeler *et al.* 2002). Senescence induction by passaging of EC was performed as previously described (Hoffmann *et al.* 2001). After detachment with trypsin, cells were grown for at least 18 h as described previously (Schroeder *et al.* 2007; Haendeler *et al.* 2009). Human embryonic kidney cells (HEK293) were cultured as described previously (Jakob *et al.* 2008).

Plasmids and Transfection

The expression vector for human GRHL-3 has been described previously (Guardiola-Serrano *et al.* 2008). For knockdown experiment plasmids from the MISSION® shRNA series (SIGMA ALDRICH, Munich, Germany) were used. GRHL3 specific reporter plasmids were constructed by inserting a double-stranded oligonucleotide encompassing two consensus GRHL3 binding sites or mutants thereof (Ting *et al.* 2005) upstream of a minimal promoter in the luciferase vector pTATALUC+ (Altschmied & Duschl 1997). Sequences for the shRNAs and the GRHL3 binding sites are available upon request.

EC were transiently transfected with Superfect (QIAGEN, Hilden, Germany) and HEK293 with Lipofectamine-PLUS (INVITROGEN, Darmstadt, Germany) as described previously (Jakob *et al.* 2008).

Luciferase assays

Two days after transfection cells were lysed with Reporter Lysis Buffer (PROMEGA, Mannheim, Germany) according to manufacturer's instructions. Equal amounts of cellular protein were made up to 20 µl with Reporter Lysis Buffer and luciferase activity was determined by automatic injection of 100 µl luciferase assay mix (20 mM

Tricine-KOH, pH 7.8, 0.1 mM EDTA, 8 mM $MgCl_2$, 33.3 mM DTT, 0.27 mM Coenzyme A, 0.53 mM ATP, 0.47 mM D-luciferin).

Migration assays

Migration assays with ECs were performed with a scratch wound assay as described previously (Spyridopoulos *et al.* 2008).

Determination of VEGF

The concentration of VEGF in cell lysates was determined using a commercially available ELISA (RAY BIOTECH, Norcross, GA, USA) according to manufacturer's specifications. For lysate samples 50 µg of total protein were used.

Reverse transcriptase PCR (RT-PCR)

RNA was isolated using Trizol (INVITROGEN, Darmstadt, Germany) according to manufacturer's specifications. RNA concentrations were determined photometrically and RNA integrity was assessed by agarose gel electrophoresis. 5 µg total RNA were digested with RQ1 RNase-free DNase (PROMEGA, Mannheim, Germany) and 2 µg of the DNAse digested RNAs were reverse transcribed using M-MLV Reverse Transcriptase or Superscript™ III (INVITROGEN, Darmstadt, Germany) according to manufacturer's specifications. Amplification was performed with gene-specific primers under standard reaction conditions, or, for quantitation with RealTime PCR using reagents supplied by the corresponding manufacturer. For comparison of relative gene expression levels the ΔΔCT method was used or semi-quantitative evaluation of band intensities using ImageJ 1.42q (Abramoff *et al.* 2004). Primer sequences are available upon request.

Immunoblotting

Immunoblotting was performed with antibodies directed against Akt (1:500), phospho-Akt (S473, 1:500), eNOS (1:1000) and phospho-Akt (S1179, 1:500) (overnight, 4°C, BECTON & DICKINSON, Karlsruhe, Germany), phospho-Src (Y418,

1:500, overnight, 4°C, CELL SIGNALING/NEW ENGLAND BIOLABS, Frankfurt, Germany), V5 (1:1000, overnight, 4°C, INVITROGEN, Darmstadt, Germany), GAPDH (1:8000), Src (1:250) (overnight 4°C, SANTA CRUZ, Heidelberg, Germany). Semi-quantitative analyses were performed on scanned immunoblots using ImageJ 1.42q (Abramoff *et al.* 2004).

S-NO content

S-NO content in EC was detected as described previously (Haendeler *et al.* 2002).

Detection of cell death by FACS

Detection of cell death was performed by FACS analysis using annexin V-APC or annexin V-FITC binding and 7-Amino-actinomycin (7AAD) staining (BD PHARMINGEN, Heidelberg, Germany). In brief, cells were trypsinized of the dish and pelleted. After washing twice with annexin binding buffer cell pellets were resuspended in annexin binding buffer and incubated with 2.5 ng/ml annexin V-APC and 2.5 ng/ml 7AAD for 20 min and analyzed in a flow cytometer. For subsequent western blot analysis cells were lysed and subjected to SDS-page.

Statistics

Statistical analyses were performed with student's T-test or Mann-Whitney-U-test using winLSTAT 2008.

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Author contributions

M. Lukosz and A. Mlynek collected data, performed statistical analyses and wrote first draft of the manuscript. P. Czypiorski passaged EC for three independent biological replicates and prepared RNA, fruitful discussion about the content of the manuscript. J. Altschmied first identified GRHL3 in the breast cancer screen, cloned all constructs, had supervision over all luciferase assays, critical and carefully reading of the manuscript, final figure set up. J. Haendeler had the idea, had supervision over all experiments in EC, helped with the EC passaging model, wrote the final manuscript.

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Figure legends

Figure 1: GRHL3 is expressed in EC and required for migration

EC were transfected with expression vectors for a GRHL3-specific shRNA (shGRHL3) or a non-specific control (shSCR). (A) GRHL3 expression was analyzed by RT-PCR, an ERK 1 RT-PCR served as control for cDNA integrity. (B) Migration was analyzed by scratch wound assay. Top: representative image of migrated cells, bottom: migrated cells per high power field were counted, data are shown as mean ± SEM (n=6, *p<0.05).

Figure 2: VEGF is not involved in GRHL3 dependent migration

EC were transfected with an expression vector for V5-tagged GRHL3 or an empty control vector (EV). (A) Overexpression of V5-tagged GRHL3 was confirmed by western blotting using a V5-antibody. Equal loading was assessed with an anti-GAPDH antibody. (B) VEGF-A was measured in cell lysates. Data are shown as mean ± SEM relative to EV (n=5).

Figure 3: NO induces GRHL3 and GRHL3 expression activates eNOS

(A) EC were treated for 19 h with the NO donor PAPA NONOate (NO, 10 µM) or left untreated (con) and GRHL3 expression was assessed by real-time PCR. Data were normalized to actin and are shown as mean ± SEM relative to untreated cells (n=3, *p=0.05). (B) EC were transfected with expression vectors for a GRHL3-specific shRNA (shGRHL3) or a non-specific control (shSCR) and treated with PAPA NONOate as described in (A). Migration was analyzed by scratch wound assay (n=3, *p<0.05). (C and D) EC were transfected with an expression vector for V5-tagged GRHL3 or an empty control vector (EV). Phosphorylation of eNOS on serine 1179 and Akt on serine 473 was analyzed by Western blot and normalized to total levels of the two proteins. Top: representative blots, bottom: densitometric quantitation (n=3, *p<0.05). (E) HEK293 were cotransfected with the indicated luciferase reporter

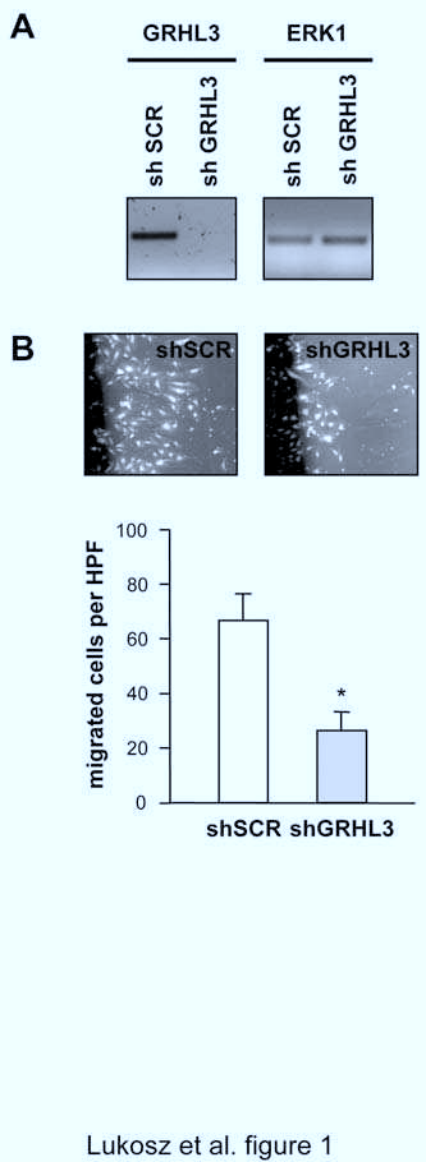
constructs and a GRHL3 expression vector (+) or an empty control vector (-). Luciferase activity was normalized to cotransfections with the empty vector (n=11, *p<0.05).

Figure 4: GRHL3 inhibits apoptosis in an eNOS-dependent manner

EC were transfected with an empty control vector (EV) or an expression vector for V5-tagged GRHL3. The latter were left untreated or treated with 1 mM L-NMMA for 19 h. Overexpression was confirmed after annexin V staining by western blotting with an anti-V5 antibody and GAPDH served as loading control (A). Apoptosis was measured by annexin V staining (B). Data are mean ± SEM (n=4, *p<0.05 vs. GRHL3).

Figure 5: Src kinases negatively regulate GRHL3 expression and EC migration

(A) EC were treated with 10 µM PP2 for 19 h or left untreated (con) and analyzed for GRHL3 expression by real-time PCR. Data were normalized to actin and are shown as mean ± SEM relative to untreated cells (n=4, *p<0.05). (B and C) EC were treated with 10 µM PP2 for 19 h or left untreated (con). (B) Phosphorylation of Src on tyrosine 418 was assessed by Western blot and compared to total Src protein levels. (C) Migration was analyzed by scratch wound assay. Data are mean ± SEM (n=4, *p<0.05). (D) GRHL3 expression in young (passage 3) and senescent (passage 11) EC was determined by RT-PCR and analyzed semiquantitatively using ImageJ. RPL32 was used for normalization. Top images: agarose gels for GRHL3 and RPL32. Lower image: semiquantitative analysis of RT-PCR products. Data are mean ± SEM (n=3, *p<0.05). (E) Migration of young (passage 3) and senescent (passage 11) EC was analyzed by scratch wound assay. Data are mean ± SEM (n=3, *p<0.05).



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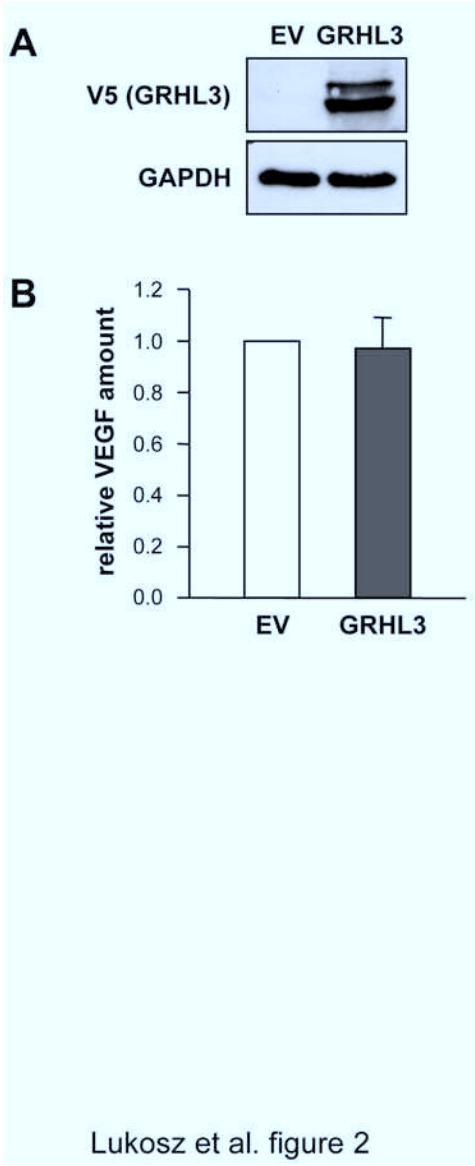

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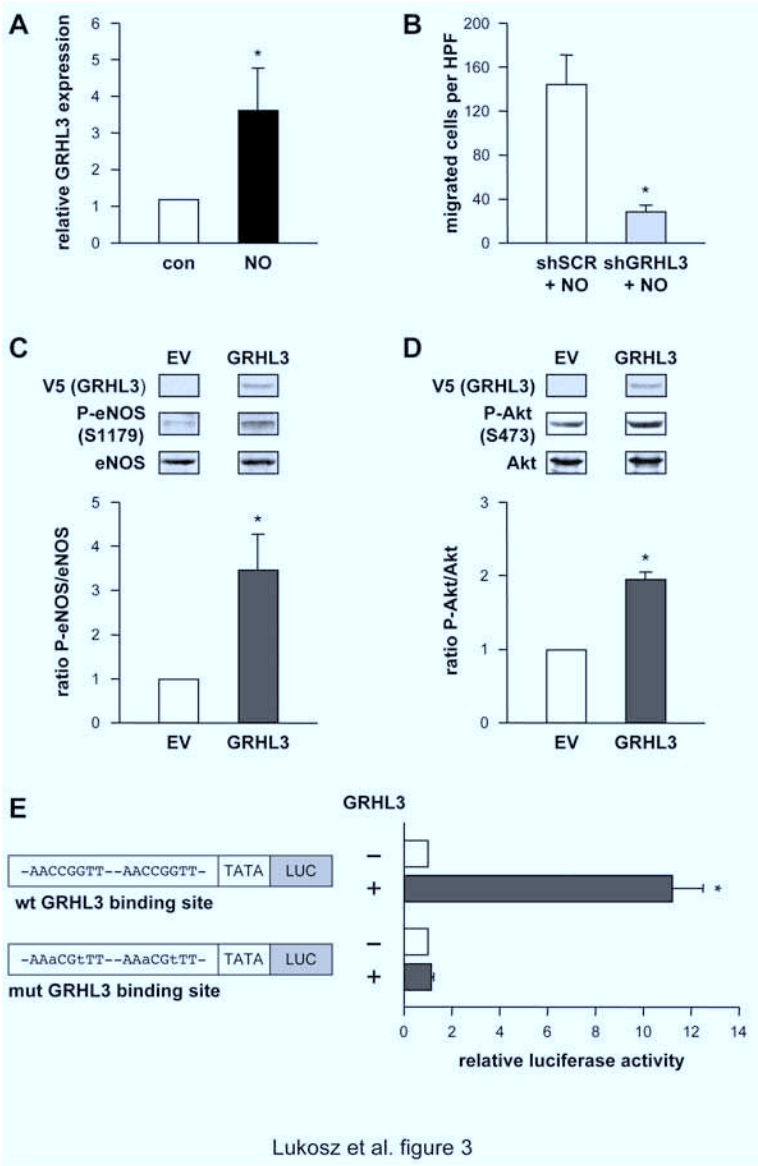

Lukosz et al. figure 3

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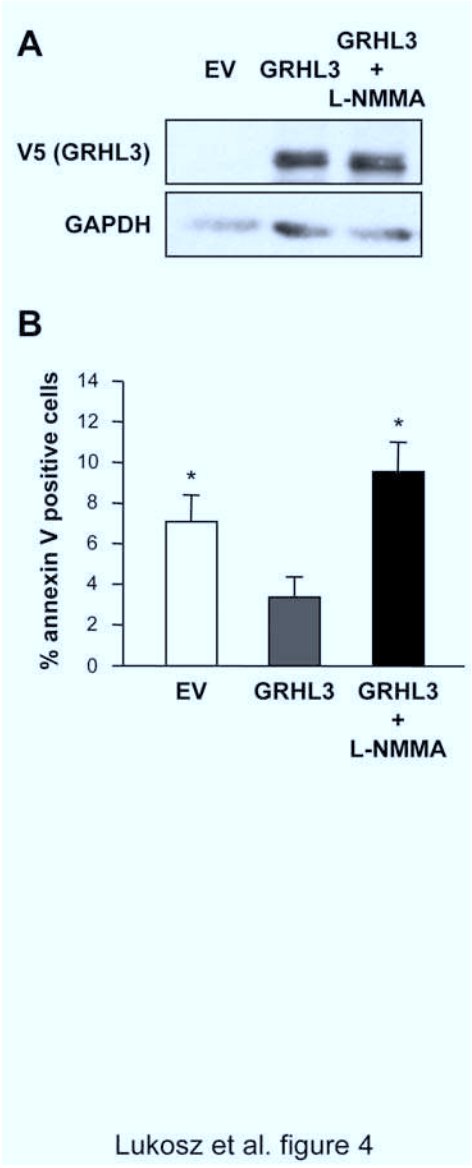

Lukosz et al. figure 4

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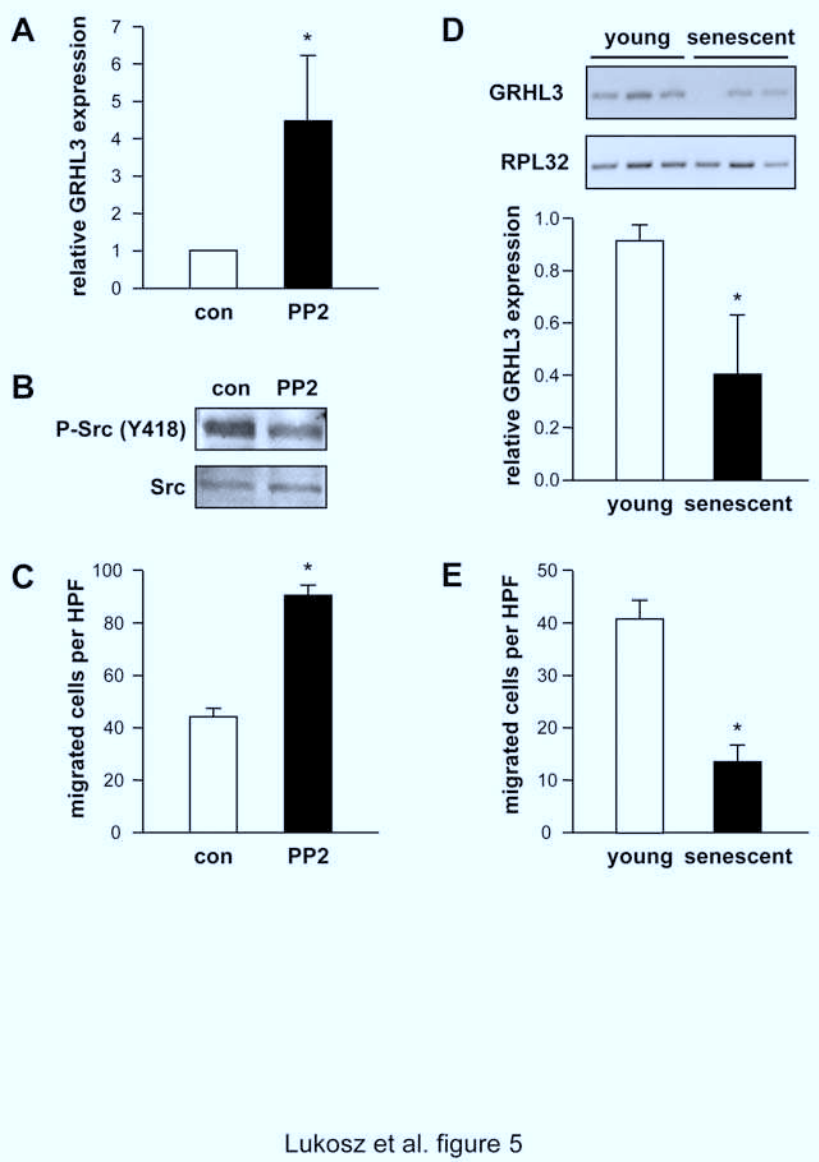

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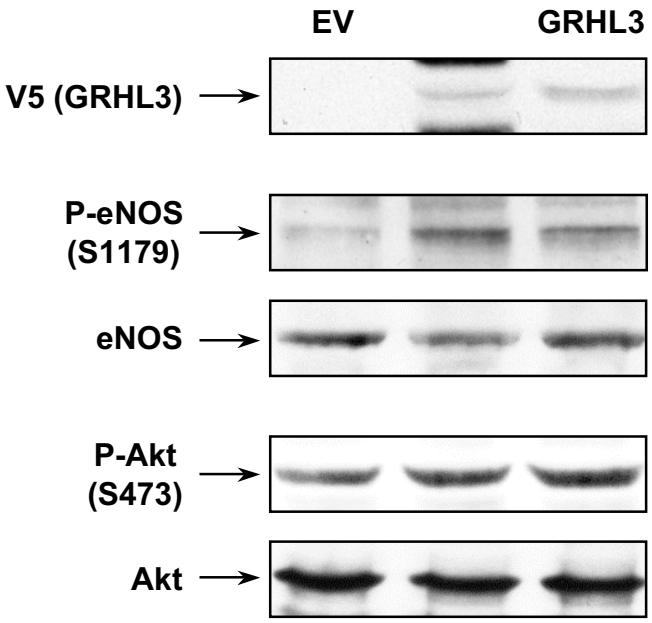

Lukosz et al. original blots from figure 3

Two isoforms of the transcription factor Sister of Mammalian Grainyhead derived from an alternatively spliced transcript have opposing functions in cells and in vivo

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Key Words:	transcription factor, isoforms, Sister of Mammalian Grainyhead, human endothelial cells, zebrafish

Two isoforms of the transcription factor Sister of Mammalian Grainyhead derived from an alternatively spliced transcript have opposing functions in cells and in vivo

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ABSTRACT

Sister of Mammalian Grainyhead (SOM) is a member of the Grainyhead family of transcription factors. In humans, three isoforms of SOM are found. SOM1 and SOM3 do not exist in mice and are encoded by splice variants of one primary transcript, whereas SOM2 is derived from a mRNA with a different first exon. SOM1 and SOM3 are co-expressed in numerous normal tissues, however, their cellular functional relevance has not been investigated so far. Here, we demonstrate that SOM1 and SOM3 are expressed in primary human endothelial cells (EC). Overexpression of SOM1 inhibits apoptosis and induces migration of EC, which is dependent on the activation of protein kinase B/Akt and endothelial nitric oxide synthase. In contrast, SOM3 has no effect on apoptosis and significantly inhibits migration. To address the in vivo relevance of the opposing effects of SOM1 and SOM3, we made use of zebrafish as a model system. SOM3, but not SOM1, induces malformations in embryos and reduces the number of normally developed embryos significantly. These results demonstrate that not only in primary human EC, but also in a whole animal SOM1 and SOM3 have opposing effects with SOM3 acting deleteriously.

INTRODUCTION

The grainyhead transcription factor family is highly conserved during evolution. The Drosophila transcription factor grainyhead (Grh) is the founder of this family (1). In vertebrates three Grh-homologues are known, Mammalian Grainyhead (MGR, GRHL1), Brother of Mammalian Grainyhead (BOM, GRHL2) and Sister of Mammalian Grainyhead (SOM, GRHL3) (2,3). SOM has been described to be required for cell migration. This is evident in SOM deficient mice. They die shortly after birth and show a pronounced spina bifida and defects in wound healing, which seemingly depends not on proliferation but on a loss of migratory capacity (4,5). Along the same lines, knock down experiments in keratinocytes and analyses of keratinocytes isolated from SOM deficient mice showed an essential role for SOM in epidermal cell migration (6,7). Recently, we identified SOM in a screen for TNFα–induced survival genes in a mammary carcinoma cell line and overexpression of the human homologue of the murine SOM in human endothelial cells (EC) strongly increased migration (8). However, in contrast to mice, in humans three isoforms of SOM are found. They are derived from different first exon usage and alternative splicing of one of the primary transcripts yielding isoforms SOM1 and SOM3, which do not exist in mice. Alternative splicing is a general phenomenon (9) and is also found in many transcription factor families, e.g. nuclear hormone receptors (10), SMADs (11), and the p53 family (12). The resulting protein isoforms can have different effects on cellular functions. SOM1 and SOM3 are co-expressed in several human tissues (2). However, their functions on the cellular level and in vivo have never been analyzed. Having shown that SOM2, the human homologue of the single mouse SOM protein, which is derived from a different transcript than SOM1 and SOM3, affects EC migration (8), we now wanted to investigate whether SOM1 and SOM3 regulate essential functions of EC, especially apoptosis, migration and nitric

oxide (NO) bioavailability. To further support our cell culture findings, we also investigated the role of SOM1 and SOM3 in zebrafish.

Here, we demonstrate that SOM1 and SOM3 are and both expressed in primary human EC and are active transcription factors. Overexpression of SOM1 inhibits apoptosis, increases the migratory capacity and the amount of bioactive NO by enhancing endothelial NO-Synthase (eNOS) activity in EC. In contrast SOM3 has no anti-apoptotic effect, reduces migration and eNOS phosphorylation/activation. Moreover, SOM3 induces severe malformations and diminishes the number of normal zebrafish embryos in vivo. Thus, we conclude that SOM3, but not SOM1 has deleterious effects in primary cells and in vivo.

MATERIAL AND METHODS

Plasmid constructs

The 5'-ends of the SOM1 and SOM3 coding sequences were amplified from MCF-7 cell cDNA with isoform specific primers and inserted into the expression vector described previously (8). The vectors for in vitro transcription were constructed by transferring BamHI/PmeI fragments from the SOM expression vectors into pCS2+ opened with BamHI and SnaBI (13). SOM specific reporter plasmids were constructed by inserting a double-stranded oligonucleotide encompassing two consensus SOM binding sites or mutants thereof (4) upstream of a minimal promoter in the luciferase vector pTATALUC+ (14). Sequences for PCR primers and the SOM binding sites are available upon request.

Cell culture

Human primary umbilical vein endothelial cells (EC) were cultured as previously described (15). After detachment with trypsin, cells were grown for at least 18 h as

described previously (16,17). Human embryonic kidney cells (HEK293) were cultured as described previously (18).

Reverse transcriptase PCR (RT-PCR)

RNA was isolated using Trizol (INVITROGEN, Darmstadt, Germany) according to manufacturer's specifications. RNA concentrations were determined photometrically and RNA integrity was assessed by agarose gel electrophoresis. 5 µg total RNA were digested with RQ1 RNase-free DNase (PROMEGA, Mannheim, Germany) and 2 µg of the DNase digested RNAs were reverse transcribed using M-MLV Reverse Transcriptase or Superscript™ III (INVITROGEN, Darmstadt, Germany) according to manufacturer's specifications. Amplifications were performed with gene-specific primers under standard reaction conditions. Primer sequences are available upon request.

Transfections

EC were transiently transfected with Superfect (QIAGEN, Hilden, Germany) and HEK293 with Lipofectamine-PLUS (INVITROGEN, Darmstadt, Germany) as described previously (18).

Luciferase assays

Two days after transfection cells were lysed with Reporter Lysis Buffer (PROMEGA, Mannheim, Germany) according to manufacturer's instructions. Equal amounts of cellular protein were made up to 20 µl with Reporter Lysis Buffer and luciferase activity was determined by automatic injection of 100 µl luciferase assay mix (20 mM Tricine-KOH, pH 7.8, 0.1 mM EDTA, 8 mM $MgCl_2$, 33.3 mM DTT, 0.27 mM Coenzyme A, 0.53 mM ATP, 0.47 mM D-luciferin).

Immunoblotting

Immunoblotting was performed with antibodies directed against Akt (1:500), phospho-Akt (S473, 1:500), eNOS (1:1000) and phospho-eNOS (S1179, 1:500) (all

overnight, 4°C, BECTON & DICKINSON, Karlsruhe, Germany), V5 (1:1000, overnight, 4°C, INVITROGEN, Darmstadt, Germany) and GAPDH (1:8000, overnight, 4°C, ABCAM, Cambridge, UK). Semi-quantitative analyses were performed on scanned immunoblots using ImageJ 1.42q (19) or Scion Image 1.6 (SCION CORP.).

Migration assays

Migration assays were performed with a scratch wound assay as described previously (20).

Apoptosis

Detection of cell death was performed by flow cytometry using annexin V-APC or annexin V-FITC binding and 7-Amino-actinomycin (7AAD) staining (BD PHARMINGEN, Heidelberg, Germany). In brief, cells were trypsinized of the dish and pelleted. After washing twice with annexin binding buffer cell pellets were resuspended in annexin binding buffer and incubated with 2.5 ng/ml annexin V-APC and 2.5 ng/ml 7AAD for 20 min and analyzed in a flow cytometer.

In vitro transcription and RNA injection into zebrafish embryos

Capped mRNA was transcribed using the mMessage mMachine SP6 Kit (AMBION/INVITROGEN, Darmstadt, Germany) with 1 µg of linearized plasmid DNA as template, extracted with Phenol/Chloroform/Isoamylalcohol, purified over RNeasy columns (QIAGEN, Hilden, Germany) and concentrated by precipitation. The quality of the synthesized mRNAs was assessed by agarose gel electrophoresis. RNAs were injected into the yolk of one-cell stage zebrafish embryos, concentrations were adjusted according to mRNA length (SOM1: 80 ng/µl, SOM3: 68 ng/µl). Injected zebrafish were incubated at 28°C in zebrafish medium over night. Unfertilized embryos were separated from fertilized embryos at 2 hours and 8 hours post-injection. Death/life ratios were determined 24 hours post fertilization (hpf) and surviving embryos were classified according to their phenotype. Uninjected wild-type

zebrafish (strain: DBS) served as controls. Photographs of embryos were taken at 34 hpf with a stereomicroscope.

Statistics

Statistical analyses were performed with student's T-test.

RESULTS

We have identified SOM in a screen for anti-apoptotic genes in a mammary carcinoma cell line. Overexpression of human SOM2, the homologue to the single murine SOM protein, in human endothelial cells (EC) enhanced their migratory capacity (8). In humans an additional first exon exists, which is not present in the mouse genome. The transcript starting with this exon is alternatively spliced resulting in two protein isoforms, SOM1 and SOM3 ((2) and figure 1A). Their role in cell functions has not been addressed so far. As a first prerequisite, we confirmed co-expression of SOM1 and SOM3 in EC by RT-PCR using three different primer combinations for each isoform (Figure 1B).

It has been proposed that SOM3 is transcriptionally inactive (2). This conclusion was based on two hybrid experiments in human 293T cells using fusion proteins between the GAL4 DNA binding domain and the N-terminal region of SOM3 or the predicted activation domain of SOM1 encoded by the exon missing in SOM3 (2). As these experiments analyzed only the predicted activation domain, it can not be excluded that other regions of the protein have a function in transcriptional activation. In addition, fusion to a heterologous DNA binding domain could mask properties of the full length protein. However, to assess cellular functions of SOM1 and SOM3, it was mandatory to us to assure whether they are both transcriptional activators or not. Therefore, we performed analogous experiments in the same cell type as Ting et al. (2) using expression vectors for full length SOM1 and SOM3 and a reporter system

with a tandem repeat of the consensus binding site for these proteins (4) fused to a minimal promoter to avoid interactions with other transcription factors (Figure 2A). As specificity controls we included a reporter in which critical residues in the SOM binding site where mutated to prevent binding of the transcription factors (Figure 2A). Cotransfection of SOM1 and SOM3 with these reporters clearly demonstrated specific transcriptional activation by both proteins. Surprisingly, SOM3 seems to be the more potent activator than SOM1 (Figure 2B).

Since it is known for several transcription factors that different splice variants have distinct and sometimes opposing functions, we investigated the role of SOM1 and SOM3 in EC. The most important properties of EC in the vessel wall are apoptosis protection, migratory capacity and supply of bioactive nitric oxide (NO). Therefore, we expressed SOM1 or SOM3 in primary human EC (Figure 3A) and subsequently analyzed all of these parameters. We first measured apoptosis using annexin V. Overexpression of SOM1 significantly inhibited apoptosis in EC, whereas SOM3 had no protective effect (Figure 3B). Next, we determined the migratory capacity by setting an artificial wound in the EC monolayer and counting the migrated cells (20). As shown in figure 3C and 3D, SOM1 dramatically induced migration. In contrast, SOM3 significantly reduced the migratory capacity of EC (Figure 3C and 3D). Next, we wanted to get mechanistical insights into the opposing effects induced by SOM1 and SOM3. The most important anti-apoptotic and pro-migratory stimulus in EC is endogenously derived nitric oxide (NO). In these cells, NO is constitutively produced by eNOS. This enzyme is constitutively activated by the protein kinase B/Akt (21,22). Thus, we determined the phosphorylation of Akt on serine 473 and of eNOS on serine 1179, which translates into the activity of the enzymes, after overexpression of SOM1 and SOM3. Corroborating our data obtained for apoptosis and migration, SOM1 induced Akt and eNOS phosphorylation (Figure 4A and 4B), whereas SOM3

did not change phosphorylation of Akt (Figure 4A and 4C), but significantly reduced eNOS activation in comparison to empty vector transfected cells (Figure 4A and 4C). Thus, SOM1 overexpression increases the amount of bioactive NO, which probably explains the anti-apoptotic and pro-migratory effects of this isoform. On the other hand, SOM3 reduced the activity of eNOS, which results in less bioactive NO in the EC and therefore in reduced protection against apoptosis and migratory capacity. This parallels our previous findings that reduced eNOS phosphorylation leads to enhanced apoptosis sensitivity in senescent EC (23) and to reduced migratory capacity (24).

To address the in vivo relevance of the opposing effects of human SOM1 and SOM3, we made use of zebrafish as a model system, because the embryos are translucent and can therefore be easily analyzed through all developmental stages. Therefore, we injected in vitro transcribed mRNA for human SOM1 and SOM3 into the yolk sac of one cell stage zebrafish embryos and analyzed the resulting phenotypes. The animals were categorized into normal, medium and severe phenotypes (Figure 5A). The medium phenotype is characterized by slightly smaller size, a bent tail, smaller eyes and obvious apoptosis in the head region. Massively deformed embryos (classified as severe or potato phenotype) have no heads and show an open spinal cord bifurcated dorsal tails and massive apoptosis.

SOM1 did not change the embryonic development significantly, when compared to control animals (Figure 5B). In contrast, SOM3 injection resulted in a dramatic increase in animals of the medium phenotype and embryos with severe malformations. In addition, the number of normally developed embryos was significantly reduced (Figure 5B). These results demonstrate that not only in primary human cells, but also in a whole animal SOM1 and SOM3 have opposing effects with SOM3 acting deleteriously.

DISCUSSION

The present study investigates for the first time the cellular functions of isoforms 1 and 3 of the human transcription factor SOM. Here, we show that SOM1 and SOM3 are both active transcription factors and that they have opposing effects on apoptosis and migration in primary human EC and that SOM3, in contrast to SOM1, has a deleterious effect on the embryonic development of zebrafish.

Over the recent years, it has become clear that isoforms of transcription factors derived from alternative splicing are often co-expressed in normal tissues and not only a typical occurrence in cancer cells (25). The inhibitor of growth family member 4 (ING4) was originally discovered in cancer cells as a candidate tumor suppressor gene (26). However, ING4 and its 3 splice variants are also present in normal tissues and in human embryonic kidney cells (27). Similarly, we found SOM1 and SOM3 both expressed in primary human EC. Interestingly, overexpression of ING4 variant 1 inhibited cell growth and cell migration, whereas ING4 variant 4 had no effect. This was ascribed to the fact that ING4 isoform 1 in contrast to ING4 isoform 4 is an active transcription factor and only ING4 isoform 1 was therefore able to induce the growth inhibitor $p21^{WAF1}$ (27). Here, we show that SOM1 and SOM3 are both active transcription factors. Thus, we have to conclude that SOM1 and SOM3 induce different target genes, which we will analyze in the future by lentiviral overexpression in primary human EC to investigate isoform specific regulation of genes, specifically in the GO clusters apoptosis and migration. SOM target genes have been identified in a microarray screen by comparing backskin from wildtype and SOM-deficient mice (28). However, because migration of keratinocytes depends on proliferation, unlike migration of EC, a completely different set of target genes might be induced. In addition, mice do not express homologues of the human SOM1 and SOM3 proteins,

as the corresponding first exon does not exist in the mouse genome, and therefore, these experiments would not have identified isoform specific targets.

Another interesting transcription factor, which is alternatively spliced and exerts opposing functions in the vasculature, is the inhibitor of DNA binding 3 (Id3). Id3 can promote entry into the cell cycle and thereby induce smooth muscle cell proliferation by inhibiting $p21^{WAF1}$ and $p16^{INK4}$ transcription (29). Id3 has an alternatively spliced isoform Id3a, which was not detected in normal carotid arteries, but upon balloon injury, was expressed throughout the neointimal layer. Overexpression of Id3a, but not Id3, induced cell loss and apoptosis induction in vascular smooth muscle cells (30). In contrast to Id3 and Id3a, SOM1 and SOM3 are co-expressed in normal tissues and in primary human EC ((2) and study here), but similar to Id3a, SOM3 also induces deleterious effects in cells and in vivo ((30) and study here). Id and SOM have a different mode of action. Whereas Id proteins act as repressors by preventing DNA binding of basic helix-loop-helix factors to their cognate response element, the E-box (29), SOM proteins are transcriptional activators. Nevertheless, the general characteristic that a deleterious protein derived from an alternatively spliced transcript is similar in that Id3a is still an active repressor and SOM3 a transcriptional activator.

A huge disadvantage for in depth in vivo analyses of SOM1 and SOM3 is their absence in mice. This makes it difficult to investigate their functions in vivo, e.g. in disease models, as no isoform specific knockouts can be made. However, since the observations concerning isoform-specific functions in primary human EC and in zebrafish are persuasive, it would be important to generate transgenic mouse models. These could be made on a SOM-proficient genetic background, since human primary EC also express SOM2, the homologue of murine SOM

(supplementary figure 1) and overexpressed SOM1 or SOM3 still exert their opposing effects.

Taken together, we have, for the first time, identified isoform-specific, opposing functions of human SOM1 and SOM3, which have an influence on important aspects of endothelial cell biology. Future analyses of gene expression programs governed by these transcription factors will uncover through which target genes these effects are mediated and might provide new targets for therapies aimed at improving endothelial function.

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FIGURE LEGENDS

Figure 1: SOM1 and SOM3 are expressed in primary human endothelial cells.

(A) Top: Schematic representation of SOM1 and SOM3 isoform-specific transcripts. The first four exons (boxes) are drawn to scale and named according to (2), translated regions are shown in black. Bottom: Primary sequences of the N-terminal

region of SOM1 and SOM3 proteins. (B) PCRs with isoform-specific primer pairs were performed on cDNA from primary human umbilical vein endothelial cells, the expected fragment sizes for SOM1 are 187 bp (lane 1), 203 bp (lane 2), 444 bp (lane 3) and for SOM3 144 bp (lane 4), 185 bp (lane 5) and 517 bp (lane 6), M is a DNA size marker.

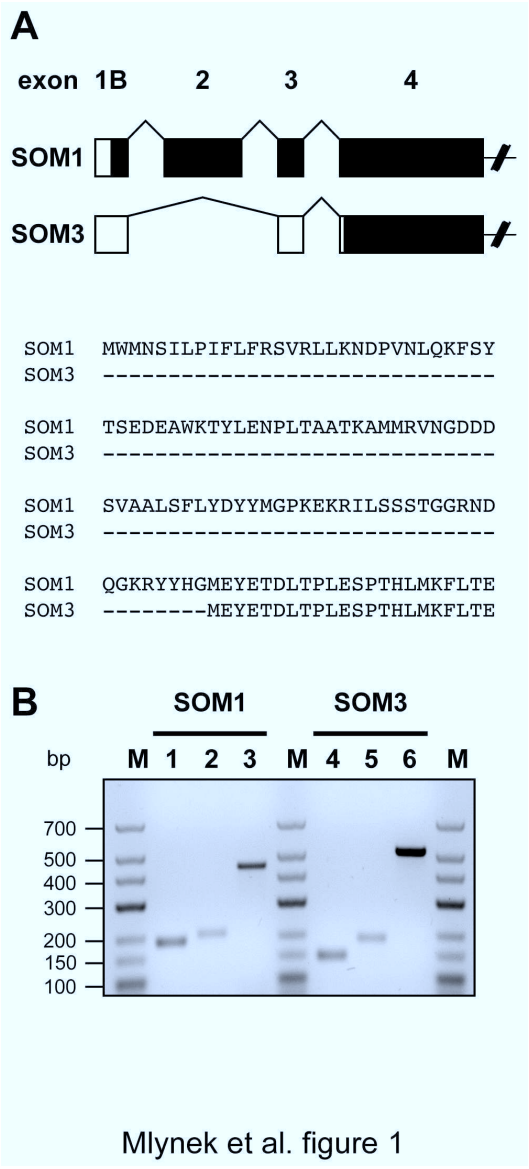
Figure 2: Both isoforms SOM1 and SOM3 are transcriptional activators. (A) Schematic structure of the SOM-specific luciferase reporter constructs. The core sequences of the SOM-binding sites with the corresponding mutations are shown. (B) Transactivation by SOM1 and SOM3. Shown are the luciferase activities after cotransfecting HEK293 cells with SOM expression vectors and reporter plasmids carrying two wildtype (wt) or mutated (mut) consensus binding sites relative to reporter expression after cotransfection with a plasmid not containing SOM, which was set to 1. Data are mean ± SEM (n=6; *p<0.05)

Figure 3: SOM1 and SOM3 have opposing effects on apoptosis and cell migration. Primary human endothelial cells were transfected with expression vectors for SOM1, SOM3 or a plasmid not containing SOM (EV). (A) Representative immunoblots of lysates from transfected cells probed with an anti-V5 antibody to detect the SOM fusion proteins (V5-SOM), proteins of the appropriate molecular weight are indicated by asterisks, GAPDH was used as loading control. (B) Apoptosis rates in transfected cells were measured with annexin V and are shown relative to EV transfected cells. Data are mean ± SEM (n=3; *p<0.05). (C and D) Migration of transfected cells was determined by scratch wound assay. (C) Representative microscopic pictures. The white line indicates the origin of migration, the migration direction is indicated by an arrow (D) Migrated cells were counted and are shown as

number of migrated cells per high power field (HPF). Data are mean ± SEM (n=4; *p<0.05).

Figure 4: SOM1 and SOM3 have opposing effects on Akt and eNOS phosphorylation. Primary human endothelial cells were transfected with expression vectors for SOM1, SOM3 or a plasmid not containing SOM (EV). (A) Representative immunoblots of lysates from transfected cells probed with antibodies directed against active Akt phosphorylated on serine 473 (P-Akt (S473)), total Akt (Akt), active eNOS phosphorylated on serine 1179 (P-eNOS (S1179)) and total eNOS (eNOS). (B and C) Semiquantitative analyses of scanned autoradiographs. Shown are the ratios of phosphorylated proteins to total proteins, respectively, induced by overexpression of SOM1 (B) and SOM3 (C) relative to cells transfected with the empty expression vector (EV). Data are mean ± SEM (n=3; *p<0.05).

Figure 5: Overexpression of SOM3, but not SOM1 induces severe malformations in zebrafish embryos. One-cell stage zebrafish embryos were injected with in vitro translated mRNA coding for SOM1 or SOM3, scored for phenotypic changes 24 h post fertilization (hpf) and compared to uninjected embryos. (A) Pictures of an uninjected control embryo and 3 embryos injected with SOM3 RNA representing the different phenotypes at 34 hpf. (B) Shown are the percentages of the four different phenotypes (mean ± SEM, n=4; 63-97 injected embryos per experiment, 160-217 uninjected control embryos per experiment; *p<0.05).



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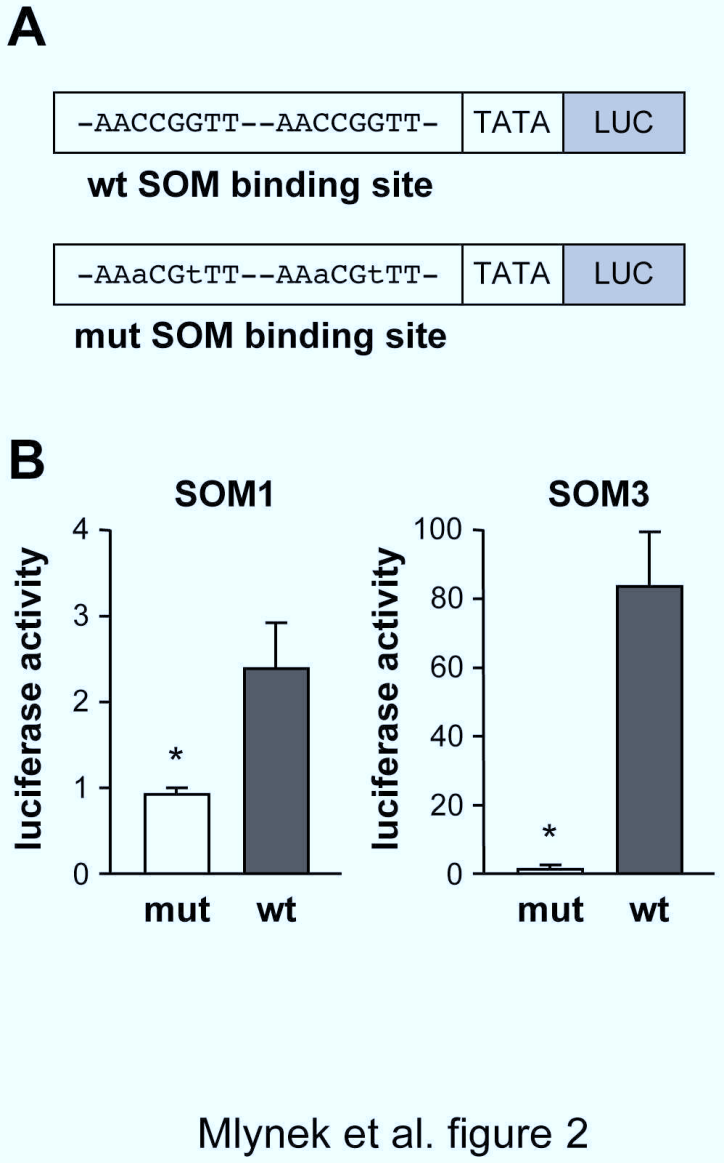

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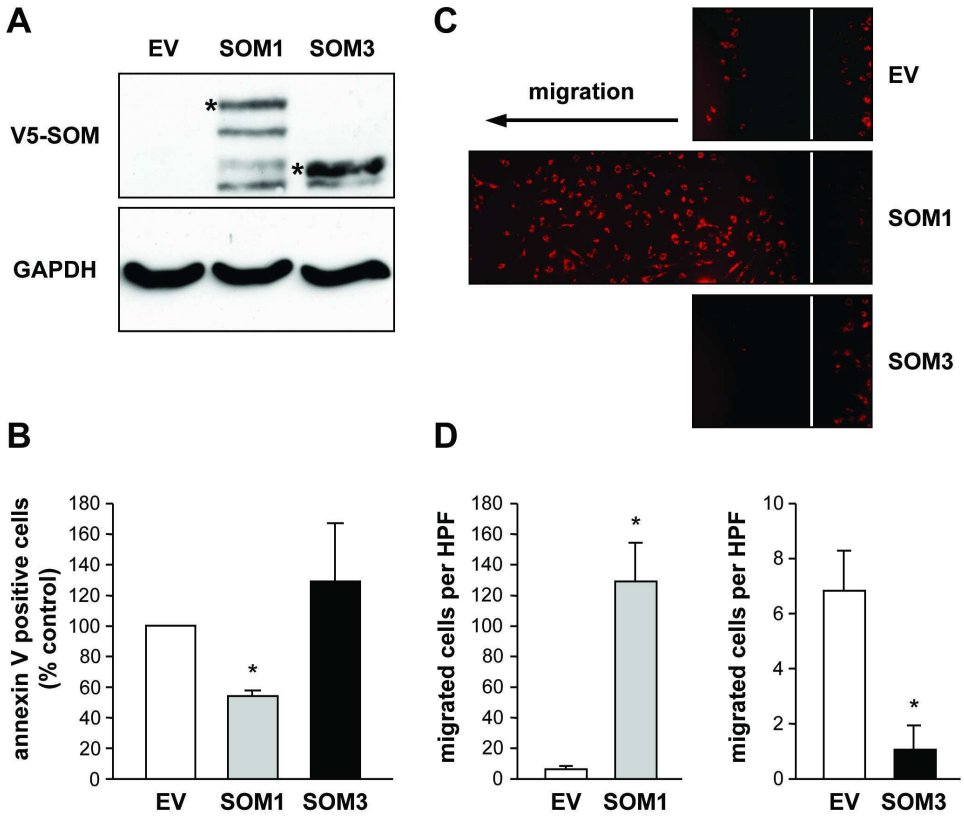

Mlynek et al. figure 3

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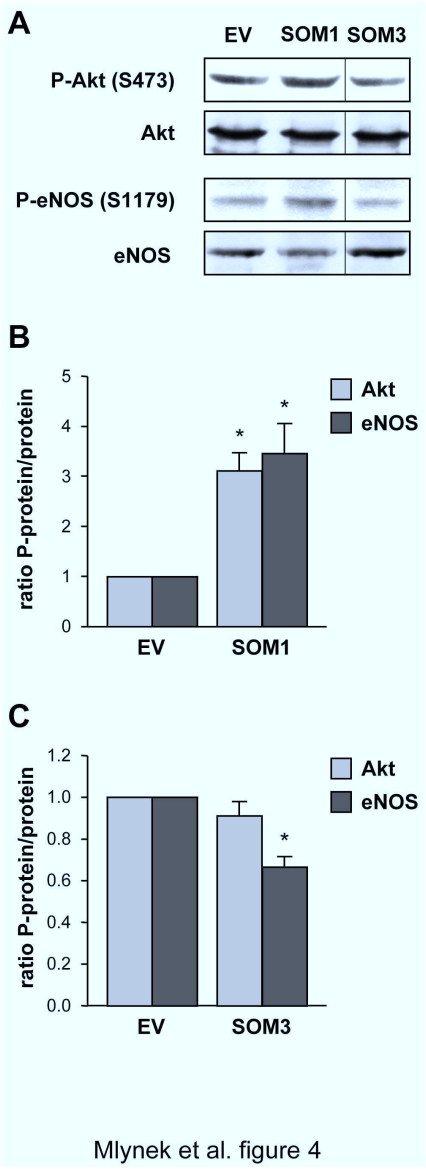

Mlynek et al. figure 4

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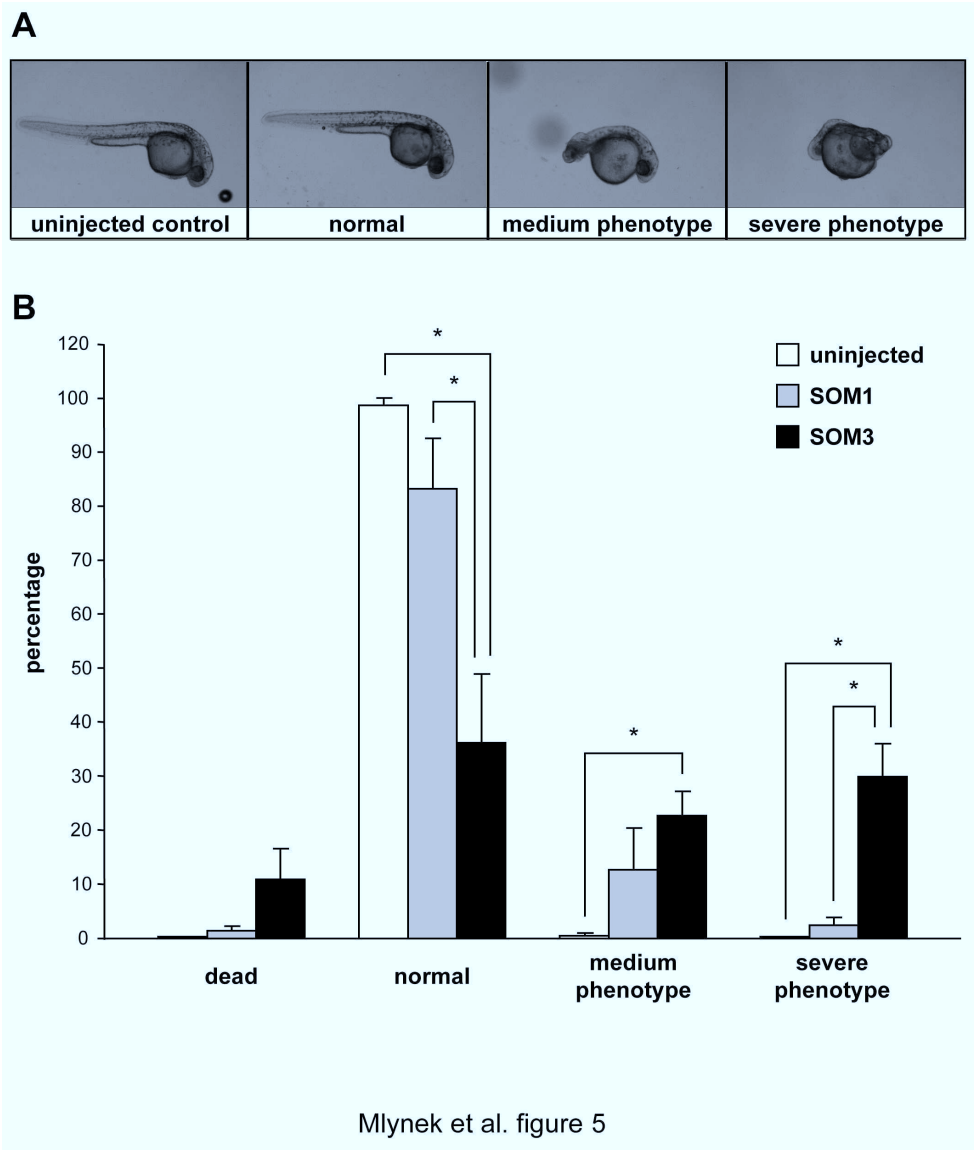

Mlynek et al. figure 5

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