Impact of genotoxins on autophagic processes in human iPSCs and thereof differentiated neural progenitor cells

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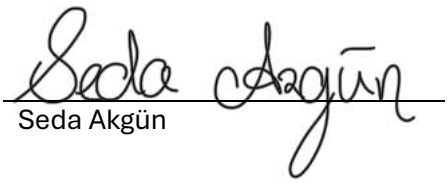

Seda Akgün

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Abbreviations

ABC	ATP-binding cassette
AhR	Aryl hydrocarbon-receptor
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ASC	Adult stem cell
ATG	Autophagy-related
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
AURKA	Aurora kinase A
$BafA_1$	Bafilomycin A_1
BaP	Benzo[a]pyrene
BDNF	Brain-derived neurotrophic factor
BECN1	Beclin1
BER	Base excision repair
BMP4	Bone morphogenic protein 4
BPDE	Benzo[a]pyrene diol epoxide
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
CDK7	Cyclin-dependent kinase 7
CHIP	Carboxyl terminus of HSC70-interacting protein
CHK	Checkpoint-kinase
CMA	Chaperone-mediated autophagy
c-Myc	Proto-oncogene c-Myc
CNS	Central nervous system
CSA	Cockayne syndrome A
CSB	Cockayne syndrome B
CSC	Cancer stem cell
CtIP	CtBP-interacting protein
CYP450	Cytochrome P450
dbcAMP	Dibutryryl cyclic adenosine monophosphate
DDR	DNA damage response
DFCP1	Zinc-finger FYVE domain-containing protein 1
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNTP	Deoxynucleoside triphosphate
DSB	Double-strand break
DSBR	DNA double-strand break repair
dsDNA	Double-strand DNA

ER	Endoplasmic reticulum
ESC	Embryonic stem cell
ESCRT	Endosomal sorting complexes required for transport
FAK	Focal adhesion kinase
FGF4	Fibroblast growth factor 4
FGF8	Fibroblast growth factor 8
FIP200	FAK family kinase-interacting protein of 200 kDa
FYCO1	FYVE and coiled- coil domain-containing protein 1
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GDNF	Glial cell line-derived neurotrophic factor
GG-NER	Global genome repair
GSK3	Glycogen synthase kinase 3
h	Hours
hiPSC	Human induced pluripotent stem cell
HNRNPK	Heterogeneous Nuclear Ribonucleoprotein K
HOP	HSP70–HSP90 organizing protein
HOPS	Homotypic fusion and vacuole protein sorting
HORMA	Hop1, Rev7, Mad2
HR	Homologous recombination
HSC70	Heat shock cognate 71 kDa protein
HSP40	Heat shock protein 40
IC	Inhibitory concentration
ICL	Interstrand crosslink
IR	Ionizing radiation
KIF	Kinesin protein
Klf4	Krüppel-Like Factor 4
LAMP2A	Lysosome-associated membrane protein type 2A
LC3	Microtubule-associated protein light chain 3
LigIV	DNA ligase IV
LIN28	LIN-28 homolog A
LIR	LC3-interacting region
mESC	Mouse embryonic stem cell
MAT1	Ménage à trois-1
MIA	Mitotic index assay
MMR	Mismatch repair
MRE11	MRE11 Homolog Double Strand Break Repair Nuclease
MRN	MRE11-RAD50-NBN
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
Nanog	Nanog homeobox

NBN	Nibrin
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NODAL	Nodal growth differentiation factor
NPC	Neural progenitor cell
NRBF2	Nuclear receptor binding factor 2
NSC	Neural stem cell
NSF	N-ethylmaleimide-sensitive-factor
NUCKS1	Nuclear ubiquitous casein kinase and cyclin dependent kinase substrate 1
OCT4	Octamer-binding transcription factor 4
OXPHOS	Oxidative phosphorylation
p53	Tumor suppressor protein 53
PAH	Polycyclic aromatic hydrocarbon
PALB2	Partner and localizer of BRCA2
PARP	Poly(ADP)ribose polymerase
PAS	Phagophore assembly site
Pax6	Paired box 6
PAXX	Paralog of XRCC4 and XLF
PE	Phosphatidylethanolamine
pH3	Phospho Histone 3
PI	Phosphatidylinositol
PI3KC3	Class III phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PIKK	Phosphatidylinositol 3-kinase-like protein kinases
PLK1	Polo-like kinase 1
qRT-PCR	quantitative real-time polymerase chain reaction
RAD23B	RAD23 homolog B
RAD50	RAD50 double strand break repair protein
RAD51	RAD51 homolog1
RAPTOR	Regulatory-associated protein of mTOR
RB1	Retinoblastoma 1
RB1CC1	RB1 inducible coiled-coil 1
RNAPII	RNA polymerase II
ROS	Reactive oxygen species
RPA	Replication protein A
SAR	Selective autophagy receptor
SC	Stem cell
Ser	Serine
SHH	Sonic Hedgehog
SMC3	Structural maintenance of chromosome 3
SNAP29	Synaptosomal-associated protein 29

SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment protein receptors
Sox2	SRY-box transcription factor
SPB	Sugar phosphate bakbone
SQSTM1	Sequestosome-1
SSB	Single-strand break
ssDNA	Single-strand DNA
STX17	Syntaxin 17
TCA	Tricarboxylic acid
TC-NER	Transcription-coupled repair
TFIIH	Transcription factor II H
TGF-β	Transforming growth factor beta
TOPO	Topoisomerase
TSC2	Tuberous sclerosis 2
t-SNARE	Target-SNARE
ULK	Unc-51-like kinase 1
UPS	Ubiquitin-proteasome system
UV-DDB	UV-DNA damage binding
UVRAG	UV radiation resistance-associated gene protein
UVSSA	UV stimulated scaffold protein A
VAMP8	Vesicle-associated membrane protein 8
V-ATPase	Vacuolar-type H^+-translocating ATPase
v-SNARE	Vesicle-SNARE
VSP34	Vacuolar protein sorting 34
WIPI	WD repeat domain phosphoinositide-interacting
WNT	Wingless-related integration site
XLF	XRCC4-like factor
XPC	Xeroderma pigmentosum group C
XRCC4	X-ray repair cross complementing protein 4
γH2AX	Phosphorylated histone protein H2A family member X
53BP1	p53-binding protein 1
8-oxo-dG	8-oxo-7,8-dihydroguanine

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Summary

The emergence of every mammalian organism begins with the fusion of two gametes leading to the development of complex, multicellular organisms relying on tissue remodeling and regeneration throughout their lifetime. The source for the genesis of a diverse range of morphological and physiological cells and complexes are stem cells with different potencies and occurrences during development. Stem cells exhibit unique features involving self-propagation, asymmetric division and differentiation and display a major breakthrough in regenerative and transplantation medicine – especially the generation and utilization of human induced stem cells. Thereby, stem cells possess strict regulatory mechanisms to maintain their pluripotency in a controlled manner. Dysregulated cells with stem cell-like characteristics are also known as cancer stem cells causing tremendous damage due to their division and differentiation potential and are associated with poor patient survival. To protect the organism from endogenous and exogenous threats, stem cells rely on several signaling pathways including the cytoprotective and survival supporting autophagic pathway.

Autophagy is a highly conserved catabolic pathway utilizing lysosomal vesicles for the degradation of superfluous and hazardous cytosolic components and interacts with several other signaling pathways, e.g., DNA damage response, to ensure cellular integrity. In cancer cells, autophagy plays a dual role depending on the stage of the cancerous pathways. It has been demonstrated that autophagy plays a crucial role in stem cell homeostasis, however, the autophagic role upon DNA damage needs further understanding. Aiming to investigate the influence of genotoxic noxae on the autophagic process in human induced pluripotent stem cells and thereof differentiated neural progenitor cells, we exposed both stem cell types to the environmental mutagen benzo[a]pyrene diol epoxide and the chemotherapeutic etoposide and despite detectable DNA damage by western blot analysis and immunofluorescence, the canonical autophagic process was not affected in stem cells and slightly in colorectal carcinoma cells HCT116, contradictory to known literature about cancer cells. Furthermore, differential proteome analysis showed no detectable changes in iPS11, but an increase in mitosis-related proteins in thereof differentiated neural progenitor cells niPS11. We propose that autophagy is rather triggered by severe DNA damage or cellular stress to support cellular survival rather than being an inevitable consequence of DNA damage. Therefore, the autophagic machinery is not affected by low dose genotoxic treatment in all tested cellular systems. Differences in sensitivity towards genotoxins might be explained by chromatic organization, cell cycle differences and the potential recruitment of p53-independent repair mechanisms. The increase in mitosis-related proteins in niPS11 emphasizes cellular differences and might indicate an impact on spindle machinery and therefore on asymmetric division.

Zusammenfassung

Die Entstehung jedes Säugetierorganismus beginnt mit der Verschmelzung zweier Gameten, welches zur Entwicklung komplexer, multizellulärer Organismen führt, die im Laufe ihres Lebens auf Gewebemodellierung und -regeneration angewiesen sind. Die Quelle für die Entstehung einer Vielzahl morphologischer und physiologischer Zellen und Komplexe sind die Stammzellen mit unterschiedlichen Potentialen und Vorkommen währen der Entwicklung. Stammzellen weisen einzigartige Eigenschaften wie Selbsterneuerung, asymmetrische Teilung und Differenzierung auf und stellen einen bedeutenden Durchbruch in der regenerativen und Transplantationsmedizin dar; insbesondere die Herstellung und Nutzung von humanen induzierten Stammzellen. Dabei verfügen Stammzellen über strenge Regulationsmechanismen, um ihr Differenzierungs-potential auf kontrollierte Weise aufrechtzuerhalten. Fehlerhaft regulierte Zellen mit stammzell-ähnlichen Eigenschaften werden auch als Krebsstammzellen bezeichnet, die aufgrund ihres Teilungs- und Differenzierungspotenzials verheerende Schäden verursachen können und mit einer schlechten Überlebensrate der Patienten in Verbindung gebracht werden. Um den Organismus vor endogenen und exogenen Bedrohungen zu schützen, stützen sich Stammzellen auf mehrere Signalwege, darunter auf die zytoprotektive und überlebensfördernde Autophagie.

Autophagie ist ein hochkonservierter kataboler Signalweg, der lysosomale Vesikel für den Abbau überflüssiger und beschädigter zytosolischer Komponenten nutzt und mit mehreren anderen Signalwegen interagiert, z. B. der DNA-Schadensantwort, um die Zellintegrität zu gewährleisten. In Krebszellen spielt die Autophagie je nach Stadium der Krebsentstehung eine zwiegespaltene Rolle in der Bekämpfung und später im Überleben der Krebszellen. Es wurde gezeigt, dass die Autophagie eine entscheidende Rolle bei der Homöostase von Stammzellen spielt, jedoch muss die Rolle der Autophagie bei DNA-Schäden noch weiter untersucht werden. Mit dem Ziel, den Einfluss genotoxischer Noxen auf den autophagischen Prozess in hiPSCs und daraus differenzierten neuralen Vorläuferzellen zu untersuchen, setzten wir beide Stammzelltypen dem Umweltmutagen Benzo[a]pyren-diol-epoxid und dem Chemotherapeutikum Etoposid aus. Dabei beobachteten wir, dass trotz nachweisbarer DNA-Schäden durch Western Blot-Analyse und Immunfluoreszenz der kanonische Autophagieprozess in Stammzellen kaum und in kolorektalen Karzinomzellen HCT116 nur leicht beeinflusst wurde. Darüber hinaus zeigte die differentielle Proteomanalyse keine nachweisbaren Veränderungen in iPS11, jedoch einen Anstieg Mitose-assoziierter Proteine in den daraus differenzierten neuralen Vorläuferzellen niPS11. Wir vermuten, dass die Autophagie eher durch folgenschwere Schäden ausgelöst wird, um das Überleben der Zelle zu unterstützen, als dass sie generell eine direkte Folge von DNA-Schäden ist. Daraus

schließend wird der autophagische Prozess in den von uns getesteten Zellsystemen durch eine genotoxische Behandlung mit niedriger Dosis nicht beeinflusst. Unterschiede in der Empfindlichkeit gegenüber Genotoxinen lassen sich möglicherweise durch die Chromatinorganisation, Unterschiede im Zellzyklus und die potentielle Rekrutierung p53-unabhängiger Reparaturmechanismen erklären. Der Anstieg Mitose-assoziierter Proteine könnte auf eine Auswirkung auf den Spindelapparat und damit auf die asymmetrische Teilung hindeuten.

1. Introduction

1.1 Stem cells

The complexity of the human body displays an amazing symphony of interaction and determination. The development of originally two fused cells to a multicellular cell system with distinct functions and characteristics enhances the importance of a faultless system and its maintenance. An important element of this system is represented by stem cells (SCs).

In 1957, patients with cancer were treated with intravenous infusions of bone marrow cells from healthy donors and a replenishment of damaged bone marrow after radiation and chemotherapy was observed. These findings indicated the existence of stem cells in the human body and paved the way for bone marrow transplantation and further research (Fernandes et al., 2013; Thomas et al., 1957). Later on, “colony-forming units” were identified after bone marrow cell transplantation in mice with the ability of self-renewal and differentiation into three different cell types (Becker et al., 1963; Till & Mc, 1961).

Stem cells are defined by their remarkable abilities of self-renewal and differentiation into a plethora of cell lineages contributing to development, tissue engineering and repair throughout an organism´s lifespan. These characteristics require an exceptional balance of maintenance, self-renewal and differentiation to preserve the stem cell pool and provide controlled regeneration relying on metabolic, transcriptional and environmental regulations (Chen et al., 2024; Keisuke Ito & T. Suda, 2014; Karam et al., 2019; Matsuoka et al., 2007). For the strict regulation of environmental influences stem cells are localized in so called stem cell niches providing specialized microenvironments. These areas within an organ support and protect the organism from stem cell depletion and uncontrolled proliferation which may lead to cancer (Moore & Lemischka, 2006; Scadden, 2006). Niches consist of cellular and non-cellular components allocating different biochemical signals to influence the stem cell´s fate and are located throughout the body accommodating distinct stem cell types which will be discussed below (Crane et al., 2017; Moore & Lemischka, 2006; Pyle & Hicks, 2022; Scadden, 2006). An important feature of maintaining the balance is the ability of asymmetric division. This process describes the division of one stem cell into two daughter cells with two different fates. While one daughter cell is identical to the mother cell and supports self-renewal and maintaining the stem cell pool, the other daughter cell is committed to lineage-specific differentiation (Bolkent, 2024; Knoblich, 2008). Interestingly, the spindle organization plays a pivotal role in this unequal division. As illustrated in Figure 1, the fate of progeny can be influenced by intrinsic or extrinsic determinants or by the combination of both factors. Intrinsically driven asymmetric division results from an unequal distribution of, e.g., a master regulator of stem cell identity leading to the depletion of this factor in the other daughter cell and its differentiation. In comparison, the positioning of the spindle poles inside or outside the microenvironmental influence of stem cell

niche contribute to the extrinsic exposure to environmental factors favoring differentiation (reviewed in Fingerhut et al., 2016). Besides asymmetrical division, stem cells can also symmetrically divide into either two new stem cells to expand the stem cell pool or both committing to differentiation after injury. The expansion or depletion of the stem cell pool plays an important role during early development and tissue repair and offers a dynamic adjustment accordingly to physiological needs (reviewed in Gimeno & Paridaen, 2022; Gorba et al., 2005; Silva-Vargas et al., 2018).

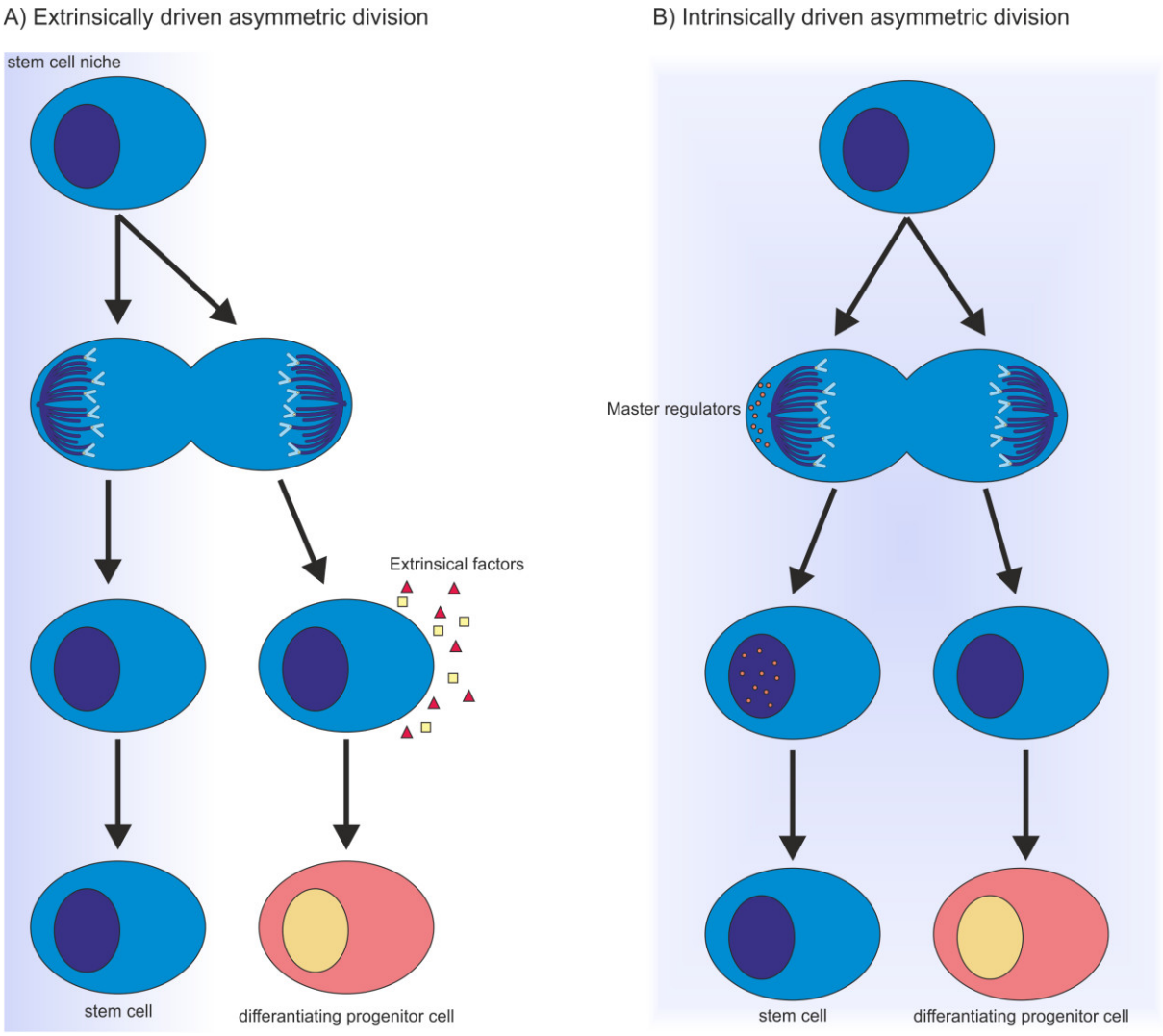

Figure 1: Asymmetric division of stem cells. Cell fate upon mitotic division can be determined by different factors. A) During spindle formation one pole is localized outside of the stem cell niche exposing the daughter cell to various environmental factors and leading to its commitment to differentiation while the other daughter cell remains in the niche and retains the original stem cell identity. B) Both spindle poles are localized in the same environment wherefore, extrinsic factors do not affect cell determination. However, intrinsic factors important to preserve the stem cell identity of the mother cell are divergently distributed resulting in different cellular compositions.

The exceptional differentiation capacity of stem cells can be distinguished by their limitations. Totipotent stem cells occur during the late zygote and blastomeres of 2-cell stage embryo in mice and have the greatest differentiation potential (Genet & Torres-Padilla, 2020; Ghazimoradi et al., 2022; Lu & Zhang, 2015; Tarkowski, 1959). They are able to differentiate into embryonic and extraembryonic tissue, such as the placenta, and therefore are capable of generating all kind of cell types to form an embryo (reviewed in Du & Wu, 2024). Cells that are able to generate different kinds of cells within the embryo, but not extraembryonic tissue, are referred to as pluripotent stem cells. These pluripotent cells are able to give rise to the three primary germ layers endoderm, mesoderm and ectoderm which are crucial for the development of the variety of cell types and organs (Tam & Loebel, 2007). The last groups of stem cells are referred to as multipotent or unipotent stem cells which are only able to differentiate into multiple or single cell types within a specific cell lineage and comprise the group of adult stem cells (Chandra et al., 2016).

1.1.1 Embryonic stem cells

Embryonic development requires a number of strictly organized processes for the emergence of a healthy organism. Following fertilization and divisions the totipotent blastomeres undergo several additional cleavage divisions resulting in cellular differentiation and segregation and the emergence of the blastocyst (Gardner et al., 1975; Papaioannou, 1982). The blastocyst is characterized by the outer layer of trophoectoderm cells and the inner cell mass (ICM) which are also known as embryonic stem cells (ESCs). ESCs have a nearly unlimited potential of self-renewal and are characterized by their pluripotent traits, allowing them to differentiate into any cell or tissue type of the body (summarized in National Institutes of Health, 2004). After gastrulation, ESCs give rise to the three germ layers: the endoderm, mesoderm and ectoderm. Subsequently, each layer is responsible for the development of certain organs underlining the transience of pluripotent ESCs. These characteristics are an exceptional feature for the emerging embryo but also offer an unrivalled tool in modern research. Generally, the human body consists of more than 200 different cell types and ESCs represent a unique type of cells with exclusively expressed proteins. The most important protein is the octamer-binding transcription factor 4 (OCT4) which is also referred to as OCT3, OCT3/4 or POU5F1 (Rosner et al., 1990; Ryan & Rosenfeld, 1997; Saijoh et al., 1996). The transcription factor OCT4 is only found in blastomeres, pluripotent ESCs and in germ cells during development and is, for example, only present in the ICM but not in the trophoectoderm (Palmieri et al., 1994). It is involved in the regulation of various target genes (Saijoh et al., 1996) including SRY-box transcription factor (Sox2) and fibroblast growth factor-4 (FGF4) displaying vital proteins for

embryonic development and survival (Ambrosetti et al., 1997; Curatola & Basilico, 1990; Nichols et al., 1998; Yuan et al., 1995).

In 1981, murine pluripotent stem cells from the pre-implantation blastocysts were successfully isolated, cultured as undifferentiated stem cells and differentiated *in vitro* (Evans & Kaufman, 1981; Martin, 1981). Consecutively, the isolation and cultivation of human embryonic stem cells from *in vitro* fertilized eggs facilitated the previously unfeasible option to investigate early embryonic development and disease modelling (Thomson et al., 1998). Amongst other aspects, *in vitro* differentiation of undifferentiated stem cells into terminally differentiated somatic cells enabled the screening of controlled protein expression during development giving insights about the emergence of certain proteins and their chronological importance (summarized in Wobus, 2001). Furthermore, differentiated cells exhibit tissue-specific functional properties, for example, spontaneously beating cardiomyocytes conducting action potentials and cell type-specific ion channels (Maltsev et al., 1993; Maltsev et al., 1994; Pich et al., 1997) or excitatory and inhibitory neurons (Lee et al., 2000; Okabe et al., 1996; reviewed in Wobus, 2001).

The possibility of culturing isolated human ESCs has been a major breakthrough in developmental and especially therapeutical research (Paolini Sguazzi et al., 2021). Genetically modified stem cells gave novel insights into disease progression and pathological outcomes that could be observed throughout cell differentiation or in different cell types. The work with the ESC system enlightened the process of early embryonic development and especially impacted the study of environmental influences and drugs on cell differentiation and physiology (Mahla, 2016). Therefore, *in vitro* work with ESCs reduced the necessity of animal testing in some cases and also eliminates the translation of animal tested outcomes on the human organism (Van Norman, 2019). However, the use and isolation of human ESCs raises ethical and political controversies due to the discussion about onset of human life and rights (Lo & Parham, 2009).

1.1.2 Adult stem cells

As described before, pluripotent ESCs play an important role in developmental processes bearing the potential to differentiate into all three germ layers. Postnatally, the majority of cells exit the cell cycle after differentiation and specialization but do need to be replaced regularly, like blood cells or the intestinal epithelium, or repaired after injury or disease, wherefore, the adult stem cells (ASCs) are responsible (Mannino et al., 2022). ASCs are undifferentiated stem cells with a limited differentiation potential and located in stem cell niches. Compared to ESCs, ASCs exhibit either a unipotent or multipotent differentiation potential capable of differentiating

into one or different cell types within a specific cell lineage, e.g., hematopoietic stem cells to various blood cells or neural progenitor cell into neurons and glial cells (Figure 2) (reviewed in Mannino et al., 2022). Therefore, stem cell niches reside in different tissues with corresponding ASCs for tissue proximity. Examples for SC niches are the bone marrow for hematopoietic stem cells (Friedenstein et al., 1968), crypts of Lieberkühn in the small intestines harboring intestinal stem cells (Jasper, 2020) and the subventricular zone of the lateral ventricle followed by the subgranular zone of the hippocampal dentate gyrus for neural stem cells (NSCs) (Andreotti et al., 2019; Kempermann et al., 2015; Riquelme et al., 2008). These niches offer a specialized microenvironment for the ASCs providing certain signals for quiescence, self-renewal and differentiation. Thereby, distinct cell fate regulations are influenced by cell-cell communication, cell-matrix interactions, intrinsic and extrinsic signaling (Ferraro et al., 2010).

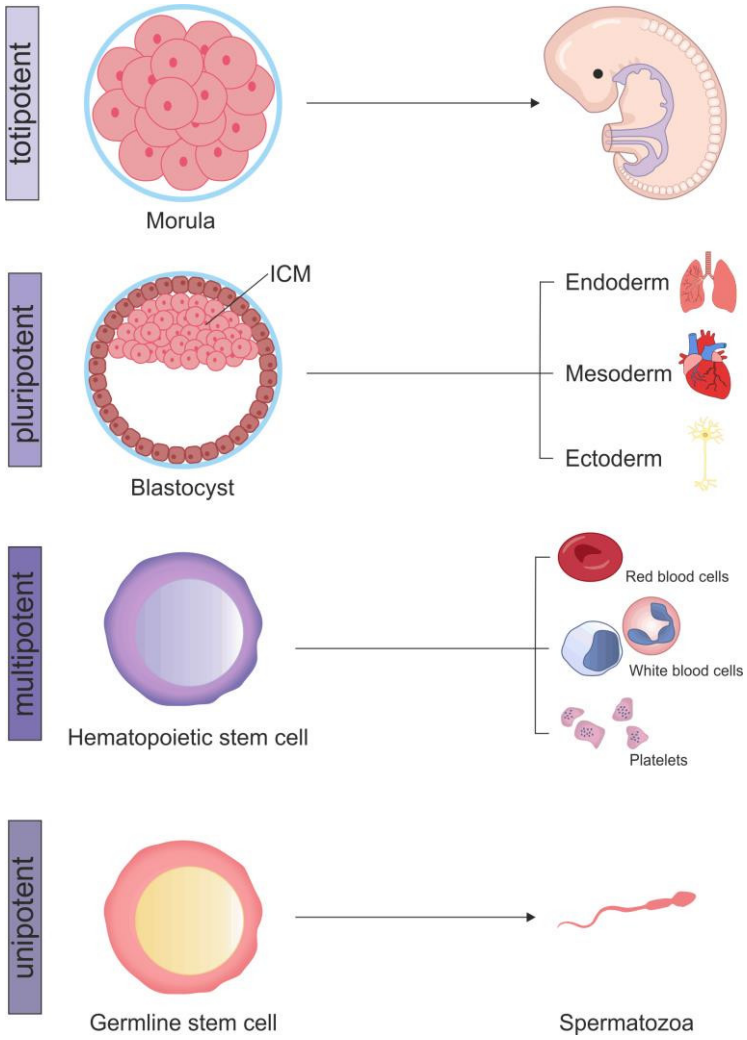

Figure 2: Stem cell potencies. In mammals, stem cells possess strictly regulated differentiation capacities. After fertilization, the morula emerges and contains totipotent stem cells able to differentiate into embryonic and extraembryonic tissue providing all essential cell types and tissue for the development of an organism. The inner cell mass (ICM) of the blastocyst contains pluripotent embryonic stem cells that are characterized by their potential

to differentiate into the three germ layers and give rise to various cell types. Multipotent stem cells are able to differentiate into different cell types within a defined cell lineage while unipotent stem cell exclusively differentiate into one cell type.

Primarily isolated human ASCs are a great opportunity for investigating disease-related effects on stem cells and thereof differentiated progeny while avoiding ethical and legal issues encountered by using ESCs. They display an important tool in therapeutical research by their potential of tissue repair, autologous use for patient-derived cells and harvesting with minimally invasive procedures depending on the tissue (Kornowski & Strauer, 2003; reviewed in Montero-Olvera & Berebichez-Fridman, 2018). However, variable quality of obtained ASCs and reduced proliferation potential with age, as well as excessive *in vitro* cultivation may hamper the usage of primarily harvested ASCs. Most importantly, not all ASCs are easy to be harvested and expanded in culture. For instance, adipose tissue-derived stem cells are easy to harvest, however, due to their limited differentiation potential they cannot substitute for other ASCs and thereof differentiated cells, e.g., neural stem cells (Montero-Olvera & Berebichez-Fridman, 2018; Slepička et al., 2018). An alternative method for reducing ethical controversies about embryonic life and still having access to the full potential of pluripotent stem cells and thereof differentiated progeny is the generation of human induced pluripotent stem cells.

1.1.3 Human induced pluripotent stem cells

In 2006, the working group of Takahashi and Yamanaka demonstrated the reprogramming of terminally differentiated mouse embryonic and adult fibroblast cells into induced pluripotent stem cells (iPSCs). These dedifferentiated cells possess the unique features of self-renewal and differentiation into the three germ layers which were exclusive characteristics of embryonic stem cells up until now (Takahashi & Yamanaka, 2006). Accordingly, iPSCs show similar morphological and growth properties and express cellular markers that only have been encountered in ESCs before. To demonstrate similar potential of iPSCs and ESCs, experiments were performed underlining the pluripotent capacity of iPSCs. For that, subcutaneous injections of iPSCs in mice verified their ability to form teratomas, tumors containing cells derived from all three germ layers and additionally, injections into blastocysts supported mouse embryonic development (Takahashi & Yamanaka, 2006).

In their initial approach, Yamanaka and colleagues investigated 24 different genes that were involved in the maintenance of pluripotency and growth in the early embryo and ESC culture including *OCT3/4* (Nichols et al., 1998; Niwa et al., 1991), *Nanog homeobox* (*Nanog*) (Chambers et al., 2003) and *proto-oncogene c-Myc* (*c-Myc*) (Cartwright et al., 2005). Subsequently, mouse embryonic fibroblasts were retrovirally transduced and factors contributing to morphology, colony formation and maintenance were selected as the four

“Yamanaka” factors. This cocktail of pluripotency markers include *OCT4*, *Sox2*, *c-Myc* and *Krüppel-Like Factor 4* (*Klf4*) (Takahashi & Yamanaka, 2006). OCT4 and Sox2 are well characterized proteins in embryonic development and have been shown to function as core transcription factors in the propagation of undifferentiated cells in cell culture and maintaining pluripotency (Boyer et al., 2005; Loh et al., 2006). The other two factors, c-Myc and Klf4, are tumor-related proteins, both essential and irreplaceable by other proteins in the approach of this working group. Thereby, c-Myc interacts with histone acetyltransferase complexes (McMahon et al., 1998) and may contribute to global chromatic changes (Fernandez et al., 2003) enabling the binding of OCT4 and Sox2 with their target sites (Takahashi & Yamanaka, 2006). Another important feature of ESCs is the low expression level of tumor suppressor p53 (p53) due to its suppression of *Nanog* expression and induction of differentiation upon DNA damage (Lin et al., 2005). Furthermore, p53 negatively affects the mesenchymal-to-epithelial transition which is required for the reprogramming of terminally differentiated somatic cells to a stem cell-like state (Brosh et al., 2013). The transcription factor Klf4 is correlated with both tumor suppression and oncogenesis and can act as a p53 suppressor when overexpressed (reviewed in Klimczak, 2015; Rowland et al., 2005). With this groundbreaking identification of reprogramming factors, Takahashi and Yamanaka have reshaped the field of stem cell research. However, while these Yamanaka factors led to the desired results in mouse-derived cells, the induction of human-derived pluripotent stem cells required adaptions since inducible c-Myc expression in human embryonic stem cells caused apoptosis and differentiation into extraembryonic endoderm and trophoectoderm lineages accompanied by decreased expression of pluripotency markers *OCT4* and *Nanog* (Sumi et al., 2007). Therefore, alternations were made, and different sets of transcriptional factors are used for the reprogramming of human-derived cells, e.g., *OCT4*, *Sox2*, *Nanog* and *LIN-28 homolog A* (*LIN28*) (Yu et al., 2007) or by using another member of the Myc family, *L-Myc* (Nakagawa et al., 2010).

For the generation of human iPSCs (hiPSCs) various induction methods have been developed. Hence, somatic cells are mainly reprogrammed by either using a viral vector or episomal delivery (reviewed in Cerneckis et al., 2024). Viral transduction can be performed with either integrating or non-integrating vectors. By that, integrating vectors are permanently inserted into the genome of the host cell by lenti- or retroviral delivery and result in a stable expression of transcription factors (Takahashi et al., 2007; Takahashi & Yamanaka, 2006; Yu et al., 2007). Nevertheless, this invasion of the genome may lead to unforeseen consequences including insertional mutagenesis, transgene reactivation and oncogenesis (Nowrouzi et al., 2011). By using a non-integrative approach the genome is not altered and vectors are gradually removed from the cell and are mainly present during the process of reprogramming by adenoviruses or Sendai viruses (Haridhasapavalan et al., 2019; Scesa et al., 2021; Stadtfeld et al., 2008; Zhou

& Freed, 2009). Non-viral delivery of the factors can be performed by transposons (Kaji et al., 2009; Woltjen et al., 2009), mRNA (Warren et al., 2010), or episomal plasmids (Okita et al., 2008).

The generation of hiPSCs has transformed the *in vitro* modeling and had a massive impact on therapeutic research and development. Using iPSCs reduce ethical concerns and circumvent the discussion about the onset of life and propose the same advantages of ESCs by their unlimited proliferation potential and differentiation into a plethora of specialized cell types. Furthermore, iPSCs are suitable for remodeling multifactorial diseases which cannot be mimicked by genetic modifications offering a great opportunity for translatable disease-in-a-dish models (Kang et al., 2017). Since somatic cells can be obtained directly from patients and reprogrammed to iPSCs, they also offer the opportunity to generate personalized therapies and transplantations without the risk of tissue rejection (Rowe & Daley, 2019). Moreover, similar to the ESCs, drug screening and impact of environmental factors can be performed in human cells with different genetic backgrounds and investigating their effects on development, differentiation and functionality (Figure 3). The ability to cultivate and differentiate iPSCs in a 2D or 3D model simulating organ-like structures, supports the application of stem cell-derived systems in toxicological screenings (Easley, 2019) and displays a standard testing system for wide-ranging applications surrogating animal-based testing.

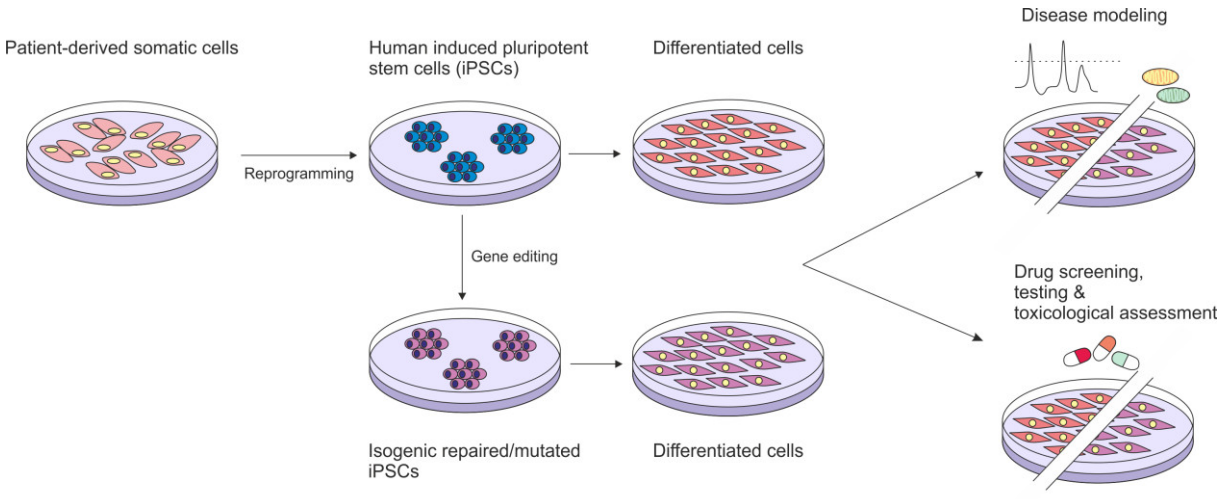

Figure 3: Biomedical assessment of human induced pluripotent stem cells. Terminally differentiated somatic cells from patients and donors allow the reprogramming to human induced pluripotent stem cells (iPSCs) and subsequent cultivation and propagation as *in vitro* cultures. Obtained stem cells can be genetically edited to repair or mutate certain genes of interest and differentiated into divergent specialized cells. Utilization of both cell models facilitates the investigation of molecular differences in healthy and diseased cells and thereupon targeting for drug screening. These opportunities may give insights about the impact of harmful substances impacting future therapeutical approaches and personalized treatments.

However, this model also has its advantages and disadvantages. Obtaining somatic cells and reprogramming them offers a great source for iPSC generation but it has to be considered that reprogrammed cells may retain epigenetic marks from the original cell. Thus, their epigenetic landscape and, consequently, their differential potential into various cell types and experimental outcomes might be influenced (Bar-Nur et al., 2011; K. Kim et al., 2011; reviewed in Scesa et al., 2021). Nevertheless, the origin of cells can also positively influence the differentiation potential if generated iPSCs are differentiated into the cell type of the original tissue (Bar-Nur et al., 2011). Additionally, cell sources and age of the donor can affect genomic heterogeneity due to prior DNA damage. For example, skin fibroblasts contain more ultraviolet (UV) light-related mutations than other cell types (Rouhani et al., 2022) and the integrity of the mitochondrial genome can be affected by age and reprogramming (Deuse et al., 2019; Wei et al., 2021). Consequently, iPSCs exhibit higher heterogeneity than ESCs that may influence the quality and differentiation potential of iPSCs. Although reprogrammed cells should therefore be utilized with caution, they nevertheless present an indispensable cell system in today´s research.

1.2 Cancer stem cells

Despite ongoing advances in cancer research and therapeutic approaches, heterogeneity and adaptation of cancer cell populations are impeding successful treatment. Tumors within a patient can show high heterogeneity attributable to their multicellular composition. One subpopulation of cells associated with poor prognosis are cancer stem cells (CSCs) (Ben-Porath et al., 2008; Leck et al., 2025). The first association of cancer and stem cells was brought to light in a study of human acute myeloid leukemia (Lapidot et al., 1994). CSCs represent the scenario of dysregulated stem cells combining the unique features of self-renewal and differentiation, and cancerous characteristics in mutagenic tolerance and adaptation. Thereby, two hypotheses are still in debate about their origin and distribution. The first one involves the emergence from normal stem/progenitor cells owing to genetic alterations and the second one states the regaining of stem cell like properties of somatic or cancerous cells (summarized in Yu et al., 2012). An overexpression of known pluripotency markers like *OCT4*, *Sox2* and *c-Myc* were identified in CSCs contributing to their stem cell-like features (Beck & Blanpain, 2013; Gidekel et al., 2003; Santagata et al., 2007). CSCs are associated with poor survival rates due to their plasticity, regeneration potential and evidence showing chemo and radiation resistance supporting tumor survival, progression, relapses and metastasis formation (Leck et al., 2025; Li et al., 2008; Yu et al., 2012; Zielske et al., 2011). Attributable to their similarities to normal stem cells and their shared signaling pathways, CSCs

are challenging to target without harming healthy stem cells, thus identifying the differences is significant for therapy.

1.3 Differentiation into neuronal lineage

The development of the central nervous system (CNS) in the human organism involves sequentially and distinctly orchestrated steps involving drastic changes on transcriptional and translational level for the emergence of a functional nervous system consisting of different cell types including neurons and glial cells (Silbereis et al., 2016; reviewed in Zhou et al., 2024). The CNS derives from the outer germ layer, the ectoderm, forming the neuroectoderm followed by the neural plate and neural tube. During neural induction, mesodermal cells produce noggin, chordin and follistatin in the dorsal mesoderm, the notochord, and cause the inhibition of bone morphogenic protein 4 (BMP4) leading ectodermal cells towards neuronal differentiation (reviewed in Sasai & De Robertis, 1997). Withal, the walls of the neural tube are sheathed by neural stem cells, proliferating and differentiating into neurons, oligodendrocytes and astrocytes (reviewed in Andrews et al., 2022). From there on, the primary and secondary brain vesicles emerge and give rise to the different brain regions accompanied by migrating and maturing neurons (Bayer & Altman, 2002; Götz & Huttner, 2005; O'Rahilly & Fabiola, 2006; Silbereis et al., 2016; Zhou et al., 2024).

Enlightenment of this complex process of neural differentiation and the investigation of human diseases and disorders became important aspects of iPSC models and differentiation. Animal models are still commonly used to mimic human diseases despite the high costs, ethical concerns and limitations in translation between animals and humans. Wherefore, iPSC-based model systems retain a great potential overcoming many of these issues, reducing the need of animal experiments and enabling high-throughput screening and neurotoxicological assessment (Schinke et al., 2021; Tukker et al., 2016; Tukker et al., 2018). For instance, patient-derived iPSCs facilitated the in-depth study of molecular mechanisms behind genetic diseases including Parkinson´s disease (Nguyen et al., 2011; Sánchez-Danés et al., 2012), Huntington´s disease (Zhang et al., 2010) and amyotrophic lateral sclerosis (Karumbayaram et al., 2009). Moreover, iPSC-based disease modeling supports the comprehension of early factors and pathology of, especially, late onset diseases and provide valuable insights for therapeutic concepts and strategies (Kim et al., 2024).

In recent years, different methods have been established for the differentiation of iPSCs into the neural lineage or directly into neurons. Thereby, cells can be cultivated and maintained as 2D cultures for fast and efficient modeling (Vigont et al., 2023) or as 3D organoids reflecting *in vivo* differentiation and organization, and providing enhanced physiological relevance

(Montgomery et al., 2015). A rapid conversion of pluripotent stem cells into specific mature neuron types can be performed by the inducible expression of neuron-specific transcription factors like *NEUROG2* (Fernandopulle et al., 2018). By using this approach, mature neurons are obtained quickly, but the developmental aspects are not considered. Another method for obtaining mature and physiologically functioning neurons is based on electrical and biochemical stimulation in microfluidic arrays (Kim et al., 2024). Well-established protocols for mimicking neural differentiation *in vitro* involve the application of small molecules (Reinhardt et al., 2013; Zink et al., 2021). These generated neural progenitor cells (NPCs) are capable of differentiating into neuron types of the CNS and neural crest cells facilitating a robust and immortal expansion only requiring small molecules for self-renewal and expansion. Therefore, dual SMAD inhibition is utilized involving molecules inhibiting BMP and transforming growth factor beta (TGF-β)/Activin/nodal growth differentiation factor (NODAL) signaling. Concomitantly, molecules that induce canonical Wingless-related integration site (WNT) and Sonic Hedgehog (SHH) signaling pathways are implemented initiating neural differentiation and resulting in the expression of neural progenitor markers Sox1, Sox2, neuroepithelial stem cell protein (Nestin) and paired box 6 (Pax6) (Reinhardt et al., 2013).

Obtained NPCs can be further differentiated into various neuron types by regulating the inhibitor concentrations or adding new small molecules for a specific direction. For undirected differentiation including neurons, astrocytes and oligodendrocytes it is sufficient to remove the small molecules. Neuronal maturation can be achieved by supplementing neurotrophic factors to promote neuronal survival and network generation (Hu et al., 2010). For instance, the differentiation of dopaminergic neurons requires additional small molecules like fibroblast growth factor 8 (FGF8), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), TGF-β3 and dibutryryl cyclic adenosine monophosphate (dbcAMP) (Reinhardt et al., 2013). Another method is the regulation of the glycogen synthase kinase 3 (GSK3) inhibitor concentration for controlling WNT signaling to obtain neural cultures with forebrain, midbrain or hindbrain identities (Kirkeby et al., 2012).

Altogether, the expansion of personalized medicine and enhancement of new therapeutic approaches are key aspects in today´s research, especially owing to the improvement of life expectancy and consequential age-related neurodegenerative diseases. Over the last two decades, the isolation and cultivation of ESCs and particularly the reprogramming of somatic cells to iPSCs were groundbreaking findings, offering valuable information about the pathology of various diseases and became an irreplaceable tool in therapeutic research. *In vivo*, stem cells are sheltered and strictly regulated by intrinsic and extrinsic factors ensuring a pool of qualitative and faultless cells. One of these cellular processes supporting stem cell function, maintenance and quality control is represented by autophagy.

1.4 Autophagy

Autophagy is a highly conserved catabolic pathway mediating the recycling of a diverse range of biomolecules, including long-lived or damaged proteins, lipids, nucleotides and even pathogens, in double membraned vesicle called autophagosomes. The term “autophagy” was first introduced by M. Anselmier, a french physiologist, in 1859 describing the survival on one´s own sources in periods of nourishment deprivation (Ktistakis, 2017). In 1963, Christian de Duve coined the term as it is interpreted today as the lysosomal degradation of cellular components (Klionsky, 2008). His earning of the Nobel Prize in Physiology and Medicine in 1974 followed by the 2016 Nobel Prize awarded to Yoshinori Ōsumi´s exceptional work in identifying the molecular mechanisms of autophagy and 15 autophagy-related proteins (ATGs) in yeast underlines the importance of this cellular process in the scientific and clinical field (Tsukada & Ohsumi, 1993).

The autophagic pathways are evolutionary conserved processes and present in different organisms including mammals, yeast and plants. The absence in prokaryotes is owed to the absence of intracellular compartmentalization (Yamamoto et al., 2023). Generally, the autophagic pathways can be subdivided into three categories characterized by their varying cargo and lysosomal uptake. Chaperone-mediated autophagy (CMA) is defined by its selectivity for cytosolic proteins bearing the KFERQ motif (Dice, 1990). This motif facilitates the binding to chaperone protein heat shock cognate 71 kDa protein (HSC70/HSPA8) and cochaperones involving carboxyl terminus of HSC70-interacting protein (CHIP), heat shock protein 40 (HSP40/DNABJ1) and HSP70–HSP90 organizing protein (HOP). After recognition, the chaperone–cargo complex is transported to the lysosomal surface for its internalization via the receptor lysosome-associated membrane protein type 2A (LAMP2A) and degradation within the lysosome (Kaushik & Cuervo, 2018). The second category comprises the process of microautophagy and its cargo uptake by endosomal or lysosomal membrane invagination while the third category describes the macroautophagic process and the sequestration of selected or unselected cargo within cytosolic double-membraned vesicles and their consecutive fusion with lysosomes generating macromolecular building blocks and energy (Feng et al., 2014; reviewed in Yamamoto et al., 2023).

Besides the ubiquitin-proteasome system (UPS) the autophagic process is involved in maintaining cellular homeostasis and adapting to cellular stress by ensuring the supply of nutrients in times of need, i.e., during development or starvation. Both systems show distinct mechanisms in their cargo recognition and handling. While the UPS mainly degrades single, unfolded polypeptides that are small enough to enter the proteasome, autophagy mainly focuses on larger, cytosolic structures including protein complexes and cellular aggregates and even entire organelles. Despite their different degradation machinery, both systems share

molecular determinants and substrates like ubiquitin and sequestosome-1 (SQSTM1/p62) (Liu et al., 2016; Pohl & Dikic, 2019). Nevertheless, the UPS is incapable of compensating for defective autophagic machinery. Non-functioning autophagy can be observed in different diseases and disorders, typically involved in the accumulation of harmful components, such as cancer (Saha et al., 2018), neurodegenerative diseases including Parkinson´s disease (Cerri & Blandini, 2019), Alzheimer´s disease (Nixon & Rubinsztein, 2024) and metabolic diseases like type 2 diabetes (Klionsky et al., 2021; Saha et al., 2018). These findings emphasize the importance of the autophagic process regarding its cytoprotective features, ensuring cellular homeostasis and quality control by engulfing superfluous and harmful components from the cytosolic compartment and underline the great significance in expanding knowledge about the importance of this pathway in pathological circumstances.

1.4.1 (Macro-)autophagy

As aforementioned, macroautophagy (herein referred to as autophagy) is a highly conserved process by which cells recycle and degrade intracellular components to ensure cellular homeostasis, survival and adaptation to stress. Autophagy can be categorized in selective and non-selective bulk sequestration. In comparison, bulk sequestration is associated with the indiscriminate engulfment of cytosolic components upon stress and nutrient deprivation, while selective autophagy is linked to the degradation of specifically targeted cellular components, i.e., mitochondria (mitophagy), protein aggregates (aggrephagy), ribosomes (ribophagy) or invading pathogens (xenophagy) and so on (W. Li et al., 2021; Shaid et al., 2012; Vargas et al., 2022).

The core machinery for the initiation of the autophagosome formation and its consecutive lysosomal delivery consists of 5 components: (i) Unc-51-like kinase 1 (ULK1) complex consisting of the serine/threonine protein kinases ULK1/2, RB1-inducible coiled-coil protein 1 (RB1CC1/FIP200), ATG13 and ATG101 (Dikic & Elazar, 2018); (ii) ATG9, the sole transmembrane protein involved in the translocation of phospholipids (Matoba et al., 2020); (iii) class III phosphatidylinositol 3-kinase (PI3KC3) complexes generating phosphatidylinositol 3-phosphate (PI3P); (iv) WD repeat domain phosphoinositide-interacting (WIPI) proteins facilitating the recruitment of ATG proteins to the phagophore and finally, (v) two ubiquitin-like conjugation complexes ATG12–ATG5-ATG16L1 and MAP1LC3 (microtubule-associated protein 1A71B light chain 3)/GABARAP (Gamma-aminobutyric acid receptor-associated protein) (Dikic & Elazar, 2018).

Autophagy is a highly adaptive process reacting to various stress stimuli including amino acid, growth factor and nutrient scarceness, and hypoxia, as well as infections, an increased ratio

of adenosine monophosphate (AMP) to adenosine triphosphate (ATP), and DNA damage (Klionsky & Emr, 2000; Rodriguez-Rocha et al., 2011; reviewed in Zoncu et al., 2011). The best characterized triggers to induce autophagy are the deprivation of amino acids and glucose regulating the serine/threonine kinases mechanistic target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK). Thereby, mTOR is implemented in two functionally distinct complexes, mTORC1 and mTORC2, whereas mainly complex 1 is involved in the autophagic regulation (Bar-Peled & Sabatini, 2014). In positively prevailing conditions, mTORC1 is activated and leads to the inhibition of autophagy (Alers et al., 2012). In contrast, AMPK displays a major energy-sensing kinase regulating cellular metabolism and energy homeostasis and is activated by metabolic stress or ATP depletion, initiating the onset of various catabolic processes including autophagy. Both kinases are able to regulate the activation and inhibition of autophagy by controlling the ULK1 complex. The ULK1 complex is the key regulator of autophagy initiation accompanied by the three above-mentioned proteins. Both FIP200 and ATG13 are essential for stability and translocation of ULK1 to the autophagosome biogenesis site additionally supported by ATG101 for autophagosome formation (Ganley et al., 2009; Jung et al., 2009). Under nutrient-rich conditions, mTORC1 facilitates the phosphorylation of ULK1 and ATG13 and interacts directly with ULK1 with its regulatory-associated protein of mTOR (RAPTOR) subunit phosphorylating ULK1 at serine (Ser) 638, the AMPK binding site Ser 758 and ATG13 at Ser 258 preventing autophagy induction (J. Kim et al., 2011; Puente et al., 2016). Interestingly, AMPK has a conflicting role in the regulation of ULK1 and autophagy induction (Kim, 2024; Park & Kim, 2024). Growing evidence suggests that AMPK does not promote autophagy upon energy depletion but rather stabilizes autophagic components and prevents their degradation (Lang et al., 2014; Nwadike et al., 2018; Park et al., 2023; Ramírez-Peinado et al., 2013). However, in the prevailing model, AMPK is activated upon glucose starvation by allosteric binding of AMP and concomitantly, is able to either indirectly inhibit mTORC1 via the activation of tuberous sclerosis 2 (TSC2) or directly phosphorylating RAPTOR (Gwinn et al., 2008; Hardie, 2007; Tripathi et al., 2013). Subsequently, ULK1 dissociates from mTORC1 and becomes dephosphorylated. This steps enables the binding of AMPK to ULK1 and ULK1´s autophosphorylation resulting in the consecutive phosphorylation of FIP200, ATG13 and ATG101 (Egan et al., 2015; Ganley et al., 2009; Poole et al., 2021). AMPK phosphorylates ULK1 at several positions, amongst others sharing Ser 638 with mTORC1, thereby supporting the activation of autophagy as depicted in Figure 4 (summarized in Wong et al., 2013). Besides the phosphorylation of its binding partners, ULK1 plays an essential role in the phosphorylation and control of various downstream components including the components of PI3KC3 complex I (PI3KC3C1), ATG9, selective autophagy receptor (SAR) SQSTM1/p62, ATG16L1 and the SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptors) proteins YTK6 and STX17

which are involved in the temporal control of autophagosome maturation and lysosomal fusion (reviewed in Pareek & Kundu, 2024). Therefore, ULK1 is not only pivotal for the initiation of the autophagic process but also for its progression.

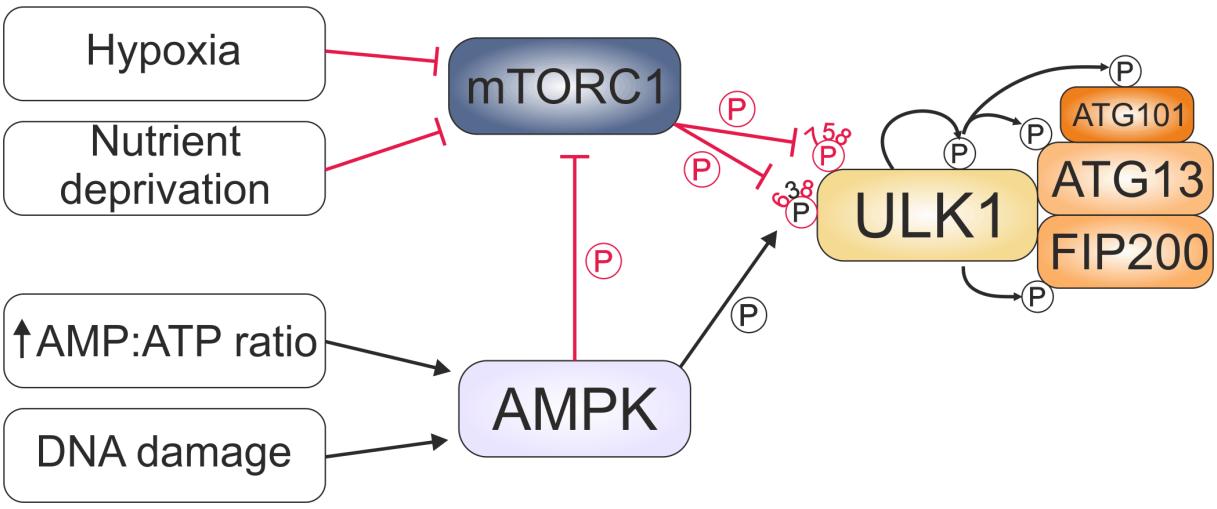

Figure 4: Regulation of ULK1 activity. Under nutrient-rich conditions, the mTORC1 subunit RAPTOR directly interacts with ULK1 and leads to the phosphorylation of ULK1 at the positions Ser 758 and Ser 638 causing the inhibition of autophagy initiation. Upon different stress stimuli including oxygen and nutrient depletion, the inhibition of mTORC1 results in the dissociation and dephosphorylation of ULK1 and subsequently to its activation. Additionally, stimuli such as DNA damage or an increased AMP:ATP ratio are able to activate AMPK which participates in the inhibition of mTOR and the direct phosphorylation of ULK1 at Ser 638. Afterwards, ULK1 undergoes autophosphorylation and phosphorylates its binding partners within the ULK1 complex promoting autophagy initiation.

As illustrated in Figure 5, the autophagic process can be subdivided into five sequential steps comprising autophagic initiation, elongation of the phagophore, autophagosomal maturation, fusion to the lysosome and degradation. Upon activation, the ULK1 complex translocates to a distinct region of the endoplasmatic reticulum (ER) known as the phagophore assembly site (PAS). Sequentially, PI3KC3 complex I becomes phosphorylated by ULK1 and is recruited to the initiation site catalyzing the production of PI3P and leading to the formation of the omegasome. The omegasome is a protrusion of the ER characterized by the accumulation of PI3P and marked by PI3P-binding protein zinc-finger FYVE domain-containing protein 1 (DFCP1), displaying the origin of a cup-shaped double-membraned structure referred to as the phagophore (Yamamoto et al., 2023). PI3P enriched regions play an important role in protein binding for subsequent vesicle formation. There are two configurations of PI3KC3 complexes both involved in the production of PI3P sharing the following subunits: the vacuolar protein sorting 34 (VPS34) lipid kinase, the VPS15 scaffold protein, and the regulatory Beclin1 (BECN1). Complex I additionally comprises the subunits ATG14 and nuclear receptor binding factor 2 (NRBF2) (Young et al., 2016) and is specifically involved in the PI3P production during

autophagy initiation (Matsunaga et al., 2010; Zhong et al., 2009). In comparison, complex II is associated with UV radiation resistance associated gene protein (UVRAG) and is mainly involved in endosomal PI3P production and plays a role in autolysosome formation (Kim et al., 2015; Liang et al., 2006). VSP15 and BECN1 primarily exhibit scaffold functions that support VSP34 kinase function and enable possible interactions with further regulatory complexes and are involved in the association of the complex with membranes. In comparison, ATG14 participates in the interaction of the PI3KC3C1 with the ULK1 complex via direct interaction with ATG13, thereby facilitating the direct phosphorylation of ATG14 by ULK1 (Park et al., 2016). Additionally, ATG14 is linked to the recognition of the PAS and the translocation of PI3KC3C1 to these sites (reviewed in Dikic & Elazar, 2018). The accessory subunit NRBF2 does not seem to be essential for PI3P production but has been shown to enhance kinase activity (Young et al., 2016). For the production of PI3P, activated VPS34 phosphorylates phosphatidylinositol at the 3′ position of the inositol ring for the generation of PI3P. The inhibition of VSP34 has been associated with the complete blockage of novel autophagosome generation (Nähse et al., 2024). The PI3P-rich regions at the omegasome support the interaction with PI3KC3C1 and PI3P-binding proteins accelerating changes in lipid composition for phagophore formation. These events are accompanied by the phosphorylation of ATG9A by ULK1 and thereby the recruitment of ATG9A-containing vesicles delivering lipids for the elongation of the phagophore (Papinski et al., 2014; Young et al., 2006; Zhou et al., 2017). ATG9 vesicle recruitment is facilitated by the interaction between the most C-terminal region of ATG9A and the HORMA-domain of the ATG13-ATG101 subcomplex (Ren et al., 2022). Moreover, ATG9A functions as a lipid scramblase facilitating the movement of lipids between the inner and outer leaflets of the membranes (Holzer et al., 2024; Maeda et al., 2020; Matoba et al., 2020). ATG9A vesicle emerging from the trans-Golgi apparatus are considered to be the initial membrane source. After initiation, ATG9A is translocated to the expanding edges of the phagophore (Matoba et al., 2020). For the expansion of the phagophore, phospholipids originating from the ER are then transferred to the expanding autophagosome membrane by ATG2, which has a rod-shaped structure with a hydrophobic pocket and binds to the ER and autophagosome with the assistance of the PI3P-binding protein WIPI4 (Chowdhury et al., 2018; Yang Wang et al., 2025; Zheng et al., 2017).

Furthermore, these clusterings of PI3P enable the interaction with WIPI2 and the recruitment of the two ubiquitin-like conjugation systems ATG12–ATG5-ATG16L1 and ATG8 family proteins LC3/GABARAP. WIPI2 is essential for the biogenesis of autophagosomes (Polson et al., 2010; Proikas-Cezanne et al., 2015) and interacts with ATG16L1, enabling the recruitment of the ATG12–ATG5-ATG16L1 complex to the phagophore (Dooley et al., 2014). ATG12 is activated by ATG7 in an ATP-dependent manner (Mizushima et al., 1998) allowing the conjugation to ATG5, which is catalyzed by ATG10 (Kaiser et al., 2012). This conjugate then

translocates to the phagophore where it binds to ATG16L1. With the support of other ATG proteins, this conjugate is responsible for the lipidation and conjugation of ATG8s to the membrane-resident phosphatidylethanolamine (PE). For the lipidation, the cysteine protease ATG4 catalyzes the exposure of a C-terminal glycine residue in ATG8s, giving rise to the unbound isoform LC3-I (Slobodkin & Elazar, 2013). For a successful PE conjugation, ATG3 binds to the ATG12–ATG5 conjugate (Metlagel et al., 2013). Afterwards, LC3-I is activated by ATG7 and conjugated to PE by ATG3 and ATG12–ATG5-ATG16L1 complex forming membrane-anchored LC3-II as depicted in Figure 5 (Dikic & Elazar, 2018; Weidberg et al., 2011). The importance of ATG8 lipidation was shown in knockout experiments causing the complete blockage of autophagy upon knockout of ATG4 or ATG5 (Fujita et al., 2008; Kuma et al., 2004). The two conjugation systems have been associated with multiple functions including membrane elongation, cargo recognition, autophagosomal closure, autophagosomal trafficking, fusion between autophagosomes and lysosomes, and inner membrane degradation (reviewed in Mizushima, 2020). For instance, a conserved motif of LC3, the LC3-interacting region (LIR), enables the direct or indirect interaction with specific interaction partners carrying this motif like the SAR SQSTM1/p62 for selective cargo recognition (Alemu et al., 2012; Pankiv et al., 2007). Since LC3-II adorns the inner and outer membrane of autophagosomes, the conjugates on the inner membrane get degraded together with the engulfed cargo upon lysosomal fusion while LC3-II on the outer membrane can be recycled by deconjugation from PE via ATG4 (Mizushima, 2020; Nakatogawa et al., 2012).

Subsequently, the closure of the phagophore is catalyzed by the ESCRT (endosomal sorting complexes required for transport) protein complexes I and III, enabling the fission of the inner and outer membrane (reviewed in Melia et al., 2020). Following cargo encapsulation and sealing of the membrane, the enclosed autophagosome undergoes maturation. This process involves the dissociation of ATGs, except for LC3-II, from the outer membrane and the recruitment of proteins involved in lysosomal delivery and fusion. Both vesicles are transported to each other by microtubule-based kinesin motors. Thereby, LC3 supports maturation by connecting the autophagosome with kinesin through autophagy-specific kinesin adaptors such as FYCO1 (FYVE and coiled- coil domain-containing protein 1) to move them along microtubules towards the plus end direction (Dikic & Elazar, 2018; Olsvik et al., 2015).

The understanding of the precise mechanisms involved in the fusion of lysosomes and autophagosomes still remains limited. Nevertheless, the fusion of lysosomes and autophagosomes involves small GTPases and tethering factors which are responsible to ensure proximity of both vesicles. Furthermore, two SNARE complexes are involved in promoting fusion. These complexes encompass i) syntaxin 17 (STX17) and synaptosomal-associated protein 29 (SNAP29) on the autophagosomal membrane and autophagosome and

vesicle-associated membrane protein 8 (VAMP8) on the lysosomal membrane and ii) autophagosomal YKT6, SNAP29 and lysosomal STX7 (Matsui et al., 2018; Zhao & Zhang, 2019). Thereby, autophagosomal SNARE proteins are referred to as target-SNARE (t-SNARE) and lysosomal ones as vesicle-SNARES (v-SNARE) forming *trans*-SNARE complexes (Diao et al., 2015; Itakura et al., 2012). The mechanism underlying STX17 recruitment to the autophagosome is still unknown, however, STX17 is exclusively present on the matured autophagosome and not the open phagophore preventing the prior interaction with the lysosomes (Itakura et al., 2012). Additionally, ATG14 interacts with STX17 stabilizing the assembly of the STX17–SNAP29 complex (Zhao & Zhang, 2019). It has been demonstrated that LC3 interacts with tethering factors like the homotypic fusion and vacuole protein sorting (HOPS) tethering complex promoting the generation of autolysosomes, together with the *trans*-SNARE complexes (Manil-Ségalen et al., 2014; Takáts et al., 2014; Zhao & Zhang, 2019). As the final step of the autophagic process, lysosomal enzymes catalyze cargo degradation, including LC3-II and the receptor p62. Moreover, the inner autophagosomal membrane is degraded by potential phospholipases. It has been shown in yeast, that Atg15 functions as a phospholipase degrading the inner membrane but not the outer one suggesting similar properties in mammalian cells (Epple et al., 2001; Teter et al., 2001). The outer membrane either does not contain the substrate for the lipases or is protected by them (Yim & Mizushima, 2020). After the degradation of the inner autophagosomal membrane, more than 60 lysosomal hydrolases acquire access to the engulfed components (Schröder et al., 2010). For optimal functionality, those enzymes require an acidic pH and rely on successful acidification of the autolysosome facilitated by proton pumping vacuolar-type H^+-translocating ATPases (V-ATPases) and chloride ion channels (Ishida et al., 2013; Mindell, 2012). Impaired acidification may result in restricted autophagic function and is associated with diseases independent from ATG functionality, for instance, in neurodegenerative diseases (Menzies et al., 2015; Rubinsztein et al., 2005) including Alzheimer´s disease (Nixon & Yang, 2011; Zare-Shahabadi et al., 2015) and Parkinson´s disease (Dehay et al., 2013). Subsequently, metabolic building blocks such as nucleotides, amino acids or other macromolecules are released via permeases in the lysosomal membrane (Wong et al., 2013).

Altogether, autophagy displays a crucial mechanism in cells for the recycling of superfluous or unwanted cellular content and participates in cell homeostasis and quality assurance due to the facts of its disease association upon dysregulation or impairment.

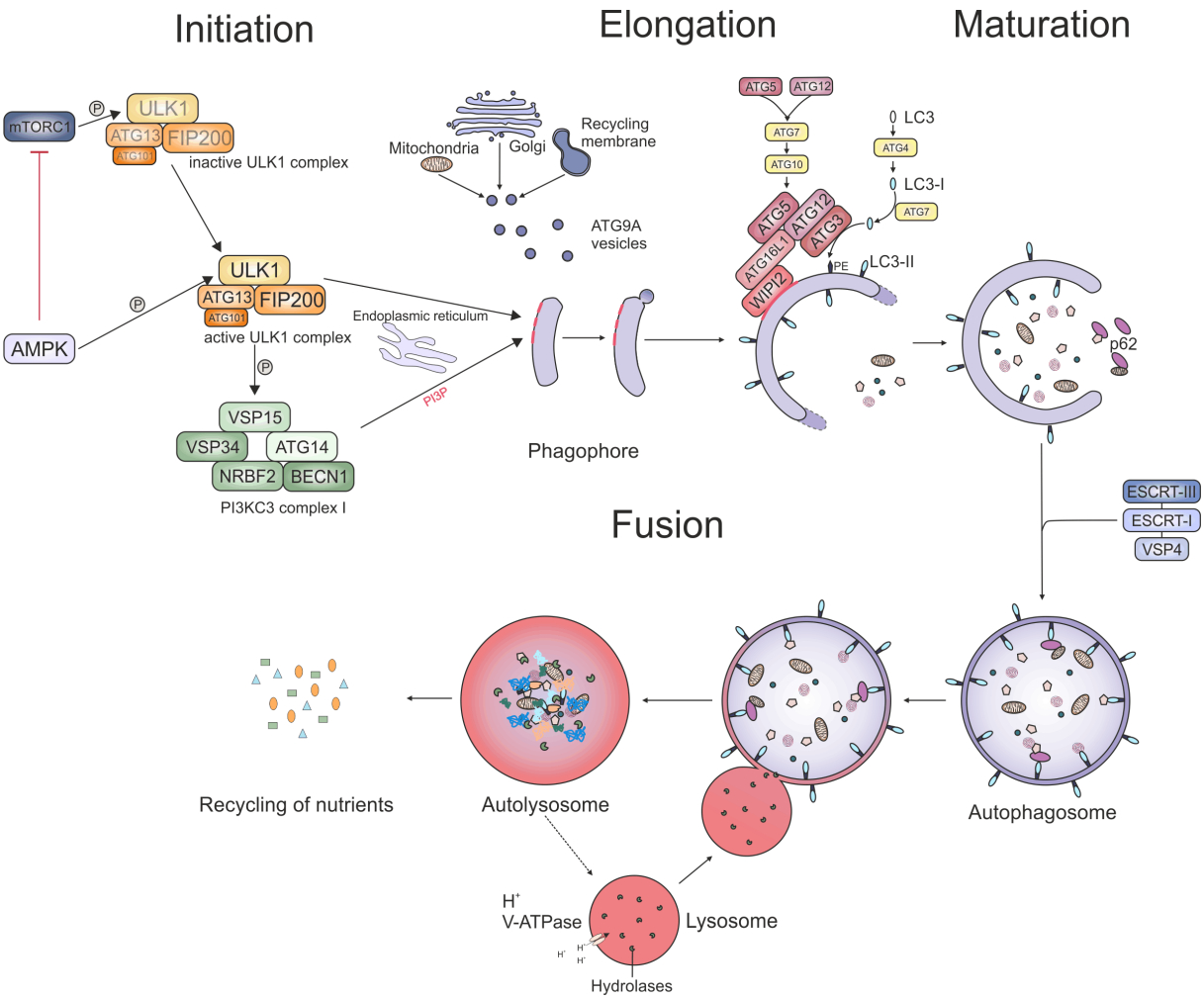

Figure 5: The (macro-)autophagic process. Under nutrient-rich conditions, the ULK1 complex, consisting of ULK1, ATG13, ATG101, and FIP200, is inhibited by phosphorylation via mTORC1. Upon cellular stress, AMPK is able to inhibit mTORC1 and subsequently, activate the ULK1 complex resulting in its autophosphorylation and the activation of the PI3KC3 complex I. This complex is involved in the generation of PI3P at a distinct site of the endoplasmatic reticulum, the phagophore assembly site (PAS) and initiating the phagophore nucleation. Expansion of the phagophore is mediated by ATG9A containing vesicles mainly and ER lipids. The enrichment of PI3P in the phagophore membrane enables the interaction with WIPI2 and the sequential recruitment of the ATG12–ATG5-ATG16L1 complex. This conjugated complex is involved in the lipidation of ATG8 proteins, including LC3. Thereby, cytosolic LC3 undergoes cleavage by ATG4 generating the floating isoform LC3-I. Subsequently, LC3-I is activated and bound to phosphatidylethanolamine (PE) forming LC3-II which supports phagophore elongation, autophagosome closure and lysosomal fusion. Upon closure, cytosolic components are either sequestrated indiscriminately or bound to selective autophagy receptors like p62 that are able to interact with inner membrane-bound LC3-II. Phagophore closure is mediated by the ESCRT complexes forming the mature double-membraned autophagosome. Finally, the autophagosome fuses with acidic lysosomes containing hydrolases responsible for the degradation of sequestered components and the release of energy sources and macromolecular building blocks.

1.5 Role of autophagy in stem cells

Autophagy is an essential process for sensing cellular status and adapting to metabolic changes. Its properties in degradation and recycling of cytosolic components play an important role in cell homeostasis and quality control and influences successful development and cell functions. As aforementioned, SCs require high quality properties since they are the fundamental element for generating and maintaining a healthy organism. These cells have distinct metabolic requirements and are strictly regulated by intrinsic and extrinsic factors. Previous studies have demonstrated that the autophagic process plays a key role in stem cell function by affecting stem cell activation, quiescence, self-renewal and differentiation (reviewed in Boya et al., 2018). Dysfunction or dysregulation of the autophagic processes have been associated with various diseases encompassing neurodegenerative diseases, cancer, muscle and heart diseases, as well as aging. Growing evidence shows that the proper metabolic homeostasis is significant for stem cell function and controlling their fate (X. Li et al., 2023; K. Liu et al., 2020). Thereby, stem cells rely mainly on energy-rich substances like glucose, fats and amino acids which are synthesized or catabolized during glycolysis, tricarboxylic acid (TCA) cycle or oxidative phosphorylation (OXPHOS). Prior to the development of the circulatory system, cells develop under oxygen-restricted conditions and this same phenomenon is also observed for adult stem cells residing in stem cell niches with low oxygen levels. Therefore, stem cells mainly rely on glycolysis and are able to switch in between different metabolic pathways adapting to different conditions (Mohyeldin et al., 2010; Shyh-Chang et al., 2013). During differentiation and leaving the niche, the cells undergo a shift to OXPHOS, thus, the balance of different metabolic pathways is crucial for stemness and differentiation (Chandel et al., 2016). Proteins involved in sensing changes in energy homeostasis are the same ones involved in autophagic activation upon cellular stress, mTOR and AMPK (X. Li et al., 2023). As mentioned before, autophagy can be divided into several subcategories depending on their selective cargo, i.e., mitophagy for the degradation of damaged or long-lived mitochondria. In regard to iPSCs, the role of the autophagic process still needs to be further investigated. However, autophagy might play an important role in reprogramming somatic cells into pluripotency by supporting mitochondrial remodeling and increasing reprogramming efficiency in early stages (Keisuke Ito & Toshio Suda, 2014; S. Wang et al., 2013). Deficiencies in ATG proteins, involving ATG7 (Mortensen et al., 2011) and ATG12 (Ho et al., 2017), in hematopoietic stem cells have shown an accumulation of reactive oxygen species (ROS) and activated mitochondria causing an increase in DNA damage, differentiation and displaying an aging phenotype. Additionally, these deficient cells resulted in a reduced stem cell and progenitor pool (Mortensen et al., 2011) which may be caused by impaired self-renewal and regenerative potential. These observations regarding increased ROS levels and consequential differentiation were also made in neural stem cells (Khacho et

al., 2016). Hence, these results support the importance of autophagy for stem cell function and maintenance by regulating the removal of activated mitochondria and oxidative metabolism (reviewed in Chang, 2020; Ho et al., 2017; Yu et al., 2013). Furthermore, hematopoietic stem cells require basal autophagy for stem cell maintenance and are capable of rapidly activating autophagy upon metabolic stress. To prevent cellular death, SCs express high levels of the transcription factor FOXOA3 which facilitates the activation of pro-autophagic genes (Mammucari et al., 2007; Warr et al., 2013). The transcription factor FOXO3 is also involved in autophagic regulation in adult neural stem cells (Audesse et al., 2019).

While autophagy is well-studied during embryonic development of the nervous system, the understanding in adult neural stem cells remains comparatively limited. Interestingly, adult neural stem cells have a high lysosomal content containing insoluble protein aggregates. Lysosomal inhibition leads to the accumulation of these aggregates and negatively affects stem cell activation (Leeman et al., 2018). In another study, the developmental deletion of *FIP200* in postnatal neural stem cells resulted in the accumulation of mitochondria, ROS and the depletion of the stem cell pool. Additionally, an accumulation of p62 aggregates was observed suggesting an involvement in superoxide control (C. Wang et al., 2013). Conversely, the same results could not be obtained by *ATG5, ATG7* or *ATG16L1* deletion. The deletion of these ATGs resulted in an accumulation of mitochondria and impaired autophagy but did not affect stem cell maintenance, differentiation or cause the accumulation of p62 aggregates. These observations implied that NSC pool depletion is affected by superoxide levels rather than by the accumulation of mitochondria and that either a non-autophagic function of FIP200 is responsible for controlling p62 and superoxide levels or that autophagy induction and phagophore elongation are weighted differently in NSCs (Wang et al., 2016).

Therefore, various studies have shown that the deletion of autophagy related proteins results in impaired differentiation, proliferation and cell survival (summarized in Chang, 2020). Autophagy is known for its cytoprotective properties, nevertheless, it can also be involved in the induction of cell death, e.g., upon insulin withdrawal and oxygen-glucose deprivation in primary rat hippocampal NSCs (Chang, 2020; Chung et al., 2018; Ha et al., 2017). In summary, a growing body of evidence supports the hypothesis that autophagy is required and crucial for preserving stem cell function and by participating in cellular remodeling and metabolism control serves as an essential quality control mechanism (Boya et al., 2018).

1.6 DNA damage response and repair in mammalian cells

During an organism´s lifetime, the most sacred mission to ensure cellular functionality contributing to a healthy organism is to ensure genomic stability, intact and unchanged

replication, transfer to progeny and to guard the genome from perpetual exposure to DNA damaging factors. The estimation of 10^5 spontaneous DNA lesions per day (Hoeijmakers, 2009) resulting from, for instance, dNTP misincorporation during DNA replication, and additional threats from endogenous (e.g., ROS) and exogenous (e.g., ultraviolet (UV) light, environmental toxins) genotoxic agents underline the significance of efficient repair mechanisms referred to as the DNA damage response (DDR) (Ciccia & Elledge, 2010). The DDR is a complex network consisting of multiple pathways that are governed by proteins responsible for surveilling DNA damage, recruiting various complexes and initiating the remodeling and repair of a divergent range of lesions based on nature.

A plethora of exogenous factors can endanger the genome, encompassing physical and chemical genotoxic agents. For instance, UV light exposure triggers the emergence of cyclobutane-pyrimidine dimers, 6-4 photoproducts and the production of ROS, leading to the oxidation of nitrogen bases and causing, e.g., 8-oxo-7,8-dihydroguanine (8-oxo-dG) (Kciuk et al., 2020; Rastogi et al., 2010). These lesions distort the DNA structure hampering with DNA replication and transcription and enable the introduction of mutations and DNA strand breaks (Greinert et al., 2012; Pfeifer et al., 2005). In comparison, ionizing radiation (IR), used for medical X-rays or radiotherapy, is able to break covalent bonds within the DNA directly causing DNA single-strand breaks (SSBs) and also, one of the most lethal damages, DNA double-strand breaks (DSBs) (Borrego-Soto et al., 2015; Ciccia & Elledge, 2010; Pastink et al., 2001; Sieber et al., 2003). Examples for chemical genotoxic agents are chemotherapeutics used for cancer therapy. Depending on the type of cancer in question, a variety of chemotherapeutic agents are available that cause diverse types of DNA damage, e.g., alkylating agents such as temozolomide; cisplatin, which is able to induce covalent crosslinks of base pairs within the same DNA strand (intrastrand crosslinks) or with the complementary strand referred to as interstrand crosslink (ICL); topoisomerase I inhibitor camptothecin or topoisomerase II inhibitor etoposide causing a distortion of DNA topology and induction of SSBs and DSBs, respectively (Ciccia & Elledge, 2010). Another exogenous factor and one of the most preventable causes of cancer might be cigarette smoking. It has been established that tobacco and tobacco smoke contain 9500 chemicals. Of these, 533 compounds have been either confirmed to be carcinogenic, or are considered probably or possibly carcinogenic to humans including polycyclic aromatic hydrocarbons forming aromatic DNA adducts such as benzo[a]pyrene (BaP) (summarized in Li & Hecht, 2022).

As mentioned above, to maintain genomic integrity and prevent DNA mutations cells possess a spectrum of DDR mechanisms specialized for the removal of damaged and modified areas, and the restoration of the DNA. These mechanisms are divided into mismatch repair (MMR) to replace and correct mismatched DNA bases (Jiricny, 2006), base excision repair (BER)

correcting DNA bases with chemical alterations or oxidations (Lindahl & Barnes, 2000), nucleotide excision repair (NER) involved in the removal of oligonucleotides after dimerization, intrastrand crosslinks or DNA adducts (Friedberg et al., 2005; Gillet & Schärer, 2006), interstrand crosslink repair and single-strand break repair. DNA double-strand breaks can be repaired by two different mechanisms: non-homologous end joining (NHEJ) and homologous recombination (HR) which will be discussed later.

Key players of the DDR orchestrating the appropriate protein recruitment and downstream cellular response are members of the phosphatidylinositol 3-kinase-like protein kinases (PIKKs) family and the poly(ADP)ribose polymerase (PARP) family. Thereby, PIKK proteins are represented by the serine kinase ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). ATM and DNA-PKcs activation is triggered by DSBs, whereas ATR is activated by both SSBs and DSBs (Cimprich & Cortez, 2008). ATM and DNA-PKcs are mainly involved in DNA DSB repair, however, exhibit different roles and impact on the repair response (Harper & Elledge, 2007). While ATM interacts with a plethora of downstream proteins and effectors, the activation of DNA-PKcs only affects a small group of proteins involved in NHEJ (Ciccia & Elledge, 2010; Meek et al., 2008). Proteins from the PARP family, PARP1 and PARP2, are also activated by SSBs and DSBs and support the recruitment of DDR components to the lesion site (Schreiber et al., 2006). After the recognition of DNA lesions, ATM and ATR interact with mediator proteins to amplify the response signaling by either directly phosphorylating effector proteins or indirectly by checkpoint-kinase (CHK) 1 and 2 (Harper & Elledge, 2007; Zhou & Elledge, 2000). One of the most studied and important effector proteins phosphorylated by ATM and CHK2 upon occurrence of DSBs is p53 (Ciccia & Elledge, 2010; Zhou & Elledge, 2000). The tumor suppressor protein p53 displays a major regulatory junction orchestrating the cellular responses including DNA repair, cell cycle arrest, autophagy, metabolism, differentiation, senescence and apoptosis (Aubrey et al., 2018; Ciccia & Elledge, 2010; Kastenhuber & Lowe, 2017; Riley et al., 2008; Vaddavalli & Schumacher, 2022; Wang et al., 2023). The fact that this protein is frequently mutated in tumor cells emphasizes its significance in ensuring proper cell function (Kastenhuber & Lowe, 2017).

Hereafter, this introduction focuses on two genotoxic noxae with different origins: the anticancer drug etoposide and the environmental genotoxin benzo[a]pyrene diol epoxide (BPDE), as well as their respective DDR mechanism involving DNA double-strand break repair (DSBR) and nucleotide excision repair (NER).

1.6.1 Etoposide

DNA replication and transcription require the unwinding of compact DNA structures for accessibility resulting in deleterious topological complications like lethal knots and genomic tangles. To prevent cellular stress and DNA damage, topoisomerases, categorized into topoisomerase I (TOPO I) and topoisomerase II (TOPO II), dissolve these topological impediments by controlling the environment for unwinding, cleaving and religating tensioned DNA segments (Wang, 2002). Thereby, these enzymes possess distinct mechanisms to amend DNA topology. The monomeric TOPO I enzyme can be further subdivided into type IA, IB and IC depending on their occurrence, requirement of co-factors and preferred DNA site. Accordingly, TOPO I facilitates the dissolvement of DNA supercoils, but not lethal knots and tangles, by introducing a SSB into one of the DNA strands allowing the transversion of the intact strand by the so called “strand passage” mechanism for type IA and “swivel” for type IB and IC (Champoux, 2001; Delgado et al., 2018; Leppard & Champoux, 2005; Wang, 2002). In comparison, the homodimeric TOPO II, categorized in IIα and IIβ, introduces a transient DSB binding both sides of the double helix to allow the passage of another double-stranded DNA segment (reviewed in Deweese & Osheroff, 2008). This DNA–enzyme intermediate is referred to as the “cleavage complex” and displays the target region for most topoisomerase II inhibitors used in chemotherapy, including etoposide.

Topoisomerase II inhibitors can be subdivided into two groups: topoisomerase poisons and topoisomerase inhibitors (Buzun et al., 2020). Accordingly, topoisomerase poisons stabilize the normally transient and occasionally occurring cleavage complex and prevent the religation of the DNA double-strands. The increase of DNA–enzyme intermediates in the cells causes a transition of an essential survival enzyme into a cytotoxic threat accompanied by different mutagenic events (Drwal et al., 2014). In comparison, topoisomerase inhibitors prevent the interaction of enzyme and DNA by either binding to the enzyme directly and attenuating its catalytic function or by intercalating into the DNA and altering its structure (Capranico et al., 1997).

The increased cancer incidences recorded over the last decades emphasizes the importance of the advancement of cancer therapies and simultaneously decreasing mortality rates reflect these endeavors (Siegel et al., 2024). Considering the significant characteristics of cancer cells involving uncontrolled proliferation and adapting cellular survival mechanisms, the opportunity to interfere with these processes by topoisomerase II inhibition, especially proliferation and survival-related subtype IIα, offers a great potential in fighting cancer. The effectiveness of topoisomerase inhibitors, of course, depends on the TOPO II concentrations in the cell. Interestingly, TOPO II can be found abundantly in highly proliferative cells like cancer cells but also stem cells. Besides the topological function of TOPO IIα, stem cells require this nuclear

enzyme for gene regulation involving proliferation, pluripotency and differentiation. It has been demonstrated that TOPO IIα inhibition affects genetic integrity, epigenetic alterations and misregulated gene expression (Thakurela et al., 2013) and knockouts of this gene resulted in impaired self-renewal and proliferation in adult neural stem cells (Qin et al., 2022). One of the most known topoisomerase II poisons used in clinical anticancer approaches is etoposide. The semi-synthetic chemotherapeutic agent is one of the earliest developed drugs targeting TOPO II and is derived from the naturally occurring podophyllotoxin which has been used for treating a variety of diseases over the last centuries due to its antimitotic properties (reviewed in Baldwin & Osheroff, 2005). As described above, TOPO II initiates a controlled and transient DSB to allow strand passage to relax under- or overwound DNA and immediately repair the DSB. As this process occurs only occasionally the cell tolerates the low concentration of cleavage complexes and DSBs (Fortune & Osheroff, 2000; Nitiss & Wang, 1988). However, etoposide captivates the broken DNA and TOPO II for a prolonged period by inhibiting the ligation activity (Robinson & Osheroff, 1990). This intervention results in an increased concentration of intermediates and breakages and subsequently provokes permanent DSBs (Figure 6) (Ross et al., 1984; Willmore et al., 1998). Depending on the severity of the damage, the cells can either activate DNA repair mechanisms involving HR or NHEJ or initiate the cell death pathway.

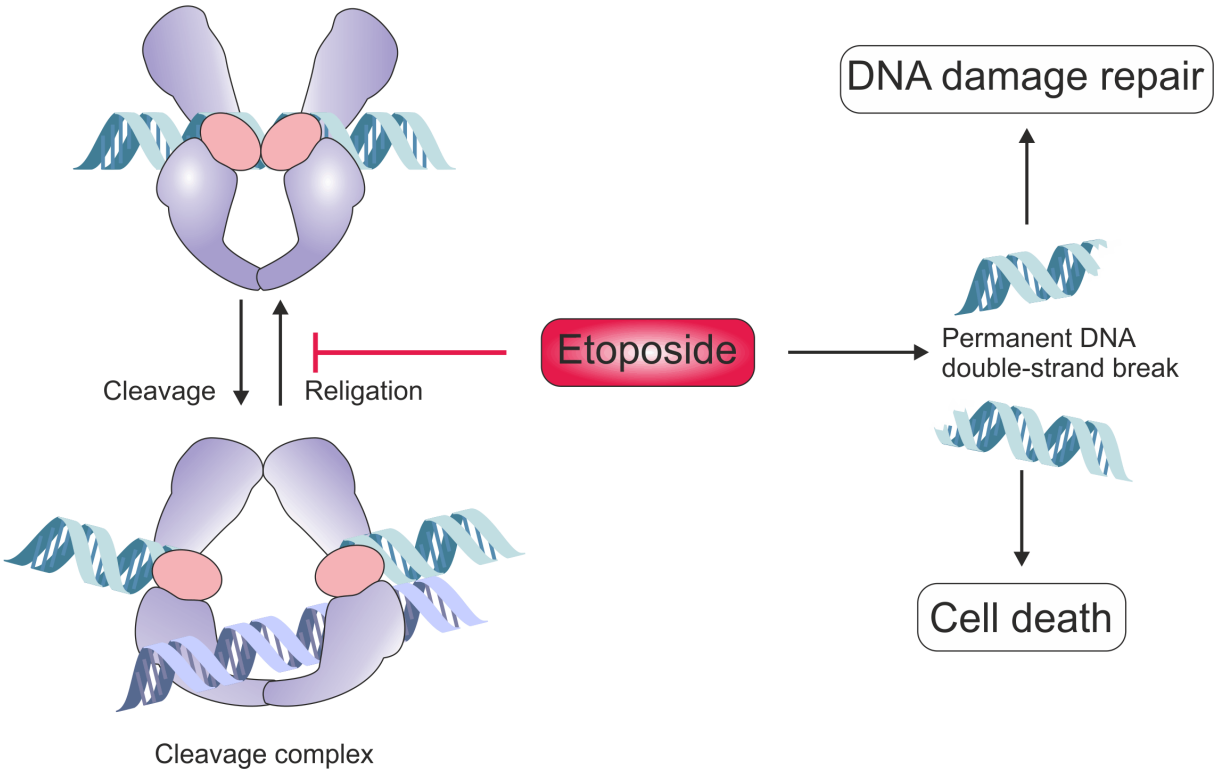

Figure 6: Mode of action of topoisomerase II poison etoposide. DNA replication and transcription can cause a topological stress situation endangering genomic integrity. To resolve topological stress, topoisomerase II (TOPO II) binds to the double-stranded DNA and induces nicks on both strands and separates both DNA segments by securely binding both ends and transiently preserving the DNA double-strand break in the so called cleavage

complex. The cleavage of the DNA allows passage of another DNA double-strand to relax the overall DNA structure. Upon passage, the broken DNA is religated and released from TOPO II. The chemotherapeutic topoisomerase II poison etoposide stabilizes the cleavage complex by attenuating the religation activity of the enzyme. The persistent cleavage subsequently causes the introduced DSBs to become permanent requiring cellular response by either aiming to repair the DNA damage or activate the cell death pathway upon increased damage occurence beyond repair.

1.6.2 DNA double-strand break repair

The most lethal form of DNA damage is the breakage of the DNA double-strand which may cause mutations or have lethal consequences if it is left unrepaired or misrepaired. These breaks can be caused under physiological conditions, i.e. DNA metabolism, or by exogenous factors as described before (Symington & Gautier, 2011). Due to its severity cells need to immediately respond to ensure genomic integrity by initiating two major DNA damage repair pathways involving homologous recombination (HR) and non-homologous end joining (NHEJ) (Ciccia & Elledge, 2010).

DSBs can be recognized by different DNA damage markers or sensor proteins and complexes, for instance, the phosphorylated histone protein H2A family member X (γH2AX) and the MRE11–RAD50–NBN (MRN) complex. H2AX phosphorylation can be performed by all PIKK proteins and functions as a marker for DNA damage. The coverage of the lesion site enables the recruitment of additional DNA damage associated proteins (Paull et al., 2000; Sharma et al., 2012). In comparison, the MRN complex consisting of MRE11 Homolog Double Strand Break Repair Nuclease (MRE11), RAD50 double strand break repair protein (RAD50) and Nibrin (NBN) functions as a damage sensor and quickly binds to the free ends at the site of the DSB and thereby initiates the activation and recruitment of ATM by direct interaction with NBN (Helt et al., 2005; Lee & Paull, 2004). The activation of ATM initiates its autophosphorylation and subsequently the phosphorylation of all three MRN complex proteins leading to a conformational change of the complex and locking to the DNA (Lee et al., 2013). Additionally, the MRN complex stimulates the kinase activity of ATM towards its downstream substrates, which include p53, CHK2, H2AX and p53-binding protein 1 (53BP1) (Lee & Paull, 2004; Podhorecka et al., 2010), as well as various cell signaling pathways regulating cell cycle arrest, checkpoint activation and apoptosis (Qiu & Huang, 2021). Mutations in this complex are associated with different cancer types and can also cause developmental deficiencies as seen in Nijmegen breakage syndrome (McCarthy-Leo et al., 2022; Varon et al., 1993). The decision of which of the two repair pathways will be employed to repair the DSB depends on the cell cycle.

HR is the most accurate way to repair DSBs since the restoration is based on the homologous counterpart located on the sister chromatid and therefore, can only be employed in either S or G2 phase (Haber, 2018; Smith et al., 2010). Besides its involvement in DSB repair, HR also plays an important role in accurate DNA replication by degrading erroneous replication forks and stalling. The process of HR can be subdivided into three steps: presynapsis, synapsis and postsynapsis (X. Li & W. D. Heyer, 2008; Michel & Leach, 2012). The first step of presynapsis encompasses the recognition and DNA end resection whereby the broken DNA ends are enzymatically degraded to obtain 3' OH single-strand DNA (ssDNA) overhangs. These overhangs offer a platform for additional proteins involved in HR preventing the onset of NHEJ (Huertas, 2010; Zhao et al., 2020). The generation of ssDNA is conducted by the MRN complex coupled with CtBP-interacting protein (CtIP) and facilitates the ensuing recruitment of various helicases and nucleases for 3´-ssDNA extension (Huertas & Jackson, 2009). Afterwards, ssDNA is rapidly covered by the replication protein A (RPA) complex which participates in all DNA processes involving ssDNA and covers that region during repair to prevent the formation of secondary structures and degradation (Eggler et al., 2002; Ma et al., 2016; Sampathkumar et al., 2024). Subsequently, to enable the interaction of HR specific proteins, RPA is removed by different RAD51 homolog1 (RAD51) mediators including breast cancer type 1 susceptibility protein (BRCA1) and breast cancer type 2 susceptibility protein (BRCA2) (Roy et al., 2011). BRCA1 exert multifactorial associations in DDR including DSB binding (Wang et al., 2007), processing via MRN/CtIP binding (Yun & Hiom, 2009) and RAD51 recruitment by CHK2 interaction (Kim et al., 2004; Roy et al., 2011). This complex of MRN–CtiP–BRCA1–CHK2 binds an additional BRCA1 protein enabling the association with partner and localizer of BRCA2 (PALB2) and BRCA2 and mediates the recruitment and assembly of the RAD51 nuclease and the transfer to the next step of synapsis (Roy et al., 2011). RAD51 is the key protein in the HR repair pathway catalyzing homologous pairing and strand invasion by forming a nucleoprotein filament scaffold (Krogh & Symington, 2004; Symington & Gautier, 2011). The RAD51–ssDNA filament searches for a homologous region on the sister chromatid forming a transient three-stranded DNA site followed by the invasion of the ssDNA into the homologous dsDNA displacing one of the strands of the sister chromatid by forming a D-loop (X. Li & W.-D. Heyer, 2008; Wright et al., 2018). The interaction of a four DNA strand intermediate is referred to as the double Holliday junction (reviewed in Song et al., 2022). This process is supported by RAD54-like (RAD54) stimulating RAD51 activity, binding ssDNA and dsDNA, and participates in its displacement after junction formation to enable DNA synthesis (San Filippo et al., 2008). After DNA synthesis and cleavage for the dissolvement of the Holliday junction, the ligation of the restored dsDNA marks the end of HR (X. Li & W. D. Heyer, 2008).

As the name implies, NHEJ does not require a homologous template and involves the direct ligation of the broken segments making this repair mechanism more erroneous compared to

HR. Due to the absented template, NHEJ takes place during interphase. As mentioned above, 53BP1 plays an important role in the pathway choice by preventing excessive DNA resection during G1 phase which antagonizes the binding of the Ku-complex (Panier & Boulton, 2014; Symington & Gautier, 2011). The Ku-complex consisting of Ku70 and Ku80, binds to the region of the DSB and recruits DNA-PKcs to the lesion site. The activation of DNA-PKcs initiates NHEJ by stabilizing the DNA ends and recruiting of endonuclease Artemis for overhang resection, DNA polymerase μ and λ, and the ligase complex consisting of X-ray repair cross complementing protein 4 (XRCC4), XRCC4-like factor (XLF), paralog of XRCC4 and XLF (PAXX), and DNA ligase IV (LigIV) (Chen et al., 2021). Subsequently, DNA-PKcs autophosphorylation causes a destabilization of DNA binding and its dissociation from the lesion site and facilitation of Artemis activation (Goodarzi et al., 2006) and the LigIV-mediated DNA end joining (reviewed in Block et al., 2004; Chen et al., 2021).

1.6.3 Benzo[a]pyrene and benzo[a]pyrene diol epoxide

The detrimental effects of conventional cigarettes are well known and proven to cause various cancer types and mutations, especially in increasing the frequency of DNA adducts by tobacco-specific nitrosamines and polycyclic aromatic hydrocarbons (PAHs) (Pfeifer et al., 2002; Sasco et al., 2004; Warren & Cummings, 2013). One of the most prevalent and precarcinogenic PAHs is benzo[a]pyrene (BaP). BaP is present or emerges from incomplete combustion of organic matter, cigarette smoke, vehicle exhausts and domestic wood burning (Boström et al., 2002; Motwani et al., 2016). Its ubiquitous environmental presence in water, air and soil results in the frequent exposure of the general public to PAHs and is mostly taken up by inhalation, skin contact or ingestion of food contaminated by the above mentioned sources (Abramsson-Zetterberg et al., 2014; IARC, 2010; Lee & Shim, 2007; Motwani et al., 2016; Zhao et al., 2024). After consumption, BaP is metabolized in the liver where it binds to the aryl hydrocarbon-receptor (AhR) nuclear complex, a transcription factor involved in the regulation of expression of genes coding for proteins involved in the xenobiotic response, such as the cytochrome protein (CYP) 450 family members (Schrenk, 1998). Together with epoxide hydrolases, especially CYP1A1 and CYP1B1 contribute to the enzymatic transformation to various electrophilic diols and benzo[a]pyrene diol epoxides (BPDEs) possessing genotoxic and carcinogenic properties (Eling et al., 1986; Shiizaki et al., 2017; Shimada, 2006; Shimizu et al., 2000). Thereby, BPDE, which is considered to be the most carcinogenic metabolite, is able to bind to the nitrogen atom of guanine within the DNA forming deleterious DNA adducts (dG-N^2–BPDE adduct) and by that distorting the DNA helix structure (Figure 7) (Boysen & Hecht, 2003; Hwa Yun et al., 2020; Phillips, 1983). If not repaired correctly, PAHs are associated with the disruption of DNA replication and somatic mutations, aging and tumorigenesis (Rose et al.,

2023; Yousefzadeh et al., 2021; Zhang et al., 2024; Zhang et al., 2025). Furthermore, studies have revealed an increased amount of guanine to thymine transversions in p53 connected with the formation of PAH-DNA adducts in cancer cells associated with smoking. (Liu et al., 2005; Pfeifer et al., 2002). With these insights, BPDE does not only cause DNA damage by forming DNA adducts and disrupt replication but also hampers the proper DDR.

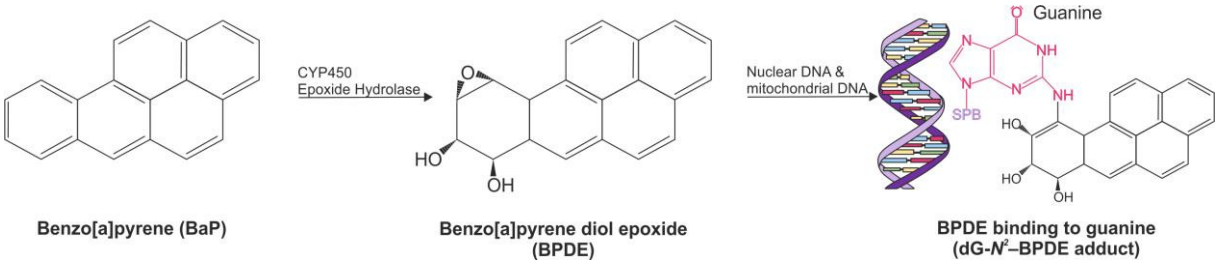

Figure 7: Chemical structure of benzo[a]pyrene and benzo[a]pyrene diol epoxide and subsequent binding to the DNA. Benzo[a]pyrene (BaP) is metabolized by the CYP450 family members and epoxide hydroxylase to the electrophilic benzo[a]pyrene diol epoxide (BPDE). BPDE is able to bind to the nitrogen atom of the DNA base guanine, which is connected to the sugar phosphate backbone (SPB), forming a covalently bound DNA adduct.

Moreover, due to its strong lipophilicity, BaP is able to cross the placenta and be metabolized by the fetus compromising fetal development (Filler & Lew, 1981; Rouet et al., 1984; Ye et al., 2020). Accordingly, the working group of Ye et al. observed a concentration-dependent incidence of abnormal murine fetuses and BPDE-associated trophoblast dysfunction by attenuating migration and elevating apoptosis in Swan 71 trophoblast cells indicating a potential link between BaP and recurrent pregnancy loss (Ye et al., 2020). Additionally, BPDE–DNA adducts were also detectable in ovarian (Zenzes et al., 1998) and sperm cells (Zenzes et al., 1999) of cigarette smokers which may also affect embryonic development and pregnancy.

Besides BaP´s ability to cross the placenta, it is also able to pass the blood-brain barrier and can be metabolized to cytotoxic metabolites within the brain (Juchau et al., 1979). In case of high exposure, BaP and thereof derived metabolites accumulate in blood and brain (Grova et al., 2008) causing neurotoxic effects (Ramesh et al., 2001; Saunders et al., 2006) and neurophysiological alterations (Grova et al., 2008). These observations are supported by different studies that have shown that BaP exposure resulted in learning and memory deficits and cognitive impairments in rodents (Cherif et al., 2021; D. Liu et al., 2020; Nie et al., 2013; Zhang et al., 2016), zebrafish (Das et al., 2020) and humans (Qiu et al., 2013). Furthermore, BaP has been associated with the enhancement and progression of Alzheimer's disease due to increased oxidative stress and neuroinflammation (Bukowska et al., 2022) indicating a potential relation to the onset of neurodegenerative diseases.

The mechanism to avoid or reduce the risk of the above-mentioned adverse events entails the removal of the DNA adducts due to the recruitment of proteins facilitating nucleotide excision repair.

1.6.4 Nucleotide excision repair

As mentioned before, several highly efficient preservation mechanisms are involved to prevent calamitous events caused by DNA structure distortion, oxidative stress and other perturbances endangering genomic integrity. One of these pathways protecting the DNA from persistent damage is the nucleotide excision repair (NER). Thereby, the NER pathway is a template-based mechanism facilitating the detection and removal of double helix-distorting lesions caused by covalently bound agents resulting in DNA adducts (Hwa Yun et al., 2020), covalently connected nucleotides giving rise to DNA crosslinks (Huang & Li, 2013) and other lesions (Kuper & Kisker, 2023). These damages can occur from endogenous processes, e.g., production of malondialdehyde by enzymatic or oxygen radical-induced lipid peroxidation (Niedernhofer et al., 2003) or from exogenous exposure to BPDE or chemotherapeutics such as cisplatin (Huang & Li, 2013). The process involves the recognition of the DNA lesion, dual incision of the DNA strand, removal of oligonucleotides, DNA synthesis and ligation (reviewed in Spivak, 2015). Furthermore, NER can be subdivided into two distinct recognition pathways: the global genome repair (GG-NER) and transcription-coupled repair (TC-NER).

In GG-NER, the DNA is constantly scanned by the xeroderma pigmentosum group C (XPC) complex encompassing the name giving XPC, the human Rad23 homolog B (RAD23B), and Centrin-2 for lesion detection. For the detection of pyrimidine dimers or 6-4 photoproducts, NER is supported by the UV-DNA damage binding (UV-DDB) proteins (reviewed in Kuper & Kisker, 2023). In comparison, TC-NER recognition is activated upon transcriptional stalling of RNA polymerase II (RNAPII) due to structure-altering lesions leading to the recruitment of Cockayne syndrome B (CSB), Cockayne syndrome A (CSA), and UV stimulated scaffold protein A (UVSSA) proteins (Duan et al., 2021; van den Heuvel et al., 2021). After lesion recognition, both pathways initiate the recruitment of the key protein complex transcription factor II H (TFIIH) consisting of the helicase xeroderma pigmentosum group D (XPD), translocase xeroderma pigmentosum group B (XPB) and p8, p34, p44, p52, and GTF2H1/p62 forming the core unit. Additional components of TFIIH are cyclin dependent kinase 7 (CDK7), Cyclin-H, and ménage à trois-1 (MAT1) forming the cyclin activating kinase complex (Roy et al., 1994). From there on both pathways share the following repair mechanism: After damage verification, dual incision around the lesions is conducted by the two nucleases xeroderma pigmentosum group F (XPF)/ERCC1 complex and xeroderma pigmentosum group G (XPG) (Riedl et al., 2003). Followed by the first incision at the 5’ end performed by the XPF/ERCC1

complex, proteins involved in DNA synthesis are recruited to the lesion site initiating DNA synthesis based on the complementary strand. Subsequently, the second incision at the 3' end is executed by XPC leading to the dissociation of TFIIH together with the removed DNA fragment (Hu et al., 2013; Staresincic et al., 2009). In the final step, the newly synthesized DNA region is ligated restoring the intact double-stranded DNA (Kuper & Kisker, 2023).

Defects in NER are associated with various pathological conditions such as xeroderma pigmentosum (XP) (Taylor et al., 1997), trichothiodystrophy (TTD) (Bergmann & Egly, 2001) and Cockayne syndrome (CS) (Fan et al., 2008). Patients suffering from these genetic disorders may show a higher risk of cancer, neurological impairments and photosensitivity (Feltes, 2022; Kraemer et al., 1994; Lehmann & Fassihi, 2020; Taylor et al., 1997).

1.7 DNA damage response in stem cells

Since healthy stem cells are crucial for proper development and regeneration, the protection of the genome and propagation of error-free DNA is significant for the generation and maintenance of a healthy organism and also play a promising role in regenerative medicine (Kimbrel & Lanza, 2015). Generally, SCs are prone to DNA damage occurrence and accumulation due to their high proliferation rate and coherent metabolic activity increasing the chances for erroneous replication or oxidative stress (Ahuja et al., 2016). An additional reason for their hypersensitivity towards DNA damage might be attributable to the euchromatic genomic organization (Ahmed et al., 2010; Gaspar-Maia et al., 2011; Williamson et al., 2018). In comparison to somatic cells, the DDR in SCs exhibits differences in damage tolerance and subsequent reactions like cell cycle arrest (Cervantes et al., 2002; Tichy & Stambrook, 2008; Vitale et al., 2017). Differentiated cells undergo an extended cell cycle of at least 16 hours and the majority of the cells reside in the G1 phase (Kapinas et al., 2013). Upon DNA damage, this constellation of the cell cycle allows the cell to have several checkpoints before transitioning to the next phase and induce cell cycle arrest to monitor the scope and severity of the DNA lesion. In contrary, stem cells have a shortened G1 phase with possibly restricted content of G1-associated regulatory mechanisms and lack the somatic cell cycle checkpoint at G1/S phase transition (Boheler, 2009; Kapinas et al., 2013; Neganova & Lako, 2008) causing the entrance of lesioned cells into the S phase, aggravating the damage and subsequently direct those cells towards apoptosis (Stambrook & Tichy, 2010). The preferred induction of apoptosis over comprising erroneous cells supports the protection and stringent quality maintenance of the stem cell pool (de Waard et al., 2003; Roos et al., 2007; van der Wees et al., 2007; Van Sloun et al., 1999). Furthermore, the key regulatory protein p53 is strongly associated with cell cycle checkpoints and activation of cell cycle arrest upon DNA damage in somatic cells, whereas in stem cells, p53 does not initiate cell cycle arrest in response to DNA damage owing

to inefficient nuclear translocation (Aladjem et al., 1998). Supporting the great potential and swift reaction upon lesion occurrence, stem cells may possess a constitutively active DDR owing to their perpetual endangerment by endogenous mechanisms and agents. These observations are reinforced by elevated γH2AX levels, chromatin-bound RPA or RAD51 compared to differentiating cells (Ahuja et al., 2016; Tichy & Stambrook, 2008; Vitale et al., 2017).

Besides the willing sacrifice of damaged cells, stem cells exhibit great DDR properties. In normal stem cells these auxiliary mechanisms of efficient damage repair and self-renewal are beneficial for tissue homeostasis and regeneration, however, they can also be detrimental when stem cells become cancerous protecting harmful cells and causing resistances towards common chemo- and radiotherapeutic agents (Cho & Clarke, 2008; reviewed in Frosina, 2010). Since stem cells mainly reside in S phase, they are able to predominantly recruit the template-based and therefore more accurate HR repair mechanism for DSBs compared to somatic cells which are mainly in G1 phase and rely on the error-prone NHEJ (Tichy & Stambrook, 2008). Moreover, microarray analyses showed an elevated abundance of DDR-related mRNA levels involved in different pathways including BER and ICL repair (Maynard et al., 2008).

In summary, to meet the expectations of stringently controlled genomic integrity stem cells exhibit a great DNA repair capacity by elevated gene expression of DDR-associated factors and rapidly induce regulated cell death or differentiation as opposed to tolerating any damage that might threaten tissue integrity. Upon differentiation, cells become more tolerable towards DNA lesions owing to reduced DDR marker expression and adaptation of the cell cycle (reviewed in Vitale et al., 2017).

1.8 Interaction between DNA damage response and autophagy

Cell homeostasis and genomic integrity are fundamental prerequisites for cell physiology and tissue function and require an interplay of a plethora of signaling pathways. As aforementioned, DDR and autophagy contribute to maintaining functionality by either repairing damage and eliminating harmful cells or removing deleterious components in the cytosol and supplying new building materials and energy. While the relationship between DDR and apoptosis has been well studied, the complex interplay between DDR and autophagy still requires further research (Gorgoulis et al., 2024). Since DDR is a highly energy-consuming process, successful activation and repair requires a reliant energy source, which has not been addressed yet. Several connections between DDR and autophagy have been reported so far (Eapen & Haber, 2013; Eliopoulos et al., 2016; Lascaux et al., 2024; Orlotti et al., 2012; Robert et al., 2011).

Recapitulating the effector proteins of DDR, ATM and p53 are both essential proteins involved in amplifying and activating downstream processes to ensure proper DNA repair. These two proteins are also important autophagy regulators. While ATM is known to positively regulate autophagy by various signaling routes, among them the inhibition of mTOR signaling via AMPK activation (Stagni et al., 2020), p53 exerts a dual role on autophagy regulation based on its subcellular localization (White, 2016). Similar to ATM, nuclear p53 supports autophagy induction by mTOR inhibition, however, cytosolic p53 instead inhibits autophagy through AMPK and mTOR activation (Eliopoulos et al., 2016; Stagni et al., 2020; White, 2016). Nevertheless, emphasizing the complexity of the relation of autophagy and DDR the autophagic process is also able to regulate DDR proteins including the repression of p53 (White, 2016). This mutual regulation shows that both pathways affect each other on different levels. Deficiencies of autophagy-related proteins have been associated with increased genomic instability, causing amongst others, an elevated proteasomal degradation of CHK1 and with that diminishing HR and shifting DNA repair towards the error-prone NHEJ causing the accumulation of genomic lesions (Liu et al., 2015).

Treatment with different chemotherapeutic agents like camptothecin (M. J. Abedin et al., 2007), etoposide (Katayama et al., 2007), and ionizing irradiation (Rieber & Rieber, 2008) demonstrated autophagy induction upon genotoxic exposure. While autophagy and DDR support cellular survival and tumor suppression in normal cells, they can turn into foes in later stages of cancer development and start promoting tumorigenesis and cellular protection of tumor cells (Cheng et al., 2022; Gorgoulis et al., 2024).

Altogether, autophagy and DNA damage response display a complex symbiotic relationship supporting and regulating each other to ensure genomic stability and cellular survival. However, both pathways can also be detrimental upon excessive activation and dysregulation and may cause the onset of tumorigenesis and support and protect the survival of cancer cells.

2. Aims of this work

Human life expectancy is ongoingly increasing, requiring the maintenance of cellular processes and prevention of diseases including neurodegeneration and cancer. The most fundamental components for successful development, tissue remodeling and regeneration, are stem cells. Ever since the cultivation of ESCs and generation of hiPSCs valuable insights into their physiology and potential medical application have been gained. Genotoxic agents endanger the integrity of the genome which has to be protected at all costs to avoid degeneration and cancer onset. This task is tackled by different cellular pathways including DDR and autophagy. However, due to stem cell´s unparalleled features the physiological interplay of various signaling pathways and protection mechanisms are not fully understood. Therefore, investigating the interconnectivity of signaling pathways upon DNA damage might give insights into the reason why stem cells are more efficient in maintaining high quality and exhibiting lower mutagenic frequencies than their differentiated counterparts.

In various cell types, including cancer cells, it has been demonstrated that upon genotoxic events autophagy and DNA damage response regulate and affect each other to either protect the cell or to induce regulated cell death (Eliopoulos et al., 2016; Gorgoulis et al., 2024; Liu et al., 2015). However, cancer cells also exploit autophagy´s cytoprotective characteristics, leading to ongoing research on targeting autophagy in cancer treatment (Chen & Karantza, 2011; Katayama et al., 2007; Niu et al., 2025). Since stem cells are crucial for tissue homeostasis and may give rise to therapy-resistant cancer stem cells, it is of great importance to understand the role of signaling pathways in normal stem cells to potentially and investigate therapeutic approaches to target cancerous cells but not the beneficial stem cells.

The main aim of this dissertation was the comparative analysis of the autophagic response upon genotoxic exposure to an environmental carcinogen, BPDE, and a commonly used and well characterized chemotherapeutic, etoposide, in hiPSCs and thereof differentiated neural progenitor cells compared to the colorectal carcinoma cell line HCT116.

First, NPCs were generated from the corresponding hiPSC lines and their autophagic capacity was investigated to gain information about their autophagic capacity and whether they show similar regulations upon starvation as other reported cell lines.

Second, cells were treated with genotoxins over a time course and their DNA damage response was analyzed and compared to HCT116 by western blot analysis and immunofluorescence.

Third, the autophagic response upon genotoxic exposure was analyzed by western blot analysis and immunofluorescence followed by differential proteome analysis to gain insights about physiological changes upon treatment in stem cells.

3. Summary of publications

3.1 Publications within the scope of this dissertation

The full original texts can be found in the appendix of this dissertation.

Publication 1

Canonical autophagy remains inactive in induced pluripotent stem cells and neuronal progenitor cells following DNA damage induced by BPDE or etoposide.

Seda Akgün, Thomas Lenz, Annika Zink, Karina Stephanie Krings, Sebastian Wesselborg, María José Mendiburo, Alessandro Prigione, Kai Stühler, Björn Stork

First version of the manuscript has been uploaded on bioRxiv.

bioRxiv 2025.05.22.655294

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Macroautophagy is a central cellular stress mechanism that degrades and recycles damaged or long-lived proteins and organelles. Upon DNA damage, autophagy seems to contribute to maintaining genomic integrity and successful damage repair which has been demonstrated in various cell types. In stem cells, it is involved in balancing quiescence, self-renewal, and differentiation, however, the interplay between DNA damage response and autophagy is rather elusive. We showed that induced pluripotent stem cells (iPSCs) and neuronal precursor cells (NPCs) have functional autophagy machinery, as evidenced by nutrient deprivation-induced autophagy flux and ULK1 activation. Using the iPSC lines iPS11 and iPS12 and their NPC derivatives, we investigated whether low doses (IC_{20}) of the genotoxic substances BPDE or etoposide, as reported in tumor cell lines, also trigger autophagy. Although both substances led to an increase in DNA damage markers and slight changes in DNA repair proteins, they did not activate autophagy flux. ULK1 activation occurred only in NPCs but was not sufficient for a complete autophagic downstream response. Differential proteome analyses revealed minimal changes in iPSCs and moderate changes, mainly associated with mitosis, in NPCs. Concluding, low doses of genotoxic agents have marginal effect on canonical autophagy in iPSCs and NPCs, despite a responsive autophagy machinery.

Author contribution: The author of this dissertation designed the experiments, performed viability assays, immunoblot analyses, fluorescence microscopy, growth curve and flow cytometry analyses. The author analyzed, interpreted, and discussed the data, and participated in writing the manuscript.

Relative contribution: ~90%

Publication 2

TOP(O)Gun: The impact of topoisomerase inhibitors on healthy and cancer stem cells

Seda Akgün, Gerhard Fritz, Björn Stork

Manuscript in preparation

Topoisomerases are a highly conserved and essential family of proteins that perform key functions in DNA topology processes such as genome stability, DNA remodeling, and repair. This makes them promising target molecules in the fight against cancer. One small subpopulation of cancer cells, referred to as cancer stem cells, exhibit stem cell like properties worsening the patient´s prognosis by enhancing tumor progression and metastasis formation. Due to their similarities to healthy stem cells, it is challenging to specifically target those cells wherefore, it is of greatest interest to understand the differences between normal and cancer stem cells. This review highlights the functions of topoisomerases, their potential as targets for cancer therapies, and the properties of normal stem cells and CSCs in the context of current research.

Author contribution: The author of this dissertation wrote the original draft manuscript, and reviewed and edited the final manuscript. All figures and tables were prepared by this author.

Relative contribution: ~90%

3.2 Publications beyond the scope of this dissertation

The author of this dissertation has contributed to multiple additional publications related to the signal transduction of autophagy or other cellular stress responses. However, these manuscripts are not discussed or attached, as they would exceed the scope of this dissertation.

Publication 1

Fin56-induced ferroptosis is supported by autophagy-mediated GPX4 degradation and functions synergistically with mTOR inhibition to kill bladder cancer cells

Yadong Sun, Niklas Berleth, Wenxian Wu, David Schlütermann, Jana Deitersen, Fabian Stuhldreier, Lena Berning, Annabelle Friedrich, Seda Akgün, María José Mendiburo, Sebastian Wesselborg, Marcus Conrad, Carsten Berndt and Björn Stork

Cell Death & Disease, Volume 12, Issue 11, 1028, October 2021

DOI: 10.1038/s41419-021-04306-2

Author contribution: The author of this dissertation gave technical support, discussed the results, and commented on the manuscript.

Relative contribution: ~2%

Publication 2

The Golgi stacking protein GRASP55 is targeted by the natural compound prodigiosin

Lena Berning, Thomas Lenz, Ann Kathrin Bergmann, Gereon Poschmann, Hannah U. C. Brass, David Schlütermann, Annabelle Friedrich, María José Mendiburo, Céline David, Seda Akgün, Jörg Pietruszka, Kai Stühler and Björn Stork

Cell Communication and Signaling, Volume 21, Issue 1, 275, October 2023

DOI: 10.1186/s12964-023-01275-1

Author contribution: The author of this dissertation gave technical support, discussed the results, and commented on the manuscript.

Relative contribution: ~2%

Publication 3

Small-molecule inhibitor of C-terminal HSP90 dimerization modulates autophagy and functions synergistically with mTOR inhibition to kill cisplatin-resistant cancer cells

Céline David, Yadong Sun, Vitalij Woloschin, Melina Vogt, Niklas Dienstbier, David Schlütermann, Lena Berning, Beate Lungerich, Annabelle Friedrich, Seda Akgün, María José Mendiburo, Arndt Borkhardt, Sebastian Wesselborg, Sanil Bhatia, Thomas Kurz and Björn Stork

First version of the manuscript has been uploaded on bioRxiv.

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Author contribution: The author of this dissertation gave technical support, discussed the results, and commented on the manuscript.

Relative contribution: ~2%

Publication 4

Identification of autophagy inhibitors selectively targeting the ATG13-ATG101 protein protein interaction

Annabelle Friedrich, Korana Mudrovcic, Anoshi Patel, Francesca Lugarini, Carina Birke, Mohanraj Gopalswamy, Stefanie Brands, Lena Berning, David Schlütermann, María José Mendiburo, Céline David, Seda Akgün, Sebastian Wesselborg, Sonja Sievers, Alex C. Faesen, Holger Gohlke and Björn Stork

Manuscript in preparation

Author contribution: The author of this dissertation gave technical support, discussed the results, and commented on the manuscript.

Relative contribution: ~2%

4. Discussion

The emergence of a well-developed conscience and self-awareness differentiates the human species from animals and is based on a complex network of neuronal connectivity. Development and maintenance throughout life requires highly qualitative and faultless cell sources to ensure proper embryonic development and tissue engineering which are executed and supported by stem cells possessing unique characteristics enabling self-renewal and differentiation. The inevitable exposure to DNA damaging agents may cause developmental deficiencies, influence the onset of neurodegenerative diseases or cancer. To prevent these calamitous events, signaling pathways like DDR contribute to maintaining genomic integrity and cell homeostasis. Another signaling pathway that is characterized by its cytoprotective features is the autophagic pathway. Autophagy is mainly involved in the sequestration and lysosomal degradation of cytosolic components but influences various signaling pathways as well as cell fate decisions and has been associated with the support of DDR upon genotoxic exposure. It is well known that autophagy plays an important role in stem cells and development regarding quality control and tissue remodeling (Boya et al., 2018), however, its implication in DDR in stem cells remains elusive. Therefore, the aim of this dissertation was to investigate the impact of low dose exposure of two distinctive genotoxins, BPDE and etoposide, on the autophagic process in hiPSCs and thereof differentiated NPCs to gain insight into similarities and differences between observed autophagic relations to DNA damage in cancer cells and stem cell physiology. These insights might help to understand the differences between healthy and cancer stem cells to contribute to cancer treatment advancement. Our investigation showed that low dose treatment with BPDE or etoposide caused detectable damage in all three cell models and revealed different regulations of DDR-related proteins in a time and stimulus-dependent manner. Furthermore, canonical autophagy did not seem to be induced upon low dose genotoxic stress in all three cell models, potentially indicating that autophagy is activated upon severe cellular stress to support cellular survival rather than being a general supporter of DDR.

4.1 Experimental set-up

Stem cells exhibit highly proliferative characteristics and therefore have high nutritional demands. To exclude any side effects caused by malnutrition or pH imbalance from our readouts, medium exchange was performed daily for all three cell types to ensure comparability.

Regarding genotoxic exposure, two different set-ups were chosen for BPDE and etoposide. BaP is one of the 16 most common PAHs in our atmosphere resulting in unavoidable exposure (Zhao et al., 2024). To investigate the impact of its most genotoxic metabolite BPDE *in vitro*,

cells were supplemented on a daily basis with the corresponding 20% inhibitory concentration (IC_{20}). Daily BaP exposure and intake are highly variable since BaP can be inhaled or consumed by food. A study with non-smoking students has revealed that dietary uptake accounts for 90% of BaP exposure (Suzuki & Yoshinaga, 2007). Hence, the daily intake of BaP depends on the area of living, dietary and food processing habits ranging from 39 ng/day in Japan (Obana et al., 1989) to 170 ng/day in Iran (Eslamizad et al., 2016). Compared to BPDE, etoposide is typically given to patients at three to five consecutive days per treatment cycle depending on the cancer type (reviewed in Schmoll, 1982) or for 24 hours (h) for stem cell mobilization (Park et al., 2019). However, 24 h low dose administration of etoposide is mainly used in trial and research settings (Slevin et al., 1989). Since *in vitro* models are often more sensitive towards stimuli owing to the lack of support and regeneration by other cells and missing tissue distribution or multicellular metabolism, consecutive stimulation with compounds causing lethal damages might have led to unphysiological conditions and distort observation in cell signaling. Therefore, we chose to expose our *in vitro* cell models to etoposide for 24 h and investigate the influence on the autophagic process posttreatment. During cancer treatment, etoposide is administered intravenously at 100-150 mg/m^2 resulting in a steady-state plasma concentration of approximately 2.1-7 µg/ml (Bennett et al., 1987; Niederle et al., 2004). Since autophagy is also a cytoprotective mechanism and contributes to cellular survival, strong stimuli, and especially drastic endangerment by DSB inducing compounds, might induce non-specific cellular responses due to the immense impact of cellular stress. Consequently, we decided to perform experiments with the IC_{20} value for each cell line to investigate equitoxic similarities and differences in cellular response and to prevent unphysiological conditions.

Since our aim was to investigate the behavior of the autophagic process upon DNA damage, two additional conditions were added to our western blot analyses for autophagic flux. The autophagic flux describes the rate at which autolysosomal degradation takes place and is detected by proteins associated with the autophagosome and cargo delivery and recognition like LC3-II and p62 (Boya et al., 2018). During high flux rates, readouts based on these markers can be hard to interpret due to the counterbalance of autophagosome generation and degradation. To analyze the efficiency of the autophagic flux, lysosomal inhibitors are used causing the accumulation of autophagosomes containing degradation-destined cargo. Bafilomycin A_1 ($BafA_1$) is a V-ATPase inhibitor preventing the acidification of lysosomes and causes inefficient hydrolase activity after short incubation periods and later on affecting autophagosome–lysosome fusion (Klionsky et al., 2008). Additionally, to ensure functional autophagic machinery during the cohorts and comparability to canonically activated autophagic response, cells were incubated in EBSS medium, a medium without amino acids and serum leading to a strong starvation-induced autophagic activation. In our experimental

set-up, $BafA_1$ and EBSS were administered two hours before cell harvesting to ensure their respective purpose without unnecessarily extending cellular stress.

Various studies in cancer cells have shown that autophagy is activated upon DNA damage to support cellular survival and DNA repair (M. Abedin et al., 2007; Liu et al., 2018; Zheng et al., 2012). Since this knowledge is rather elusive in stem cells, we decided to implement the colorectal cancer cell line HCT116 as a control cell line to investigate if stem cells show similar reactions to DNA damage like in different reported studies.

4.2 Differences in sensitivity of iPSC, NPC and HCT116 towards genotoxic noxae might be a result of cell cycle differences

To identify equitoxic doses for all three cell types the cell toxicity assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was chosen. The positively charged and lipophilic MTT reagent enters the cell and inner mitochondrial membrane and is metabolized to the violet-blue water-insoluble formazan (Berridge et al., 2005; Ghasemi et al., 2021; Stockert et al., 2018). There, kinetic analysis revealed that iPS11 have the highest sensitivity towards etoposide and BPDE, followed by niPS11 and HCT116. The fact that pluripotent stem cells have the highest expression level of DDR associated genes (summarized in Frosina, 2010; Maynard et al., 2008) might lead to the assumption that iPSCs are less sensitive towards genotoxic noxae. Our results and literature (Becker et al., 2024; Federmann et al., 2025; Westerhoff et al., 2025) demonstrated that pluripotent stem cells are more sensitive towards genotoxic exposure than their progeny. An explanation for these observations might be the differences in chromatic organization and cell cycle. As described in chapter 1.7, hypersensitivity of stem cells towards DNA damage might be owed to their euchromatic genomic organization (Ahmed et al., 2010). Generally, chromatin fibers are organized as eu- or heterochromatin. The majority of the Metazoan genome is organized in heterochromatic domains that encourage genomic integrity. Those areas of the genome are densely compacted, resulting in decreased DNA accessibility, and contain less transcriptionally active or repetitive sequences (Fortuny et al., 2021; Grewal, 2023). In comparison, euchromatic areas are less condensed and easier to access and therefore, harbor transcriptionally active and gene-rich regions (reviewed in Morrison & Thakur, 2021). The working group of Ahmed et al. has demonstrated in mouse embryonic stem cells (mESCs) that pluripotent epiblast cells mainly consist of uncompacted fibers with increasing compaction upon differentiation. *In vivo* experiments verified the onset of chromatin compaction in *OCT4* knockout cells (Ahmed et al., 2010). With that, the open reading frame in pluripotent stem cells may be one explanation why iPS11 is more sensitive to genotoxins compared to niPS11 and

HCT116. Since NPCs forfeit the pluripotency marker after differentiation, condensation of chromatin is initiated and consequentially, the DNA is less accessible. Another hypothesis might be that euchromatic organization facilitates quick damage recognition and repair, whereas heterochromatin has a delayed response due to additionally required processes like chromatin relaxation (Watts, 2016). This might contribute to a delayed induction of apoptosis since the cells with higher heterochromatic organization need more time to evaluate the damage and proper response. An additional explanation for the disparities in drug sensitivity between iPS11 and niPS11 might be the different expression of ATP-binding cassette (ABC) transporter associated with the export of chemotherapeutics (Zhou et al., 2001). While pluripotent stem cells express the ABCG2 transporter (Erdei et al., 2014) which is involved in the export of various drugs, e.g., imatinib (Orlando & Liao, 2020), topotecan or tyrosine kinase inhibitors (Xiao et al., 2021), NPCs additionally express ABCB1 (Lin et al., 2006) which was shown to facilitate the export of doxorubicin and etoposide (Jiang et al., 2019).

Another stem cell characteristic that potentially influences the hypersensitivity of pluripotent stem cells compared to their progenitor cells is their shortened cell cycle. Somatic cells mostly reside in G1 phase preparing for DNA replication. To ensure genomic integrity, different checkpoints are established between phase transitions. These include checkpoints between G1 and S phase, G2 and M phase and M and G1 phase (Cavalu et al., 2024). In contrast, mESCs spend less time in the gap phases (G1 and G2) and mainly reside in S phase and have a compromised G1/S checkpoint (Aladjem et al., 1998; Kapinas et al., 2013). For instance, the tumor suppressor protein retinoblastoma, which is crucial for the G1/S checkpoint and prevents S phase entrance of damaged cells and cell cycle progression (Giacinti & Giordano, 2006), is inactive in mESCs compared to differentiated cells (Savatier et al., 1994). These circumstances favor the advancement of the cell cycle despite unrepaired DNA damage. The absence of the G1/S checkpoint and entrance of damaged cells into S phase might also be beneficial for faultless DNA repair. Presumably, stem cells try to avoid error-prone NHEJ repair and rather rely on accurate HR repair. HR-associated proteins can quickly recognize DNA damage and repair the DNA during S phase. If the damage is beyond repair, the damage is exacerbated and cells are directed towards apoptosis (Stambrook & Tichy, 2010). Hence, results obtained from this dissertation support the observations made in previous studies in mESCs. The euchromatic organization of pluripotent SCs potentially contributes to the increased sensitivity towards DNA damage and the adapted cell cycle supports the decision between efficient damage repair or apoptosis.

G1 phase lengthening in NPCs during cerebral cortex development has been observed in mammalian, mouse and chick cells (Arai et al., 2011; Molina et al., 2022; Takahashi et al., 1995). Thereby, shorter G1 length is associated with NPCs involved in pool expansion and

lengthening with the commitment of neuronal differentiation (Arai et al., 2011; Calegari et al., 2005; Takahashi et al., 1995). This fate-dependent difference might be a factor that has to be considered in *in vitro* studies. Lineage analysis in chick NPCs has revealed that after division the daughter cells may have a longer G1 phase compared to the mother cells indicating a direction towards neurogenesis over time (Molina et al., 2022). Accordingly, spontaneous differentiation and prolonged cultivation periods might influence the impact of genotoxic agents in cell viability assay. Both genotoxins used in this dissertation mainly act in or from S phase on. TOPO II concentrations increase during S phase and peak at G2 and M phase to ensure proper chromosome condensation and segregation before cell division. After exiting M phase, the protein abundance decreases (Adachi et al., 1997; Larsen et al., 1996; Lee & Berger, 2019; Wells et al., 1995). Etoposide captures TOPO II in the cleavage complex wherefore, cells residing in G1 are not yet affected by this genotoxic agent. Various studies have shown that BPDE mainly causes replication stress during S phase (Ji, 2015; Shinozaki et al., 1998). The quantifications of the MTT assays revealed that iPS11 showed high decreases in cell viability after 24 h by both compounds while niPS11 and HCT116 cell viability decreased over time in a concentration-dependent manner indicating a delayed response to the genotoxins. Therefore, due to the differences in cell cycle length and composition, a direct kinetic comparison of iPSCs, NPCs and HCT116 in regard to cell cycle-dependent genotoxins is not recommended.

Interestingly, both iPS11 and niPS11 show a higher sensitivity towards etoposide compared to BPDE, whereas HCT116 have a lower IC_{20} for BPDE than for etoposide. This observation might be a consequence of MTT assay´s limitations of being unable to distinguish cytostatic and cytotoxic effects. Treatment with etoposide resulted in a growth-arrest response and the emergence of enlarged cells after 24 hours of treatment in HCT116 which can be seen in immunofluorescence images. These senescence-like cells were also detected in other cancer cells treated with cisplatin (Mirzayans et al., 2017). This phenotype could not be observed upon BPDE treatment. Senescence was not measured within this thesis but Nicoletti assay revealed a G2 arrest in etoposide-treated HCT116 after 24 h (data not shown). Therefore, etoposide first causes cytostasis with high metabolic activity and later on induces cell death in HCT116. Furthermore, besides growth arrest, those enlarged cells might be able to secrete growth promoting factors for nearby cells, which would contribute to increased cell numbers (Mirzayans et al., 2017). The secretion of growth promoting factors would also explain the lower IC_{20} after 48 h compared to 72 h. Accordingly, results obtained by MTT assay have to be evaluated cautiously as a cytotoxic readout due to its suggestibility and indistinguishability of cellular status.

To investigate cellular differences in our iPSC models and thereof differentiated progenies, cell cycle analyses could be performed. First, cell cycle synchronization may be utilized by using nocodazole which is an G2/M phase inhibitor preventing microtubule polymerization (Blajeski et al., 2002). Additionally, nocodazole does not seem to affect pluripotency or the differentiation potential of hiPSCs (Yiangou et al., 2019). Afterwards, cell cycle kinetics could be investigated by incorporating DNA-binding dyes and utilizing flow cytometry to analyze the DNA content of individual cells. Another aspect for further investigations is the recruitment of DDR proteins upon genotoxic treatment by either analyzing specific transcripts or proteins of interest by quantitative real-time polymerase chain reaction (qRT-PCR) or western blot analysis. To gain information in a broader spectrum, differential transcriptome and proteome analyses shortly after administration and at later timepoints may be utilized.

Altogether, iPS11 have the lowest observed IC_{20} for both investigated genotoxins over time which may be a result of chromatic organization coupled with cell cycle adaptations. These features may contribute to hypersensitivity towards DNA damage but also to a fast response and decision making of subsequent cellular response, whereas niPS11 and HCT116 may have a delayed temporal response owing to an extended G1 phase and cell cycle checkpoints.

4.3 Differential DNA damage response in stem cells and HCT116

Upon DNA damage, cells recruit DDR-associated proteins with a diverse range of functions. It is well known that stem cells, especially pluripotent stem cells, have elevated levels of DDR gene expression (Frosina, 2010). Therefore, understanding the physiological and regulatory differences between stem cells and cancer cells might support the advancement of cancer therapy with decreased side effects.

Within this thesis, we were able to demonstrate that iPSCs and NPCs showed a more moderate DDR to genotoxic noxae compared to HCT116. Due to the nature of NPCs to grow as multilayered colonies, immunofluorescence quantification could not be performed, which is why analysis for this specific cell type is solely based on qualitative images and western blot. One of the cell´s first reactions to DNA damage is the phosphorylation of H2AX. In contrast to HCT116, iPS11 showed only a slight increase in DDR markers upon equitoxic treatment after 4 h. The moderate reaction of iPS11 might be explained by the constitutively active DDR. It is known that pluripotent stem cells generally have an elevated level of γH2AX foci caused by endogenous factors, so that the presence of a low dose genotoxin might not trigger an excessive recruitment of additional DDR markers. Interestingly, we observed decreased levels of all DNA damage markers after 24 h in iPS11 below the basal level of the control which was not observed in etoposide-treated HCT116 or any BPDE-treated cells. Presumably, damage

caused by etoposide might occur faster and predominantly cause DSBs compared to BPDE. With that, a low dose treatment in iPSCs might slightly increase the already activated DDR resulting in the efficient repair of genotoxin-induced damage but also of preexisting damage and thereby decreasing the amount of DNA damage in the cell and the requirement for DDR markers. This phenomenon is known as hormesis and describes an adaptive response which is triggered by a low dose stressor positively influencing physiological functions of the cell (Wan et al., 2024). Low dose stimulation of cancer cells, in this case HCT116, resulted considerably in the primary activation of the DDR, wherefore this hormesis-effect may not be observed. Despite the fact, that BPDE mainly causes damage during S phase, it does not trigger one specific type of DNA damage like etoposide. DNA-adducts cause distortions of the DNA double-helix structure leading to replication and transcriptional stress which may result in DSBs or induction of mutations. Additionally, BPDE does not only affect the genomic DNA but also interacts with mitochondrial DNA (Pavanello et al., 2013). Subsequently, mitochondrial dysfunction causes an increase of ROS and oxidative stress (Xu et al., 2024; Yang et al., 2019). The variety of threats endangering genomic integrity might hinder the occurrence of the hormesis-effect in iPSC when treated with BPDE. Therefore, an increase of DDR markers after 24 h BPDE treatment owing to the emergence of different genotoxic sources and damage underlines the different cellular response to etoposide and BPDE. Furthermore, iPS11 show a proportional increase of γH2AX, 53BP1 and co-localized foci possibly indicating the emergence of DSBs, while HCT116 quickly upregulate γH2AX eventually implying the increase of other DNA damage after BPDE treatment.

In contrast to immunofluorescence, western blot analysis of iPSCs did not show the decrease of γH2AX after 24 h etoposide treatment but rather an increase. This discrepancy may be a result of accuracy and limitations of western blot analysis. Independent from DNA damage, H2AX phosphorylation also takes place during mitosis (McManus & Hendzel, 2005; Tu et al., 2013) and apoptosis (Lu et al., 2008). In regard to apoptosis, the phosphorylation is induced by p38 which regulates serum starvation-induced apoptosis (Lu et al., 2008). This effect could also be observed in our EBSS-treated samples, where γH2AX abundance was significantly increased. In western blot analysis, the proteome of the whole cell is analyzed, wherefore the total γH2AX abundance does not give clear information about the origin. In immunofluorescence, distinct foci per nucleus are counted for analysis and the co-staining with 53BP1 is used to verify the existence of DNA damage in form of DSBs. Therefore, quantification of γH2AX abundance by immunofluorescence is considered to be more reliable than by western blot.

Interestingly, administration of $BafA_1$ caused an accumulation of γH2AX in iPSCs and NPCs. Similar approaches were followed by the working group of Jobst et al. in T24 urothelial

carcinoma cells. They demonstrated that $BafA_1$ induces rearrangements of the mitochondrial network accompanied by DNA damage resulting from nuclear deformation. While the majority of their experiments were performed with a 24 h incubation time, 4 h were sufficient to initiate mitochondrial rearrangement and elevation of γH2AX levels. Additionally, this working group performed their experiments in HCT116 and did not see mitochondrial rearrangement and γH2AX increase after 4 h of incubation with $BafA_1$. The authors hypothesized that observed heterogeneity between cancer cell lines might be contributable to morphological and physiological differences such as cell stiffness (Jobst et al., 2025). In comparison to the data obtained in this thesis, γH2AX levels were also not strongly affected in HCT116 whereas iPSCs and NPCs showed a more prominent upregulation of γH2AX upon $BafA_1$ incubation. In any case, considering the interplay of autophagy and DNA damage, the usage of bafilomycin A_1 might have to be re-evaluated since autophagy induction could also be caused by bafilomycin A_1-associated DNA damage or cellular stress.

Another important DDR protein we investigated in western blot analysis was p53 and its phosphorylation status. As described above, p53 is a key regulator and signal amplifier upon DNA damage and is phosphorylated by ATM at Ser 15 and Ser 46 (Saito et al., 2002). While Ser 46 is mainly phosphorylated in response to apoptosis initiation, the phosphorylation site Ser 15 represents an early event in DDR facilitating the transcriptional activation of proteins involved in cell cycle arrest, co-factor recruitment (Loughery et al., 2014) and primes p53 for further modifications (Nicolaou et al., 2022). After 24 h etoposide treatment, only iPS11 showed an increase of pp53 at Ser 15, whereas iPS12 and NPC did not seem to be affected. The differential response between iPSC lines can be explained by their different origins as explained in chapter 1.1.3. Interestingly, NPCs did not show any upregulation of pp53 Ser 15 but showed slight increases in p53 levels. These observations might indicate that p53 may be targeted for other modifications. With reference to literature, besides the p53 phosphorylation at Ser 15, p53 was additionally phosphorylated at Ser 20 in mouse NPCs upon etoposide treatment (Nam et al., 2010). Mouse NPC were shown to be highly sensitive to DNA damage and have an ATM- and p53-dependent DDR rapidly inducing proliferation arrest, apoptosis or differentiation after IR (Barazzuol et al., 2017; Li et al., 2016) or S phase accumulation upon etoposide exposure (Nam et al., 2010). These studies were mouse *in vivo* experiments performed with higher IC values. Since we did not see any p53 regulations upon genotoxic treatment in our NPC model, the question occurred if non-detectable changes might indicate species differences or the requirement of a stronger stimulus. Experiments with higher inhibitory concentrations or longer incubation times could be performed in human NPCs to investigate if literature-based observations can be reproduced in our NPC model. Further investigation is required to pinpoint whether the discrepancy is based on translational issues or on results obtained from *in vitro* vs. *in vivo* studies.

Overall, all three cell models did not show significant regulations in pp53 Ser 15 upon BPDE treatment. It has to be considered that pluripotent stem cells do have a comparatively low amount of p53 abundance due to p53's characteristic to repress pluripotency markers and initiate differentiation (Lin et al., 2005). Moreover, ESCs have been shown to inefficiently translocate p53 from the cytoplasm to the nucleus where phosphorylation takes place. These findings support the hypothesis that stem cells might rely on a p53-independent DDR when encountering mild damage whereas severe damage causes the translocation and phosphorylation of p53. It has been demonstrated that mESCs can utilize p53-independent DDR upon UV-induced DNA damage and loss of p53 did not alter the response to DNA damage (Prost et al., 1998). An increase of p53 and its phosphorylated form might indicate severe stress and the initiation of pluripotency loss to retain the damage and the induction of apoptosis or differentiation. This hypothesis is supported by the pp53 Ser 15 levels in iPSCs and NPC after 2 h serum starvation. Elevated levels of pp53 levels were detected in iPSCs and NPCs and which cannot be observed as prominent in HCT116. Considering that stem cells require strict cellular homeostasis, 2 h of nutrient-deprivation might be a severe endangerment for these cells compared to HCT116. However, besides p53, another marker in stem cells for p53-independent apoptosis and differentiation upon DNA damage has been identified which is not a protein but a long non-coding RNA called *Lnc956* (Ma et al., 2023). For investigating the hypothesis that p53 phosphorylation only takes place upon severe genotoxic stress, a serial dilution of both genotoxins could be performed to analyze the timepoint and required concentration to induce p53-dependent cellular response. Concomitantly, levels of ATM and ATM phosphorylation could be analyzed by western blot, and *Lnc956* methylation could be investigated by ELISA assay.

The working group of Lu et al. performed similar experiments as in this thesis to investigate the differential response of hiPSCs and cancer lines with the environmental carcinogen Chromium (VI), superoxide and doxorubicin (Lu et al., 2013). They compared hiPSCs with the tumor cell line Tera-1 (teratoma origin) and BEAS-2B (lung epithelial origin) and observed different regulations of p53 in hiPSCs compared to cancer cell lines. While doxorubicin and superoxide resulted in an upregulation of pp53 Ser 15 levels at 4-8 h, Chromium (VI) did not seem to affect pp53 Ser 15 levels. In comparison, both cancer cell lines showed a constant upregulation of pp53 Ser 15 treated with Chromium (VI) but also showed divergent regulations towards doxorubicin and superoxide. Furthermore, they have demonstrated that different genotoxic stimuli caused an increased abundance of different p53 phosphorylation sites. These findings support the hypothesis of differential regulation of DDR-associated proteins in iPSCs and NPCs compared to cancer cells. Concluding from the results obtained in this thesis and the previous study (Lu et al., 2013), p53 and its phosphorylation might not be an optimal readout for DNA damage in pluripotent SCs and thereof differentiated progenitor cells when

investigating the impact of low dose genotoxins due to its distinct function in SCs. These results emphasize the differences in DDR in SCs and cancer cells and the requirement for further investigation.

Besides p53 and γH2AX levels, we additionally investigated the impact of both genotoxins on the NER protein XPC. Since NER is responsible for the removal and repair of DNA adducts, we surprisingly saw only a slight upregulation of XPC within 48 h BPDE treatment in iPSC. In comparison, niPS11 and HCT116 had elevated XPC levels after 72 h. These differences between iPSCs and the other cell lines might again originate from differences in chromatin organization. NER repair has limited access to heterochromatic structure requiring additional processes to allow repair whereas, euchromatic organization facilitates quick repair (Adar et al., 2016). The faster DDR reflected in obtained data supports the aspects of euchromatic organization in iPSCs and ongoing chromatin condensation upon pluripotency loss.

Furthermore, treatment with etoposide also resulted in an increase of XPC abundance. It was previously described that treatment of non-small cell lung cancer cells results in an increase of XPC levels upon etoposide treatment as a cytoprotective reaction mediated by AKT signaling since XPC knockout or AKT inhibition caused sensitization of cells towards etoposide (Chen et al., 2018). In our experimental set-up, etoposide was administered for 24 h and removed from the medium which explains the decrease of XPC abundance after 48 h as seen in pp53 Ser 15 levels. These results might reflect the distinctive DDR mechanisms of stem cells by either efficiently repairing the damage or consistently eliminating cells with remaining damage. To investigate the apoptotic rate, the Nicoletti assay via flow cytometry or TUNEL assay using either flow cytometry or fluorescence microscopy could be utilized for kinetic experiments. The gradual increase of XPC protein levels in NPCs and HCT116 in contrast to pluripotent stem cells underlines the cellular differences as stem cells are either able to repair the damage posttreatment due to DNA accessibility or are potentially more tolerant towards damage and possible mutations.

In conclusion, iPSCs, NPCs and HCT116 execute different DDR to low dose exposure to etoposide and BPDE. According to literature, iPSCs and NPCs strongly rely on ATM and p53 signaling, however, most studies were performed with higher cytotoxic concentrations in contrast to the experimental set-up used in this dissertation. These high concentrations potentially cause severe DNA damage and therefore cellular stress, which might trigger different cellular responses than by encountering mild damage. Low dose treatment elicited a rather p53-independent cellular response in iPSCs and NPCs compared to HCT116 emphasizing the importance of comprehending cellular differences between stem cells, progenitor cells and cancer cells to optimize treatment approaches. To gain more knowledge regarding cellular organization of different cell types and its impact to genotoxic outcomes,

analysis of chromatic organization by high-throughput chromatin conformation capture and cell cycle composition by flow cytometry offer perspectives for comparative analysis. Additionally, obtained and previous data have shown that pluripotent SCs and NPCs have a different response to DNA damage than somatic cells which can be further investigated by dilution and kinetic experiments in corresponding cell line by transcriptome or proteome analysis. Stem cells may possess great DDR capacity, however, data about DNA organization, cell cycle differences and exclusive DDR mechanisms might support the protection of adult stem cells during cancer treatment. Various studies have shown that anticancer treatment by radiation therapy or drug administration can cause neurological and cognitive defects such as memory processing, attention, concentration and information processing (Armstrong et al., 2010; Gibson & Monje, 2012; Langer et al., 2002; Surma-aho et al., 2001). However, data about the specific impact of anticancer treatment on adult NPCs in particular remains elusive.

4.4 Low dose genotoxic treatment shows no to moderate effect on canonical autophagy

Since autophagy has been implicated with cytoprotection in regard to genomic integrity and support of DDR, we investigated the impact of both genotoxic noxae on early autophagic events by ULK1 phosphorylation and protein levels of downstream effectors LC3 and p62. The phosphorylation status of ULK1 was not significantly affected by neither BPDE nor etoposide in iPSCs and NPCs. The same pattern was also observed for LC3 and p62 abundance upon treatments. These observations might support the unique characteristics of stem cells to either repair DNA damage quickly or to induce apoptosis. In somatic cells, DNA damage causes cell cycle arrest followed by the evaluation of the damage and its potential repair. The prolongation of the repair process might cause accelerated cellular stress and energy demands requiring the activation of autophagy since autophagy supports cellular survival. Assumedly, stem cells do not require autophagic support for cellular survival owing to the quick evaluation of the damage and subsequent decision to repair or eliminate the cells. As described in chapter 1.7, subcellular localization of p53 either supports autophagy activation by inhibiting mTOR or inhibiting autophagic initiation by AMPK and mTOR activation. Thereby, ESCs poorly translocate p53 into the nucleus (Aladjem et al., 1998) which might also be the reason why there was no significant difference in p53 phosphorylation as discussed before. This could result in the accumulation of p53 in the cytosol and an inhibition of autophagy. As stated above, a stronger stimulus and severe damage potentially cause the accelerated translocation of p53 and its ATM-dependent phosphorylation. Consequently, autophagy might be activated due to p53 translocation and severe cellular stress to either support cellular survival or contribute to the activation of cell death pathways rather than being a response to DNA damage in general.

Another reason why autophagy might not be activated in stem cells unless encountering severe stress, might be the distinct involvement of the autophagic process in stem cell quiescence. High autophagic activity and mTOR inhibition are involved in the induction of quiescence in stem cells and CSCs (Baquero et al., 2018; Borsa et al., 2023; Wang et al., 2018). Consequentially, acceleration of autophagic response upon genotoxic stress could induce cell cycle exit to protect the stem cell pool. The hypothesis of autophagy's involvement in cellular adaptation rather than being a consequence of DNA damage is supported by the autophagic response seen in HCT116. Different studies have shown an activation of canonical autophagy upon genotoxic stress in cancer cells (Eliopoulos et al., 2016; Katayama et al., 2007; Orlotti et al., 2012). The majority of studies focused on the autophagic reaction upon high dose genotoxic treatment, for example with etoposide (Katayama et al., 2007) or investigated the consequences of modulating a multifunctional pathway upon genotoxic treatment rather than the direct impact on the autophagic process (Galati et al., 2019). Our experiments in HCT116 showed slight changes in ULK1 phosphorylation status but only marginal impact on downstream effectors. With the low dose treatment accumulation of LC3-II in western blot analysis could not be observed as seen in the previous studies (Katayama et al., 2007; Orlotti et al., 2012) supporting the hypothesis that autophagy might rather be activated upon severe cellular stress to support cellular survival in cancer cells than being directly affected by genotoxic events. Nevertheless, we saw an increase of LC3 puncta after 24 h etoposide treatment in immunofluorescence. It has to be considered that immunofluorescence displays the total amount of LC3B while western blot analysis focuses on the lipidated LC3-II form in this dissertation. This difference might indicate that lipidation of LC3 may be affected by etoposide treatment although no increase in LC3-I can be qualitatively detected in western blot analysis.

Low dose treatment with BPDE did not affect LC3-II levels and showed a slight decrease in LC3 abundance in iPS11 and HCT116 in immunofluorescence, respectively. A study in mouse ovary luteal and luteinized granulosa cells has demonstrated that BaP or BPDE exposure inhibited autophagosome formation and impaired LC3 lipidation (Li et al., 2022). The decrease in total LC3 might imply impairment in lipidation or degradation by non-canonical autophagy.

As discussed in Publication 1, CMA (Park et al., 2015) and Golgi membrane-associated degradation (GOMED) (Sakurai et al., 2023) have been associated with DNA damage. Abundance of CMA-associated protein HSC70 has been analyzed by western blot analysis but did not seem to be affected by neither BPDE nor etoposide (data not shown). GOMED is described as an ATG5-independent alternative autophagy pathway (Nagata et al., 2018; Nishida et al., 2009; Torii et al., 2020). For GOMED activation, the dephosphorylation of pULK1 Ser 638 by protein phosphatase 2A (Wong et al., 2015) and protein phosphatase, Mg^{2+}/Mn^{2+}-

dependent 1D (PPM1D) (Torii et al., 2016) is required which was not observed in our cell systems. The dephosphorylation by PPM1D is mediated in a p53-dependent manner (Torii et al., 2016), which might be attenuated in stem cells due to their low p53 abundance. Furthermore, receptor-interacting protein kinase 3 (RIPK3)-dependent phosphorylation of ULK1 at Ser 746 upon genotoxic stress and its exclusive localization at the Golgi apparatus has been reported to be required for GOMED but not canonical autophagy. Experiments in mouse embryonic fibroblasts showed etoposide-induced increase of Ser 746 (Torii et al., 2020). For investigating the role of alternative autophagy pathways in iPSCs and NPCs, dose- and time-dependent western blot or immunofluorescence could be performed.

Furthermore, it has to be taken into consideration that DDR and autophagy do have a complex relationship that is still poorly understood. The review of Juretschke & Beli summarizes the impact of different genotoxins on autophagy initiation and thoroughly described that the initiation is altered depending on the genotoxin. While different stimuli including cisplatin, etoposide, camptothecin and UV light can trigger autophagy initiation, only the last one results in mTOR inactivation in an ATR-CHK1-dependent manner (Juretschke & Beli, 2021). Additionally, it has been suggested that ATM and DNA-PKcs could have DDR-independent functions influencing autophagy initiation (Alexander et al., 2010; Juretschke & Beli, 2021).

Another interesting aspect to investigate is the impact of genotoxic noxae on the autophagic process in postmitotic neurons compared to NPCs. Terminally differentiated neurons require exceptional quality control and cell homeostasis to last throughout lifetime. Especially, neurons are compartmentalized into soma, dendrites and axon with specific autophagic functions (Stavoe & Holzbaur, 2019). For instance, autophagosome biogenesis mainly takes place in the distal axon and is transported towards the soma and dendrites and are also generated under nutrient-rich conditions (Maday & Holzbaur, 2014). Impaired or dysregulated autophagic function is associated with various neurodegenerative diseases as stated before. Autophagy may play a dual role in either executing neuroprotection (Menzies et al., 2017) function or initiating autophagic cell death if excessively activated or dysregulated (Gao et al., 2022; Hou et al., 2019). Neurons are not able to dilute hazardous components by dilution and may rely more on the efficient removal by autophagy. Especially the impact of our investigated agents may reveal acquired adaptations during development. As described before, etoposide is used as an anticancer drug due to the inhibition of TOPO IIα in proliferating cells. Furthermore, patients treated with etoposide may show peripheral neuropathies as a side effect (Imrie et al., 1994). Since neurons are postmitotic, TOPO IIα abundance might be comparatively low, nevertheless, they strongly rely on TOPO IIβ. TOPO IIβ is essential for neuronal differentiation, transcriptional gene activation and chromatin remodeling (Tsutsui et al., 2001). Disruption of TOPO IIβ's functions may lead to impaired axonal growth, reduced chromatin accessibility and

premature neuronal death (Tiwari et al., 2012). It has been demonstrated that BPDE induces ferroptosis and consequently causes neuronal loss which is linked to cognitive deficits and may contribute to neurodegenerative onset (He et al., 2025; Zhou et al., 2025). Further investigations may involve the impact of genotoxins on the differentiation potential of iPSCs and NPCs and if thereof differentiated progeny shows impairment or physiological alterations. Moreover, the impact on the autophagic process could be assessed by immunofluorescence or western blot. To investigate the influence on functionality in mature neurons, microarray assays could be utilized. Another interesting aspect to investigate would be the impact on genotoxins on neurons upon autophagic modulation since it is associated with a dual role. In case of beneficial outcomes for neuronal survival autophagy modulation could be additionally utilized for targeted-drug delivery for either protecting a certain area during anticancer treatment or potentially supporting treatments for neurodegenerative diseases.

In summary, low dose treatment with BPDE and etoposide did not seem to activate canonical autophagy in hiPSCs and NPCs and only showed a marginal autophagic response in regard to ULK1 phosphorylation in HCT116 despite divergent reports in literature. Hypotheses explaining these observations might be that autophagy mainly supports cellular survival upon severe damage or cellular stress wherefore, low dose treatment does not require autophagy recruitment. The aspect of supporting cellular survival upon stress might not be transferable to stem cells since high autophagic activation induces dormancy. In stem cells, the subcellular localization of p53 might play a role in autophagy repression. EBSS treatment resulted in an increased p53 phosphorylation, however, since starvation is the main trigger for autophagy activation the relationship between p53 translocation and phosphorylation and its impact on autophagy initiation cannot be determined in our experimental set-up. Consequentially, to address the hypothesis of autophagy inhibition due to the cytosolic localization of p53 in stem cells, immunofluorescence could be performed to investigate p53's subcellular localization upon genotoxic treatment in a dose-dependent manner and co-stained with LC3. For investigating the canonical autophagic process, reporter cell lines with tandem green fluorescent protein (GFP) and red fluorescent protein (RFP)-tagged LC3 can be utilized. In these cell lines, RFP-LC3 has a deleted C-terminal glycine which is required for ATG4 processing. While GFP-LC3 is sequestrated and degraded in autolysosomes, the RFP-LC3ΔG remains in the cytosol. This GFP-LC3-RFP-LC3ΔG supports the localization of LC3 and investigation of the autophagic flux (Kaizuka et al., 2016).

4.5 iPS11 and niPS11 show different proteomic changes after genotoxic exposure

Since we saw that the majority of typically investigated proteins in western blot analysis were not significantly affected by genotoxic treatments, we performed differential proteome analysis to gain more information about physiological changes in iPS11 and niPS11 upon treatment with genotoxins. Thereby, treatment with etoposide and BPDE did not reveal major proteomic changes in iPS11 which, again, might support the hypothesis that stem cells display efficient decision making regarding cellular fate since we looked at late timepoints with regard to DDR. One protein that was upregulated upon BPDE exposure was nuclear ubiquitous casein kinase and cyclin dependent kinase substrate 1 (NUCKS1). NUCKS1 has been identified to maintain HR repair in human cells and acts as a tumor suppressor protein in mice by directly interacting with RAD54, a protein important for proper HR repair (Maranon et al., 2020). Since BPDE was administered daily to the cells and DSBs are rather a secondary effect of occurring DNA adducts, the accumulation of damage and delayed emergence of DSBs might cause the upregulation of this HR-associated protein. Interestingly, in p53-dependent DDR, NUCKS1 is downregulated upon DNA damage by p53, and an upregulation indicates disruption in p53 signaling. Besides NUCKS1´s participation in HR repair, it also promotes S phase entry by increasing S phase Kinase-associated Protein 2 (SKP2) and subsequently decreasing cyclin-dependent kinase (CDK) inhibitors p21 and p27 (Hume et al., 2021). As discussed before, the lack of G1/S checkpoint potentially leads to the entry of unrepaired cells into S phase resulting in the repair by HR or induction of apoptosis. This observation supports that hypothesis since NUCKS1 upregulation upon DNA damage is normally repressed to initiate cell cycle arrest and upregulation is rather associated with cancer (Hume et al., 2021). Additional proteins upregulated upon BPDE treatment were amongst others structural maintenance of chromosome 3 (SMC3), the core protein of cohesion-rings involved in maintaining genomic stability during cell division and upon DSBs (Hou et al., 2022; Wu & Yu, 2012), RAD23A, which is involved in NER (Renaud et al., 2011), Heterogeneous Nuclear Ribonucleoprotein K (HNRNPK), a protein involved in the regulation of transcription and translation, and epigenetic regulation of cancer cell proliferation (D. Li et al., 2023). Proteins upregulated upon BPDE treatment in iPSCs were mainly associated with cell proliferation or with DDR (Renaud et al., 2011; Wu & Yu, 2012). However, their regulation pattern was rather similar to cancer cells as opposed to somatic cells (D. Li et al., 2023; Wang et al., 2020) which underlines the similarity of stem and cancer cells. The parallel upregulation of DDR-associated and cell proliferation promoting proteins supports the previously made observation by Stambrook and Tichy in mESCs that pluripotent stem cells enter S phase with unrepaired damage.

The treatment of niPS11 by both genotoxins resulted in an upregulation of a plethora of mitosis-associated proteins including aurora kinase A (AURKA), polo-like kinase 1 (PLK1) and various

kinesin proteins (KIF). Amongst other detected proteins, these proteins are involved in proper mitotic spindle formation. An upregulation of KIF11, which was identified in both genotoxin-treated proteomes, has been associated with mitotic catastrophe and micronuclei formation (Dale et al., 2022). In our immunofluorescence analysis we could also detect micronuclei formation after 24 h treatment. Another KIF protein identified as upregulated in both niPS11 data sets was KIF2C. KIF2C has been reported to be involved in DSB repair in a PARP- and ATM-dependent manner (Zhu et al., 2020). The upregulation of mitotic or DDR-related proteins in NPCs underlines the physiological differences between pluripotent SCs and thereof derived progenitor cells. An upregulation or reorganization of spindle assembly could also consequentially lead to cell differentiation as described in chapter 1.1. Asymmetric division is an important feature of stem and progenitor cells to maintain the cell pool and provide progeny for tissue regeneration and remodeling. Upon stress and DNA damage, damaged cells are eliminated by inducing apoptosis or differentiation. Further investigation is required to identify the consequences of low dose genotoxic treatment and whether cells tend to induce apoptosis or differentiation. Induction of differentiation supports the maintenance of the stem cell pool by differentiating into postmitotic cells. A tendency towards differentiation might also explain the results obtained from the mitotic index assay (MIA) and cell growth curve. Both assays show a slight decrease in phospho Histone 3 (pH3) signaling and relative cell growth after 48 h etoposide treatment. With that, during 24 h recovery period damaged cells could have been sorted out while recovered cells started proliferating again as seen in the cell growth curve. Furthermore, pH3 is a marker for G2/M phase transition (Hans & Dimitrov, 2001). In our proteome data, we saw an upregulation of CDK1 and CDKN1a/p21, both important regulators of the cell cycle. CDK1 is a regulator for G2/M phase transition and its upregulation together with cyclin B has been associated with apoptosis induction after 2-methoxyestradiol-induced mitotic catastrophe (Choi & Zhu, 2019). Together with the upregulation of CDK inhibitor p21, low dose treatment with etoposide in NPCs might affect the cell cycle and cause either cell cycle arrest or other interfering mechanisms in contrast to iPSCs. As discussed before, mouse NPCs showed p53-dependent cell cycle arrest upon genotoxic exposure. Our data might indicate impact on the cell cycle, but we do not see an upregulation of p53 in western blot or proteome analysis. Furthermore, these fluctuations in MIA and cell growth curve could only be detected in etoposide samples but not BPDE. Presumably, effects on the cell cycle after BPDE treatment might require more time in comparison to etoposide since, for instance, regulation of XPC was observed after 72 h potentially implying ongoing damage repair and evaluation. Autophagy-related proteins were not regulated in both cellular systems and genotoxins despite ongoing DNA repair and mitotic effects supporting the hypothesis as autophagy being more of a cytoprotective “bystander” effect.

Altogether, differential proteome analysis has revealed that autophagy-related proteins and the canonical autophagic process are not influenced in etoposide or BPDE-treated iPS11 and niPS11 at designated timepoints. Furthermore, iPS11 did only show marginal proteomic regulation with both genotoxins potentially indicating that pluripotent stem cells quickly evaluate and handle genotoxic stress so that the analyzed timepoints might be too late to see regulations. In comparison, niPS11 showed DDR-related and mitosis-associated protein upregulations under equitoxic conditions underlining cellular differences between pluripotent stem cells and NPCs. Figure 8 summarizes the discussed cellular differences and similarities which might explain obtained results within this thesis and from previous literature. To further investigate the genotoxic impact on iPSCs, earlier timepoints could be analyzed by either western blot or transcriptome and proteome analyses. Furthermore, to gain more insights into NPC regulations regarding induction of apoptosis or differentiation the Nicoletti assay could be performed to gain information about the cell cycle and the amount of apoptotic cells. Protocols involving neuronal differentiation from NPC require a longer face of approximately 8 days until cells are not proliferative anymore (Reinhardt et al., 2013). This aspect has to be considered for further analyses to extend the investigated time period if the differentiation potential of NPCs is analyzed. Cells could be treated either with acute or chronic exposure and changes in transcriptome analyzed by qRT-PCR over time.

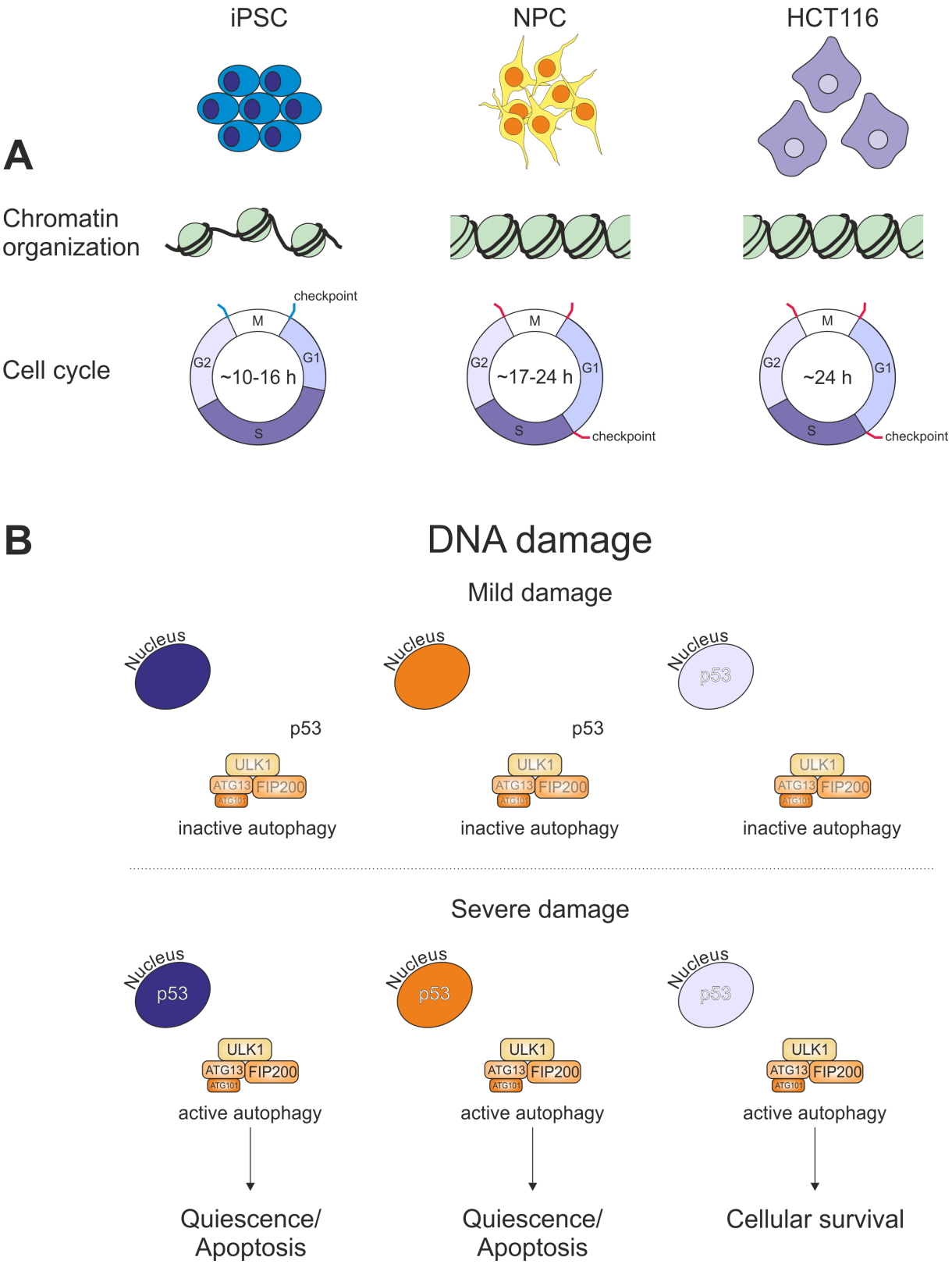

Figure 8: Cellular differences of iPSCs, NPCs and HCT116. A) iPSCs show distinct physiological features compared to NPCs and HCT116. Their euchromatic organization allows quick DNA accessibility for transcription and proliferation while NPCs and HCT116 mainly contain heterochromatic structures. Furthermore, iPSCs display a shortened cell cycle for fast proliferation and mainly reside in S phase accompanied by absent or compromised cell cycle checkpoints (marked in blue). These features might result in a higher sensitivity of iPSCs towards genotoxic stress but are also potentially beneficial to repair and eliminate erroneous cells. B) Upon mild DNA

damage, p53 is located in the cytoplasm potentially contributing to the inhibition of autophagic activation in iPSCs and NPCs, whereas p53 translocates to the nucleus in HCT116 but does not activate canonical autophagy. After severe damage, p53 might translocate to the nucleus in all three cell systems and activate autophagy. While autophagic activation supports cellular survival in HCT116, upregulated autophagy potentially causes the induction of quiescence or apoptosis in iPSCs and NPCs.

4.6 Cancer stem cells

As described in chapter 1.2 and Publication 2, the origin of CSCs is still debated while one potential source might be mutated stem cells. Understanding the difference between somatic and stem cell physiology will help to improve therapeutic treatment and potentially help reduce side effects. CSCs share distinct characteristics with healthy stem cells including pluripotent features and the ability to induce dormancy and reactivation. The aspect of dormancy complicates the application of many replication-dependent anticancer drugs. Both pluripotent and multipotent SCs can induce quiescence *in vivo* and *in vitro*. Investigating the differences of cycling and quiescent stem cells might support the understanding of the differences between these states and in regard to CSCs to protect healthy stem cells during anticancer treatment. Autophagy inhibition offers a great approach to induce cell cycle entry and sensitize cells towards replication-dependent anticancer drugs (Tiwari et al., 2024). However, since healthy stem cells would also be affected by autophagy inhibition, the targeted delivery of inhibiting compounds is essential to protect healthy stem cells. Targeted drug delivery is a rapidly advancing field aiming to overcome drug resistance and reduce side effects. This can be achieved by, for instance, nanoparticle-based delivery or exosome-based systems coupled with antibodies recognizing specific CSCs surface markers (Ertas et al., 2021; Wang et al., 2017). This aspect of specific targeting can be challenging since the majority of cell surface markers found in CSCs are also present on healthy stem cells or differentiated cells. It has been proposed that tumors might have tumor-specific glycans due to altered stem cell marker glycosylation (reviewed in Karsten & Goletz, 2013). Cycling CSCs might be more sensitive to anticancer drugs accompanied by euchromatic organization owing to pluripotency marker expression and proliferation. Nevertheless, CSCs have elevated chromatic plasticity allowing the fast activation or repression of gene expression and adaption to therapeutic approaches (Feng et al., 2021; Yinu Wang et al., 2025). Furthermore, CSCs also show the upregulated gene expression of DDR-related proteins accompanied by the tolerance and accumulation of genetic mutations (Al-Hajj et al., 2003; Shackleton, 2010).

Despite cellular similarities, CSCs exhibit obstacles in anticancer approaches due to their plasticity and mutation tolerance but still possess beneficial characteristics of healthy stem cells for propagation and survival. Understanding every aspect of stem cell and CSC features is crucial for fighting CSCs and protecting healthy stem cells. While CSCs display chemo

resistance (Y. Li et al., 2021), healthy stem cells are strongly affected by chemotherapeutics causing mutations, impaired proliferation and stem cell depletion (Li et al., 2004; Upadhyaya et al., 2019). To deepen the knowledge about cellular differences, iPSC models can be utilized to understand physiology during stem cell dormancy by Myc depletion (Scognamiglio et al., 2016) or mTOR inhibition (Alhasan et al., 2021). Additionally, thorough analyses of cell surface markers of different adult stem cells and CSCs need to be performed to enable specific targeting of CSCs.

4.7 Conclusion

Within this thesis, we investigated the impact of BPDE and etoposide on hiPSCs and thereof differentiated NPCs in regard to DDR and their influence on the canonical autophagic process. Autophagy plays a significant role in stem cell homeostasis and has been repeatedly associated with DDR in cancer cells, however, the interplay of the autophagic process and DDR in stem cells has not been analyzed so far. We propose that autophagic activity does have a different function in stem cells than in cancer cells upon genotoxic treatment and is rather activated by fatal damage supporting the elimination of damaged cells as opposed to cellular survival as seen in cancer cells.

In our studies we identified that iPSCs and NPCs show similarities in the recruitment of DDR-associated proteins, however, differ in their temporal activation. Reasons substantiating these differences might be the chromatic organization, the cell cycle or drug efflux. Differential proteome analysis showed that NPCs had elevated level of mitosis-related proteins and specifically in etoposide treated cells an elevation of proteins affecting the cell cycle which could not be observed in iPSCs. NPCs treated with BPDE also showed upregulations in spindle arrangement-related proteins but did not have elevated levels of cell cycle inhibitors as observed in etoposide-treated NPCs. These results support the reorganization of cell cycle-dependent components and the efficiency of pluripotent stem cells to decide if cells are worth repairing or need to be eliminated.

In regard to autophagy, the canonical autophagic process was not affected in all three tested cell systems implying that autophagic activation might be a result of severe damage and potentially also be a consequence of non-DNA damage related functions of DDR proteins. The lack of autophagy initiation in stem cells might be the result of its distinct function in regard to the induction of quiescence in this subpopulation. To further investigate the role of the autophagic process and additionally the implication of non-autophagic functions of autophagy-related proteins, autophagy modulators could be utilized to understand the impact of altered canonical autophagy, especially in the context of dormant stem cells. Furthermore, the aspect

of reproducibility has to be considered, and more iPSC lines need to be studied. Another interesting aspect to investigate is the impact of different inhibitory concentrations on iPSCs, NPCs and postmitotic neurons and especially the impact on their differentiation potential. Overall, this dissertation contributed to fundamental research to understand mechanisms and differences in stem cell physiology, potentially providing information to support stem cell maintenance and survival and contributes to translating the obtained knowledge for anticancer treatment improvements.

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6. Licensing & Copyright

7. Appendix

Publication 1

Canonical autophagy remains inactive in induced pluripotent stem cells and neuronal progenitor cells following DNA damage induced by BPDE or etoposide.

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Canonical autophagy remains inactive in induced pluripotent stem cells and neuronal progenitor cells following DNA damage induced by BPDE or etoposide.

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Keywords:

Autophagy; iPSC; NPC; etoposide; BPDE

Abbreviations:

AMPK, AMP-activated protein kinase; ATG, autophagy-related; AURKA, aurora kinase A; $BafA_1$, bafilomycin A_1; BECN1, beclin 1; DDR, DNA damage response; DSB, double-strand breaks; FIP200, focal adhesion kinase family interacting protein of 200 kDa; IC_{50}, half maximal inhibitory concentration; iPSC, induced pluripotent stem cells; (MAP1)LC3, (microtubule-associated proteins 1A/1B) light chain 3; mTOR, mechanistic target of rapamycin; NPC, neural progenitor cell; NUCKS1, nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate 1; PLK1, polo like kinase 1; RB1CC1, retinoblastoma 1 inducible coiled-coil 1; SQSTM1, sequestosome 1; V-ATPase, vacuolar-type H^+-ATPase

ABSTRACT

(Macro-)Autophagy is a key cellular stress response mediating the recycling of long-lived or damaged proteins and organelles. In stem cells, autophagy is essential for the decision between quiescence, self-renewal and differentiation. We observed that induced pluripotent stem cells (iPSCs) and thereof derived neural progenitor cells (NPCs) have a functional autophagy machinery, as shown by starvation-induced autophagic flux and ULK1 activation. Using the human iPSC lines iPS11 and iPS12 and thereof derived NPCs (niPS11 and niPS12), we investigated whether genotoxic stress induced by low doses (IC_{20}) of benzo[a]pyrene diolepoxide (BPDE) or etoposide can similarly activate autophagy, as previously reported for cancer cell lines. While both BPDE and etoposide induced the DNA damage markers phospho-p53 Ser15 and γH2AX and slightly altered the expression of DNA repair proteins such as XPC, they did not trigger autophagic flux in either iPSCs or NPCs. After genotoxin treatment, ULK1 activation was only observed in NPCs, but this was not sufficient to trigger a significant downstream autophagic response. Mass spectrometry revealed minimal proteomic changes in iPSCs and moderate changes in NPCs, mainly involving mitotic regulators. These results suggest that low doses of genotoxic agents do not strongly affect canonical autophagy in pluripotent stem cells or their neural derivatives despite an otherwise responsive autophagic system.

INTRODUCTION

(Macro-)autophagy represents an intracellular stress response mediating the recycling of long-lived or damaged proteins and organelles. During this process, the cargo to be degraded becomes engulfed within double-membraned vesicles termed autophagosomes. The outer membranes of these autophagosomes fuse with lysosomes, forming autolysosomes. Within lysosomes, lysosomal hydrolases degrade the cargo, and the resulting building blocks such as amino or fatty acids are transferred back to the cytosol where they are available again for ATP production, protein synthesis, etc. Autophagy occurs at basal levels in most cell types and ensures cellular homeostasis. However, an autophagic response can also be induced upon stress conditions such as nutrient deprivation, protein aggregation, infection with intracellular pathogens, or DNA damage. Autophagy is executed by autophagy-related (ATG) proteins and non-ATGs, mediating all steps of the autophagy pathway, i.e., initiation, nucleation of the phagophore, expansion of the autophagosomal membrane, maturation of autophagosomes, and fusion with lysosomes. The initiation of autophagy is centrally regulated by the autophagy-inducing ULK1 complex, containing the Ser/Thr kinase unc51-like kinase 1 (ULK1) and the associated factors ATG13, ATG101, and FIP200 (Yamamoto et al., 2023). Two frequently used autophagy marker proteins are the microtubule-associated proteins 1A/1B light chain 3 (MAP1LC3, in brief LC3) and sequestosome-1 (SQSTM1)/p62, which function in autophagosome biogenesis and cargo recruitment (Mizushima & Murphy, 2020).

Autophagy plays a particularly important role in stem cell populations, as they are dependent on intracellular quality control and the maintenance of cellular homeostasis. Autophagic processes have been studied in various stem cell types, including embryonic stem cells, various tissue stem cells (e.g. hematopoietic or neural stem/progenitor cells, NSPCs), cancer stem cells and induced pluripotent stem cells (iPSCs) (Rodolfo et al., 2016). Previous research suggests that autophagy plays a central role in the decision between quiescence, self-renewal and differentiation (Rodolfo et al., 2016). In NSPCs, cytoprotective autophagy is involved in both maintenance and neuronal differentiation (Casares-Crespo et al., 2018). It has been shown that FIP200, a component of the autophagy-inducing ULK1

kinase complex, is essential for these two functions, especially in the postnatal brain (Wang et al., 2013). On the other hand, it could be shown in the mouse model that inhibition of autophagy reduces the irradiation-induced loss of NSPCs (Wang et al., 2017).

The DNA damage response (DDR) is a cellular stress response usually initiated upon genotoxic stress. Generally, a DDR is initiated with the detection of the DNA lesion and the recruitment of factors mediating DNA repair. DNA repair in turn can be executed by five different pathways, depending on the type of DNA lesion. These pathways are 1) base excision repair (BER), 2) nucleotide excision repair (NER), 3) mismatch repair (MMR), 4) homologous recombination (HR) and 5) non-homologous end joining repair (NHEJ) (Sadoughi et al., 2021). In vertebrate cells, the DDR is controlled by three related kinases: ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), and DNA-dependent protein kinase (DNA-PK) (Blackford & Jackson, 2017). On the level of these three kinases, the crosstalk between the DDR and autophagy is initiated. It is generally assumed that autophagy provides the metabolic resources to enable DNA repair. On the molecular levels, it has been demonstrated that ATM, ATR and DNA-PK regulate autophagy signaling via transcriptional or post-translational control (Juretschke & Beli, 2021). The transcriptional control might be executed via the activation of p53 or TFEB, or the nuclear exclusion of FOXK (Juretschke & Beli, 2021). The posttranslational control mainly involves the inactivation of the mammalian target of rapamycin (mTOR) and/or the activation of the AMP-activated protein kinase (AMPK) (Juretschke & Beli, 2021), two key upstream regulators of the above-described autophagy-inducing ULK1 complex. In turn, autophagy modulates DNA repair pathways (Gomes et al., 2017). Finally, it has recently been demonstrated that autophagy exerts a direct role in the repair of DNA lesions, via TEX264-mediated selective autophagy of topoisomerase 1 cleavage complexes (TOP1cc) DNA lesions (Lascaux et al., 2024).

Autophagy induction by anticancer drugs has been reported for several cell lines, but the effects in stem cells are largely unknown. In this study, we aimed at investigating how low-concentration genotoxins affect autophagy signaling in iPSCs and thereof differentiated NPCs. We utilized

benzo[a]pyrene diolepoxide (BPDE) and etoposide as model compounds. BPDE is a metabolite of benzo[a]pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH) found in tobacco smoke, smog and other combustion products, and it forms adducts with nitrogen-containing bases of the DNA (Zhao et al., 2024). Etoposide in turn is a potent topoisomerase II poison, causing double-stranded DNA breaks (DSBs) (Bailly, 2023). We found that neither compound elicited a significant autophagic response in iPSCs and NPCs, albeit the autophagic machinery is both present and functional in these cells.

RESULTS

Characterization of iPSCs and NPCs

In this study, we made use of the iPSC lines iPS11 (derived from human foreskin fibroblasts, Alstem) and iPS12 (human mesenchymal stromal cells, Alstem). Both iPSC lines ectopically express OCT4, SOX2, KLF4, and L-MYC. OCT4 expression was confirmed by immunoblotting (Suppl. Figure S1A) and by immunofluorescence microscopy (Suppl. Figure S1B). Differentiation of iPSCs to neural progenitor cells (NPCs) was done as previously described (Zink et al., 2021) and as depicted in Suppl. Figure S1C, and the resulting cell line was designated niPS11 or niPS12. Expression of NPC marker proteins Pax6 and Nestin was confirmed in niPS11 by immunoblotting and immunofluorescence, respectively (Suppl. Figure S1A and S1B).

iPSCs and NPCs reveal starvation-inducible autophagic capacity

In order to evaluate genotoxin-induced autophagy in iPSCs and thereof differentiated NPCs, we first investigated the general starvation-inducible autophagic capacity of these two cell models. For that, we starved the cells in the absence or presence of bafilomycin A_1, which is a vacuolar-type H^+-translocating ATPase (V-ATPase) inhibitor blocking autolysosomal degradation, and analyzed autophagy by immunoblotting for the autophagy markers phospho-ULK1 (Ser758), LC3, and p62/SQSTM1. The autophagy-inducing kinase ULK1 is phosphorylated at Ser758 by mTOR complex 1 (mTORC1) and thus kept in an inhibited state. Dephosphorylation of this site correlates to the induction of autophagy (Dorsey et al., 2009; Kim et al., 2011; Shang et al., 2011). Lipidated LC3 (LC3-II) decorates the inner and outer surfaces of autophagic membranes and recruits both components of the autophagic machinery and cargo to be degraded. p62 is an autophagy receptor mediating the recruitment of autophagic cargo. In both cell lines and in both differentiation states, starvation induced autophagic flux as determined by these three markers, i.e., reduced phosphorylation of ULK1 at Ser758, increased LC3-II turnover (difference in LC3-II levels with and without bafilomycin A_1), and

increased p62 turnover (difference in p62 levels with and without bafilomycin A_1) (Figure 1A-D). Collectively, these data indicate the "general" autophagic capacity of both iPSCs and NPCs.

BPDE and etoposide induce DNA damage in iPSCs and NPCs

In order to determine suitable concentrations of the genotoxins BPDE and etoposide for our autophagy assays, we first determined IC_{20} values. For that, we performed MTT assays in iPS11 and niPS11, and analyzed viability after 24, 48 and 72 hours, respectively (Suppl. Figure S2). As treatment scheme, cells were exposed to etoposide for 24 h and subsequent medium exchanges were without etoposide. In contrast, BPDE was freshly supplemented to the cells every 24 hours. As control, we used the colon carcinoma cell line HCT116. For both genotoxins, IC_{20} values were lower in iPS11 as compared to niPS11 over all time points, indicating a higher sensitivity of iPS11 for DNA-damaging agents (Suppl. Figure S2). Next, we assessed DNA damage by immunoblotting for phospho-p53 (Ser15) and phospho-H2AX (Ser139; γH2AX). Ser15 of p53 can become phosphorylated by ATM, ATR, and DNA-PK, and this phosphorylation prevents p53 from associating with MDM2, ultimately leading to the accumulation and activation of p53 following DNA damage (Shieh et al., 1997; Tibbetts et al., 1999). Similarly, phosphorylation of H2AX at Ser139 is also mediated by the mentioned kinases upon DNA damage (Burma et al., 2001; Rogakou et al., 1999). In iPS11 and iPS12, both phospho-substrates were detectable upon both treatments (Suppl. Figures S3A and S4A). Of note, γH2AX was also clearly induced by starvation (EBSS) in both iPSC lines. This was also the case for niPS11, niPS12 and HCT116 (Suppl. Figures S3B/S3C and S4B), and this observation might be attributed to a p38-dependent phosphorylation of H2AX (Lu et al., 2008). In iPSCs and NPCs, γH2AX appeared to be partially sensitive to bafilomycin A_1 treatment (Suppl. Figures S3A/S3B and S4A/S4B). Generally, the DNA damage-induced phosphorylation of p53 and H2AX was clearly observable in HCT116, and also more distinct in comparison to untreated controls (Suppl. Figure S3C).

We next analyzed Xeroderma pigmentosum group C protein (XPC) levels. XPC generally functions as an initiator of global genome nucleotide excision repair (GG-NER), which repairs lesions generated by

BPDE (Piberger et al., 2018). Although DNA double strand breaks are the main type of damage induced by etoposide, NER proteins have also been linked to topoisomerase II inhibitors (Rocha et al., 2016). XPC levels were clearly increased by both genotoxins in control HCT116 cells. In iPS11/iPS12 and niPS11/niPS12 cells, effects on XPC levels were less prominent, with the exception of BPDE-treated niPS11 and niPS12 cells (Suppl. Figures S3D and S4C).

We also investigated induction of DNA damage by immunofluorescence microscopy (Figure 2). iPS11, niPS11 and HCT116 cells were treated with genotoxins for 4 and 24 h, and stained for γH2AX and 53BP1 (Figure 2A-C). Co-localization of these two proteins is indicative for DSB (Schultz et al., 2000; Ward et al., 2003). Quantification revealed that etoposide induces γH2AX/53BP1-double positive foci in iPS11 and HCT116 cells after 4 h (Figure 2D and 2F). These is also the case for HCT116 cells after 24 h (Figure 2F). Interestingly, in iPS11 cells single and double-positive foci are reduced in etoposide-treated cells after 24 h (Figure 2D). This might indicate an "overshooting" repair and thus a hormesis effect. Please note that image-based quantification for niPS11 cells is hampered by their overlapping growing behavior. With regard to BPDE treatment, γH2AX/53BP1-double positive foci were observed in iPS11 and HCT116 cells at both time points (Figure 2E and 2G).

Collectively, these data indicate that both genotoxins generally induce a DNA damage response in iPS11/iPS12 and niPS11/niPS12 cells.

ULK1 activation status is not affected by BPDE or etoposide in iPSCs

Since the main goal of our project was to investigate genotoxin-induced autophagy in stem cells and thereof differentiated cells, we next investigated the activation status of the autophagy-inducing kinase ULK1. For that, we analyzed ULK1 phosphorylation at Ser758 and Ser638 (another mTOR/AMPK-dependent phospho-site) upon etoposide or BPDE treatment. In iPS11 and iPS12, no alterations of ULK1 activation status were observable, neither for etoposide nor for BPDE (Figures 3A and 4A, Supplemental Figures S5A and S6A), although responsiveness towards starvation could be confirmed (see also figure 1A and 1B). In contrast, ULK1 activation (i.e., dephosphorylation at Ser758 and Ser638)

was detected in niPS11 and niPS12 (Figures 3B and 4B, Supplemental Figures S5B and S6B), although the extent of ULK1 activation was not as strong as with starvation. Of note, the effect of etoposide treatment was indifferent in HCT116, with rather decreased Ser758 phosphorylation and increased Ser638 phosphorylation (Figure 3C). With regard to BPDE, a consistent pattern of ULK1 activation was observed in HCT116 cells (Figure 4C). These data indicate that early autophagy events such as the activation of ULK1 do not occur in iPSCs upon genotoxin treatment, whereas this appears to be the case in NPCs.

BPDE or etoposide do not induce autophagic flux in iPSCs or NPCs

Next to early ULK1 activation, we also investigated the effect of the two genotoxins on autophagic flux. For that, we monitored again turnover of LC3 and p62. Neither in iPS11/iPS12 nor in niPS11/niPS12 cells autophagic turnover of LC3 or p62 were significantly induced (Figure 5A and 5B, Supplemental Figure S7A and S7B). Of note, this was also the case for the cancer cell line HCT116 (Figure 5C). All three cell models remained responsive to bafilomycin A_1 (indicating basal autophagy) and to starvation, as indicated by an EBSS-dependent reduction of p62. Next to immunoblot-based detection of LC3, we again employed immunofluorescence microscopy in order to monitor LC3 puncta formation (Figure 6A-C). Quantification for iPS11 cells confirmed the lack of genotoxin-induced LC3 puncta formation (Figures 6D and 6E). In contrast to the immunoblot data, etoposide treatment significantly increased the number of LC3 puncta in HCT116 cells after 24 h (Figure 6F). BPDE, however, was also ineffective in this cell line (6G).

Taken together, these observations lead to the conclusion that low doses (IC_{20}) of the genotoxins BPDE and etoposide do not mount a canonical autophagic response in iPSCs or NPCs.

Genotoxin treatment of iPSCs and NPCs does not result in altered expression profiles of autophagy-relevant proteins

In order to get a global overview of the genotoxin-mediated alterations of the cellular proteome, we performed mass spectrometry. In iPS11, proteome changes with regard to biological relevance and statistical significance remained minimal for both treatments (Figure 7A). This was also true for a list of autophagy-relevant genes (orange in Figure 7A). One of the slightly enriched proteins upon BPDE treatment was the transcription factor nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1), which has been implicated in the regulation of both S phase entry and double-strand break repair (De Angelis et al., 2018; Hume et al., 2021; Maranon et al., 2020; Parplys et al., 2015; Yue et al., 2016). This upregulation was also confirmed by immunoblotting of the samples analyzed by mass spectrometry (Figure 7B). Similar to iPS11, the proteome alterations with regard to autophagy-relevant proteins remained at low levels in niPS11 (possibly except for a downregulation of PRKAR2A upon BPDE treatment and an upregulation of SESN2 upon etoposide treatment). In niPS11, specifically proteins involved in the regulation of mitosis appeared to be upregulated (Figure 7C). Again, this was confirmed by immunoblotting for the candidate proteins polo like kinase 1 (PLK1) and aurora kinase A (AURKA) (Figure 7D). Interestingly, for BPDE treatment, an AURKA downregulation was observed. We next aimed at determining whether these observed changes in protein abundance translated into mitotic alterations. For that we performed immunoblotting (acetylated tubulin), mitotic index assays, and growth curves (Figures 7D and 7E). During mitosis, microtubules become acetylated, and this posttranslational modification is important for proper spindle function and chromosome segregation (Piperno et al., 1987; Rasamizafy et al., 2021). We observed increased levels of acetylated tubulin upon BPDE treatment, but this was not the case for etoposide. We also analyzed AURKA, PLK1

and acetylated tublin levels in niPS12, and observed increases for all three proteins upon treatment with both genotoxins except for AURKA upon etoposide treatment (Supplemental Figure S8). Phosphorylation of histone H3 at Ser10 is linked to chromosome condensation during mitosis (Hendzel et al., 1997; Wei et al., 1998; Wei et al., 1999). However, we did not detect any differences of H3 Ser10 phosphorylation upon treatment with genotoxins. Finally, proliferation rates appeared to be similar between untreated and treated niPS11 (Figure 7E).

In summary, our data indicate that the environmental genotoxin BPDE and the topoisomerase II inhibitor etoposide do not activate the autophagic program in iPSCs and NPCs. Although niPS11/12 show a slight activation of ULK1 upon treatment, this does not result in increased autophagic flux. Generally, both cell types mount an autophagic response upon starvation, indicating that a functional autophagy machinery is present in these cells.

DISCUSSION

Several previous works indicate that DNA-damaging drugs induce an autophagic response (Bordin et al., 2013; Eliopoulos et al., 2016; Galati et al., 2019; Juretschke & Beli, 2021; Katayama et al., 2007; Rodriguez-Rocha et al., 2011; Vanzo et al., 2020). However, the vast majority of these analyses has been performed in cancer cell models, and the effect of DNA damage on stem cells remains largely unknown. Here, we aimed at investigating how induced pluripotent stem cells and thereof differentiated neural progenitor cells react to low-concentration genotoxin treatment with regard to the autophagy signaling pathway. We observed that both iPSCs and NPCs are generally capable of mounting a strong starvation-induced autophagy, confirming the functionality of the autophagy machinery in these cell models. However, the environmental genotoxin BPDE and the topoisomerase II inhibitor etoposide do not induce a canonical autophagy response. We observed that these compounds only moderately alter the global cellular proteome in iPSCs. In NPCs, mitosis-regulatory proteins were differentially expressed, but this does not result in changes of the mitotic index or the proliferation rates.

Autophagy generally represents a cytoprotective stress response. It has been demonstrated by several groups that DNA damage-inducing agents or treatments induce an autophagic response. Katayama et al. reported that temozolomide and etoposide induced an autophagy-dependent increase in ATP production in multiple glioma cell lines (Katayama et al., 2007). Autophagy induction has also been reported for several other DNA-damaging compounds or treatments (Bordin et al., 2013; Galati et al., 2019; Vanzo et al., 2020). In all mentioned works, cancer cell lines were used. Accordingly, we made use of HCT116 cells as control cell line for our analyses. We observed significantly increased numbers of LC3 puncta upon etoposide treatment in this cancer cell line, but this was not the case for BPDE. In immunoblot-based quantifications of autophagic flux, also the HCT116 cells remained rather unresponsive. Generally, additional differential parameters such as duration of treatment or concentration of compounds need to be considered. We have deliberately chosen IC_{20} concentrations for our studies in order to avoid too extensive cell death and to enable subsequent further

differentiation of stem cells. Although we confirmed that these concentrations led to significant DNA damage, we cannot exclude that higher concentrations would result in a more pronounced autophagy activation. With regard to the treatment, it has been previously reported that radiation-induced DNA damage induces autophagy in HCT116 (Alotaibi et al., 2016; Qased et al., 2013). However, the extent and persistence of DNA damage might again differ between the different treatments. In several reports it has been described that autophagy inhibition enhances the cytotoxic effects of DNA-damaging chemotherapy. However, a sensitization of cells to genotoxic drugs by pharmacological inhibition of the autophagic pathway does not necessarily imply that the genotoxic drugs themselves directly induce autophagy. In other words, autophagy might represent a cytoprotective "bystander" effect that rather acts as a counter-mechanism to cell death induction. In this case, non-lethal concentrations of a DNA-damaging drug might not be sufficient to elicit an autophagy response.

A further level of complexity arises from the large number of canonical and non-canonical autophagy signaling pathways. Park et al could demonstrate that chaperone-mediated autophagy (CMA) is upregulated in response to DNA damage and mediates the regulated degradation of CHK1 (Park et al., 2015). Notably, they also reported that cells were more sensitive to several genotoxins (methylmethanesulfonate, cisplatin, paraquat, hydroxyurea, etoposide, camptothecin) when CMA was blocked, whereas blockade of canonical autophagy sensitized cells only to alkylating agents (methylmethanesulfonate and cisplatin) (Park et al., 2015). Accordingly, it might be worthwhile to analyze CMA and/or alkylating agents in our cellular model systems. Another autophagic signaling pathway that has been associated with DNA damage is Golgi membrane-associated degradation (GOMED) (Sakurai et al., 2023). This pathway requires 1) ULK1 and 2) dephosphorylation of ULK1 at Ser638 (Sakurai et al., 2023; Torii et al., 2016). We do not observe a significant alteration of ULK1 Ser638 phosphorylation status—at least in iPSCs—we currently do not think that GOMED plays a major role in this cellular system.

One central aspect of our study was the usage of iPSCs and NPCs and to analyze DNA damage-induced autophagy in stem cells. Generally, autophagy is supposed to provide energy in order to maintain both

cell cycle arrest and DNA repair activities. Possibly, this is not desired in stem cells, as the genome is "too valuable". Accordingly, cytoprotective stress responses are not preferred over cell death mechanisms, since for a whole organism it is beneficial that single damaged cells become depleted and are replenished by non-harmed cells. In contrast, tumor cells do not have to be so stringent with regard to their genomic integrity. In this regard, it would be interesting to investigate induction of autophagy in neuronal cells differentiated from iPSCs and NPCs, since they are rather post-mitotic and likely rely on adaptive stress responses in order to avoid undesired cell loss.

Our proteome analysis revealed that mitosis-regulatory factors are upregulated in NPCs upon treatment with genotoxic compounds. We speculate that these alterations might represent an attempt to counteract the G2/M arrest caused by the genotoxins. Ultimately, we do not observe differences in the mitotic index or the proliferation rate. Even these "adjustments" of mitosis-regulating factors were not observed in iPSCs, again indicating that the stem cell pool is strictly controlled with regard to genomic integrity and mitosis.

On the molecular level, a direct crosstalk between DNA damage response factors and the autophagy signaling machinery is well established. Our analyses so far have addressed early (ULK1 activation status) and late autophagic events (LC3 and p62 turnover). However, the direct crosstalk between DNA damage-sensing factors and autophagy initiation in our cellular model systems awaits further clarification. We observe that treatment with both genotoxins induces phosphorylation of p53 and H2AX, respectively, indicating that the DDR-inducing kinases become activated. However, this does apparently not translate into a sustained autophagy activation. With regard to transcriptional control of the autophagic response via p53 or other factors (e.g., TFEB or FOXK), we at least do not observe any differences on the proteomic level. However, we have not yet analyzed alterations on the transcriptional level. Meira de Amorim et al. recently reported that BPDE exposure enhances gene expression of cell cycle arrest related genes, but the authors did observe an impact on the cell cycle (Meira de Amorim et al., 2024). Specific alterations of autophagy-related genes were not reported. Future analyses will also focus on the ATM/ATR/DNA-PK-dependent control of the autophagy

regulators AMPK and mTOR, respectively. At the moment we speculate that—although a DNA damage response is initiated—the signaling cascade towards autophagy is blocked at an early stage. Notably, we detect an upregulation of SESN2 in NPCs upon etoposide treatment, a protein that has been shown to regulate autophagy via mTOR, AMPK, and ULK1 (Lu et al., 2023). As we do not observe significant ULK1 activation upon genotoxin treatment, future studies will need to address if inhibition of mTOR or activation of AMPK are affected, or if the blockade might occur on the level of the interaction between SESN2 and ULK1.

In summary, it appears that neither the environmental toxin BPDE nor the topoisomerase II inhibitor etoposide elicit a significant autophagic response in iPSCs or thereof differentiated NPCs, although these cell models mount a “regular” autophagic response upon starvation. These observations indicate that stem and progenitor cells do not tolerate adaptive cellular stress responses if genomic stability and integrity is endangered. Future studies need to address 1) how an autophagic response to genotoxic stress is suppressed and 2) whether alternative or non-canonical forms of autophagy are executed instead.

MATERIAL AND METHODS

Antibodies and reagents

Antibodies against ULK1 (Cell Signaling Technology, Danvers, MA, USA, #8054, 1:1000), phospho ULK1 Serine 757 (Cell Signaling Technology, Danvers, MA, USA, #6888, 1:1000), phospho ULK1 Serine 638 (Cell Signaling Technology, Danvers, MA, USA, #14205, 1:1000), GAPDH (abcam, Cambridge, UK, #ab8245, 1:5000), LC3B (MBL, Woburn, MA, USA, #M-152–3, 1:200 for IF and Cell Signaling Technology, Danvers, MA, USA, #2775, 1:1000 for WB), SQSTM1/p62 (PROGEN, Heidelberg, Germany, #GP62-C, 1:1000), Vinculin (Sigma-Aldrich, St. Louis, MO, USA, #V9131, 1:2000), XPC (Cell Signaling Technology, Danvers, MA, USA, #14768, 1:1000), p53 (Cell Signaling Technology, Danvers, MA, USA, #9282, 1:1000), phospho p53 Serine 10 (Cell Signaling Technology, Danvers, MA, USA, #9284, 1:1000), OCT4a (Cell Signaling Technology, Danvers, MA, USA, #2840, 1:1000), Nestin (Merck Millipore, Darmstadt, Germany #MAB5326, 1:200), PAX6 (Biolegend, San Diego, CA, USA, #901302, for WB: 1:1000, for IF: 1:100) , NUCKS1 (Proteintech, Chicago, IL, USA, #12023-2-AP, 1:1000), γH2AX (For WB: Cell Signaling Technology, Danvers, MA, USA, #2577, 1:1000 and for IF: Merck Millipore, Darmstadt, Germany #05-636, 1:100), 53BP1 (Novus bio, Centennial, CO, USA, 1:2500), Aurora A (Cell Signaling Technology, Danvers, MA, USA, #144755, 1:1000), acetylated Tubulin (Sigma-Aldrich, St. Louis, MO, USA, #312701, 1:20000), PLK1 (abcam, Cambridge, UK, #ab189139, 1:1000), phospho Histone 3 Serine 10 (Cell Signaling Technology, Danvers, MA, USA, #3377, 1:1600) were used. For WB, IRDye 800- or IRDye 680-conjugated secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE, USA, #926-68077, #926-32211 and #926-32210). Secondary antibodies for immunofluorescence analyses and mitotic index assay were purchased from Jackson ImmunoResearch (Alexa Fluor 488-AffiniPure Goat Anti-Mouse IgG, 1:500, #115–545-003; Alexa Fluor 647-AffiniPure Goat Anti-Mouse IgG, 1:500, #115–605-003; Alexa Fluor 647-AffiniPure Goat Anti-Rabbit IgG, 1:500, #111–605-144 and Alexa Fluor 488-AffiniPure Goat Anti-Rabbit IgG, 1:500, #111–545-003). Other reagents used were bafilomycin A_1 (Sigma-Aldrich, St. Louis, MO, USA, #B1793), DMSO (PanReac AppliChem, Darmstadt, Germany, #A3672), 70% ethanol (VWR, Radnor, PA, USA, #85825.360), thiazolyl blue tetrazolium

bromide (MTT, ROTH, Karlsruhe, Germany, #4022.3), Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA, #23225), DRAQ7™ (abcam, Cambridge, UK, #ab109202, 1:100), Benzo[a]pyrene diol epoxide (Santa Cruz, Dallas, TX, USA, #sc-503767) and etoposide (abcam, Cambridge, UK, #ab120227).

Cell lines and cell culture

iPSCs were cultured in mTeSR Plus (Stemcell Technologies, Vancouver, Canada, #100-0276) supplemented with 100 units/mL Penicillin-Streptomycin (P/S; 10,000 U/ml Penicilin, 10,000 µg/ml Streptomycin) (Thermo Fisher Scientific, Waltham, MA, USA, Gibco, #15140122). Neural progenitor cells were differentiated and cultured in self-prepared medium as previously described (Zink et al., 2021). Maintenance medium (sm-) consists of Neuralbasal A medium (Thermo Fisher Scientific, Waltham, MA, USA, #10888022), DMEM/F12 HEPES (Thermo Fisher Scientific, Waltham, MA, USA, #31330038), B27 supplement without vitamin A (Thermo Fisher Scientific, Waltham, MA, USA, #12587010), N2 supplement (Thermo Fisher Scientific, Waltham, MA, USA, #17502048) and L-Glutamine (200 mM) (Thermo Fisher Scientific, Waltham, MA, USA, Gibco, #25030081). Medium was stored up to 2 weeks at 4°C or aliquoted and frozen at -20°C. Before usage, 3 µM CHIR99021 (Cayman Chemical, Ann Habor, MI, USA, #Cay13122-5), 500 nM Purmorphamine (Miltenyi Biotec, Bergisch Gladbach, Germany, #130-104-465) and 150 µM (+)-Sodium L-ascorbate (Vitamin C) (Merck, Sigma-Aldrich, St. Louis, MO, USA, #A4034) were supplemented to the maintenance medium (sm+). Both cell types were cultivated in 6 well plates coated with Geltrex (Thermo Fisher Scientific, Waltham, MA, USA, #a1413302). Coated plates were incubated at 37°C for 1 h before usage. After thawing of iPSCs and NPCs, cells were supplemented with 10 µM Rock inhibitor/Y-27632 (Dihydrochloride) (Stemcell Technologies, #72304) for 24 h. For passaging and seeding, iPSCs were treated with ReLeSR (Stemcell Technologies, Vancouver, Canada, #100-0483) accordingly to manufacturer´s instructions and NPC were passaged by Accutase (Stemcell Technologies, Vancouver, Canada, #07922). HCT116 were cultured in McCoy medium (Thermo Fisher Scientific, Waltham, MA, USA, Gibco, #36600-021)

supplemented with 100 units/mL Penicillin-Streptomycin and 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA, #F9665, LOT 0001655439) and passaged with Trypsin/EDTA 0.05% (Thermo Fisher Scientific, Waltham, MA, USA, Invitrogen, #2530096). All cells were cultured and treated at 37 °C and 5% CO_2 in a humidified atmosphere.

Stimulation

To circumvent autophagy induction by starvation due to rapid proliferation of the stem cells the medium was exchanged on a daily basis. Thereby, cells were exposed to etoposide for 24 h and subsequent medium exchanges were without etoposide. In comparison, BPDE was freshly supplemented to the cells every day. On the day of sample harvesting, the medium was exchanged 4 h before lysis and 40 nM bafilomycin A_1 were supplemented to the cells 2 hours before lysis. For showing autophagic response in the experiments, cells were washed with DPBS and incubated with EBSS (Thermo Fisher Scientific, Waltham, MA, USA, #24010-043) and corresponding medium control for 2 h. A detailed treatment scheme is depicted in supplemental Figure S9.

For the analysis of differential proteome, cells were treated like described above. Thereby, analysis for etoposide treated cells were performed at 24 h and treated with BPDE after 48 h.

Cell viability assay

For assessment of cytotoxicity the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed. Cells were seeded in 96-well plates (density per well: iPS11: 2500 cells; niPS11: 15000 cells; HCT116: 300-1250 cells). 3-4 days after seeding, cells were treated with different concentrations of BPDE or etoposide, 0.1% DMSO as a solvent control for 24, 48 and 72 h. After the incubation time, 20 µL of a 5 mg/mL MTT stock solution (ROTH, Karlsruhe, Germany, #4022.3) were added to the cells and they were incubated at 37 °C and 5% CO_2 in a humidified atmosphere for 30 min. Upon removal of the MTT-containing medium 100 µL DMSO per well were added for extraction of the formazan. Absorbance was measured at 570 nm and 650 nm (reference) with a microplate reader

(SynergyMx, BioTek, Winooski, VT, USA). After subtraction of the reference value, the mean of the absorbance of the solvent control was set as 100% and the relative viability was calculated for each sample.

Immunoblotting

For SDS PAGE and western blotting, cells were washed with DPBS and lyzed with RIPA buffer (150 mM Sodium cloride, 50 mM Tris-HCl, 1% Nonidet-40, 0.5% Sodium deoxycholate [w/v], 0.1% SDS [w/v] X PhosSTOP [Roche, Basel, Switzerland, #4906837001]), 1X protease inhibitor cocktail [Roche, Basel, Switzerland, #4693132001]) for 20 min on ice and the lysates were cleared by centrifugation at 18000 rcf and 4 °C for 20 min and quickly frozen in liquid nitrogen. Protein concentration was determined by BCA assay and sample buffer was added (62.5 mM Tris, 8.6% [v/v] glycerol, 2% [w/v] SDS, 33.3 µg/mL bromophenol blue, 1% [v/v] β-mercaptoethanol). Samples were heated at 95 °C for 5 min and then equal amounts of protein (25 µg) were subjected to SDS-polyacrylamide gels. After separation by SDS-PAGE, proteins were transferred to PVDF membranes (Merck, Darmstadt, Germany, #IPFL00010), blocked with 5% milk powder in TBST or EveryBlot Blocking Buffer (Bio-Rad, Hercules, CA, USA, #12010020) and analyzed using the indicated primary antibodies followed by appropriate IRDye 800- or IRDye 680-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA). Fluorescence signals were detected using an Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA) and signals were quantified with Image Studio Lite 5.2 (LI-COR Biosciences, Lincoln, NE, USA).

Immunofluorescence

For immunofluorescence microscopy, cells were seeded on glass coverslips in 24-well plates. Coverslips for iPSCs and NPCs were coated with geltrex and incubated at 37°C for 1 h. After treatment, cells were fixed in 4% paraformaldehyde for 15 min on room temperature, quenched with 50 mM NH_4Cl for 15 min and permeabilized with either 50 µg/mL digitonin (Sigma-Aldrich, St. Louis, MO, USA, #D141)

or 0.5% Triton X-100 for 10 min. Fixed samples were blocked with 3% BSA (Roth, Karlsruhe, Germany, #8076) for 30 min and incubated with primary antibodies diluted in 3% BSA for 1 h at RT. Samples were then washed three times with DPBS, incubated with the appropriate secondary antibodies and 2 µg/mL DAPI (Roth, Karlsruhe, Germany, #6335.1) diluted in 3% BSA for 1 h and washed three times with DPBS. Afterwards, cells were embedded in ProLong Glass Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA, #P36980). Images were recorded with an Axio Observer 7 fluorescence microscope (Carl Zeiss Microscopy, Oberkochen, Germany) using a 40x/1,4 Oil DIC M27 Plan-Apochromat objective (Carl Zeiss Microscopy, Oberkochen, Germany) and an ApoTome 2 (Carl Zeiss Microscopy, Oberkochen, Germany).

Mitotic Index Assay

For determination of the mitotic index, cells were detached and fixed in cold 70% ethanol and stored at 4°C for up a week. During sample preparation, cells were permeabilized in 0.25% Triton X-100 on ice for 15 min and incubated with rabbit anti-H3 phospho-Ser10 antibody in wash buffer, consisting of 1% BSA in DPBS, overnight at 4˚C in constant rotation. All samples were washed twice in wash buffer and incubated anti-rabbit Alexa 488 diluted in wash buffer for 30 min at RT in the dark. After a final wash in washing buffer each cell pellet was re-suspended in 500 µL DPBS containing 3 µM Draq7 for DNA staining, filtered through a cell strainer, processed in a BD LSR Fortessa flow cytometer with FACSDIVA software and analyzed with FlowJo software.

Growth curve analysis

To investigate the impact on proliferation, niPS11 were treated and cultured as described above and detached by using Accutase. Cells were incubated for 5 min at 37°C and subsequently centrifuged at 60 x g for 3 min. Cell pellet was resuspended in 250 µL sm+ medium. Afterwards, 20 µL of cell suspension were mixed with 20 µL Trypan Blue Stain (0.4%) (Thermo Fisher Scientific, Waltham, MA,

USA, Gibco, #15250-061) and measured by Luna Automated Cell Counter (Biocat, Heidelberg, Germany, Model #L10001). Number of daily measured cells were divided by the cell number at 0 h.

Mass Spectrometry (MS)-based Proteomics

SAMPLE PREPARATION

Sample preparation was performed as described (Sinatra et al., 2022; Sprengel et al., 2025).

LC-MS ANALYSIS

LC-MS analysis was performed essentially as described (Sinatra et al., 2022) using a QExactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, software versions: Xcalibur software: version 4.5.474.0, LC: Thermo Scientific SII for Xcalibur 1.7.0.468, MS: Q Exactive Plus - Orbitrap MS 2.12 build 3134), operated in positive mode and coupled with a nano electrospray ionization source connected with an Ultimate 3000 Rapid Separation liquid chromatography system (Dionex/Thermo Fisher Scientific, Idstein, Germany) equipped with an Aurora Ultimate C18 column (75 µm inner diameter, 25 cm length, 1.7 µm particle size from Ion Opticks) as separation column and an Acclaim PepMap 100 C18 column (75 µm inner diameter, 2 cm length, 3 µm particle size from Thermo Fisher Scientific) as trap column, using a 120 min LC gradient. Capillary temperature was set to 250 °C and source voltage to 1.5 kV.

For iPS11 sample set analysis, using a data-dependent acquisition (DDA) top ten method, MS survey scans were carried out over a mass range from 350 to 2000 m/z at a resolution of 140 000. The automatic gain control target was set to 3 000 000, and the maximum fill time was 80 ms. The 10 most intensive peptide ions with charge states +2 and +3 were selected (2 m/z isolation window, 1700 intensity threshold, minimum automatic gain control target 1000), fragmented by high-energy collisional dissociation (normalized collision energy 30), and fragments were analyzed (scan range 200–

2000 m/z, resolution 17,500, target for automatic gain control 10,000, maximum injection time 60 ms). Selected precursors were dynamically excluded for 100 s.

For the niPS11 sample set, data-independent acquisition (DIA) was used on the same instrument with otherwise same parameters. One survey scan was followed by six DIA scans, respectively, all with 35,000 resolution and 3,000,000 as target for automatic gain control. Survey scans were carried out over a mass range from 400 to 1650 m/z and the maximum fill time was 200 ms. DIA scans had 200 m/z as fixed first mass and the normalized collision energy set to 30 with automatic maximum injection time, and were performed on 27 isolation windows, each of 20 m/z width, with equidistant centers (19 m/z distance) starting at 410 m/z and ending at 904 m/z.

DATA ANALYSIS

For the iPS11 sample set, data analysis was performed as described (Sinatra et al., 2022; Sprengel et al., 2025) using MaxQuant (version 2.5.2.0, Max Planck Institute for Biochemistry, Planegg, Germany) and a human sequence database (UniProtKB, downloaded on 12/21/2023, 82685 entries). For the niPS11 sample set, data analysis was performed using DIA-NN (version 1.9.2, (Demichev et al., 2020)) and a human sequence database (UniProtKB, downloaded on 07/08/2024, 82518 entries). For both analyses, methionine oxidation and N-terminal acetylation as well as carbamidomethylation at cysteine residues were considered as variable and fixed modifications, respectively, and a false discovery rate of 1% on protein and peptide levels was set as identification threshold. Statistical analysis was performed as described (Sinatra et al., 2022; Sprengel et al., 2025) but using a -lg(p-value) >= 3 significance cutoff instead of SAM 5% FDR.

Statistical analysis

All IC_{20} values were calculated with non-linear regression using GraphPad Prism 8.0.2. For quantification of immunoblotting experiments, the signal of each protein band was divided by the

average signal of all protein bands of the respective protein and furthermore normalized to the ratio of the loading control. These normalized ratios were divided by the average normalized ratio of the DMSO controls of all biological replicates to calculate fold changes. The background signal for each membrane was subtracted ahead of quantification. All p-values were calculated with ordinary one-way ANOVA (Tukey´s multiple comparison test) and student´s t-test if not indicated otherwise. For immunofluorescence analyses, puncta, nuclei and co-localization were quantified and analyzed using Biovoxxel ImageJ v1.54p. A punta/foci to nuclei ratio was calculated for each image to determine the average number of punta/foci per cell, and were normalized by dividing through the mean dot number of the solvent control. All macros used for quantifications are provided in Supplementary Table xxx. 10 representative images from three biological replicates per experiment were analyzed. For all immunofluorescence analyses, results are shown in scatter plot diagrams visualized as mean with standard deviation and p-values were determined by student´s t-test and are shown in the diagrams. All p-values are shown as *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no competing financial interests in relation to the work described.

AUTHOR CONTRIBUTION STATEMENT

S.A. designed the experiments, performed viability assays, immunoblot analyses, fluorescence microscopy, growth curve and flow cytometry analyses. A.Z. and A.P. gave technical and theoretical support for the cultivation and/or differentiation of iPSCs and NPCs. T.L. and K.S. performed and analysed mass spectrometry experiments. K.S.K., S.W. and M.J.M. gave technical and theoretical support. S.A., T.L., and B.S. analyzed the data and wrote the manuscript. B.S. supervised the project. All authors discussed the results and commented on the manuscript.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Björn Stork (bjoern.stork@hhu.de).

LEGENDS TO THE FIGURES

Figure 1: Both iPSCs and NPCs show canonical autophagic capacity. (**A-D**) All cell types (A: iPS11, B: iPS12, C: niPS11, D: niPS12) were treated with either cultivation medium or serum- and amino acid-free EBSS for 2 h. For the accumulation of the lysosome-associated proteins LC3-II and p62, V-ATPase inhibitor bafilomycin A_1 was additionally supplemented to each medium. Afterwards, cells were lysed, and cellular lysates were immunoblotted for ULK1, phospho-ULK1 Ser758, Vinculin, SQSTM1/p62, LC3 and GAPDH. One representative immunoblot is shown. Results show mean + SD of three independent experiments. For statistical analysis, ordinary one-way ANOVA (Tukey´s multiple comparisons test) was utilized to compare means of genotoxin-treated samples to DMSO. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2: iPSCs, NPCs and cancer cells show different response patterns in DNA damage markers after genotoxic treatment. (**A**) iPS11, (**B**) niPS11 and (**C**) HCT116 were treated with corresponding genotoxin for 4 or 24 h and subsequently fixed. Afterwards, cells were stained for γH2AX and 53BP1 and visualized by immunofluorescence. Scale bar: 10 µm. (**D-G**) Positively stained foci for γH2AX, 53BP1 and double-positive foci (γH2AX /53BP1) were counted per nucleus and normalized to corresponding control after 4 or 24 h in (D-E) iPS11 and (F-G) HCT116. Please note that image-based quantification for niPS11 cells is hampered by their overlapping growing behavior. Results show mean + SD of three independent experiments whereby 10 images per cohort were analyzed. For statistical analysis, Student´s t-test were utilized to compare means of genotoxin-treated samples to DMSO. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3: ULK1 activation status is not affected in iPS11 after etoposide treatment. (**A**) iPS11, (**B**) niPS11 and (**C**) HCT116 were treated with corresponding IC_{20} dose of etoposide and lysed after 24, 48 or 72 h. Cellular lysates were immunoblotted for ULK1, phospho-ULK1 Ser758 and phospho-ULK1

Ser638 respectively. One representative immunoblot is shown. Results show mean + SD of three independent experiments. For statistical analysis, ordinary one-way ANOVA (Tukey´s multiple comparisons test) and Student´s t-test were utilized to compare means of genotoxin-treated samples to DMSO. Significance bars are highlighted in different colors accordingly to involved condition: dark blue: control, light blue: genotoxin; orange: control + bafilomycin A_1 and genotoxin + bafilomycin A_1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4: BPDE exposure does not influence ULK1 phosphorylation. (**A**) iPS11, (**B**) niPS11 and (**C**) HCT116 were treated with corresponding IC_{20} dose of BPDE and lysed after 24, 48 or 72 h. Cellular lysates were immunoblotted for ULK1, phospho-ULK1 Ser758 and phospho-ULK1 Ser638 respectively. One representative immunoblot is shown. Results show mean + SD of three independent experiments. For statistical analysis, ordinary one-way ANOVA (Tukey´s multiple comparisons test) and Student´s t-test were utilized to compare means of genotoxin-treated samples to DMSO. Significance bars are highlighted in different colors accordingly to involved condition: dark blue: control, light blue: genotoxin; orange: control + bafilomycin A_1 and genotoxin + bafilomycin A_1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 5: Autophagic flux is not affected by genotoxic treatment. (**A**) iPS11, (**B**) niPS11 and (**C**) HCT116 were treated with corresponding IC_{20} dose for 24, 48 or 72 h. Cells were lysed, and cellular lysates were immunoblotted for SQSTM1/p62, LC3 and GAPDH. One representative immunoblot is shown. Results show mean + SD of three independent experiments. For statistical analysis, ordinary one-way ANOVA (Tukey´s multiple comparisons test) and Student´s t-test were utilized to compare means of genotoxin-treated samples to DMSO. Significance bars are highlighted in different colors accordingly to involved condition: dark blue: control, light blue: genotoxin; orange: control + bafilomycin A_1 and genotoxin + bafilomycin A_1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 6: LC3 puncta formation is not affected in iPS11 but in HCT116 after etoposide treatment. (**A**) iPS11, (**B**) niPS11 and (**C**) HCT116 were treated with corresponding genotoxin for 4 or 24 h and subsequently fixed. Afterwards, cells were stained for LC3B and visualized by immunofluorescence. Scale bar: 10 µm. (**D-G**) Positively stained puncta for LC3B were counted per nucleus and normalized to corresponding control after 4 and 24 h in (D-E) iPS11 and (F-G) HCT116. Please note that image-based quantification for niPS11 cells is hampered by their overlapping growing behavior. Results show mean + SD of three independent experiments whereby 10 images per cohort were analyzed. For statistical analysis, Student´s t-test was utilized to compare means of genotoxin-treated samples to DMSO. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 7: Differential Proteome Analysis of BPDE- and etoposide-treated iPS11 and niPS11. (**A**) Volcano plots based on intensity values for MS-based proteomics of BPDE- or etoposide-treated iPS11. Autophagy-relevant proteins are indicated by orange data points. Proteins with a -lg(p-value) >= 3 significance cutoff are displayed as red (down-regulated) or green (up-regulated) data points. (**B**) Samples from differential proteome analysis were prepared with sample buffer and cellular lysates were immunoblotted for NUCKS1 and GAPDH. The blot with all samples is shown. Results show mean + SD of five independent experiments. For statistical analysis, Student´s t-test was utilized to compare means of genotoxin-treated samples to DMSO. (**C**) Volcano plots for MS-based proteomics of BPDE- or etoposide-treated niPS11. Autophagy-relevant proteins are indicated by orange data points. Proteins with a -lg(p-value) >= 3 significance cutoff are displayed as red (down-regulated) or green (up-regulated) data points. Proteins upregulated by etoposide were submitted to a functional enrichment analysis using STRING (v12.0, https://string-db.org) yielding the displayed protein-protein interaction network, for which the gene ontology term GO:190304 (Mitotic cell cycle process) was most prominent. (**D**) Samples from differential proteome analysis were prepared with sample buffer and cellular lysates were immunoblotted for Aurora kinase A (AURKA), polo like kinase 1 (PLK1), acetylated

tubulin (K40), and GAPDH as loading control. The blot with all samples is shown. Results show mean + SD of five independent experiments. For statistical analysis, Student´s t-test was utilized to compare means of genotoxin-treated samples to DMSO. (**E**) For mitotic index assay, niPS11 were treated with 50 nM etoposide and 100 nM BPDE for 24, 48 or 72 h. Cells were fixed, permeabilized and stained for phospho-H3 Ser10 and DNA marker DRAQ7 and were analyzed by flow cytometry. 50,000 cells per experiment were quantified and results show mean + SD of three independent experiments. For statistical analysis, Student´s t-test was utilized to compare means of genotoxin-treated samples to DMSO. Growth curve analysis was performed by Trypan Blue Assay. niPS11 were treated with 50 nM etoposide and 100 nM BPDE for 24, 48 or 72 h. Cells were detached and stained by Trypan Blue Stain to count the number of living cells. Results show mean + SD of three independent experiments. For statistical analysis, Student´s t-test was utilized to compare means of genotoxin-treated samples to DMSO. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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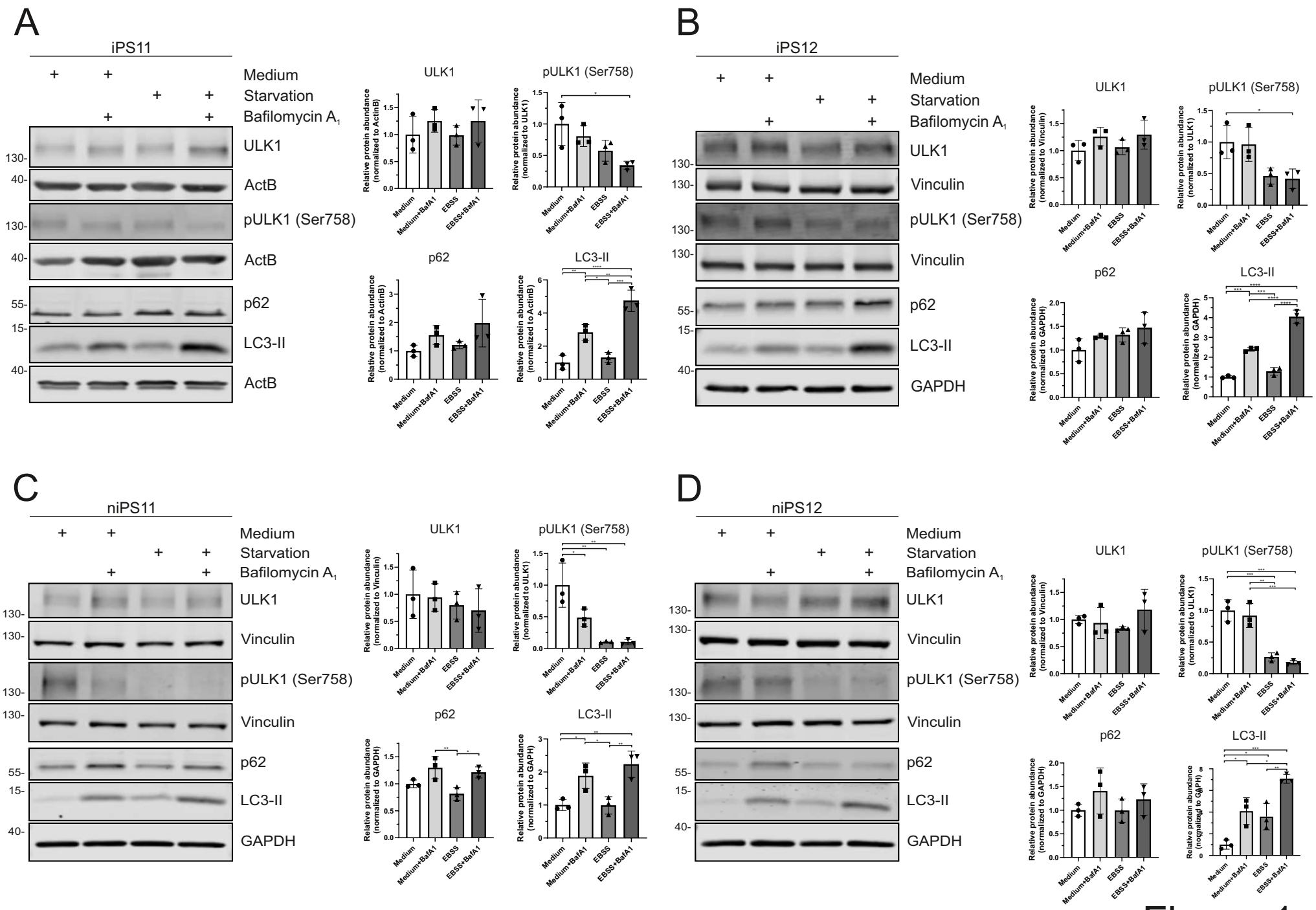

A
iPS11
Medium
Starvation
Bafilomycin A1
ULK1
ActB
pULK1 (Ser758)
ActB
p62
LC3-II
ActB
130-
40-
130-
40-
55-
15-
40-
ULK1
pULK1 (Ser758)
p62
LC3-II
Relative protein abundance (normalized to ActinB)
Relative protein abundance (normalized to ULK1)
Medium
Medium+BafA1
EBSS
EBSS+BafA1
B
iPS12
Medium
Starvation
Bafilomycin A1
ULK1
Vinculin
pULK1 (Ser758)
Vinculin
p62
LC3-II
GAPDH
130-
130-
130-
55-
15-
40-
ULK1
pULK1 (Ser758)
p62
LC3-II
Relative protein abundance (normalized to Vinculin)
Relative protein abundance (normalized to ULK1)
Relative protein abundance (normalized to GAPDH)
C
niPS11
Medium
Starvation
Bafilomycin A1
ULK1
Vinculin
pULK1 (Ser758)
Vinculin
p62
LC3-II
GAPDH
130-
130-
130-
55-
15-
40-
ULK1
pULK1 (Ser758)
p62
LC3-II
Relative protein abundance (normalized to Vinculin)
Relative protein abundance (normalized to ULK1)
Relative protein abundance (normalized to GAPDH)
D
niPS12
Medium
Starvation
Bafilomycin A1
ULK1
Vinculin
pULK1 (Ser758)
Vinculin
p62
LC3-II
GAPDH
130-
130-
130-
55-
15-
40-
ULK1
pULK1 (Ser758)
p62
LC3-II
Relative protein abundance (normalized to Vinculin)
Relative protein abundance (normalized to ULK1)
Relative protein abundance (normalized to GAPDH)

Figure 1

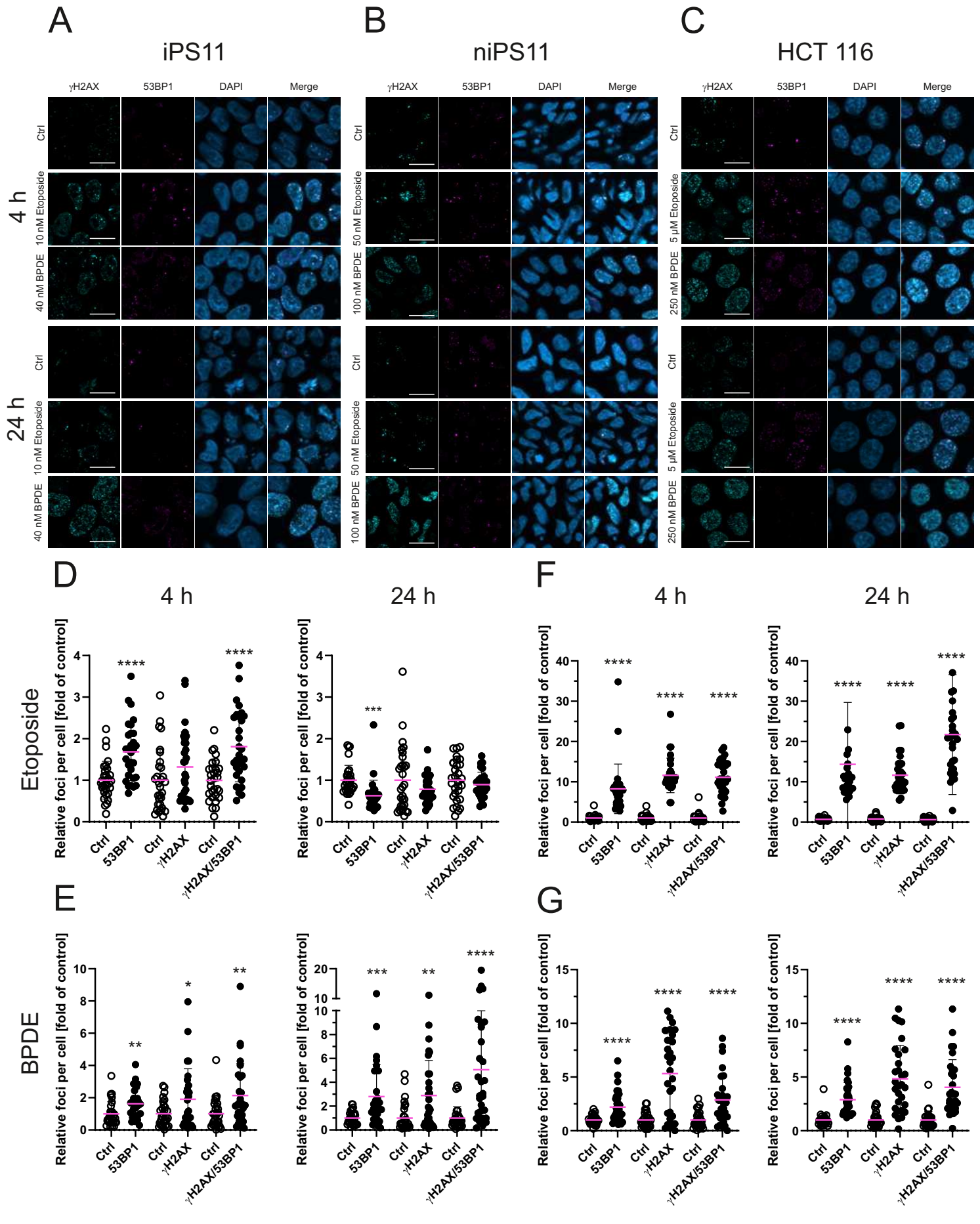

A
iPS11
B
niPS11
C
HCT 116
γH2AX
53BP1
DAPI
Merge
4 h
24 h
Ctrl
10 nM Etoposide
40 nM BPDE
50 nM Etoposide
100 nM BPDE
5 μM Etoposide
250 nM BPDE
D
F
E
G
Etoposide
BPDE
Relative foci per cell [fold of control]
Ctrl
53BP1
γH2AX
γH2AX/53BP1
iPS11
HCT116

Figure 2

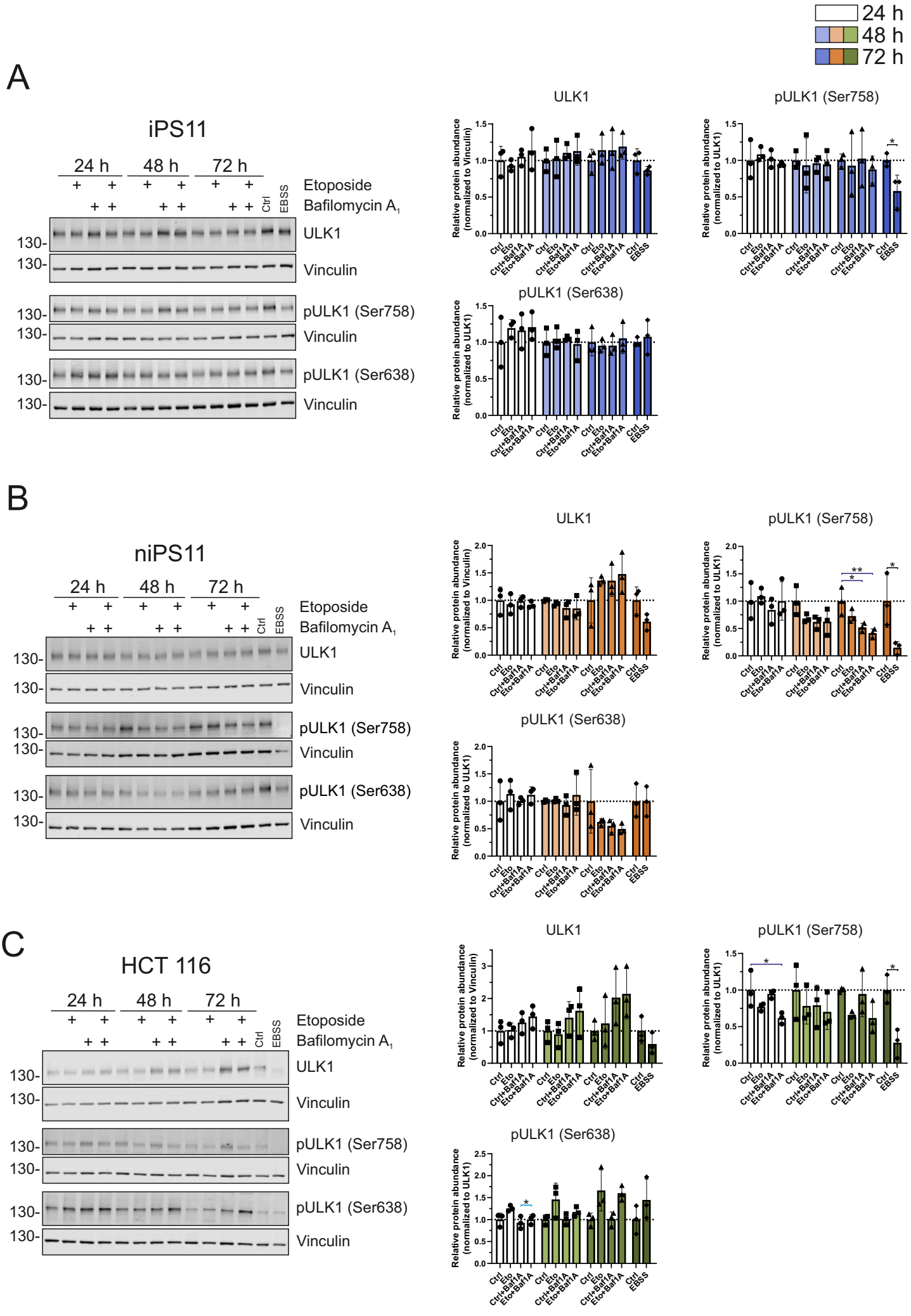
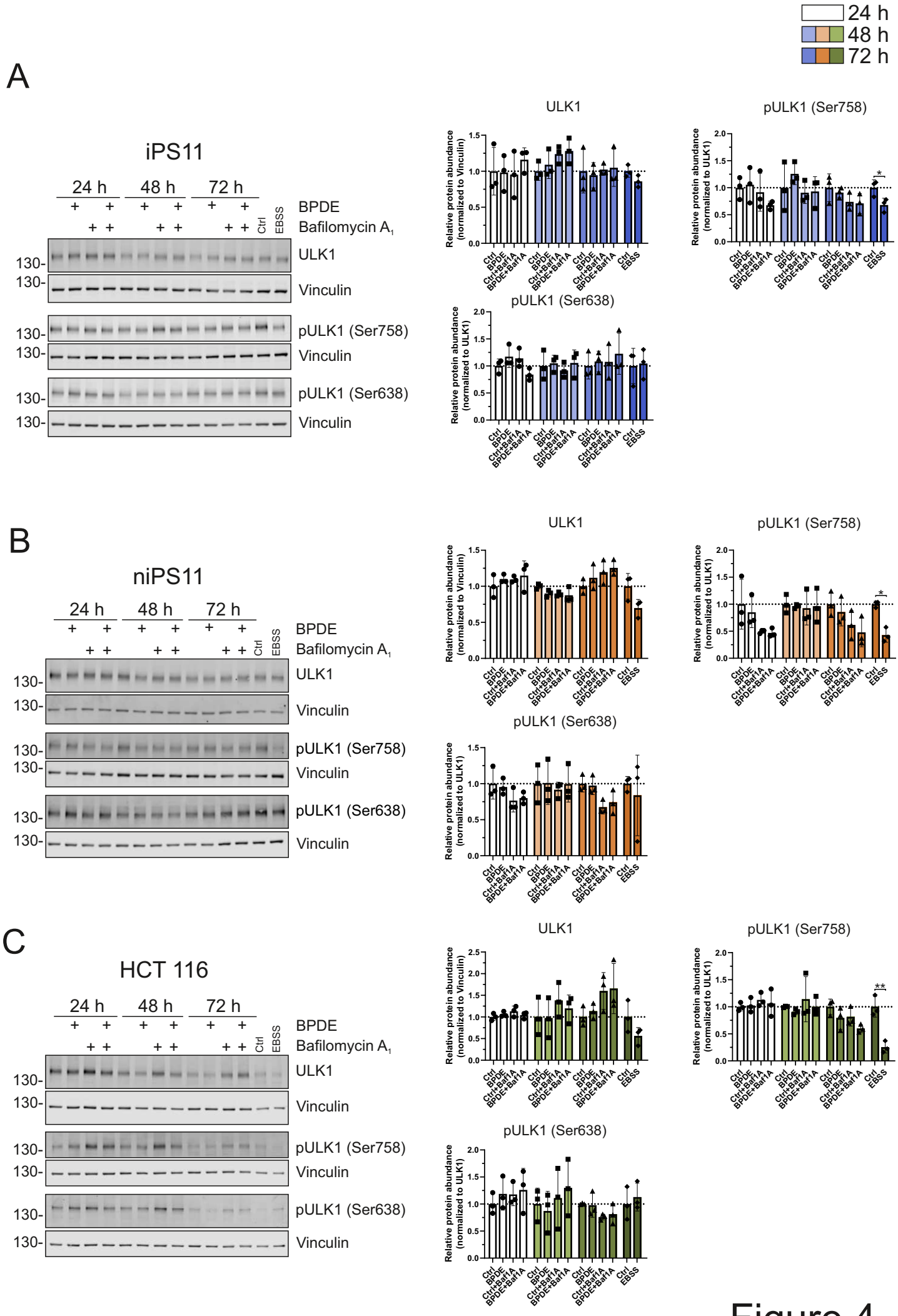

24 h
48 h
72 h
A
iPS11
24 h
48 h
72 h
Ctrl
EBSS
Etoposide
Bafilomycin A1
130-
ULK1
Vinculin
pULK1 (Ser758)
pULK1 (Ser638)
ULK1
pULK1 (Ser758)
pULK1 (Ser638)
Relative protein abundance (normalized to Vinculin)
Relative protein abundance (normalized to ULK1)
Ctrl
Eto
Ctrl+Baf1A
Eto+Baf1A
B
niPS11
C
HCT 116

Figure 3


24 h
48 h
72 h
A
iPS11
24 h
48 h
72 h
BPDE
Bafilomycin A1
Ctrl
EBSS
130-
ULK1
Vinculin
pULK1 (Ser758)
pULK1 (Ser638)
ULK1
pULK1 (Ser758)
pULK1 (Ser638)
Relative protein abundance (normalized to Vinculin)
Relative protein abundance (normalized to ULK1)
Ctrl
BPDE
Ctrl+Baf1A
BPDE+Baf1A
EBSS
B
niPS11
C
HCT 116
Figure 4

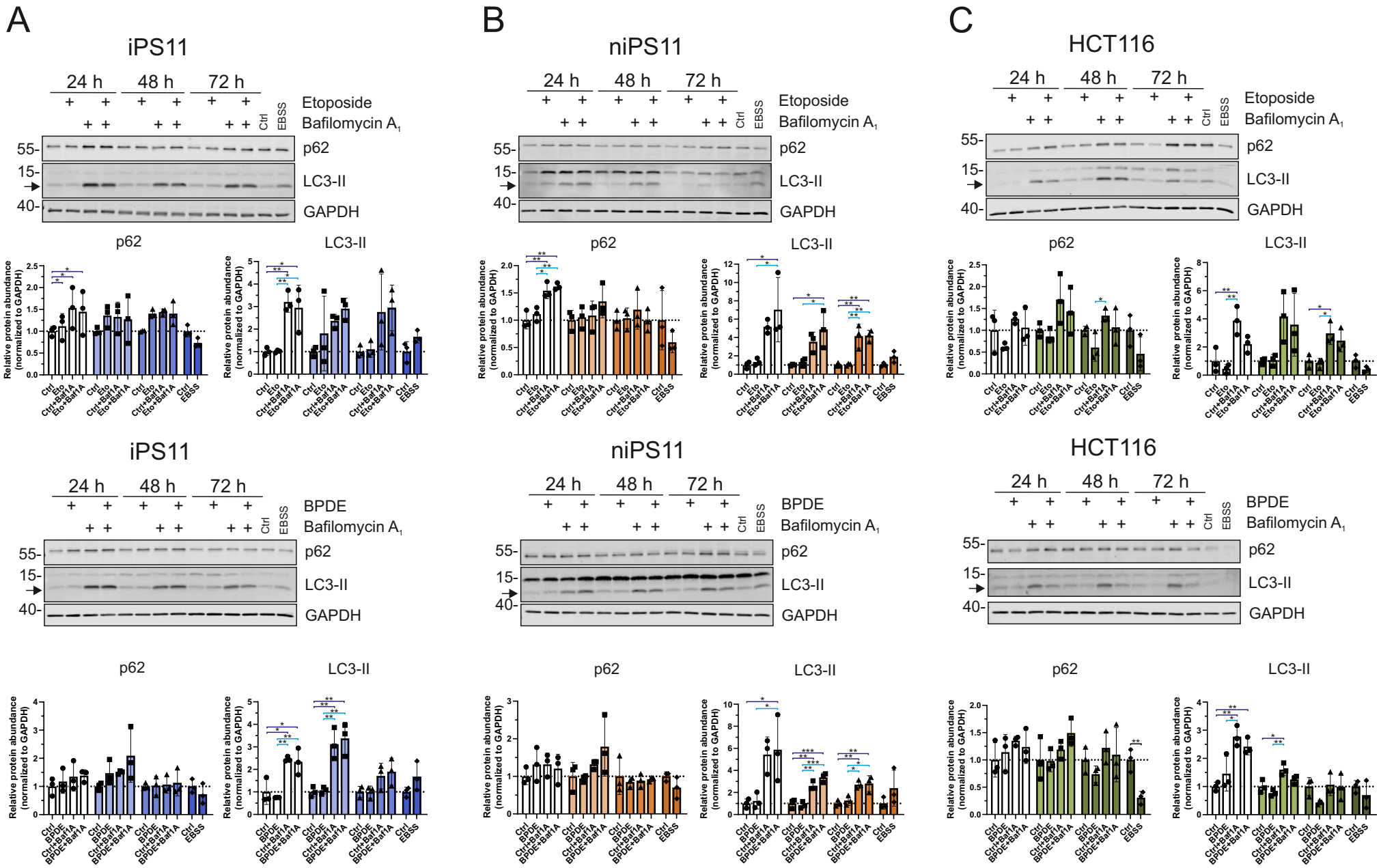
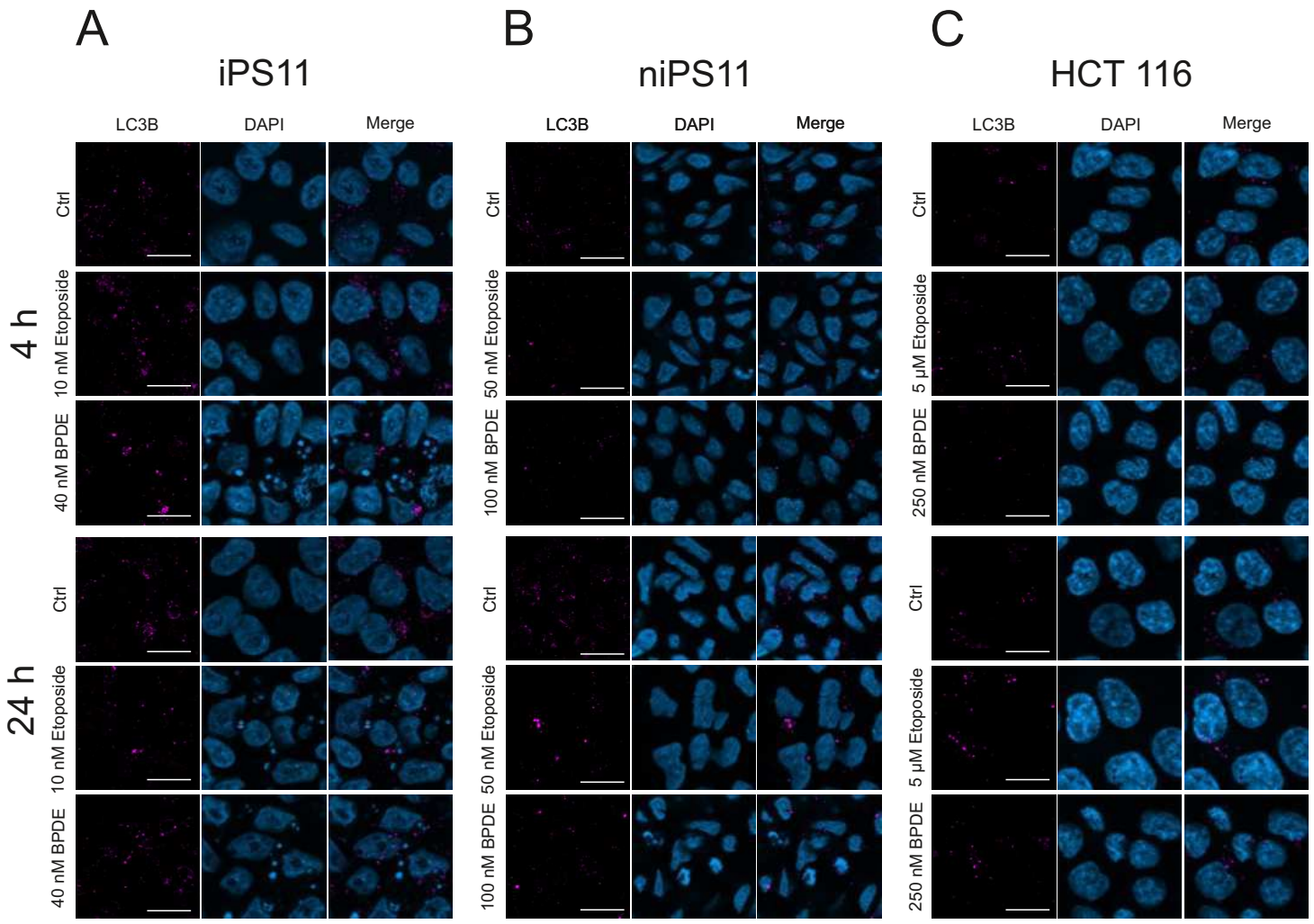

A
iPS11
24 h
48 h
72 h
Etoposide
Bafilomycin A1
Ctrl
EBSS
55-
15-
40-
p62
LC3-II
GAPDH
Relative protein abundance (normalized to GAPDH)
Ctrl
Eto
Ctrl+Baf1A
Eto+Baf1A
B
niPS11
C
HCT116
BPDE
BPDE+Baf1A

Figure 5


A
iPS11
B
niPS11
C
HCT 116
LC3B
DAPI
Merge
4 h
24 h
Ctrl
10 nM Etoposide
40 nM BPDE
50 nM Etoposide
100 nM BPDE
5 µM Etoposide
250 nM BPDE

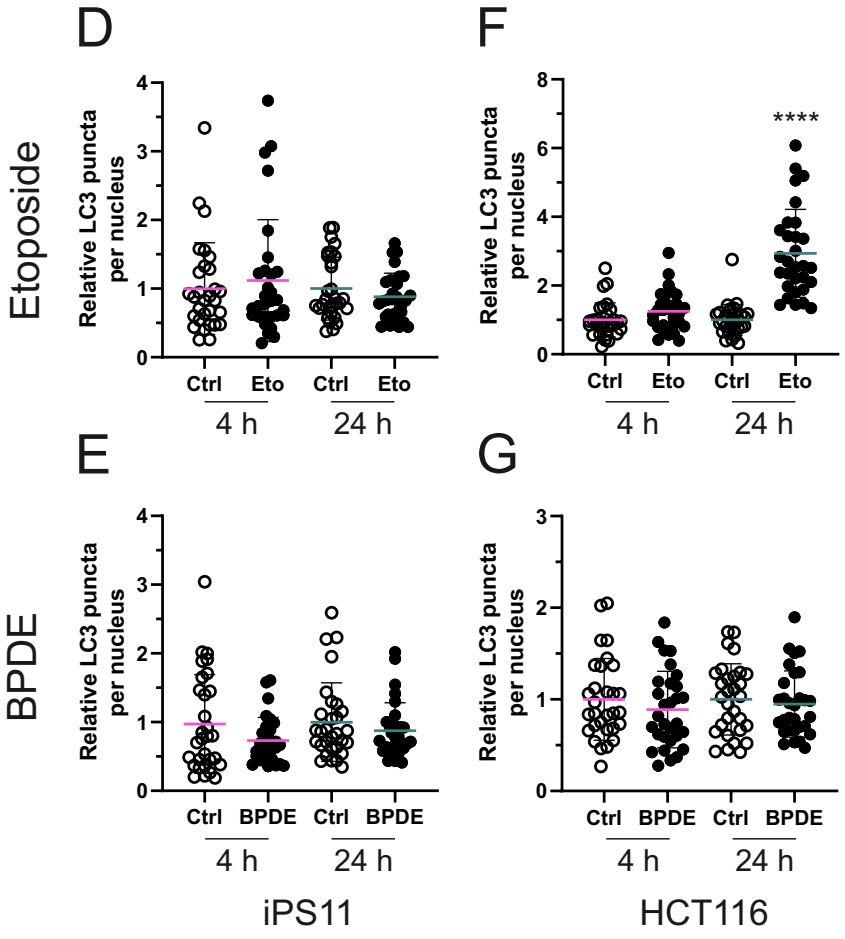

D
F
E
G
Etoposide
BPDE
Relative LC3 puncta per nucleus
Ctrl
Eto
BPDE
4 h
24 h
****
iPS11
HCT116

Figure 6

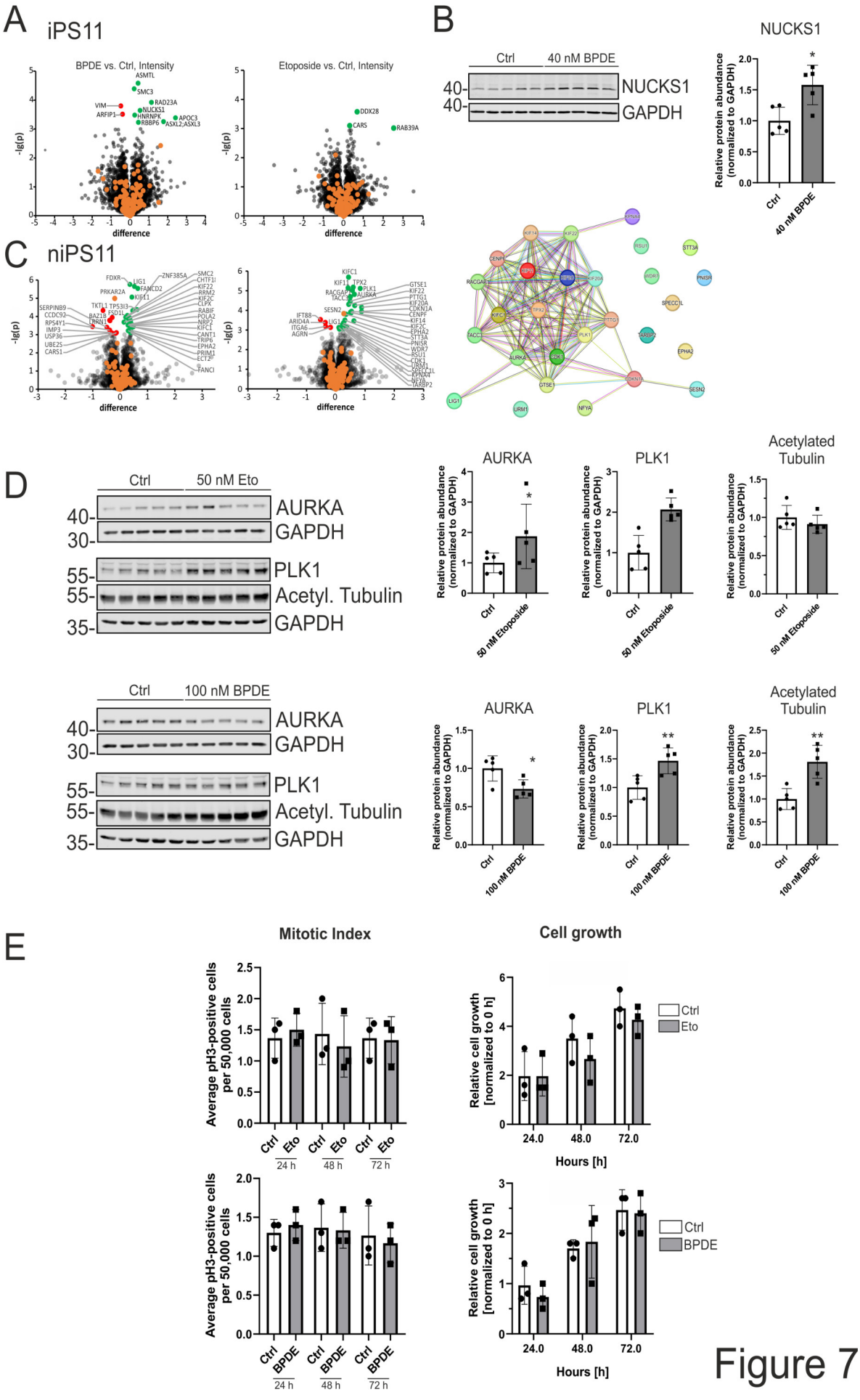

A
iPS11
BPDE vs. Ctrl, Intensity
Etoposide vs. Ctrl, Intensity
-lg(p)
difference
B
Ctrl
40 nM BPDE
NUCKS1
GAPDH
Relative protein abundance (normalized to GAPDH)
Ctrl
40 nM BPDE
C
niPS11
D
Ctrl
50 nM Eto
AURKA
GAPDH
PLK1
Acetyl. Tubulin
Acetylated Tubulin
50 nM Etoposide
100 nM BPDE
E
Mitotic Index
Cell growth
Average pH3-positive cells per 50,000 cells
Relative cell growth [normalized to 0 h]
Hours [h]
Ctrl
Eto
BPDE
24 h
48 h
72 h

Figure 7

Supporting Information

Canonical autophagy remains inactive in induced pluripotent stem cells and neuronal progenitor cells following DNA damage induced by BPDE or etoposide

AUTHORS/AFFILIATIONS

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Björn Stork, Universitätsstr. 1, Building 22.03, 40225 Düsseldorf, Germany

Tel.: +49 (0)211 81 11954, E-mail: bjoern.stork@hhu.de

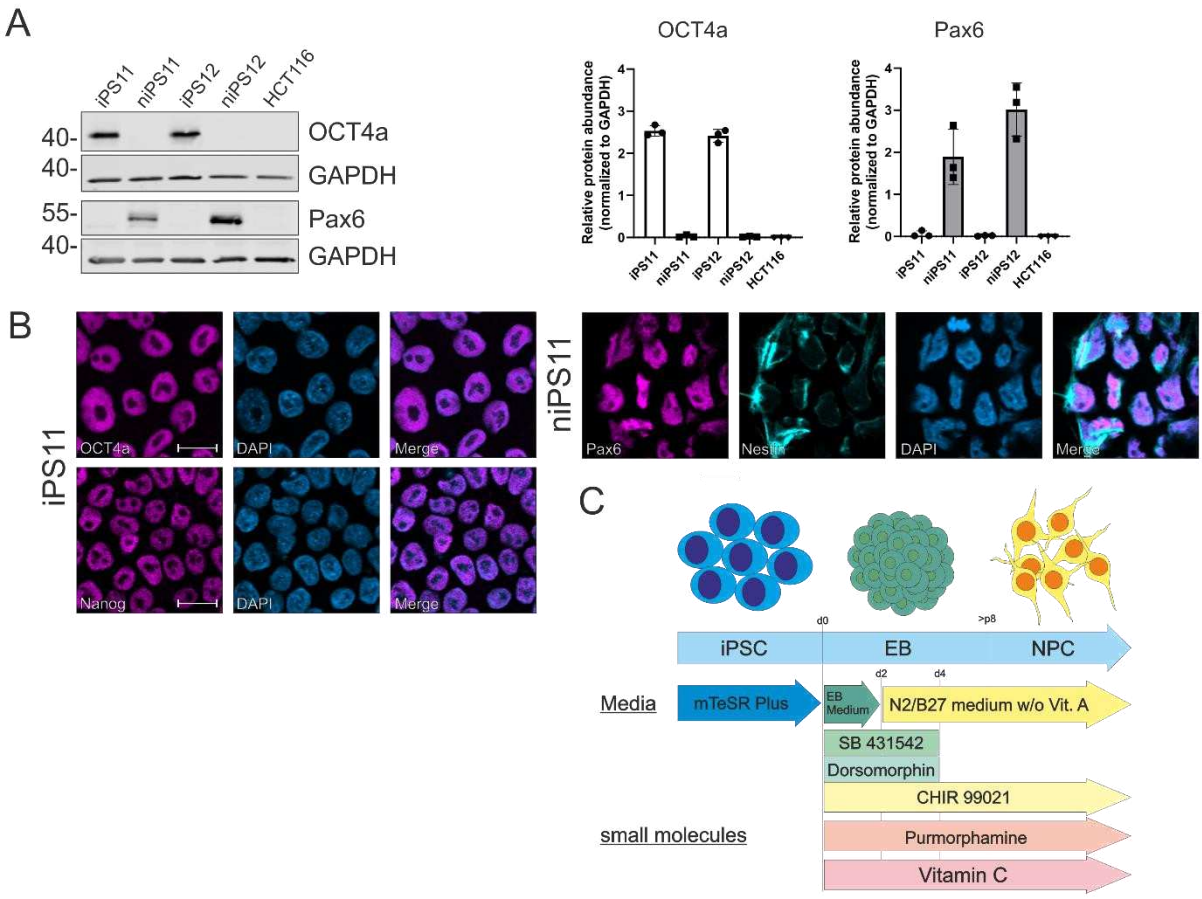

Figure S1: Verification of induced pluripotent stem cells and thereof differentiated neural progenitor cells. (**A**) Induced pluripotent stem cells (iPS11/12), neural progenitor cells (niPS11/12) and HCT116 were lysed, and cellular lysates were immunoblotted for OCT4a, Pax6 and GAPDH respectively. One representative immunoblot is shown. The quantifications of indicated ratios are from three independent experiments (means ± SD). (**B**) Cells were fixed and stained for pluripotency markers OCT4a and Nanog in iPS11 and neural markers Pax6 and Nestin in niPS11 and visualized by immunofluorescence. Scale bar: 10 µm. (**C**) Differentiation of iPSCs into NPCs was achieved by SMAD and AMPK inhibition via SB 431542 and Dorsomorphin, directed and sustained as neural progenitor cells by supplementation of above mentioned compounds.

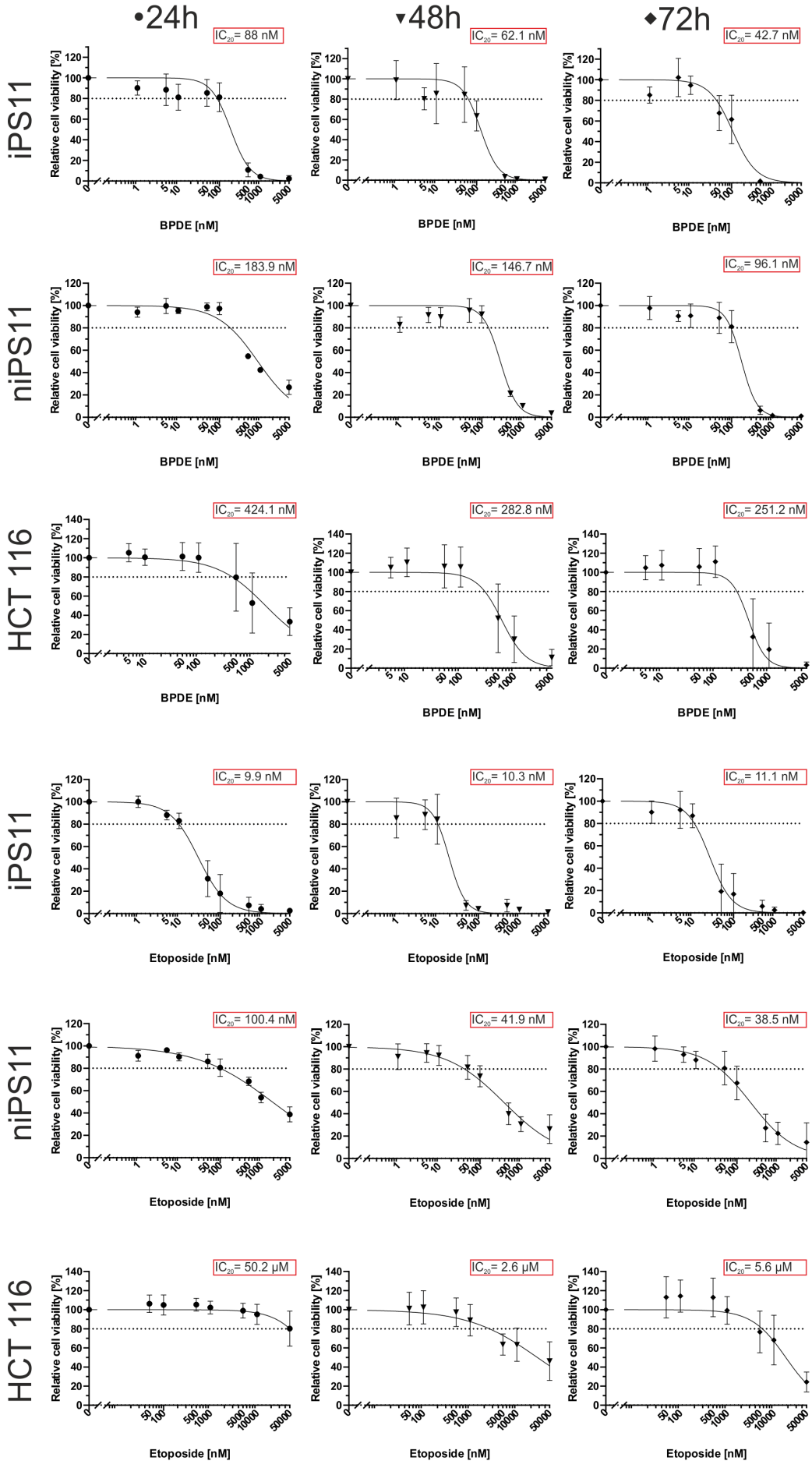
•24h
▾48h
◆72h
iPS11
niPS11
HCT 116
IC20= 88 nM
IC20= 62.1 nM
IC20= 42.7 nM
IC20= 183.9 nM
IC20= 146.7 nM
IC20= 96.1 nM
IC20= 424.1 nM
IC20= 282.8 nM
IC20= 251.2 nM
IC20= 9.9 nM
IC20= 10.3 nM
IC20= 11.1 nM
IC20= 100.4 nM
IC20= 41.9 nM
IC20= 38.5 nM
IC20= 50.2 µM
IC20= 2.6 µM
IC20= 5.6 µM
Relative cell viability [%]
BPDE [nM]
Etoposide [nM]

Figure S2: Definition of IC_{20} values for iPS11, niPS11 and HCT116.

To identify a sublethal IC_{20} dose, cells were treated with different concentrations of BPDE and etoposide for 24, 48 or 72 h while etoposide was only supplemented for 24 h and BPDE was given daily to the cells. After treatment, cell viability was measured using a thiazolylblue (MTT) assay. Results are shown as the mean ± SD of 3-5 independent experiments performed in triplicates for each treatment.

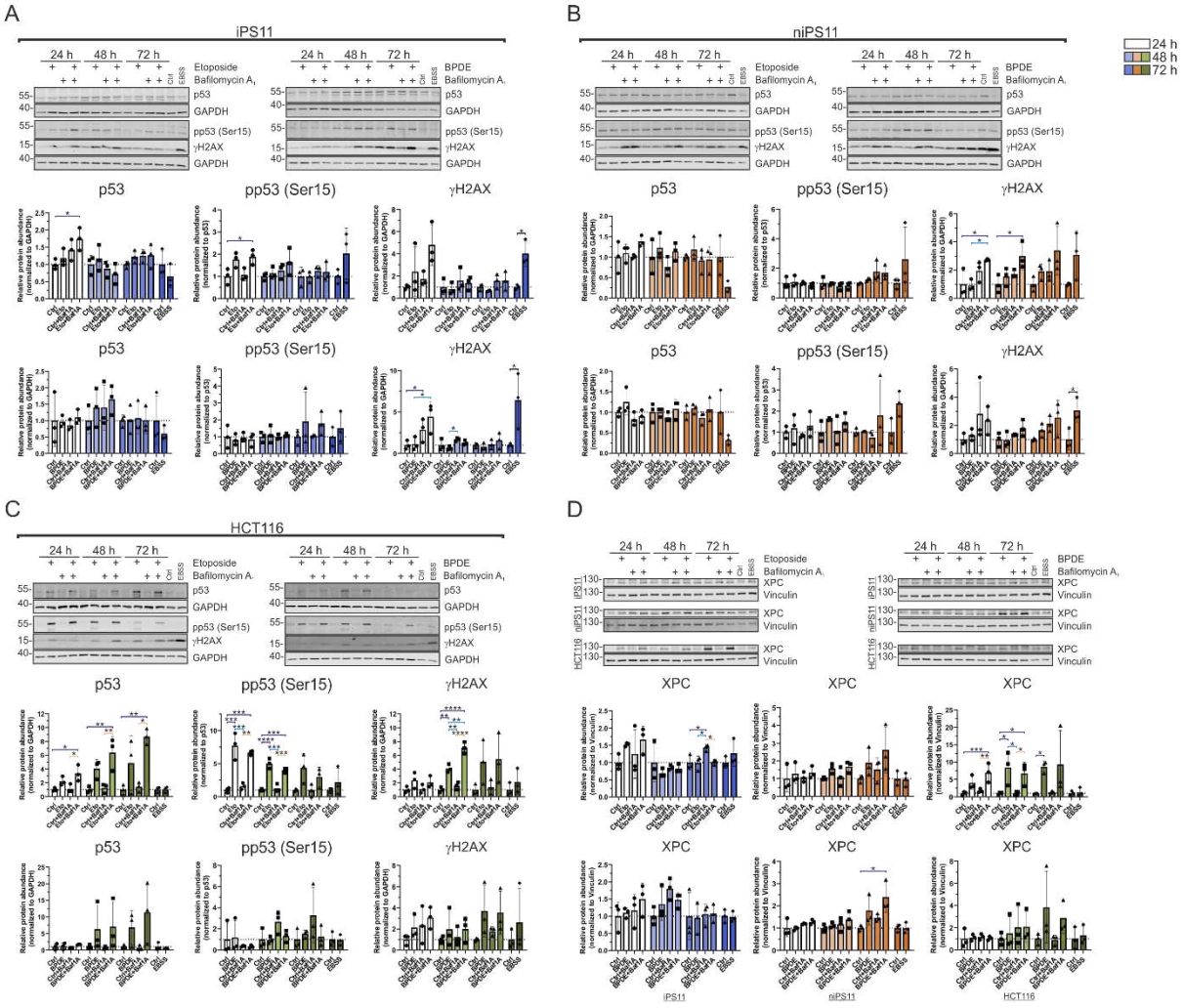

Figure S3: Stem cells show a more moderate impact on DNA damage markers compared to HCT116.

(**A-D**) General DNA damage response proteins were investigated by immunoblotting. (**A**) iPS11, (**B**) niPS11 and (**C**) HCT116 were treated with corresponding IC_{20} dose for 24, 48 or 72 h. Therefore, etoposide was supplemented for 24 h and exchanged with genotoxin-free medium for 48 h and 72 h while BPDE was supplemented daily. 4 hours before harvesting medium was exchanged and 40 nM bafilomycin A_1 was supplemented to labeled samples 2 h before lysis. Cells were lysed, and cellular lysates were immunoblotted for p53, phospho-p53 Ser15, phospho-H2AX Ser139 (γH2AX) and GAPDH. (**D**) NER protein XPC was immunoblotted as described above. One representative immunoblot is shown. Results show mean + SD of three independent experiments. For statistical analysis, ordinary one-way ANOVA (Tukey´s multiple comparisons test) and Student´s t-test were utilized to compare

means of genotoxin-treated samples to DMSO. Significance bars are highlighted in different colors accordingly to involved condition: dark blue: control, light blue: genotoxin; orange: control + bafilomycin A_1 and genotoxin + bafilomycin A_1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

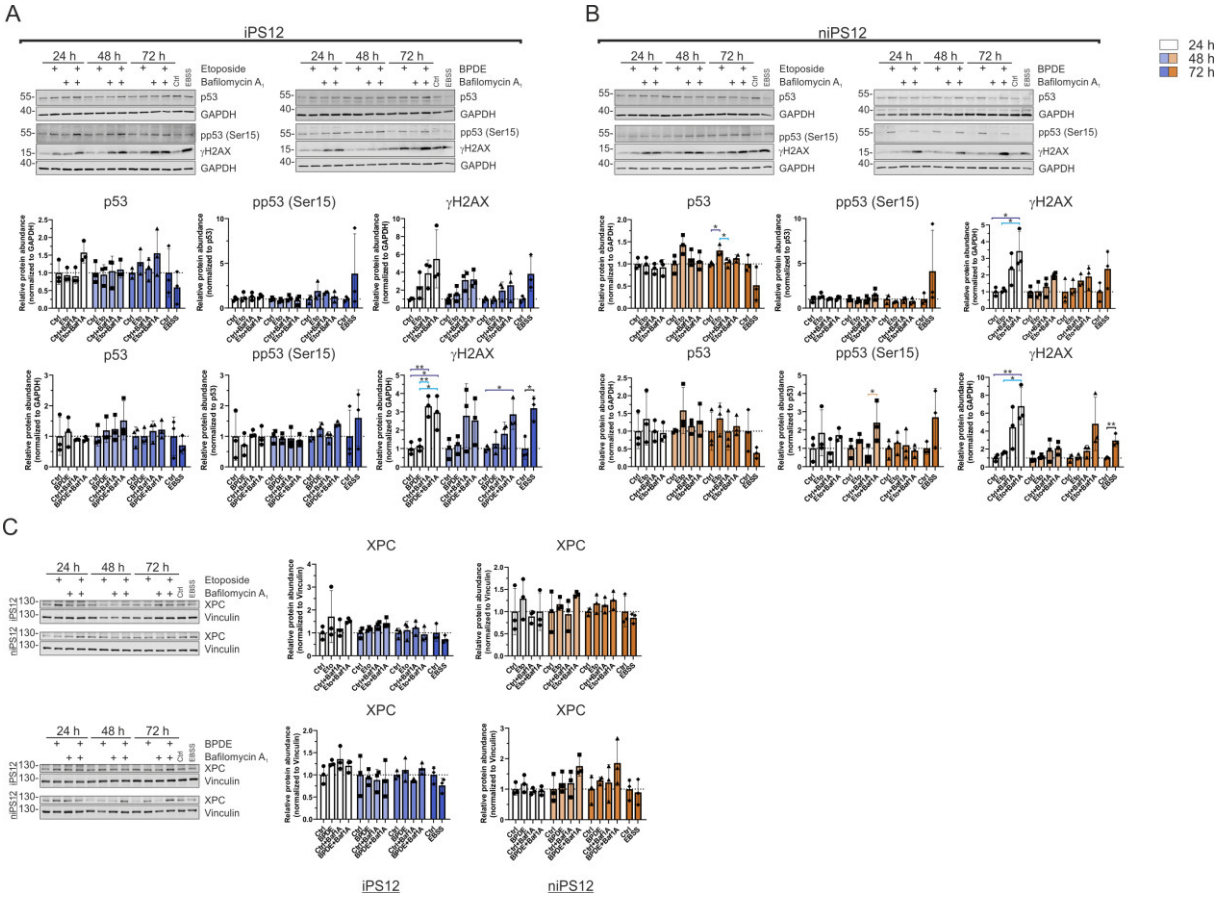

Figure S4: iPS12/niPS12 show no significant effect on p53 but an increase of γH2AX after genotoxic treatment. (**A-C**) General DNA damage response proteins were investigated by immunoblotting. (**A**) iPS12 and (**B**) niPS12 were treated with corresponding IC_{20} dose for 24, 48 or 72 h. Therefore, etoposide was supplemented for 24 h and exchanged with genotoxin-free medium for 48 h or 72 h while BPDE was supplemented daily. 4 hours before harvesting medium was exchanged and 40 nM bafilomycin A_1 was supplemented to labeled samples 2 h before lysis. Cells were lysed, and cellular lysates were immunoblotted for p53, phospho-p53 Ser15, phospho-H2AX Ser139 (γH2AX) and GAPDH. (**C**) NER protein XPC was immunoblotted as described above. One representative immunoblot is shown. Results show mean + SD of three independent experiments. For statistical analysis, ordinary one-way ANOVA (Tukey´s multiple comparisons test) and Student´s t-test were utilized to compare means of genotoxin-treated samples to DMSO. Significance bars are highlighted in different colors accordingly to involved condition: dark blue: control, light blue: genotoxin; orange: control + bafilomycin A_1 and genotoxin + bafilomycin A_1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

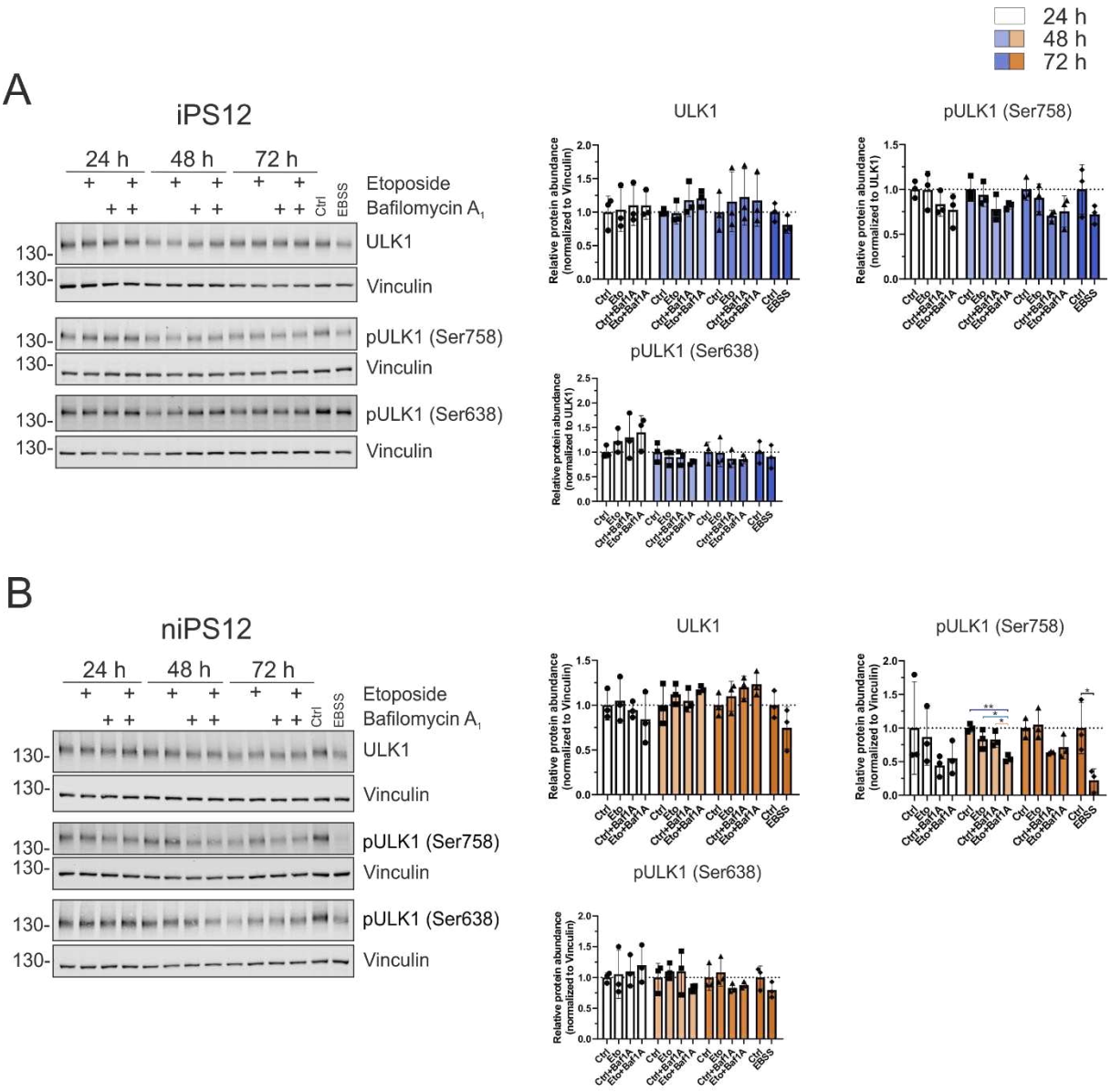

Figure S5: ULK1 activation status is not affected in iPS12 after etoposide treatment. (**A**) iPS12 and (**B**) niPS12 were treated with corresponding IC_{20} dose of etoposide and lysed after 24, 48 or 72 h. Cellular lysates were immunoblotted for ULK1, phospho-ULK1 Ser758 and phospho-ULK1 Ser638 respectively. One representative immunoblot is shown. Results show mean + SD of three independent experiments. For statistical analysis, ordinary one-way ANOVA (Tukey´s multiple comparisons test) and Student´s t-test were utilized to compare means of genotoxin-treated samples to DMSO. Significance bars are highlighted in different colors accordingly to involved condition: dark blue: control, light blue: genotoxin; orange: control + bafilomycin A_1 and genotoxin + bafilomycin A_1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

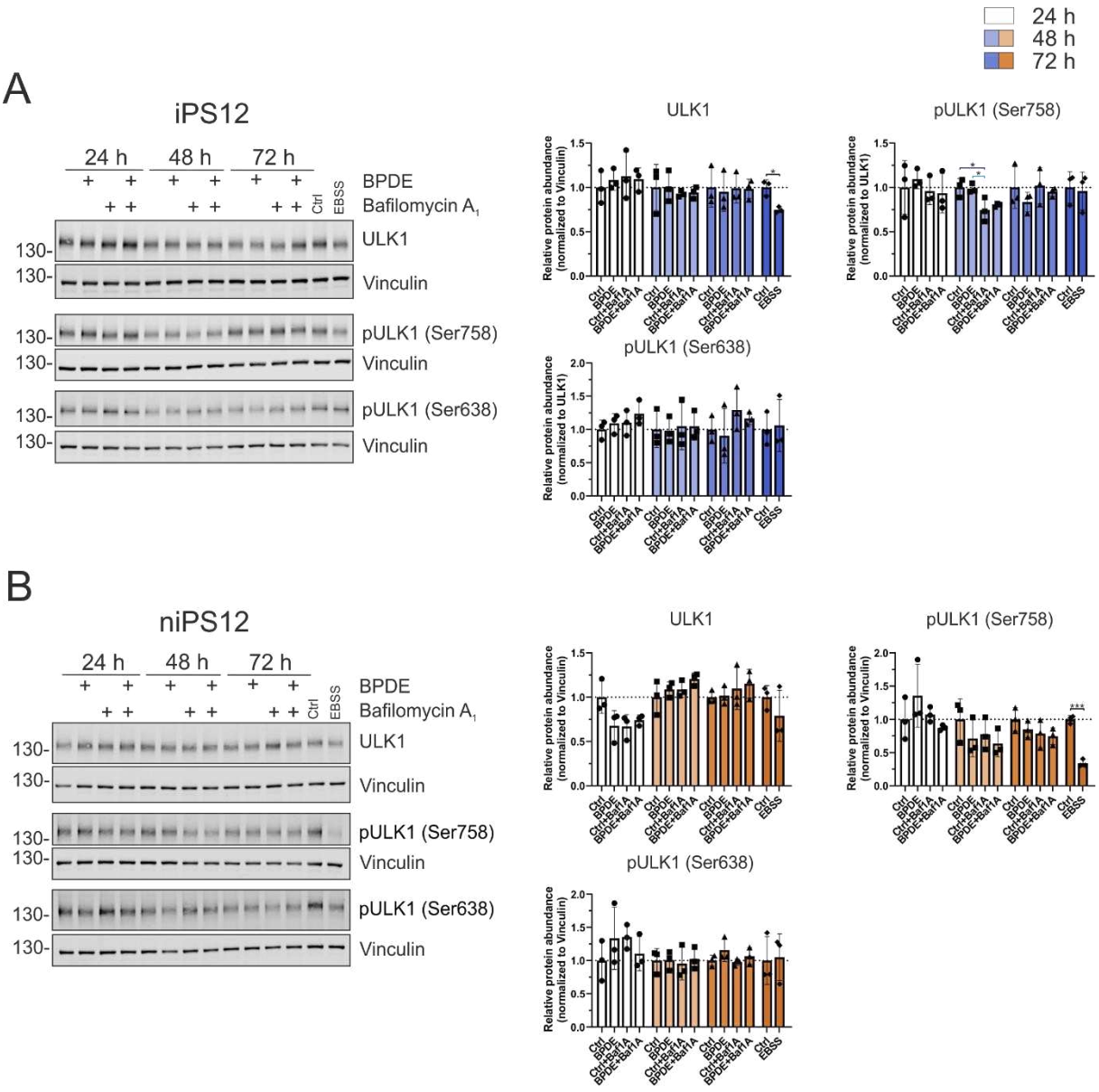

Figure S6: BPDE exposure does not influence ULK1 phosphorylation. (**A**) iPS12 and (**B**) niPS12 were treated with corresponding IC_{20} dose of BPDE and lysed after 24, 48 or 72 h. Cellular lysates were immunoblotted for ULK1, phospho-ULK1 Ser758 and phospho-ULK1 Ser638 respectively. One representative immunoblot is shown. Results show mean + SD of three independent experiments. For statistical analysis, ordinary one-way ANOVA (Tukey´s multiple comparisons test) and Student´s t-test were utilized to compare means of genotoxin-treated samples to DMSO. Significance bars are highlighted in different colors accordingly to involved condition: dark blue: control, light blue: genotoxin; orange: control + bafilomycin A_1 and genotoxin + bafilomycin A_1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

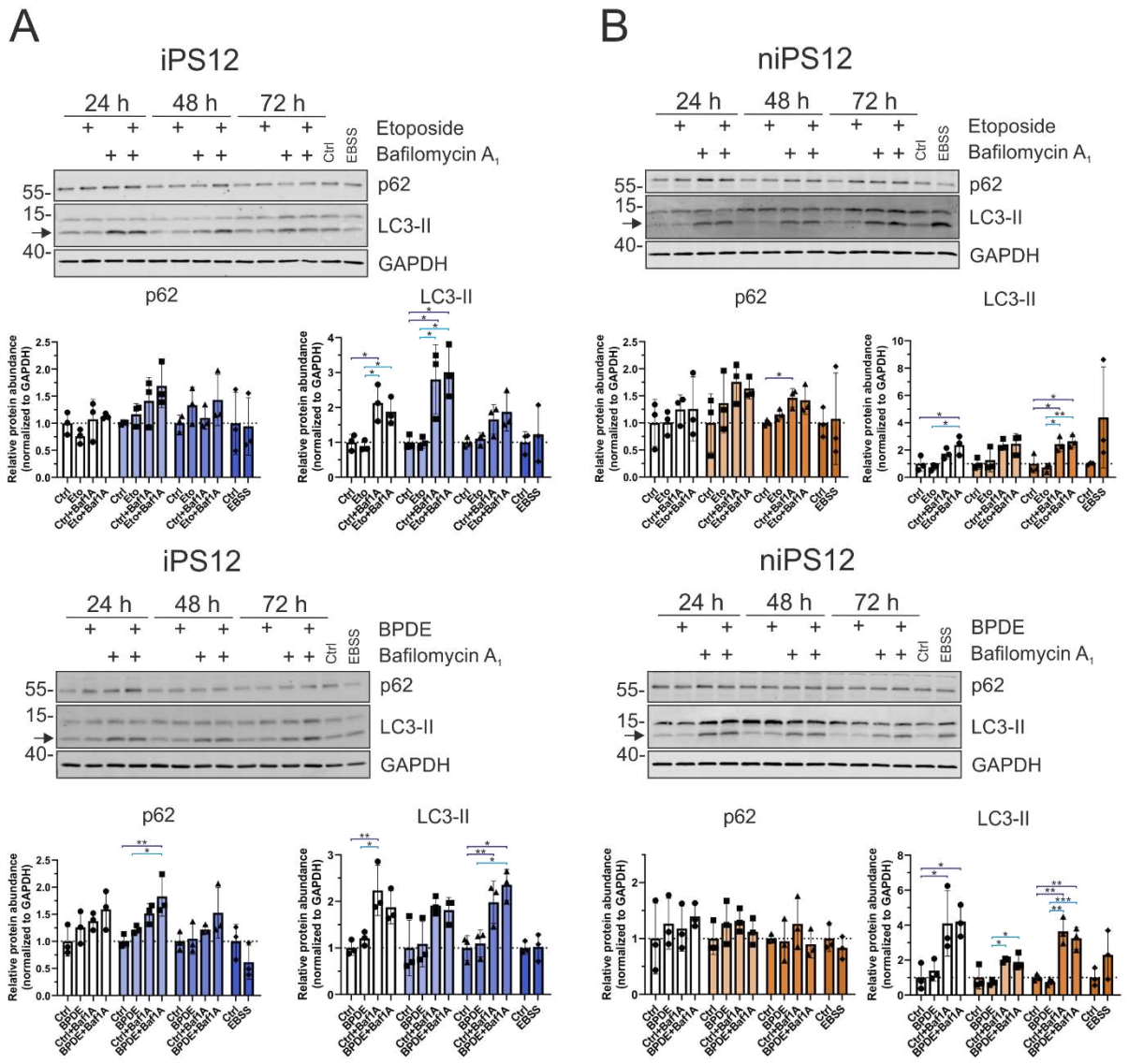

Figure S7: Autophagic flux is not affected by genotoxic treatment. (**A**) iPS12 and (**B**) niPS12 were treated with corresponding IC_{20} dose for 24, 48 or 72 h. Cells were lysed, and cellular lysates were immunoblotted for SQSTM1/p62, LC3 and GAPDH. One representative immunoblot is shown. Results show mean + SD of three independent experiments. For statistical analysis, ordinary one-way ANOVA (Tukey´s multiple comparisons test) and Student´s t-test were utilized to compare means of genotoxin-treated samples to DMSO. Significance bars are highlighted in different colors accordingly to involved condition: dark blue: control, light blue: genotoxin; orange: control + bafilomycin A_1 and genotoxin + bafilomycin A_1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

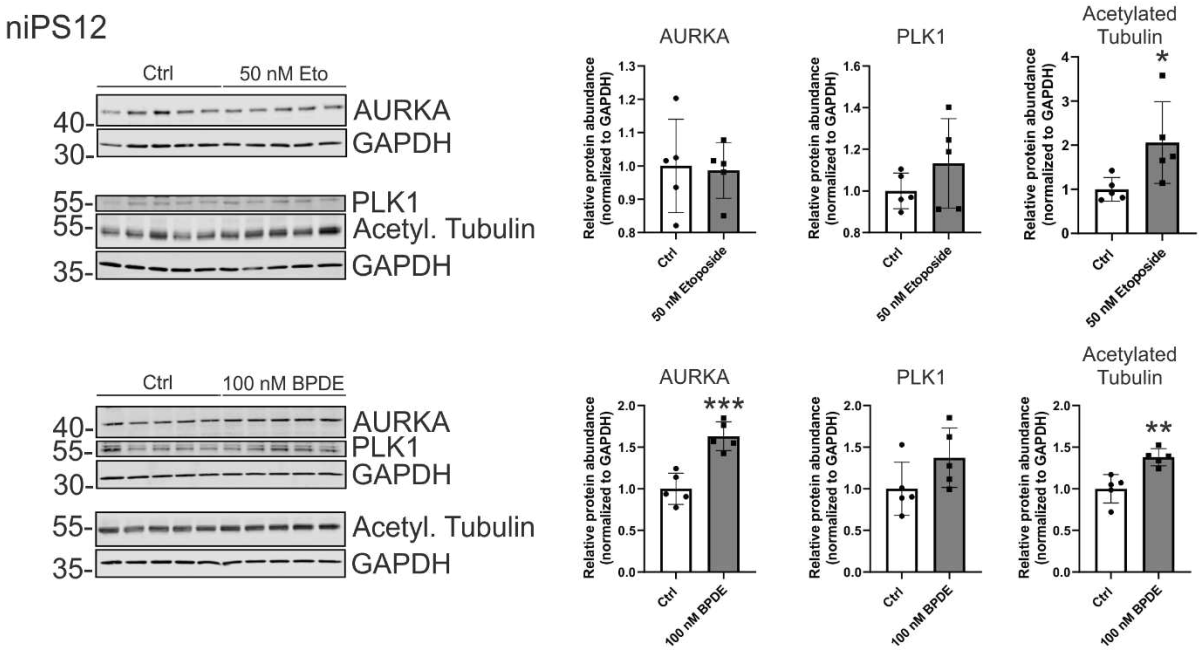

Figure S8: Mitosis-related proteins are affected upon genotoxic treatment in niPS12. niPS12 were either treated with etoposide for 24 h or BPDE for 48 h and afterwards lysed. Cellular lysates were immunoblotted for Aurora kinase A (AURKA), polo like kinase 1 (PLK1), acetylated tubulin (K40), and GAPDH as loading control. The blot with all samples is shown. Results show mean + SD of five independent experiments. For statistical analysis, Student´s t-test was utilized to compare means of genotoxin-treated samples to DMSO. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

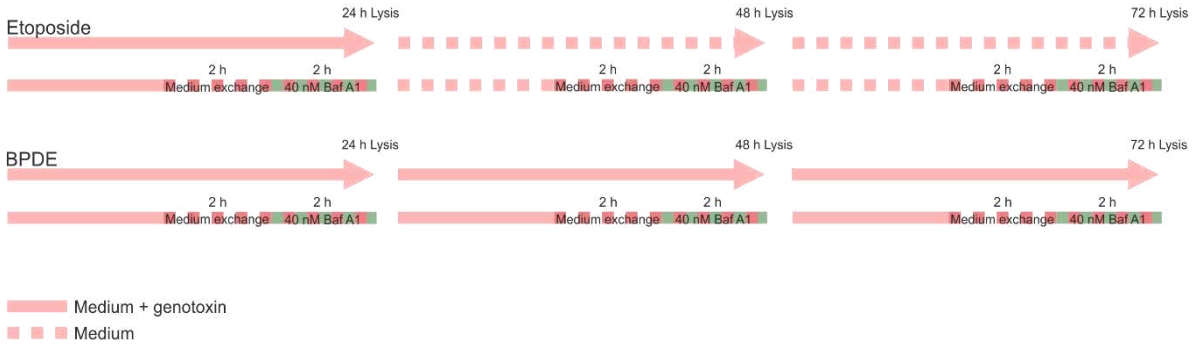

Figure S9: Treatment scheme used in this study.

The medium was exchanged every 24 h to exclude starvation-induced autophagy. On the day of lysis, medium was exchanged 4 h and 40 nM bafilomycin A_1 was supplemented 2 h prior to lysis. Cells treated with etoposide were incubated with the genotoxin for 24 h (solid line). In following medium exchanges etoposide was not included (dotted line). In case of BPDE treatment, BPDE was supplemented daily to the cells corresponding to the IC_{20} value, and cells were processed as described before on the day of lysis.

Publication 2

TOP(O)Gun: The impact of topoisomerase inhibitors on healthy and cancer stem cells

Seda Akgün, Gerhard Fritz, Björn Stork

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TOP(O)Gun: The impact of topoisomerase inhibitors on healthy and cancer stem cells

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Abbreviations:

ASC, adult stem cell; ALDH, aldehyde dehydrogenase; BER, base excision repair; CPT, camptothecin; CSC, cancer stem cell; DDR, DNA damage response; DSB, DNA double strand break; ESC, embryonic stem cell; ICL, interstrand crosslink repair; iPSC, induced pluripotent stem cell; MMR, mismatch repair; MSC, mesenchymal stem cell; NSC, neural Stem cell; NSCLC, non-small-cell lung cancer; γH2AX, phosphorylated H2AX; TOPO I, topoisomerase I; TOPO II, topoisomerase II

ABSTRACT

Topoisomerases display a highly conserved and crucial protein family in many different biological processes involving DNA topology. Their participation in the maintenance of genomic integrity, remodeling and DNA repair offered a great target in the fight against the ongoing and increasing crisis against cancer. In 2020, around 19.3 million incidences and 10 million deaths could be assigned to cancer diseases worldwide emphasizing the importance for anticancer treatments and overcoming drug resistance. The most common and known cancer cell types are mainly composed of mutated terminally differentiated cells with proliferation potential, for example, epithelial cells lining the airways and large intestine. Besides these differentiated cells, a small subgroup of cancer cells within different tumorous tissue possesses stem cell like properties regarding self-renewal and the potential to differentiate in different cell types, designated as cancer stem cells (CSCs). Their origin and emergence are still highly controversial, but CSCs might play a crucial role in the severity of cancer, drug resistance, relapse and metastasis. This review focusses on the function of topoisomerases and their role as target for anticancer treatment and highlights the characteristics of normal stem cells and cancer stem cells in regard to current research findings.

Topoisomerase I and II

Naturally, DNA is underwound, negatively supercoiled, allowing an easier unwinding of complementary DNA strands in the processes of replication and transcription, respectively. During these processes, the DNA gets separated but not unwound by the helicases resulting in overwound, positively supercoiled, DNA. These deleterious overwindings complicate the separation of the DNA double strand and may facilitate lethal knots and tangles in the genome (summarized in McClendon & Osheroff, 2007).

Topoisomerases are involved in altering DNA topology by controlled unwinding, separating and religating DNA strands allowing the effortless solution of topological problems during DNA replication, transcription, and other cellular processes (Jain et al., 2015; Wang, 2002).

The monomeric subtype topoisomerase I (TOPO I) is further divided into types IA, IB, and IC (Champoux, 2001). These three subcategories show similarities in their execution, however, distinguish in finer prosecutions and occurrence. TOPO I causes a single strand breakage by attacking the DNA phosphate group in the backbone by its active tyrosyl oxygen resulting in a covalently bound DNA-enzyme intermediate (Figure 1) (Wang, 2002). Type IA is known to be a bacterial topoisomerase and, in comparison to the other subcategories, causes a single strand break and forms a transient covalent bond with its active-site tyrosine to a 5'-phosphoryl group of the DNA (Kirkegaard & Wang, 1985; Tse & Wang, 1980). The DNA scission by this subtype requires the presence of divalent metal ions while type IB has no need of divalent metal ions for this process (Champoux, 2001; Leppard & Champoux, 2005; Wang, 1996). The intact DNA strand can now be passed through the nick to amend the DNA topology. This process of DNA passage through the break site is referred to as the "strand passage" mechanism. Subsequently, the nicked DNA strand is religated, thus reforming the intact DNA double strand (Delgado et al., 2018; Jain et al., 2015; reviewed in Yakkala et al., 2023). Due to its mechanistical properties working with single strand breaks, TOPO I is able to unwind and solve DNA supercoils during transcription but cannot remove lethal DNA knots and tangles (i.e., catenated DNA) (Bjornsti & Kaufmann, 2019; Deweese & Osheroff, 2009; Pommier et al.,

2022; Pommier et al., 2016; Vos et al., 2011). Duplex DNA might form catenanes during replication and chromatin loop formation, whereas DNA knots have been proposed to contribute to chromatin organization and recombination in yeast models (Pommier et al., 2022).

While type IA covalently attaches to 5'-phosphoryl groups of the DNA, type IB and type IC also covalently bind to a single strand DNA but to its 3'-phosphoryl group resulting in a mechanism called "swivel" to relax the DNA supercoils. By this, the broken DNA strand rotates around the complimentary intact strand followed by the religation of the nick. While both types share a similar mechanism for DNA relaxation, type IB is present in both prokaryotes and eukaryotes, including the human topoisomerase I, and type IC was discovered in archaea and also found in bacteria and viruses (Buzun et al., 2020).

The second category describes the topoisomerase II (TOPO II) subtypes IIα and IIβ. In contrast to TOPO I, TOPO II causes transient double strand breaks in order to solve under- or overwound DNA (Deweese & Osheroff, 2009). TOPO II functions as a homodimer and requires, like TOPO IA, divalent metal ions and additionally ATP for its catalytic activity (Fortune & Osheroff, 2000; McClendon & Osheroff, 2007). The enzyme causes a double strand break with a four-base overhang by covalently binding the 5'-phosphoryl group with its active site tyrosine forming a DNA-enzyme intermediate stabilizing the genomic integrity (Berger et al., 1996; Deweese & Osheroff, 2009; Liu, 1983; Wang, 1996). This intermediate state is also referred as "cleavage complex" and plays an important role in later discussed pharmacological inhibition of topoisomerases. The DNA is passaged by the double stranded passage mechanism and thus, able to dissolve supercoils, knots and tangles (Deweese & Osheroff, 2009). While TOPO IIα is essentially involved in the survival of proliferating cells, TOPO IIβ rather plays a role in post-mitotic cells and neural development (Chen & Beck, 1995; Dereuddre et al., 1997; B. N. Singh et al., 2003; Yang et al., 2000). During proliferation phases an increasing protein abundancy of TOPO IIα can be observed (Hsiang et al., 1988; Woessner et al., 1991). Additionally, it shows dependence and regulation through the cell cycle with

TOPO IIα peaking in the G_2/M phase which indicates an involvement in DNA replication (reviewed in Deweese & Osheroff, 2009; Grue et al., 1998; Heck & Earnshaw, 1986). In contrast, TOPO IIβ expression is independent of the cell cycle and does not play a role in proliferation or chromatin association during mitosis (Austin & Marsh, 1998; Isaacs et al., 1998; Linka et al., 2007) but is potentially linked to transcriptional regulation of hormonally or developmentally regulated genes (Haince et al., 2006; Ju et al., 2006). These two subtypes have a distinct expression pattern and seem not to be able to compensate for each other (Austin & Marsh, 1998; reviewed in Deweese & Osheroff, 2009; Grue et al., 1998).

Topoisomerase Inhibitors

Since the first discovery of topoisomerase targeting substances, the number of anticancer drugs attenuating efficiently topoisomerase function has increased rapidly. Thereby, topoisomerase inhibitors can be classified into two group; topoisomerase poisons and catalytic inhibitors (Buzun et al., 2020). While topoisomerase poisons are characterized by facilitating the stabilization of the DNA-enzyme intermediate preventing the religation of the single strand break (Drwal et al., 2014), the catalytic inhibitors focus on preventing the interaction of DNA and enzyme by either binding to the DNA or the topoisomerase (Capranico et al., 1997). Under physiological conditions the DNA-enzyme intermediate represents only a short-lived and occasionally occurring event, displaying a great target for disruption and causing various mutagenic events (Fortune & Osheroff, 2000; Nitiss & Wang, 1988; Sabourin et al., 2003). The majority of inhibitors convert the crucial enzymes to cytotoxic foes of the cell, with both classes of topoisomerase inhibitors leading to cell death via apoptosis triggered by the formation of DNA double strand breaks (Figure 1) (reviewed in Buzun et al., 2020).

Topoisomerase I inhibitors are characterized by the sustained covalently bound DNA-enzyme intermediate impeding the religation of the nick (Chen et al., 2013; Pommier et al., 2015). A commonly known TOPO I inhibitor class are the camptothecins (CTPs). CPT is a plant alkaloid from the Chinese tree *Camptotheca acuminata* and was confirmed in 1985 to have anticancer

activity regarding TOPO I (reviewed in Chen et al., 2013; Wall et al., 1966). Thereby, CPT itself does not cause lethal DNA damages since the single strand breaks are fully reversible. The damage is caused due to the prolonged cleavage complex occurrence affecting the cell´s ability to properly replicate and causing irreversible DNA double strand breaks (DSBs) by stalling the replication fork (Hsiang et al., 1985; Hsiang et al., 1989; Pizzolato & Saltz, 2003). Additionally, CPT is also able to induce DSBs S-phase-independently by a transcription-dependent manner and also shows cytotoxicity in non-proliferative cells (Nishida et al., 2022; Sakasai et al., 2010). Camptothecin derivatives like topotecan are successfully used in the treatment of different cancer variations like metastatic ovarian cancer or relapsed platinum-sensitive small-cell lung cancer (Armstrong et al., 2005; Asai et al., 2014; Bookman et al., 1998; Garst, 2007; Pawel et al., 1999; Rodriguez & Rose, 2001).

Topoisomerase II inhibitors can be divided into three categories, i.e. compounds 1) that, similarly to TOPO I inhibitors, bind non-covalently to the DNA-enzyme intermediate, 2) that covalently modify the enzyme and 3) that change DNA structure by covalently binding to it (McClendon & Osheroff, 2007).

The most commonly known examples for non-covalent inhibitors are etoposide and doxorubicin which are widely used in clinical anticancer therapies. Etoposide is a derivative of the natural compound podophyllotoxin, which is known for its antimitotic properties and used against different illnesses over centuries, and is one of the earliest anticancer drugs targeting topoisomerase II (reviewed in Baldwin & Osheroff, 2005). While etoposide mainly interacts with the enzyme and shows poor DNA intercalating properties, doxorubicin represents high affinity to free DNA also in the absence of the enzyme. Accordingly, during DNA replication etoposide functions as a canonical TOPO II poison and traps TOPO IIα on newly replicated DNA behind the replication fork resulting in fork stalling and preventing the resolution of topological stress (Van Ravenstein et al., 2022). In contrast, doxorubicin intercalates parental DNA ahead of the fork and causes replication fork stalling independently from TOPO II (Van Ravenstein et al., 2022). Experiments performed in *Xenopus* egg extracts and HCT 116 cells

showed that, surprisingly, only etoposide causes DSBs during DNA replication but both compounds exhibit TOPO II-dependent cytotoxicity, decoupling the effect of doxorubicin on cell viability and DNA replication. The authors speculate that doxorubicin might act as a TOPO II poison during transcription but not replication (Van Ravenstein et al., 2022).

The most prominent compounds in the class of covalently bound inhibitors are quinone-based TOPO II poisons, such as N-acetyl-p-benzoquinone imine, benzoquinone, and several polychlorinated biphenyl (PCB) quinones (Vann et al., 2021). Interestingly, they display a dual inhibitory function by acting as a topoisomerase poison when incubated with the DNA-TOPO II intermediate and as an inhibitor when exclusively encountering TOPO II. These properties might be a result of the ability of quinones to cross-link the N-terminus of the enzyme, which in one hand enables the entrapment of the cleavage complex, and on the other hand, blocks the interaction of TOPO II and DNA (Bender et al., 2006; Bender et al., 2004; Lindsey et al., 2004; McClendon & Osheroff, 2007).

The third class of TOPO II inhibitors describes DNA lesion-induced TOPO II poisoning. Abasic sites, generated by different DNA damaging methods including ionizing radiation, DNA reactive chemicals and also by the cell´s base excision repair pathways themselves, are amongst the most common lesions and strongest topoisomerase poisons (Kingma et al., 1997; Kingma & Osheroff, 1998; Sabourin & Osheroff, 2000). The position of the DNA lesion plays a crucial role in its influence on topoisomerase activity. As described above, TOPO II induces one incision on each complementary strand with 4 base pairs apart from each other. A lesion located within these 4 base pairs can often induce TOPO II-mediated DNA cleavage while lesions outside of the scission frame rather inhibit or slightly affect DNA cleavage (Kingma et al., 1997; Kingma & Osheroff, 1998; McClendon & Osheroff, 2007).

Playing an indispensable role in clinical treatments for various malignancies and solid tumors, the emergence of drug-resistant cancer cells displays calamitous problems in terms of the treatment of this disease and advancing in the field of cancer therapy (Ganapathi & Ganapathi, 2013). These resistances can be based on physiological properties, genetic alteration or

enhanced drug efflux. Physiological levels and activity of topoisomerase II defines the sensitivity towards the drugs. Cells with lower levels or less active topoisomerase show resistance to inhibitors while cells with the opposite properties are rather hypersensitive (reviewed in Deffie et al., 1989; Fortune & Osheroff, 2000; Robert & Larsen, 1998). Furthermore, cells displaying mutations in different positions in the *TOPI* gene show resistance to variant camptothecins (summarized in Nishida et al., 2022). Additionally, topoisomerase inhibitors can be removed from the cell by ABC transporters. Five out of seven subfamilies of ABC transporters are known for causing chemoresistance (Choi, 2005; Dean, 2009; summarized in Wtorek, 2018). For example, ABCB1 (also known as P-glycoprotein 1), shows increased expression levels in different organs like liver, kidney, brain and colon in the occurrence of cancer development (Hilgendorf et al., 2007; Juliano & Ling, 1976; Laberge et al., 2014; Wtorek, 2018). This transporter pump is observed in doxorubicin resistance (Januchowski et al., 2013; Mirzaei et al., 2022). Another pump, ABCG2, mediates resistance to doxorubicin, etoposide, and camptothecin (Brangi et al., 1999; Doyle et al., 1998).

Therapeutical research facilitated groundbreaking advancement in fighting various types of cancer disease over the last decades. However, the ongoing neck-and-neck race between fighting these diseases and new defense mechanisms of cancer cells leads to encounters of novel problems for curing the different facets of cancer. An important aspect in this ongoing war is to understand the differences between healthy and malignant cells and their distinction in cellular functionality and physiology.

Healthy Stem cells (embryonic and adult stem cells)

Stem cells display distinctive developmental and regenerative capacities by their ability of self-renewal by asymmetric cell division and differentiation into various cell lineages (Figure 2). Over the last decades, their therapeutic potential was utilized to investigate different disease models and developmental disorders by primary cell culture or re-differentiated induced pluripotent stem cells (iPSCs).

Naturally occurring stem cells can be attributed totipotent, pluripotent or multipotent properties and can be divided into embryonic and adult stem cells. Totipotent cells are located in the late zygote and blastomeres of 2-cell stage embryo and are defined by their differentiation potential into embryonic and extra embryonic tissue being able to form an entire embryo (Genet & Torres-Padilla, 2020; Ghazimoradi et al., 2022; Lu & Zhang, 2015; Tarkowski, 1959). In comparison, pluripotent stem cells are only able to differentiate into embryonic tissue, which means that they can differentiate into all three germ layers. Naturally pluripotent stem cells are exclusively existent during early developmental stages, known as embryonic stem cells (ESCs) (Evans & Kaufman, 1981; Martin, 1981; Thomson et al., 1998; Wobus & Boheler, 2005). These cells represent a great opportunity for repair and regeneration research due to their potential to differentiate into any cell type in the body and have a nearly unlimited potential of self-renewal, however, are also interconnected with ethical issues since ESCs are obtained from blastocytes. In 2006, the group of Yamanaka at Kyoto University was able to identify specific transcription markers for reprogramming somatic cells, finally differentiated cells, into induced pluripotent stem cells enabling new aspects and possibilities in the field of disease and stem cell research by circumventing ethical issues (Takahashi et al., 2007; Takahashi & Yamanaka, 2006; Yu et al., 2007). iPSCs show many similarities to ESCs regarding their expression of cell markers, chromatin methylation pattern and embryonic body formation (Ye et al., 2013). De-differentiation of terminally differentiated adult cells had a major impact on disease research, since the obtained iPSCs have nearly identical genetic properties of the donor enabling research in various diseases including multifactorial ones that were hard to mimic by genetical modification. The only group of stem cells existent postnatally are referred to as adult stem cells (ASC) and show multipotent properties with a limited potential in differentiation. Compared to the embryonic stem cells, adult stem cells are only able to differentiate into all cell types within one particular lineage, for example, neural stem cells for neurons and glial cells, hematopoietic stem cells for blood and immune cells, or mesenchymal stem cells (MSCs) for cartilage and bones.

Since stem cells are characterized by their unlimited potential of self-renewal, quality control and error elimination play a crucial role in preserving their distinctive ability in a healthy organism.

Besides spontaneous mutations, endogenous (e.g., oxidative stress) or exogenous (e.g., UV light, environmental pollution) noxae may cause damage to the genome by introducing different types of DNA alterations including single- and double strand breaks, DNA adducts, or oxidative damage. Non-repaired and perpetuating damages could be passed on to self-renewing progenitor cells resulting in cancerous tissue or cause malfunctioning after differentiation. Prevention of these calamitous events are facilitated by fast and efficient DNA repair mechanisms (Frosina, 2010; Maynard et al., 2008). Microarray analysis showed that ESCs contain elevated levels of different DNA damage repair proteins compared to their differentiated cells including base excision repair (BER), interstrand crosslink repair (ICL-repair) and mismatch repair (MMR) genes (summarized in Frosina, 2010; Maynard et al., 2008; Saretzki et al., 2008). Additionally, previous studies have shown that ESCs even have enhanced repair capacities regarding the removal of DNA adducts and resealing strand breaks compared to mature and also progenitor cells (Bracker et al., 2006). Besides their efficiency in DNA damage repair, ESCs also show a higher apoptotic response which may reduce irreparable damages and their inheritance by eliminating these cells (de Waard et al., 2008; Roos et al., 2007; van der Wees et al., 2007). These observations were also made for adult stem cells and partially for MSCs and were well tabularly summarized by Frosina 2010, showing that stem cells in general own great DNA repair potential and have other mechanisms including apoptotic responses to maintain intact genomic material for self-renewal and differentiation.

Highly proliferative stem cells display higher abundancy of topoisomerase IIα regulating the ESC transcriptome including their pluripotency and differentiation potential (Thakurela et al., 2013). The importance of TOPO IIα during development was demonstrated by genetical deletion of this protein resulting in embryonic lethality (Akimitsu et al., 2003; Carpenter &

Porter, 2004). Previous works in rodents have shown that during neuronal differentiation and maturation cells shift their reliance from TOPO IIα to TOPO IIβ supporting the pivotal role of this subtype for successful neurogenesis. (Capranico et al., 1992; Tiwari et al., 2012; Watanabe et al., 1994; Yang et al., 2000). Mutations in TOPO IIβ showed normal neurogenesis but with axon growth defects causing impairments in innervation and resulting in breathing complications and death shortly after birth (Yang et al., 2000). As previously described, TOPO IIβ is known for its contribution in transcriptional regulation during development. Regulating the activation or repression of genes involved in late neurogenesis support the neuronal maturation and functionality (Neha & Dholaniya, 2021). Due to their increased proliferation rate and similarities to cancerous tissue, stem cells might be experiencing unwanted side effects during chemotherapy. However, the available data regarding the effects of topoisomerase inhibitors on “normal” stem cells and how they circumvent irreversible damage is quite elusive. A previous study has shown that hESCs are hypersensitive to topoisomerase I inhibition and rather undergo p53-dependent apoptosis upon camptothecin exposure (García et al., 2014). A study from 2009 showed that continuous etoposide exposure of hESC induces *MLL* rearrangements and other chromosomal abnormalities including trisomies and translocations. They have also observed increased cell death occurrence after a single dose treatment and that surviving cells exhibited no morphological differences to DMSO-treated hESCs (Bueno et al., 2009). Compared to these findings, another study displayed the resistance of mesenchymal stem cells towards topoisomerase I and II inhibitors showing survival levels equivalent to differentiated fibroblasts maintaining their proliferation and differentiation properties during treatment and no increased apoptosis appearance. The authors hypothesized that the resistance towards topoisomerase inhibitors is due to their efficient implementation of DNA damage repair (Nicolay et al., 2016). Additionally, Nicolay et al. demonstrated that TOPO II inhibitors resulted in a higher number of phosphorylated H2AX (γH2AX) foci compared to TOPO I inhibitors, which has been previously reported for A549 lung cancer cells (Zhao et al., 2008). For human $CD34^{+}$ hematopoietic stem cells, it has been

reported that multiple pro-survival and pro-apoptotic pathways are simultaneously activated in order to arrest cell cycle, to halt proliferation, and to induce apoptosis (Tao et al., 2003).

Recently it was shown that equitoxic doses of different types of genotoxins stimulated distinctive and complex phosphorylation signaling cascades in mouse embryonic stem cells (Sampadi et al., 2020). Etoposide triggers a DDR through both replication stress-related ATR kinase and DSB-associated ATM kinase, whereas DDR activation by cisplatin mainly occurs via ATR kinase (Sampadi et al., 2020). This difference might reflect differences in the rate at which DSBs are generated: etoposide can induce replication-independent DSB caused by the collision of the transcription machinery with trapped topoisomerase II, whereas replication stress-induced DSBs (by etoposide or cisplatin) only occur during the S phase (Sampadi et al., 2020). Along these lines, a cell cycle-dependent susceptibility to etoposide has also been reported for cord blood hematopoietic stem cells (HSCs) (Becker et al., 2024). Whereas quiescent HSCs are largely unaffected by etoposide, cycling HSCs employ both DNA damage repair and apoptosis mechanisms to prevent the accumulation of damage (Becker et al., 2024), confirming the results described above (Tao et al., 2003). Current findings of the effect of topoisomerase inhibitors on stem cells are summarized in Table 1.

Cancer Stem cells

As described before, stem cells show strict control mechanisms to ensure healthy and error free progenies. In 1994, the first connection of stem cells with cancer were found in a study of human acute myeloid leukemia and later on also found in solid tumors in brain and breast (Al-Hajj et al., 2003; Lapidot et al., 1994; S. K. Singh et al., 2003; Yu et al., 2012). Cancer stem cells (CSCs) represent a subpopulation of stem cells with dysregulated properties uniting the features of both stem cells and cancer cells. They possess the ability of unlimited self-renewal combined with tumorigenicity if planted into animal hosts proposing the possibility to be the origin of metastasis (Rosen & Jordan, 2009; Yu et al., 2012) (Figure 2). Thereby, the concepts of CSCs does not refer to the emergence of cancer but rather to the potential of self-

propagation (Dick, 2008). The origin and distribution of CSCs is still debated with two hypotheses regarding their emergence. The first theory is that CSCs emerge from normal stem/progenitor cells and gain tumorigenicity by genetic mutations while the second theory claims their origin from somatic cells acquiring stem cell like properties (summarized in Yu et al., 2012). CSCs were highly discussed over the last decade due to their stem cell-like properties and showing resistance to known chemo- and irradiation therapy. These concerning characteristics have evoked more attention to new methods regarding anticancer treatments since CSCs are also indicated to play a role in cancer relapse and metastasis formation (Bao et al., 2006; Hermann et al., 2007; Lathia et al., 2015; Qin et al., 2012). Acquired drug resistance due to genetic/epigenetic alterations could be gained by intrinsic mechanisms resulting in enhanced expression of transporters, supporting stemness pathways or increased DNA repair and quiescence (summarized in Gillespie et al., 2023; Li et al., 2021; Phi et al., 2018; Prieto-Vila et al., 2017; Rezayatmand et al., 2022). CSCs express different ATP-binding cassette transporters that, for example, eliminate drug efficiency and protect leukemia and some solid tumor cells (Gottesman et al., 2002). Another acquired resistance strategy is contributed by the aldehyde dehydrogenase (ALDH) that is mainly present in CSCs. ALDH efficiently reduces DNA damage by eliminating oxidative/electrophilic stress and free radicals and enhances the resistance to radiation and several drugs including platinum drugs (Singh et al., 2013; reviewed in Yang et al., 2020). Generally, a high DNA repair capacity has been described for CSC populations in different tumors including glioblastoma, prostate, lung and breast cancers, and mainly attributed to the activation of the ATR-Chk1 and ATM-Chk2 pathways (Krause et al., 2017). Yang et al. exceptionally summarized therapeutical targets of CSCs displaying an immense variety of surface markers and signaling pathways inhibitors and their combinational use in different tumor forms (Yang et al., 2020). Regarding topoisomerase gene expression levels in cancer cells, higher levels of TOPO IIα were found in different cancer types including non-small-cell lung cancer (NSCLC), colorectal cancer and breast cancer. These findings of higher expression levels were associated with higher disease stages and worse survival rates (Coss et al., 2009; Hou et al., 2017; Villman et al., 2002). Simultaneously,

an increased level of TOPO I was found to be correlated with increased survival rate in NSCLC patients (Hou et al., 2017). These insights demonstrate the influence of dysregulated topoisomerase expressions in cancer cells and emphasize the importance of gene expression analyses in CSCs.

Dysregulation of certain proteins may mediate resistance to therapeutical approaches. Normal neural stem cell proliferation shows a dependency on the tumor suppressor p53 while glioma stem cells, cancerous cells originating from neuronal progenitor cells located in the subventricular zone, display a downregulation of p21, which is a downstream effector of p53, and a cyclin-dependent kinase inhibitor normally mediating G_1 cell cycle arrest (Abel et al., 2009; reviewed in Frosina, 2010; Medrano et al., 2009; Meletis et al., 2006). Generally, gliomas are the most common brain cancer type in adults and show high variations in classifications and survival rates and require a combination of different treatment approaches including radiotherapy, surgery and chemotherapy (Lee & Wee, 2022). Temozolomide, an oral alkylating agent with the potential of passing the blood-brain-barrier, is one of few drugs positively affecting the survival chances when applied to the most aggressive form of gliomas, the glioblastoma multiforme (Jia et al., 2023). For glioblastoma CSCs, Hong et al. found higher TOPO IIα expression in CSCs than in non-CSCs, and TOPO IIα silencing resulted in apoptosis induction of glioblastoma CSCs, again emphasizing the importance of TOPO IIα in proliferative cells (Hong et al., 2012). Kenig et al. reported that TOPO IIβ is increased in glioblastoma stem cells compared to glioblastoma primary cells and that TOPO IIβ mediates the resistance of glioblastoma stem cells to replication stress-inducing drugs, such as cisplatin, methyl-methanesulfonate, hydrogen peroxide, and temozolomide (Kenig et al., 2016).

A study has shown that the *in vitro* application of topotecan, a semisynthetic camptothecin derivative, resulted in a decreased glioma cell and glioma stem cell growth by inducing cell cycle arrest in G_0/G_1 and S-phase and an increase of p21 abundancy finally initiating apoptosis (Zhang et al., 2013). Topotecan´s ability to penetrate the blood-brain barrier paved the way for

its therapeutical application for brain tumors and metastasis (Baker et al., 1995; Sung et al., 1994; Zamboni et al., 2001). Despite its cytotoxicity to glioma cells, it was ineffective with systemic delivery in a phase 2 clinical trial (Friedman et al., 1999). However, topotecan's safety and feasibility was reported for a short-term, single-dose convection-enhanced delivery (CED) in a clinical phase 1b trial for patients with refractory malignant gliomas (Bruce et al., 2011), and for a chronic CED in a clinical phase 1b trial for patients with recurrent glioblastoma (Spinazzi et al., 2022). Interestingly, another preclinical approach for treating gliomas is the infiltration of therapeutic stem cells. Thereby, neural stem cells (NSCs), MSCs and induced NSCs show tumor-trophic behavior and are capable of migrating throughout the tumor. These properties were used for transporting a variety of therapeutic agents to tumors like toxins, antibodies and viruses (Bagó et al., 2016; Bagó et al., 2017; summarized in Calinescu et al., 2021).

A study in MCF7-derived cancer stem-like cells described decreased TOPO I and increased TOPO II activity in suspension-cultured sphere-like structures referred to as mammospheres, which consist of cancer stem-like cells compared to the adhesive parental MCF7 cells (Peleg, Romzova, et al., 2014). Dissociated mammosphere-derived cells show resistance to TOPO I inhibitors camptothecin or topotecan and an increased hypersensitivity to TOPO II inhibitor etoposide by resulting in reduced cell viability after 24 h, while intact mammospheres rather display a chemoresistance to both types of topoisomerase inhibitors (Peleg, Romzova, et al., 2014). Previous studies have shown that a co-treatment of mammospheres with topoisomerase inhibitors and tyrosine kinase antagonists resulted in an increased cytotoxic effect (Chen et al., 2007; Ciardiello et al., 1999; Koizumi et al., 2004; Peleg, Bobilev, et al., 2014; Peleg, Romzova, et al., 2014). From a therapeutic point of view, it would be desirable that TOPO I/II expression in CSCs serves as prognostic marker for the responsiveness of tumors towards TOPO inhibition. In a screen for compounds selectively targeting breast CSCs, Zhao et al. discovered five small molecules that preferentially inhibited the growth of CSC-like cells (Zhang et al., 2012). One of these compounds was β-lapachone, for which TOPO I inhibition was one reported mode of action (Li et al., 1993). Remarkably, Zhao et al. also

observed high TOPO I expression in breast CSC-like cells and in primary breast CSCs, and found other TOPO I inhibitors from distinct chemical classes also exhibited preference for the CSC-like cells, letting them hypothesize that TOPO I indeed represents a potential CSC marker (Zhang et al., 2012). In contrast, the CSC-like cells were more sensitive towards etoposide and doxorubicin treatment compared to their matched non-CSC-like cells.

As previously described, stem cells showed high γH2AX foci numbers after topoisomerase II inhibition. These observations were also made in prostate cancer stem-like cells. Upon etoposide treatment, prostate cancer stem-like cells show a significantly higher survival rate compared to non-prostate cancer cells maintaining their self-renewal properties (Yan & Tang, 2014). These resistances might be associated with their increased sensitivity for DNA damage response checkpoint activation and their slow proliferation rate giving cells more time to remove chemotherapeutics and repair damages since many therapeutical drugs rely on the cell proliferation for their cytotoxic unfolding (McDonald et al., 2010). Aforementioned, TOPO IIα is regulated by the cell cycle and its arrest might explain the increased resistance of CSCs.

CONCLUSION

Altogether, cancer stem cells are the result of the strict and efficient quality-maintaining mechanisms of stem cells, which are the source of every developmental and regenerative process for a healthy organism, turning into malignant and lethal calamity. Cancer displays one of the leading premature deaths in the world constantly changing and adapting to modern medical approaches (Bray et al., 2024; Bray et al., 2021). Due to their resistance to several therapeutical approaches, the presence of CSCs can be used to determine the responsiveness to chemo- and radiotherapy and recurrence of cancer in patients (reviewed in Chu et al., 2024; de Jong et al., 2010; Fu et al., 2018; He et al., 2011). Compared to its cancerous counterpart, healthy stem cells also show excellent DNA repair mechanisms but mainly undergo apoptosis after encountering severe and/or irreparable DNA damage, emphasizing the essential difference between healthy and cancer stem cells. Both healthy and cancer stem cells show interesting, but still widely unexplored characteristics when exposed to the most commonly used topoisomerase inhibitors. The gene expression of topoisomerases was investigated in “normal” cancer cells and showed distinct dysregulations in their topoisomerase expressions and were associated with improved or worsened survival of patients (Hou et al., 2017). With respect to currently available research, this information is quite elusive for CSCs, however, the majority of CSCs types referenced in this review showed an occurrence of elevated TOPO IIα levels (Hong et al., 2012; Peleg, Bobilev, et al., 2014). Therefore, future research needs to determine the differences in topoisomerase expression levels in healthy and cancer stem cells and the impact of their dysregulations to investigate and predict sensitivity to TOPO inhibitors and ensure the best possible treatment to fight cancer and reprieve healthy stem cells. The impact of therapeutical drugs on normal stem cells and their strategies for maintaining functionality or inducing cell death might play an important role in understanding the differences between normal stem cells and cancer stem cells to advance in prospective cancer therapy possibilities without harming our valuable regenerative sources of stem cells.

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LEGENDS TO THE FIGURES

Figure 1: Mode of action of DNA topoisomerases and their inhibitors. The active tyrosine site of topoisomerases executes a nucleophilic attack on the phosphoryl group of the DNA, thus forming a transient covalent phospho-tyrosine bond and generating strand breaks by disrupting the phosphodiester bond within the DNA backbone. Depending on the topoisomerase subtype and isoform, the enzyme either binds to the 5´- or 3´end of the phosphoryl group. After covalently binding to the DNA, topoisomerase I induces a single strand break to allow the complementary strand to pass through leading to the relaxation of DNA supercoils. Subsequently, the nicked DNA is rejoined by reversing the above shown reaction. Topoisomerase II induces controlled double strand breaks (DSB) into a double helix structure allowing another double helix to pass through and thereby enabling the resolution of lethal DNA tangles compared to topoisomerase I. Every step in these topological altering processes can be targeted by different drugs. However, the most common topoisomerase inhibitors target the religation step in both topoisomerases leading to a trapped cleavage complex and resulting DNA double strand breaks.

Figure 2: Comparison of healthy stem cells and cancer stem cells. Healthy stem cells are characterized by their ability of self-renewal and asymmetric division. By this, stem cells are able to control and maintain the stem cell pool and generate a daughter cell undergoing further differentiation (Bu, Chen, Lipkin, et al., 2013). Depending on the potency of the stem cell, derived progenitor cells then can differentiate into various cell types displaying their key functions in development and regeneration. To facilitate qualitative and error free progenies, stem cells possess a higher abundance of different DNA damage response protein to ensure stability and integrity of DNA. Stem cells containing severe damages beyond repair undergo differentiation or apoptosis (reviewed in Al Zouabi & Bardin, 2020). Some stem cells reside in quiescence to protect the maintenance of the stem cell pool and can be reactivated upon injury and tissue remodeling. In comparison, CSCs can also divide asymmetrically in early stages

but rather symmetrically divide in late stages contributing to higher proliferative capacity and hyperplasia (Bu, Chen, Chen, et al., 2013). CSCs also display multipotent properties enabling the differentiation into different cell types and possibly promoting heterogeneity of tumorous tissue. Similar to healthy stem cells, CSCs have an elevated DNA damage repair contributing to the increased resistance towards common anticancer treatments. However, while healthy stem cells do not tolerate any genetic alteration, CSCs tolerate genetic mutations facilitating a higher survival rate. Additionally, upon stress stimuli including DNA damage CSCs can undergo quiescence. Since quiescence displays a reversible exit of the cycling state, CSCs can cause primary and secondary malignancies and also circumvent common drugs used in chemotherapy targeting proliferating cells (summarized in Chen et al., 2016; Cheung & Rando, 2013; Turdo et al., 2019).

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Table 1: Effects of topoisomerase inhibitors on healthy and cancer stem cells.

Analyzed stem cells	Inhibitor	Effects	Reference
Healthy stem cells (*in vitro*)			
Embryonic stem cells (ESCs)	Camptothecin	• Hypersensitive towards TOPO I inhibition • p53-dependent apoptosis induction	(García et al., 2014)
Embryonic stem cells (ESCs)	Etoposide	• Low dose exposure induces *MLL* rearrangements and other chromosomal abnormalities	(Bueno et al., 2009)
Mesenchymal stem cells (MSCs)	Irinotecan Etoposide	• Resistance to inhibitors • No effect on survival, proliferation, migration, differentiation	(Nicolay et al., 2016)
Mouse embryonic stem cells	Etoposide	• DDR through both replication stress-related ATR kinase and DSB-associated ATM kinase	(Sampadi et al., 2020)
Cord Blood Hematopoietic Stem Cells (HSCs)	Etoposide	• Quiescent HSCs: unaffected by etoposide • Cycling HSCs: both DNA damage repair and apoptosis	(Becker et al., 2024)
Hematopoietic Stem Cells (HSCs)	Etoposide	• Activation of multiple pro-survival and pro-apoptotic pathways	(Tao et al., 2003)
Cancer stem cells (*in vitro*)			
Breast cancer cells MCF7 mammospheres	Camptothecin Topotecan Etoposide	Dissociated mammospheres • Decreased TOPO I inhibitor sensitivity • Increased TOPO II inhibitor sensitivity Intact mammospheres • Decreased TOPO I/II inhibitor sensitivity	(Peleg et al., 2014)
Breast CSC-like cells	β-lapachone Camptothecin Topotecan Etoposide Doxorubicin	• Growth inhibition by TOPO I inhibitors • Resistance to TOPO II inhibitors	(Zhang et al., 2012)
Glioma stem cells	Topotecan	• Reduced cell growth • Apoptosis induction	(Zhang et al., 2013)
Glioblastoma CSCs	TOPO IIα siRNA	• Apoptosis induction	(Hong et al., 2012)

Prostate cancer stem-like cells	Etoposide	• Higher survival rate compared to non-stem-like cancer cells	(Yan & Tang, 2014)

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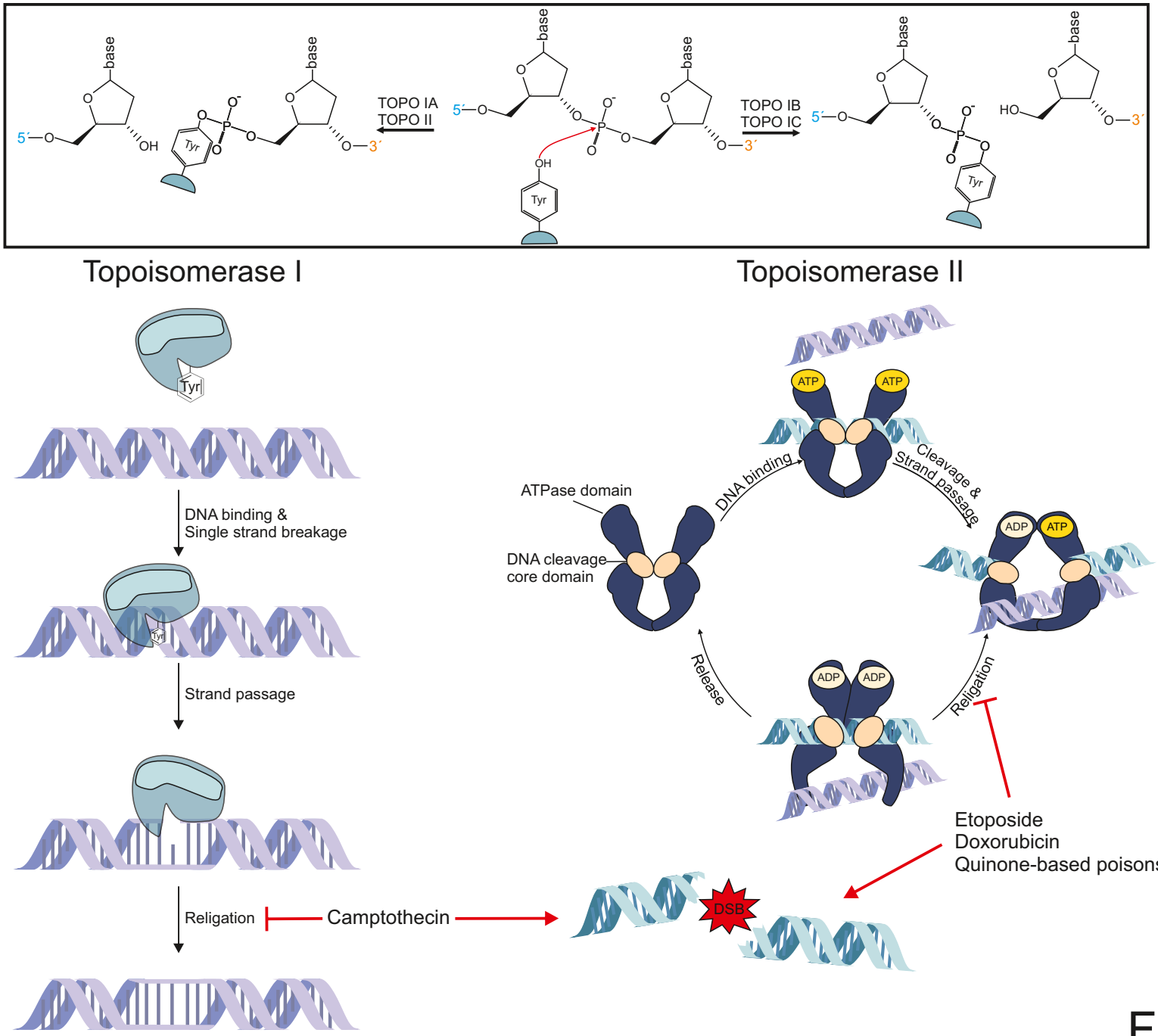
base
5′
O
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HO
Topoisomerase I
Topoisomerase II
DNA binding &
Single strand breakage
Strand passage
Religation
Camptothecin
DSB
ATP
ATP
ADP
ADP
ADP
ATP
ATPase domain
DNA cleavage
core domain
DNA binding
Cleavage &
Strand passage
Release
Religation
Etoposide
Doxorubicin
Quinone-based poisons

Figure 1

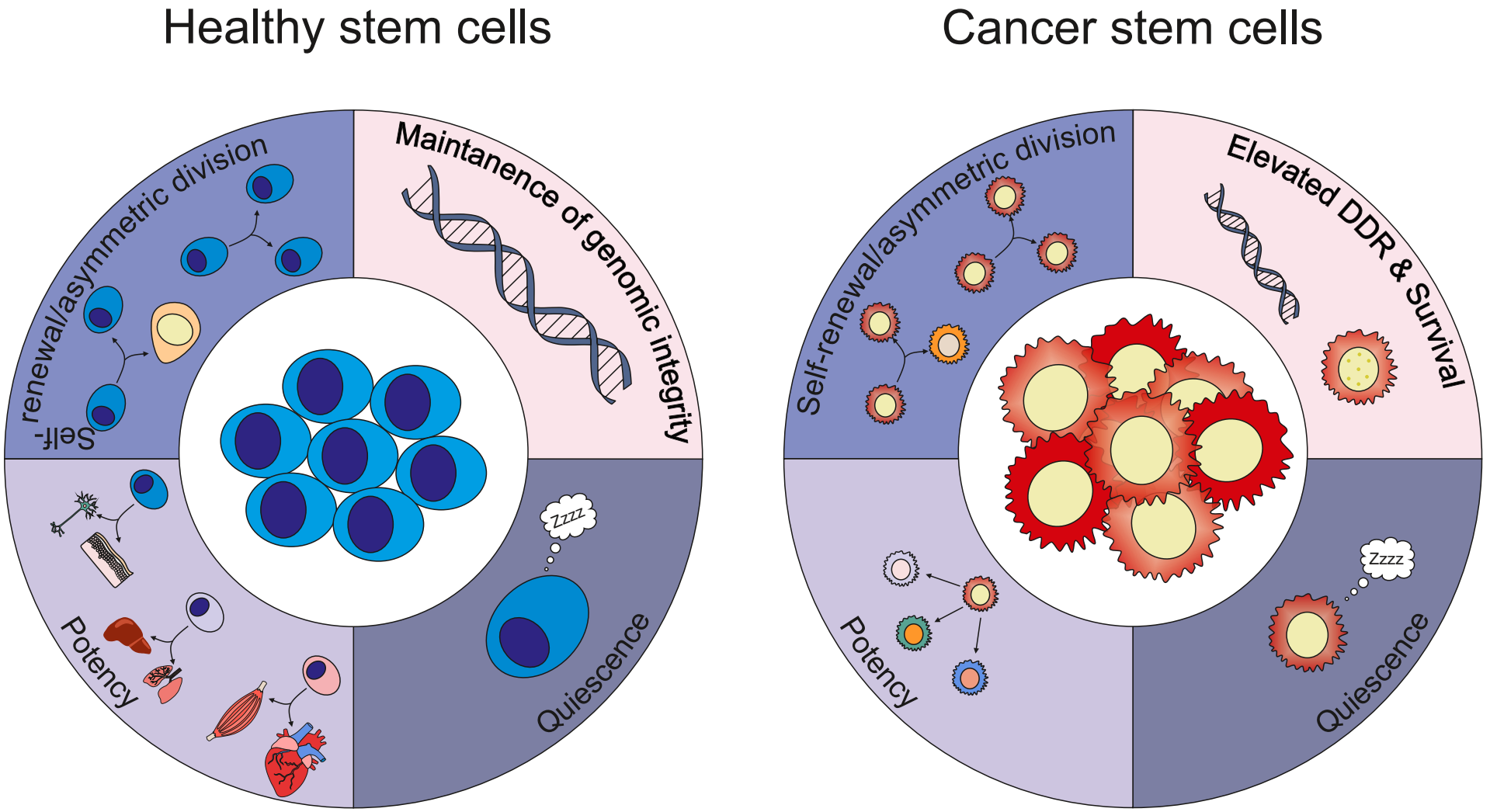
Healthy stem cells
Cancer stem cells
Self-renewal/asymmetric division
Maintanence of genomic integrity
Potency
Quiescence
Zzzz
Self-renewal/asymmetric division
Elevated DDR & Survival
Potency
Quiescence
Zzzz

Figure 2