

Molecular mechanisms of aneuploidy and glioblastoma aggressiveness induced by DIAPH3 loss

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إلى أمي وأخي، قلبي على الأرض
إلى أبي، قلبي في السماء

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ABSTRACT

DIAPH3, member of the formin family of proteins, regulates actin and microtubule dynamics and localizes to the centrosome during mitosis, where it contributes to spindle organization and chromosomal segregation. In the mouse brain, loss of Diaph3 disrupts karyokinesis in neural progenitors, leading to aneuploidy and apoptosis, underscoring its essential role in mitotic fidelity. Given the high level of chromosomal instability in glioblastoma, we investigated whether DIAPH3 deregulation contributes to tumor aggressiveness and therapy response.

Using publicly available transcriptomic datasets (TCGA and single-cell RNA-seq), we found that DIAPH3 expression is specifically increased in grade 4 gliomas and strongly co-expressed with genes regulating mitotic progression and chromosome segregation. DIAPH3 was predominantly expressed in dividing cells, suggesting a mitosis-related function.

To explore its mechanistic role, we silenced DIAPH3 in human GBM cell lines with distinct p53 status, U87 (wild-type *p53*) and U251 (mutant *p53*), using siRNA or pharmacological inhibition. Loss of DIAPH3 induced aneuploidy, DNA damage and activation of the DNA damage response in p53-mutated U251 cells, while promoting G2/M cell cycle arrest in both U251 and U87 cells and decreasing cell survival in U87 cells. In U87 cells, co-suppression of p53 and DIAPH3 reproduced the phenotype observed in U251 cells, confirming that DIAPH3 status influences the cellular outcome observed. Functionally, DIAPH3 loss enhanced radiosensitivity response in p53-mutated U251 cells.

Together, these findings identify DIAPH3 as a key regulator of chromosomal stability and DDR in GBM suggesting its potential as a therapeutic target to improve radiotherapy outcomes.

Keywords: Aneuploidy, Chromosome Segregation, Glioblastoma, DNA Damage Response, Cell Cycle

LIST OF ABBREVIATIONS

Adh : Adherent Cells
aNPCs : Apical Neural Progenitor Cells
APC/C : Anaphase-Promoting Complex /Cyclosome
Apc : Adenomatous Polyposis Coli
ATM : Ataxia Telangiectasia Mutated
ATR : ATM and Rad3-Related
ATRIP : ATR-Interacting Protein
AUNA1 : Auditory Neuropathy Type 1
BSC : Best Supportive Care
BER : Base Excision Repair
BUB1B (BubR1) : Mitotic Checkpoint Serine/Threonine Kinase
CAF : Cancer-Associated Fibroblast
CDC20 : Cell Division Cycle 20
CDC25 : Cell Division Cycle 25 Phosphatase
CDK : Cyclin-Dependent Kinase
CDKN2A : Cyclin-Dependent Kinase Inhibitor 2A
CENP-A : Centromere Protein A
CHK1 / CHK2 : Checkpoint Kinase 1 / 2
ChIP-seq : Chromatin Immunoprecipitation Sequencing
CIN : Chromosomal Instability
CNV : Copy Number Variation
CPC : Chromosomal Passenger Complex
CRM1 : Chromosome Region Maintenance 1 (Exportin 1)
DAD : Diaphanous Autoregulatory Domain
DAPI : 4',6-Diamidino-2-Phenylindole
DDR : DNA Damage Response
DIAPH3 : Diaphanous-Related Formin 3
DID : Diaphanous Inhibitory Domain
DiOC₆(3) : 3,3'-Dihexyloxacarbocyanine Iodide
DIP : Dia-Interacting Protein
DRF : Diaphanous-Related Formin
DSB : Double-Strand Break
E2F : E2 Factor (Transcription Factor Family)
EdU : 5-Ethynyl-2'-Deoxyuridine
EGFR : Epidermal Growth Factor Receptor
EMT : Epithelial-to-Mesenchymal Transition

FH1 : Formin Homology Domain 1
FH2 : Formin Homology Domain 2
GBD : GTPase-Binding Domain
GBM : Glioblastoma
GO : Gene Ontology
GSE : Gene Series Expression
GTE_x : Genotype-Tissue Expression
H2AX (γ H2AX) : Phosphorylated Histone Variant H2AX
HGG : High-Grade Glioma
HJURP : Holliday Junction Recognition Protein
HR : Homologous Recombination
IDH : Isocitrate Dehydrogenase
INF2 : Inverted Formin 2
IR : Ionizing Radiation
KU70/80 : Ku Heterodimer (DNA End-Binding Complex)
Mad1/Mad2 : Mitotic Arrest Deficient 1 / 2
MCC : Mitotic Checkpoint Complex
MDC1 : Mediator of DNA Damage Checkpoint 1
MGMT : O6-Methylguanine-DNA Methyltransferase
MNP : Molecular Neuropathology (Classifier System)
MMR : Mismatch Repair
MRN : MRE11-RAD50-NBS1 Complex
MPS1 / TTK : Monopolar Spindle 1 Kinase / Threonine-Tyrosine Kinase
MRTF1 : Myocardin-Related Transcription Factor 1
MT : Microtubule
mDia : Mammalian Diaphanous (Formin)
NBS1 : Nijmegen Breakage Syndrome Protein 1
NER : Nucleotide Excision Repair
NHEJ : Non-Homologous End Joining
NES : Nuclear Export Signal
NLS : Nuclear Localization Signal
p21 : Cyclin-Dependent Kinase Inhibitor 1
p53 : Tumor Suppressor Protein p53
PDZ : PSD95-Dlg1-ZO-1 Domain
PHH3 : Phospho-Histone H3
PI : Propidium Iodide

PLK1 : Polo-like Kinase 1
PTEN : Phosphatase and Tensin Homolog
RAC1 / RAC2 : Ras-Related C3 Botulinum Toxin Substrate 1/2
RB / RB1 : Retinoblastoma Protein / Retinoblastoma 1 Gene
RhoA / RhoC : Ras Homolog Family Member A/C
ROCK : Rho-Associated Protein Kinase
RPA : Replication Protein A
RT : Radiotherapy
RTK : Receptor Tyrosine Kinase
SAC : Spindle Assembly Checkpoint
siRNA : Small Interfering RNA
SMIFH2 : Small-Molecule Inhibitor of Formin Homology 2
SN : Supernatant
SPAG5 : Sperm-Associated Antigen 5
SSB : Single-Strand Break
TCGA : The Cancer Genome Atlas
TF : Transcription Factor
TMZ : Temozolomide
TSS : Transcription Start Site
TTF : Tumor Treating Fields
UPR : Unfolded Protein Response
WHO CNS5 : World Health Organization Classification of Tumors of the Central Nervous System, 5th Edition

INTRODUCTION

CHAPTER 1

DIAPH3: A Cytoskeletal and Mitotic Regulator

Diaphanous-related formin 3 (DIAPH3, also known as mDia2) belongs to the Formin family of proteins. The name *diaphanous* derives from the Greek *diaphanēs*, meaning “translucent.” The gene was first identified in *Drosophila melanogaster*, where mutations caused defective cytokinesis and resulted in abnormally thin, translucent spermatids, hence the descriptive name *diaphanous* (Castrillon & Wasserman, 1994).

Formins consist of dimeric multidomain proteins conserved among species (animals, plants and fungi). They are known for being regulators of actin dynamics suggesting diverse actin-related functions such as nucleation, elongation and organization (Innocenti, 2023). Besides, their role in microtubules regulation has also been demonstrated. Formins have been recognized as critical regulators of the microtubule (MT) cytoskeleton during interphase and mitosis independently of actin (Bartolini & Gundersen, 2010). As both actin and MT networks are remodeled downstream of signaling pathways

governing cell shape and division, formins represent important candidates for coordinating cytoskeletal responses. Formins can control microtubule stability either directly or through cooperation with other microtubule-binding proteins. Hence, they play a key role in remodeling the cytoskeleton and thereby regulate fundamental cellular processes, including division, adhesion, motility and polarity.

A defining feature of formin proteins is the presence of conserved formin homology (FH) domains that strengthen their diverse cellular functions. Formins in mammals consist of 15 members which are divided into 7 subfamilies. Formin subfamilies are defined according to sequence homology within the conserved formin-homology domain 2 (FH2), which is essential for actin nucleation. These subfamilies can be distinguished between each other due to domain architecture, regulatory mechanisms, subcellular localization and specialized cellular roles (Fig.1) (Kawabata & Kengaku, 2018).

Formins can be broadly divided into Diaphanous-related formins (DRF) and non-DRF, each with distinct regulatory mechanisms. Non-DRF formins are less well characterized but appear to fulfill roles in actin

cytoskeleton remodeling that are not dependent on canonical autoinhibition. In contrast, DRFs are typically regulated through autoinhibition that is relieved by upstream signaling inputs, allowing them to dynamically couple actin filament nucleation and elongation with the regulation of microtubule stability. Through this mode of regulation, DRFs contribute to essential cellular processes including polarity establishment, migration and division (Das et al., 2025).

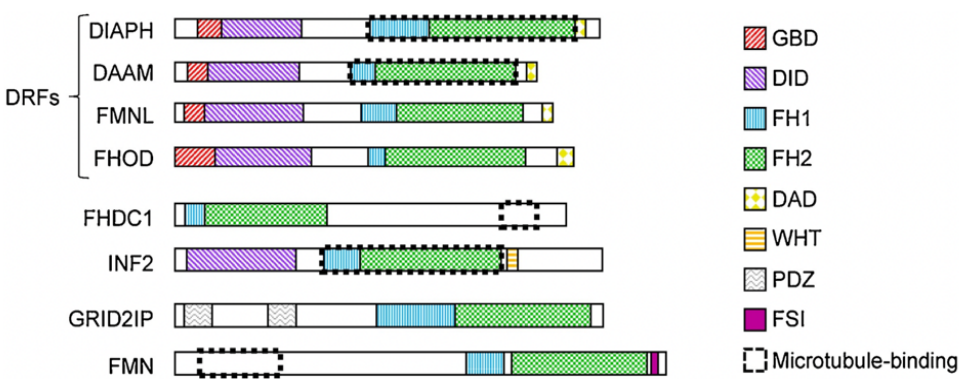


Figure 1. Domain organization of the seven mammalian formin subfamilies. GBD, GTPase-Binding Domain; DID, Diaphanous Inhibitory Domain; FH1, Formin Homology 1; FH2, Formin Homology 2; DAD, Diaphanous Autoregulatory Domain; WHT, Wiskott–Aldrich Homology 2; FSI, Formin–Spire Interaction domain; PDZ, PSD95–Dlg1–ZO-1 domain. Adapted from (Kawabata & Kengaku, 2018).

Structural organization of DIAPH3

This thesis focuses on DIAPH3, a member of the DRF subfamily, which exemplifies the multifunctional nature of formins and their capacity to integrate actin and microtubule regulation in the context of cellular physiology and disease.

Although the overall protein architecture differs between formin subfamilies, they all share two highly conserved domains: the formin homology 1 (FH1) and FH2 domains. These domains are typically located in the C-terminal half of the protein, with the exception of inverted formins, where they are positioned at the N-terminus, for example FHDC1 (Fig 1.). In addition, most formins contain further regulatory modules which play a crucial role in controlling their actin-related activities. Notably, these regulatory elements are absent in non-DRFs as mentioned earlier (Kawabata & Kengaku, 2018; Labat-de-Hoz et al., 2024).

1. Core Formin homology domains (FH1/FH2)

The structural domains FH1 and FH2 are the most critical for the cytoskeletal functions of formins. The FH1 domain is rich in proline residues and binds profilin-actin complexes, thereby serving as a platform to recruit actin monomers and deliver them to the FH2 domain (Courtemanche, 2018). The FH2 domain binds to actin monomers, catalyzing both nucleation and processive elongation of actin filaments. During elongation, the FH2 domain remains processively attached to the fast-growing (barbed) end of the filament, protecting it from termination by capping proteins while allowing the incorporation of new actin subunits (Aydin et al., 2018). Beyond nucleation and elongation, some FH2 domains confer additional actin-regulatory activities. For example, certain formins can promote filament bundling (as shown for DIAPH3) (Harris et al., 2006), while others are capable of severing pre-existing filaments under specific conditions (for example, the inverted formin INF2) (Chhabra & Higgs, 2006). These diverse activities emphasize the versatility of formins as master regulators of actin cytoskeleton organization. In addition to their actin-related roles, several formins also

influence MT dynamics. A particularly well-characterized example is DIAPH3, which has been shown to bind directly to microtubules (Bartolini et al., 2008), thereby enhancing microtubule stability independently of its actin-regulatory activity. Through this dual regulation of actin and MT cytoskeletons, DIAPH3 exemplifies the multifunctionality of formins in coordinating cytoskeletal architecture and dynamics.

2. Regulatory modules (DID/DAD)

The activity of formins must be tightly regulated to ensure the precise organization of actin filaments within the cell. A well-established regulatory mechanism involves an autoinhibitory interaction between two conserved domains: the diaphanous inhibitory domain (DID) located in the N-terminal region, and the diaphanous autoregulatory domain (DAD) situated at the C-terminus. In the inactive conformation, the intramolecular binding of DID to DAD prevents the FH2 domain from exerting its actin nucleation and elongation activity. This autoinhibition can be relieved by upstream signaling pathways, most prominently

those involving Rho family GTPases. When bound to GTP, members of the Rho family (for example, RhoA, RhoC, Cdc42) interact with the adjacent GTPase-binding domain (GBD), disrupting the DID-DAD interaction and thereby activating the formin to promote actin filament assembly. This switch between inactive (Rho-GDP) and active (Rho-GTP) states provides a direct link between extracellular signals and cytoskeletal remodeling (Fig. 2) (Lammers et al., 2008; Rose et al., 2005; Thumkeo et al., 2013).

In addition to Rho-GTPase-mediated control, alternative regulatory mechanisms have been described, reflecting the functional diversity of the formin family. For example, DIAPH3 can be activated independently of Rho through phosphorylation of residues within the DAD domain by the Rho-associated protein kinase (ROCK), highlighting the integration of kinase signaling with formin regulation (Staus et al., 2011). Conversely, DIAPH3 activity can be suppressed by Dia-interacting protein (DIP), which directly binds to the FH domain and interferes with actin filament elongation (Eisenmann et al., 2007; Wyse et al., 2012). As mentioned earlier, not all formins are subject to this canonical

autoinhibition/activation cycle. For instance, Delphinin, a non-DRF, lacks the classical DID and DAD modules altogether, implying that alternative regulatory strategies govern its activity (Miyagi et al., 2002; Silkworth et al., 2018).

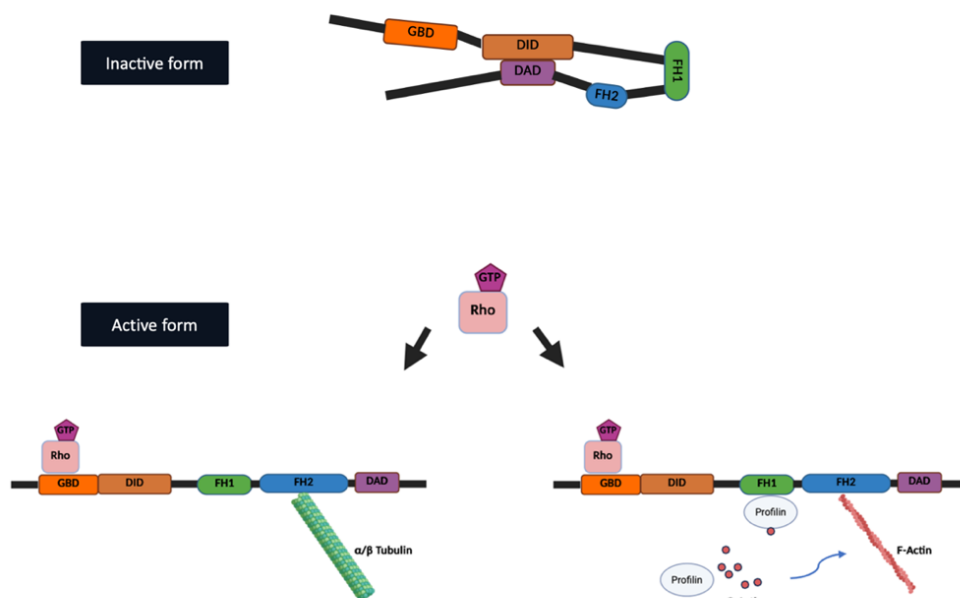


Figure 2. Autoinhibition and activation of diaphanous-related formins. In the inactive form, intramolecular DID-DAD binding prevents actin and microtubule interactions. Upon Rho-GTP binding to the GBD, this autoinhibition is relieved, allowing the FH1 domain to recruit profilin-actin complexes and the FH2 domain to drive actin filament elongation and stabilize microtubules through direct binding to α/β -tubulin. Created using BioRender.

Cellular functions of DIAPH3

In 1998, DIAPH3 was first identified by Alberts and colleagues as a novel effector of Rho-GTPases (Alberts et al., 1998). Since then, evidence has shown that this formin contributes to several essential cellular processes by linking upstream signaling pathways to cytoskeletal regulation. Its activity is not restricted to actin nucleation and elongation but also extends to the control of MT stability and to functions within the nucleus, such as genome maintenance (Chiereghin et al., 2022; Kazama et al., 2020; Morley et al., 2015). Through this versatility, DIAPH3 emerges as a multifunctional regulator involved in cell division, nuclear organization and cytoskeletal dynamics.

1. Regulating mitosis

The eukaryotic cell cycle is traditionally divided into interphase and mitosis. During interphase, cells undergo growth, duplication of organelles and replication of the genetic material to prepare for division. Mitosis then follows as a highly orchestrated sequence that ensures faithful transmission of the replicated genome. It is subdivided into six

stages: prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. While prophase through telophase collectively drive the accurate segregation of chromosomes to opposite poles of the cell (karyokinesis), cytokinesis physically partitions the cytoplasm to generate two daughter cells. Successful completion of these events relies on precise coordination between signaling pathways and cytoskeletal networks (Uzbekov & Prigent, 2022; Z. Wang, 2021). In this context, DIAPH3 has emerged as a critical mediator by promoting actin filament nucleation and elongation and by associating with MTs, thereby contributing to the structural and regulatory framework that underpins mitotic progression (Lau et al., 2021).

1.1. Karyokinesis

Accurate chromosome segregation during karyokinesis requires the formation of a properly organized mitotic spindle. Recent work from Tissir's lab has demonstrated that DIAPH3 localizes to centrosomes during mitosis and that its depletion disrupts spindle bipolarity, leading to defective attachment of microtubules to kinetochores. This

disruption is associated with decreased levels of kinetochore-associated proteins such as SPAG5 and KNSTRN both of which are essential for the stability of kinetochore-microtubule attachments (Fig. 3) (Lau et al., 2021). In addition to its structural contribution to spindle assembly, DIAPH3 influences the spindle assembly checkpoint (SAC). Loss of DIAPH3 results in reduced expression of BUB1B (BubR1), a key SAC component, thereby compromising checkpoint activation and increasing the likelihood of premature anaphase onset and chromosome missegregation (Damiani et al., 2016). The SAC normally safeguards chromosomal fidelity by inhibiting the anaphase-promoting complex/cyclosome (APC/C) until kinetochores of sister chromatids are stably attached to opposite spindle poles, and thus its attenuation following DIAPH3 depletion provides a direct mechanistic link to mitotic errors (McAinsh & Kops, 2023). DIAPH3 plays a critical role in coordinating spindle architecture and checkpoint signaling during karyokinesis, and more broadly highlight the importance of formin activity for faithful chromosome segregation.

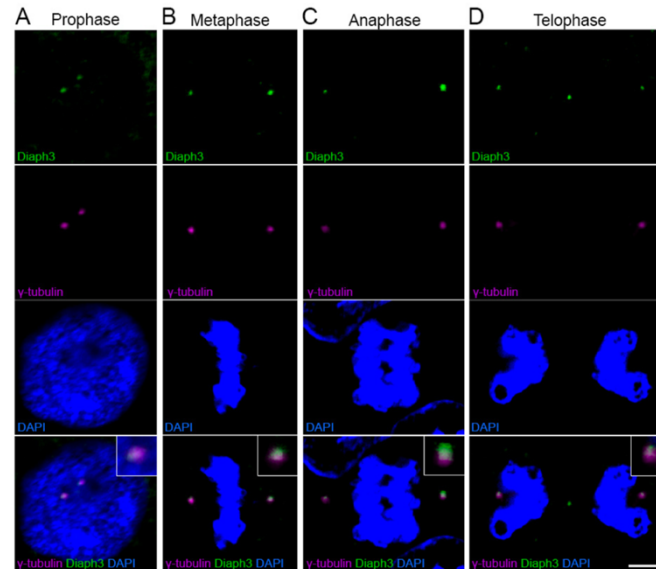


Figure 3. DIAPH3 localizes to centrosomes throughout mitosis. Immunofluorescence staining of U2OS cells at (A) prophase, (B) metaphase, (C) anaphase, and (D) telophase. DIAPH3 (green) and γ -tubulin (magenta) were detected using specific antibodies, and DNA was counterstained with DAPI (blue). Adapted from (Lau et al., 2021).

1.2. Cytokinesis

Cytokinesis, the terminal step of cell division, is the process by which the cytoplasm and plasma membrane are partitioned to generate two daughter cells. It can be subdivided into three main phases: central spindle assembly, cleavage furrow ingression and abscission (Fig. 4). A central event is the assembly of the actomyosin contractile ring which provides the mechanical force for cell constriction. Formins, including

DIAPH3, play a crucial role in this process by supplying β -actin filaments necessary for RhoA-mediated contractile ring formation (A. Chen et al., 2017). Beyond nucleation, DIAPH3 also stabilizes the position of the contractile ring at the equatorial cortex, ensuring proper cleavage furrow ingression. In its absence, contractile forces become mislocalized, resulting in failed cytokinesis (Watanabe et al., 2008). Thus, perturbation of DIAPH3-dependent microtubule regulation can impair contractile ring positioning and abscission, ultimately leading to mitotic errors and altered cell fate (Lau et al., 2021).

DIAPH3 is recruited to the cleavage furrow in late mitosis, where its activity is coordinated by RhoA and the scaffolding protein anillin. DIAPH3 depletion disrupts this process, leading to mislocalized actin and spindle components at the cleavage furrow, as shown by abnormal contractile ring organization (Fig. 4) (Watanabe et al., 2010). Interestingly, in the specialized case of erythroblast enucleation, this process does not rely on RhoA but instead depends on RAC1 and RAC2, highlighting the ability of DIAPH3 to integrate distinct Rho-family GTPase signals in different cellular contexts (Ji et al., 2008). These findings

demonstrate that DIAPH3 functions as a key regulator of contractile ring assembly ensuring the fidelity of cytokinesis.

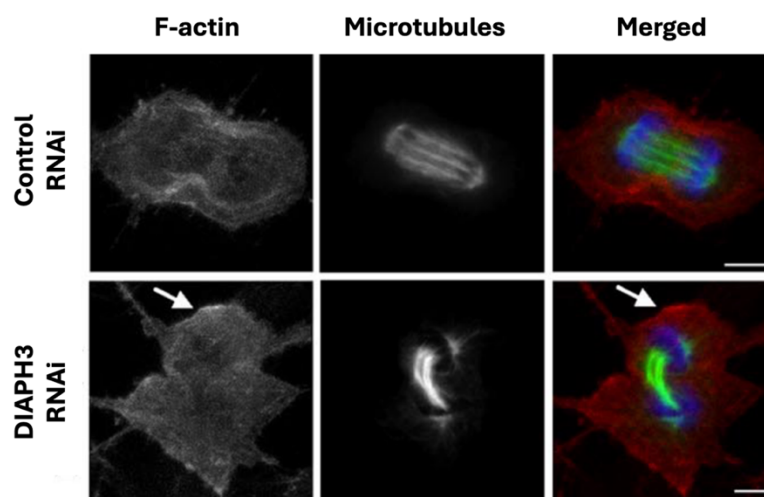


Figure 4. Disruption of contractile ring organization following DIAPH3 depletion. Immunofluorescence images of NIH 3T3 cells treated with control RNAi (top row) or DIAPH3-targeting RNAi (bottom row). F-actin (left, grayscale), microtubules (middle, grayscale), and merged channels (red = F-actin, green = microtubules, blue = DNA) are shown. Adapted from (Watanabe et al., 2008).

2. Modulating cytoskeletal dynamics

Cytoskeletal remodeling is fundamental to diverse cellular processes such as migration, adhesion and invasion. Formins like DIAPH3 have emerged as pivotal regulators of these events. By nucleating, elongating and protecting actin filaments, these proteins

provide the structural framework necessary for the formation of dynamic protrusive structures at the leading edge of migrating cells. DIAPH3 has been shown to orchestrate both lamellipodial and filopodial assembly, underscoring its dual contribution to branched and linear actin networks (Watanabe et al., 2008).

Targeting DIAPH3 demonstrates that disrupting its activity impairs cytoskeletal remodeling and thereby reduces invasive potential of cancer cells (Pettee et al., 2019). When DIAPH3 is silenced, cells lose the ability to maintain stable lamellipodia and filopodia, instead they adopt a rounded morphology characterized by membrane blebbing. This switch reduces reliance on protrusion-based migration and favors an amoeboid mode of motility. In contrast, re-expression of DIAPH3 suppresses blebbing and restores lamellipodial structures, indicating that DIAPH3 normally supports protrusive actin dynamics, and its loss reprograms the cytoskeleton toward amoeboid behavior (Hager et al., 2012).

2.1. Lamellipodial extensions

Directed cell migration relies on a finely coordinated cycle of morphological changes driven by the actin cytoskeleton. At the front of migrating cells, the sheet-like protrusions lamellipodia emerge, powered by the polymerization of actin filaments beneath the plasma membrane. This process is largely dependent on the Arp2/3 complex, which nucleates and organizes branched actin networks (Papanikolaou et al., 2022; Rottner & Stradal, 2011). Nevertheless, evidence indicates that formins also contribute to lamellipodia dynamics. In particular, DIAPH3 has been implicated in sustaining lamellipodial growth by elongating barbed ends and reinforcing the protrusive actin meshwork. Lamellipodia initiation requires Arp2/3 and its full extension and stabilization depend on the action of the nucleators DIAPH3 (K. Lee et al., 2015).

2.2. Filopodial protrusions

In parallel with lamellipodia, many cells generate thin finger-like extensions known as filopodia, which are formed by tightly bundled actin filaments. These structures serve as sensory organelles that probe the extracellular environment, guiding migration and adhesion (Yang & Svitkina, 2011). Multiple mechanisms have been described for filopodia biogenesis, reflecting the diversity of cell types and contexts in which they arise. DIAPH3 emerges as one of the essential proteins that are implicated in filopodium formation due to its actin nucleation and elongation capabilities. DIAPH3 was observed to be present at the leading edge of lamellipodia during filopodia initiation, but progressively gather into a compact cluster at the tip of the nascent filopodium, suggesting that the initial step of filopodia formation is driven by the formin (Fig. 5) (Yang & Svitkina, 2011).

We next turn to its nuclear functions, where DIAPH3 extends its influence beyond classical cytoskeletal regulation.

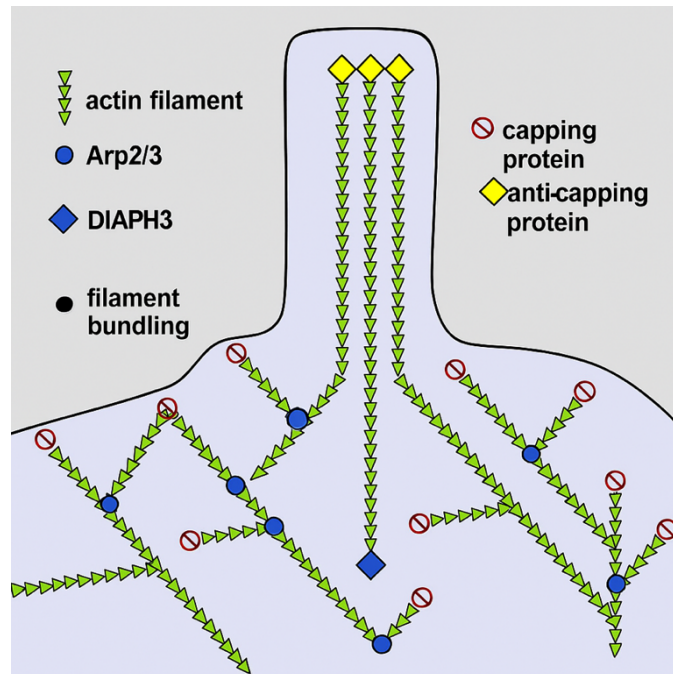


Figure 5. DIAPH3-mediated regulation of protrusive actin networks. Schematic representation of DIAPH3 function in actin nucleation and elongation, highlighting its role in promoting long, unbranched actin filaments within filopodia while coexisting with Arp2/3-dependent branched networks. Adapted from (Lundquist, 2009).

3. Governing nuclear functions

3.1. Nuclear actin polymerization

DIAPH3 is not restricted to the cytoplasm, it can shuttle into and out of the nucleus through nuclear localization (NLS) and nuclear export signals (NES), mediated by the Importin- α/β complex and the nuclear export factor (exportin 1) CRM1, respectively (Fig. 6) (Miki et al., 2009).

Evidence supporting a key role for DIAPH3 in regulating nuclear actin dynamics shows that upon targeting DIAPH3 by siRNA, the basal actin polymerization activity of nuclear extracts is reduced. Conversely, expression of the DIAPH3-DAD domain is sufficient to enhance actin filament formation within the nucleus, making nuclear filaments detectable in living cells (Baarlink & Grosse, 2014). These findings demonstrate that DIAPH3 undergoes dynamic nucleo-cytosolic transport and can directly promote nuclear actin filament assembly.

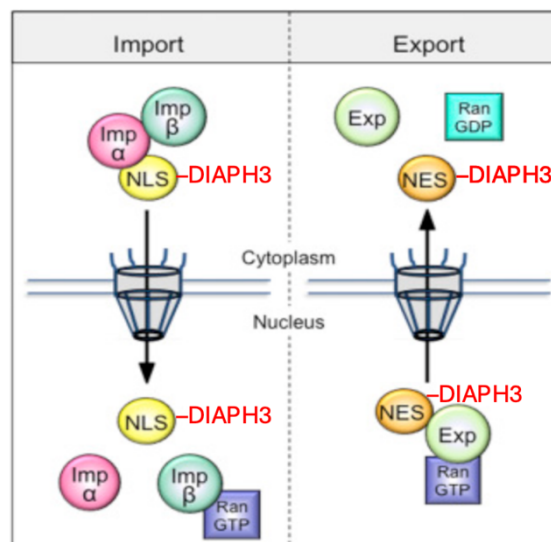


Figure 6. Model of NLS- and NES-dependent nucleocytoplasmic transport of DIAPH3. NLS-containing DIAPH3 is transported into the nucleus via importin α/β , whereas NES-containing DIAPH3 is exported through exportin in a Ran-GTP-regulated manner. This framework illustrates the potential trafficking routes relevant for DIAPH3 regulation. Adapted from (Miyamoto et al., 2018).

3.2. Microtubule regulation during mitosis

During interphase, microtubules remain cytoplasmic, as the nuclear envelope forms a physical barrier that prevents their polymerization within the nucleus. Only after nuclear envelope breakdown at mitotic entry do microtubules penetrate the nuclear region to assemble the bipolar spindle required for chromosome segregation (Dey & Baum, 2021). In this context, DIAPH3 associates with centrosomes and spindle poles, contributing to microtubule stabilization and proper spindle architecture. DIAPH3-depleted cells exhibit disorganized or multipolar spindles and lagging chromosomes, phenotypes indicative of defective microtubule anchoring and kinetochore attachment (Fig. 7) (Lau et al., 2021). This evidence positions DIAPH3 as a key modulator of microtubule dynamics during mitosis, acting in concert with its roles in actin regulation.

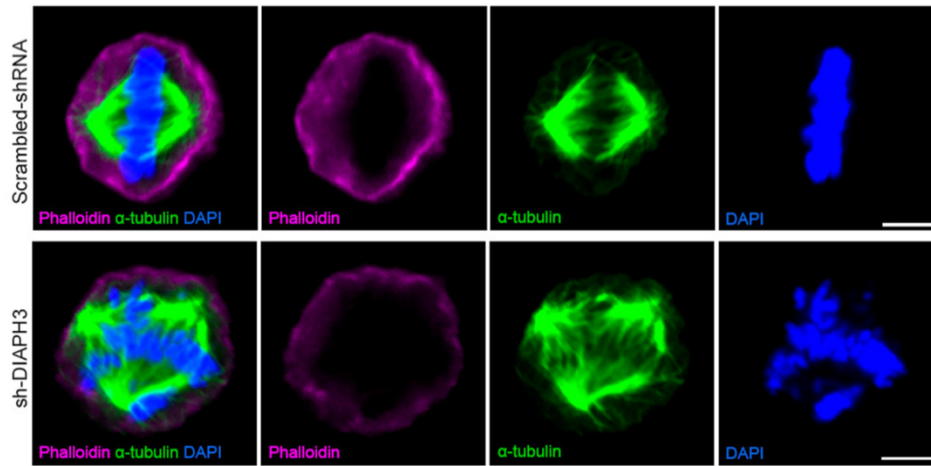


Figure 7. DIAPH3 depletion disrupts spindle organization during mitosis. Fluorescent images of mitotic cells expressing scrambled shRNA (top row) or DIAPH3-targeting shRNA (bottom row). Cells were stained with phalloidin (magenta), α -tubulin to visualize spindle microtubules (green), and DAPI to label chromosomes (blue). Compared to control cells, DIAPH3-depleted cells display disorganized spindle structures and abnormal chromosome alignment. Adapted from (Lau et al., 2021).

3.3. Chromatin remodelling

DIAPH3 plays a pivotal role in regulating chromatin architecture through its ability to drive actin polymerization within the nucleus. By promoting the formation of short nuclear actin filaments, DIAPH3 facilitates the binding of lamin B1 to chromatin, thereby maintaining a compact and transcriptionally repressive state. Sustaining adequate

levels of lamin B1 is essential for proper DNA replication and repair (Butin-Israeli et al., 2015).

As in the case of mesenchymal stem cells, DIAPH3 facilitates the association of lamin B1 with chromatin, thereby maintaining a transcriptionally inactive state that prevents the activation of osteogenesis-related genes. Experimental silencing of DIAPH3 leads to loss of lamin B1 integrity at the nuclear envelope, increased nuclear height and reduced nuclear stiffness (Fig. 8). Thus, DIAPH3 emerges as a key regulator of nuclear mechanics and chromatin organization, with its actin-driven influence on the lamin network linking cytoskeletal regulation to transcriptional control and cell fate decisions (Sankaran et al., 2020).

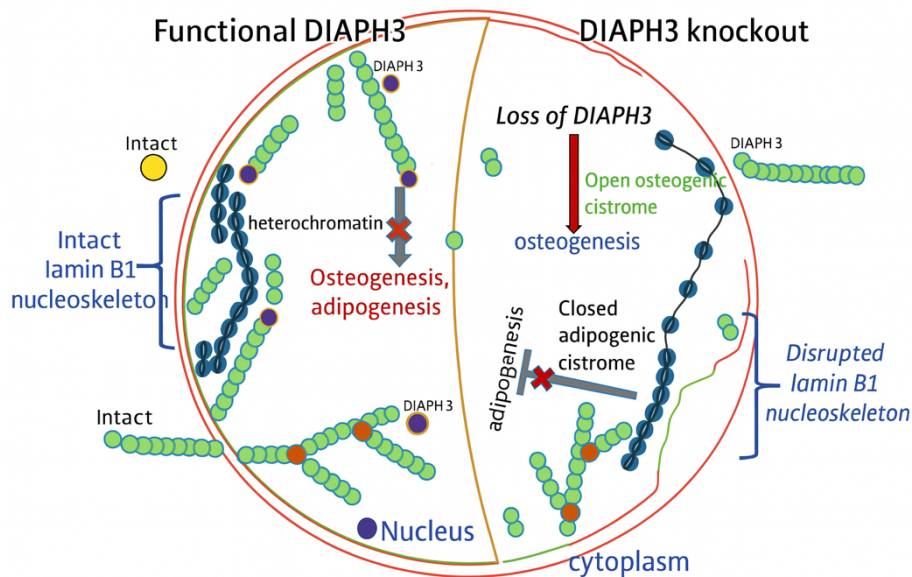


Figure 8. DIAPH3-dependent regulation of nuclear architecture and lineage-specific chromatin programs. Schematic representation illustrating the role of DIAPH3 in maintaining lamin B1-dependent nucleoskeletal integrity and chromatin organization. In cells with functional DIAPH3 (left), DIAPH3-driven actin structures support an intact lamin B1 nucleoskeleton, compact heterochromatin and balanced osteogenic and adipogenic transcriptional programs. In contrast, loss of DIAPH3 (right) leads to disruption of the lamin B1 nucleoskeleton, reorganization of chromatin, opening of the osteogenic cistrome and closure of the adipogenic cistrome, thereby altering cell fate decisions. Adapted from (Sankaran et al., 2020).

3.4. Centromere stability

DIAPH3 also plays a role in preserving genome stability through regulation of centromeres. Short actin filaments nucleated by DIAPH3 in the nucleus restrict centromere mobility in early G1 phase. This

confinement facilitates efficient loading of the centromeric histone variant CENP-A by its dedicated chaperone HJURP (C. Liu et al., 2018). Centromere identity is defined through the incorporation of CENP-A, a specialized histone H3 variant that replaces canonical H3 within centromeric nucleosomes. The presence of CENP-A is critical because it provides a unique structural platform required for the assembly of the kinetochore. Loss or mislocalization of CENP-A disrupts this process, leading to chromosome mis-segregation and genomic instability (De Rop et al., 2012). Loss of DIAPH3 by inhibition of nuclear actin assembly increases centromere motility and reduces CENP-A incorporation, compromising centromere identity and leading to errors in chromosome segregation in subsequent mitoses (C. Liu et al., 2018). These findings reinforce the idea that DIAPH3 integrates nuclear actin dynamics with mechanisms safeguarding genome integrity.

Physiological and developmental roles of DIAPH3

The implication of DIAPH3 in fundamental cellular mechanisms such as cytokinesis, cytoskeletal remodeling and nuclear polymerization extends beyond the single-cell level to shape tissue and organ development. Evidence from genetic models and patient studies highlights essential functions for DIAPH3 in hematopoiesis, cortical neurogenesis and auditory system maintenance.

1. Hematopoietic development

Erythropoiesis is the process by which hematopoietic stem cells give rise to mature red blood cells through successive differentiation into erythroblasts. During the final stages, erythroblasts undergo nuclear condensation, enucleation and remodeling of the plasma membrane before maturing into reticulocytes and erythrocytes (Cha, 2024). DIAPH3 plays a critical role in these late stages. Loss of DIAPH3 in mice results in embryonic lethality around embryonic day 12.5 due to failed cytokinesis in late erythroblasts due to reduced actin accumulation at the cleavage furrow, leading to multinucleated cells, ineffective erythropoiesis and severe anemia (Fig. 9) (Watanabe et al., 2013).

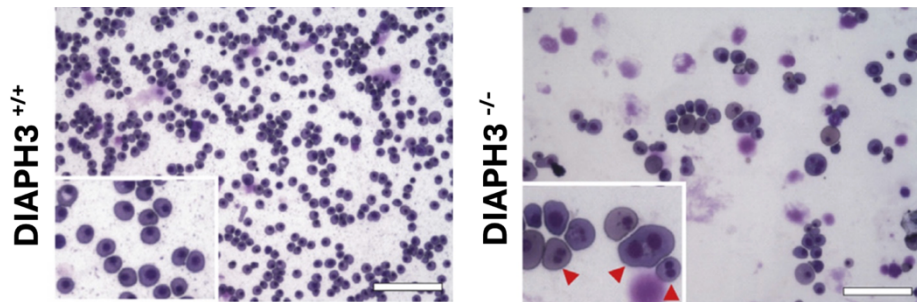


Figure 9. Loss of DIAPH3 leads to defective erythropoiesis and multinucleated erythroblasts. Wright-Giemsa-stained peripheral blood smears from wild-type (DIAPH3^{+/+}, left) and DIAPH3 knockout (DIAPH3^{-/-}, right) embryos. While wild-type erythroblasts display normal nuclear morphology, DIAPH3-deficient embryos show numerous multinucleated erythroblasts (arrowheads), indicative of failed cytokinesis during late erythroid differentiation. Adapted from (Watanabe et al., 2013).

Similarly, in adult mice, conditional deletion of DIAPH3 in the hematopoietic compartment causes anemia, arising from aberrant cytokinesis and defective enucleation of late erythroblasts (Mei et al., 2016). At the mechanistic level, the membrane skeleton becomes abnormally rigid and disorganized, preventing it from properly remodeling and thereby failing to release the extruded nucleus. Thereby, these findings establish DIAPH3 as a key determinant of red blood cell production and function (Y. Liu et al., 2021).

2. Neurodevelopment

During corticogenesis, apical neural progenitor cells (aNPCs) in the ventricular zone generate excitatory neurons through proliferative and neurogenic divisions (Petros et al., 2015). DIAPH3 is specifically expressed in these progenitors, where it ensures accurate karyokinesis (Damiani et al., 2016; McAinsh & Kops, 2023). Loss of DIAPH3 compromises spindle organization, induces chromosomal instability and aneuploidy, and triggers progenitor cell death, particularly among aNPCs. In addition, when DIAPH3 is absent, neural progenitor cells no longer maintain a proper balance between self-renewal and differentiation. Instead of undergoing sufficient proliferative divisions to expand and preserve the progenitor pool, these cells are pushed prematurely toward neurogenic divisions, producing neurons earlier and in greater numbers than normal. This results in cortical thinning and reduced progenitor pools in cortex-specific knockout mice, which in adulthood manifest autistic-like behaviors. Consistently, a double-hit alteration in the human DIAPH3 gene, comprising a focal deletion and a missense mutation, has been associated with autism spectrum disorder

(Lau et al., 2021; Vorstman et al., 2011). Together, these observations underscore the importance of DIAPH3 in maintaining progenitor survival and proper cortical development.

3. Auditory system maintenance

Auditory neuropathy type 1 (AUNA1) is a disease in the cochlea consisting of a progressive form of hearing loss. Deafness in patients is expressed in a gradual manner throughout their lives. Patients typically experience hearing decline starting in the second decade of life, progressing to profound sensorineural deafness within two decades (T. Kim et al., 2004; Starr et al., 2004). The only difference between heterozygotes compared to homozygous carriers, is that the latter's phenotype is more rapidly expressed. However, the severity does not change. A distinct function of DIAPH3 has been uncovered in this disease, as a gain-of-function mutation of DIAPH3 causes AUNA1 (Schoen et al., 2013).

Auditory neuropathy is characterized by dysfunction of the inner hair cells of the organ of Corti, while the outer hair cells remain intact. (Starr et al., 2004).

In animal models, DIAPH3 overexpression in the organ of Corti results in progressive hearing loss due to structural defects in the inner hair cell stereocilia. These defects appears to originate from DIAPH3-mediated accumulation of MTs in the cuticular plate at the stereocilia base, destabilizing their architecture (Surel et al., 2016).

DIAPH3 in cancer

Because of their fundamental ability to nucleate and elongate actin filaments, formins are widely recognized as central regulators of cytoskeletal remodeling, influencing processes such as cell adhesion, polarization and migration. These functions are not only essential for normal cellular physiology but also closely linked to pathological conditions. In particular, the same cytoskeletal dynamics that enable cells to move and interact with their environment are frequently utilized during tumor development. It is therefore unsurprising that formins, through their control of actin organization, contribute to key aspects of

cancer progression, including enhanced motility, invasive behavior and metastatic potential.

1. Dysregulation in solid tumors

Aberrant cytoskeletal organization is a hallmark of cancer progression, and DIAPH3, as a key actin nucleator, has emerged as an important player in this process. By regulating actin filament assembly and interacting with microtubules, DIAPH3 influences essential features of tumor biology in their capacity to metastasize (Xiong et al., 2025). In many tumors, this includes promoting the epithelial-to-mesenchymal transition (EMT), a developmental program hijacked by cancer cells to enhance motility and invasiveness (Ribatti et al., 2020). Subsequent to an EMT, the 'amoeboid' phenotype has emerged as a migratory mechanism that facilitates metastasis. Amoeboid behavior is prominent at the invasive front of tumors, confers rapid migration rates and enables survival within the vasculature (Morley et al., 2015). However, studies evaluating the role of DIAPH3 in cancer have reported conflicting results. The major example would be in breast cancer progression where depletion of DIAPH3 promotes cell migration and metastatic spreading

(Hager et al., 2012). In contrast, a recent study showed that repression of DIAPH3 in breast cancer reduced migration, particularly under conditions of MRTF1 overexpression, a regulator of the expression of genes relevant to cytoskeleton organization (Eder et al., 2024). Similarly, in osteosarcoma, DIAPH3 knockdown suppressed tumor proliferation and metastatic spread, suggesting that in this context, DIAPH3 provides essential signals for maintaining tumor growth and invasiveness (Zhang et al., 2021).

Contrarily, in several cancers, including prostate (Di Vizio et al., 2009a) and hepatocellular carcinoma (Su et al., 2023), depletion of DIAPH3 has been linked to EMT, which enhances motility and metastatic potential. For example, in prostate cancer, silencing DIAPH3 disrupts actin and microtubule organization, driving a switch from mesenchymal migration to a bleb-based amoeboid mode of motility. Clinically, low DIAPH3 expression correlates with metastatic prostate disease in patients (Morley et al., 2015). Altogether, these data point to the multifaceted and sometimes contradictory role of DIAPH3 in solid

tumors, acting either as a promoter of invasion and metastasis or, in certain contexts, as a suppressor of aggressive cell phenotypes (Fig 10).

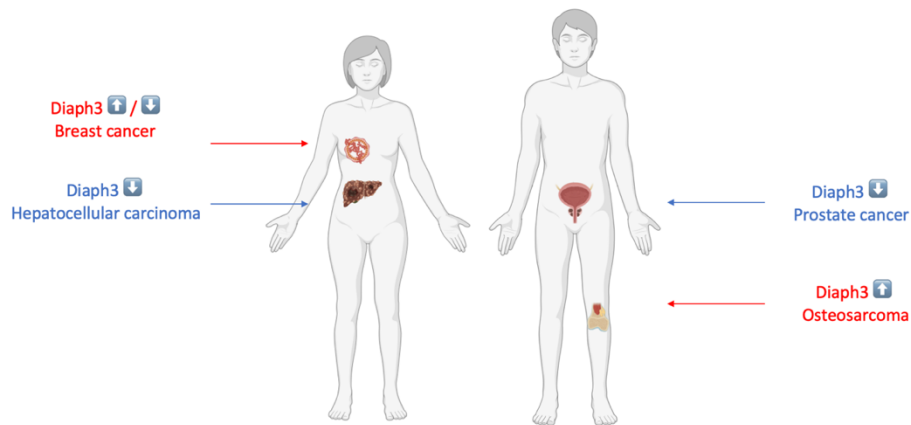


Figure 10. Correlations of DIAPH3 expression in various tumor types. Schematic illustration showing cancer types associated with *DIAPH3* dysregulation. Both *DIAPH3* upregulation and downregulation have been implicated in tumor development and progression. In breast cancer, *DIAPH3* can be either upregulated or downregulated, whereas it is predominantly downregulated in prostate and hepatocellular carcinoma, and upregulated in osteosarcoma. Created using Biorender.

2. Role in glioblastoma

The contribution of DIAPH3 to glioblastoma (GBM) biology has been investigated in many experimental models, but the findings remain somewhat paradoxical. Both pharmacological activation of DIAPH3 and targeted knockdown using siRNA produced a similar outcome: in both cases, tumor cells displayed reduced invasive behavior and shifted towards a more epithelial-like morphology. These observations suggest

that modulating DIAPH3 activity, whether by enhancing or suppressing it, disrupts the mesenchymal-like invasive features that typically characterize GBM cells (Arden et al., 2015; Heuser et al., 2020). The clinical significance of DIAPH3 expression in GBM is also still debated. Earlier studies did not find a robust association between DIAPH3 levels and patient prognosis. However, more recent evidence from Tissir’s lab of a well-characterized cohort of 73 GBM patients showed a different trend. In this study, patients with high *DIAPH3* expression exhibited a markedly better overall survival compared to those with low expression (20.2 months versus 11.7 months, respectively) (Fig. 11) (Chehade et al., 2024; Stoyanov et al., 2022). These results highlight the complexity of DIAPH3 function in GBM, pointing to a dual role that may depend on the cellular context, mode of regulation and disease stage.

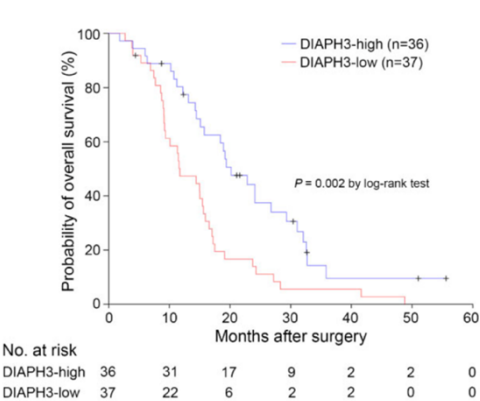


Figure 11. Kaplan–Meier analysis of overall survival (OS) according to DIAPH3 expression. Patients with high DIAPH3 expression showed significantly longer median OS (20.2 months, 95% CI: 14.9–25.5, n=36) compared to those with low DIAPH3 expression (11.7 months, 95% CI: 7.3–16.2, n=37; $P = 0.002$, log-rank test). Adapted from (Chehade et al., 2024).

CHAPTER 2

Chromosomal Instability and Aneuploidy

Aneuploidy refers to an abnormal number of chromosomes in a cell, meaning that the total chromosome count is either greater than normal (hyperploidy) or lower than normal (hypoploidy). In healthy human cells, the correct number of chromosomes is known as euploidy and any deviation from this balance can have major consequences. Aneuploidy most often arises as a result of chromosomal instability (CIN), which occurs when chromosomes do not separate correctly during cell division. Although the two terms are closely related, they are not identical: CIN describes the ongoing process of chromosome mis-segregation, whereas aneuploidy describes the state or outcome of having an abnormal chromosome number (Fig. 12). Importantly, a cell can be aneuploid without being chromosomally unstable. For example, some aneuploid cells maintain a stable abnormal karyotype over time (van Jaarsveld & Kops, 2016).

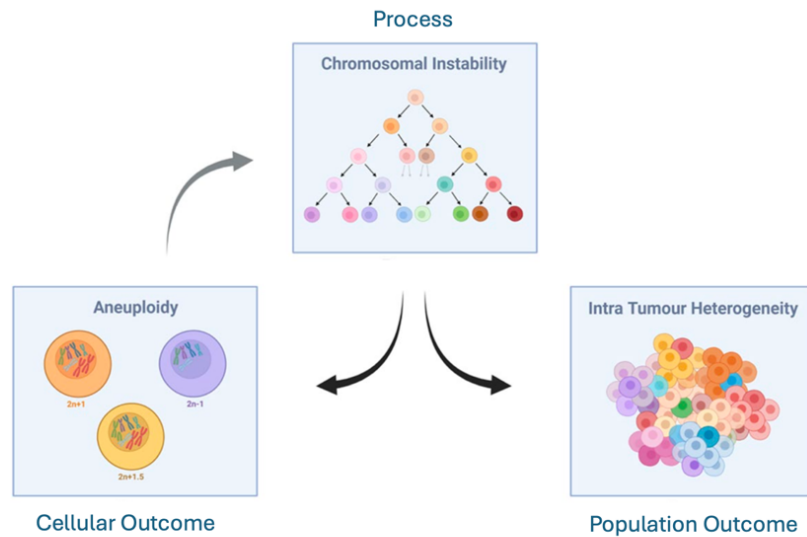


Figure 12. Chromosomal instability vs Aneuploidy. CIN is the process driving heterogeneous aneuploid populations and intratumor heterogeneity. While aneuploidy and Intra Tumour Heterogeneity represent cellular and population states, respectively, CIN generates them, and aneuploidy itself can further promote CIN, highlighting their interdependence. Adapted from (Andrade et al., 2023).

In embryonic tissues, very few forms of constitutional aneuploidy (present in all cells from birth) are compatible with life but are far less compatible with long-term survival. This highlights how disruptive aneuploidy can be for embryonic development. In adult tissues, aneuploidy can also occur in a mosaic or somatic fashion, meaning that only certain cells are affected. Early studies suggested that tissues such as the brain or liver might harbor a surprisingly high proportion of

aneuploid cells (Duncan et al., 2012; McConnell et al., 2013). However, more precise single-cell analyses have shown that aneuploidy is actually much rarer in healthy tissues than initially thought, indicating that cells with abnormal chromosome numbers are usually eliminated under normal physiological conditions (Knouse et al., 2014).

In contrast, cancer cells frequently tolerate or even thrive with aneuploidy. In fact, the presence of an abnormal number of chromosomes is considered one of the defining hallmarks of many tumors, particularly those that grow and divide rapidly (Taylor, Shih, Ha, Gao, Zhang, et al., 2018).

For the purposes of this manuscript, the term aneuploidy will be used in its more classical sense, referring specifically to changes involving whole chromosomes rather than partial alterations such as gains or losses of chromosome arms or smaller segments.

A. Origins of aneuploidy

Aneuploidy most often arises from chromosomal instability, and the primary source of this instability is mistakes that occur during mitosis. For this process to be accurate, several tightly controlled mechanisms

must work together in perfect coordination. When even one of these control systems fails, chromosomes can be unequally distributed, leaving one daughter cell with too many and the other with too few. Among the best-studied causes are, first, defects in the SAC, which normally functions as a safeguard to prevent premature chromosome separation (J. H. Teixeira et al., 2014). Second, problems in the attachment and stability of kinetochore-microtubule interactions, which are essential for guiding chromosomes to the correct poles (Tanaka & Zhang, 2022). Third, the weakening or loss of cohesion that normally holds sister chromatids together until the right moment (Moura-Castro et al., 2021). Fourth, errors in centrosome number or function can disrupt the formation of a bipolar spindle, further increasing the chance of chromosome mis-segregation (Levine & Holland, 2018). Fifth, beyond these mitosis-specific defects, it has become clear that broader cellular stresses can also promote chromosomal instability, thereby increasing the likelihood of aneuploidy (R. Li & Zhu, 2022).

1. Spindle assembly checkpoint defects

SAC serves as a key surveillance mechanism that preserves chromosomal stability during mitosis. By ensuring that all chromosomes are correctly attached to spindle microtubules before anaphase onset, the SAC prevents premature segregation and the formation of aneuploid daughter cells (Lima et al., 2025; Musacchio, 2015). This checkpoint functions as a molecular “brake”: when even a single kinetochore remains improperly attached, whether in monotelic, syntelic or merotelic configurations, it halts progression into anaphase (Fig. 13).

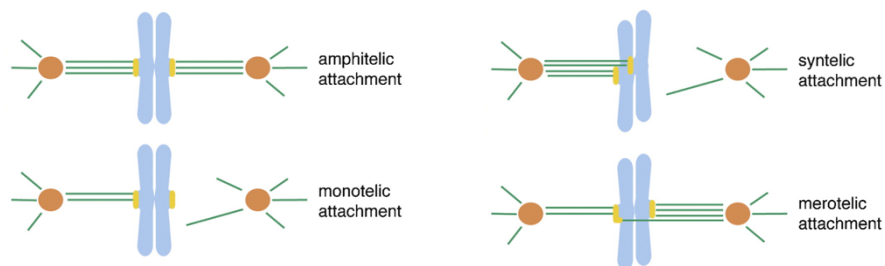


Figure 13. Schematic of kinetochore–microtubule attachments. Illustration of monotelic, amphitelic, and syntelic attachment states during mitosis. Amphitelic attachments are correct, whereas monotelic and syntelic configurations represent erroneous interactions. Adapted from (Kelly & Funabiki, 2009).

For accurate segregation, sister kinetochores must attach to microtubules coming from opposite spindle poles, a configuration called amphitelic attachment. If only one kinetochore is attached (monotelic) or both kinetochores connect to the same spindle pole (syntelic) or one of the sister kinetochores is attached to microtubules from both centrosomes (merotelic), the SAC remains active. The tension generated at properly attached kinetochores is sensed by the chromosome passenger complex (CPC), composed of Aurora kinase B, survivin, borealin and INCENP, which in turn promotes assembly of the mitotic checkpoint complex (MCC) (McVey et al., 2021).

The MCC, containing MAD2, BUBR1, BUB3 and sequestered CDC20, inhibits the anaphase-promoting complex (APC/C) to prevent premature chromatid separation (Sarkar et al., 2021). Once proper attachments are achieved, the SAC is silenced, allowing APC/C-CDC20 activation, which drives degradation of securin and cyclin B, thereby enabling chromatid separation and mitotic exit (D. Lu et al., 2014) (Fig. 14). When SAC components are weakened or deregulated, this control system becomes leaky, allowing cells to enter anaphase with misattached

chromosomes: a major route to chromosome mis-segregation and aneuploidy.

In normal cells the SAC is robust, whereas many cancers retain a weakened but still essential checkpoint that contributes to aneuploidy and tumour progression. This partial dependence of tumour cells on a compromised SAC provides a therapeutic window. The checkpoint can be exploited either by sustaining its activation to arrest mitosis, as achieved by taxanes, or by overriding it to induce catastrophic chromosome mis-segregation and cell death. Accordingly, small-molecule inhibitors of SAC kinases such as MPS1/TTK, Aurora B and Bub1 have been developed to trigger lethal chromosomal instability, with several candidates advancing into clinical trials. Notably, preclinical studies have also highlighted glioblastoma as a promising setting for SAC-directed therapies. Inhibition of key mitotic regulators linked to SAC function, such as Aurora kinases and Polo-like kinase 1 (PLK1), has been shown to profoundly reduce GBM cell proliferation, impair tumour growth in xenograft models and sensitise cells to

radiation and chemotherapy. (Lang & Yang, 2025; Van Brocklyn et al., 2014).

Although early ATP-competitive inhibitors suffered from off-target effects, next-generation compounds are being optimized to improve specificity and potency. These findings underscore the SAC as an attractive therapeutic target for selectively compromising mitotic fidelity in cancer cells while sparing normal cells.

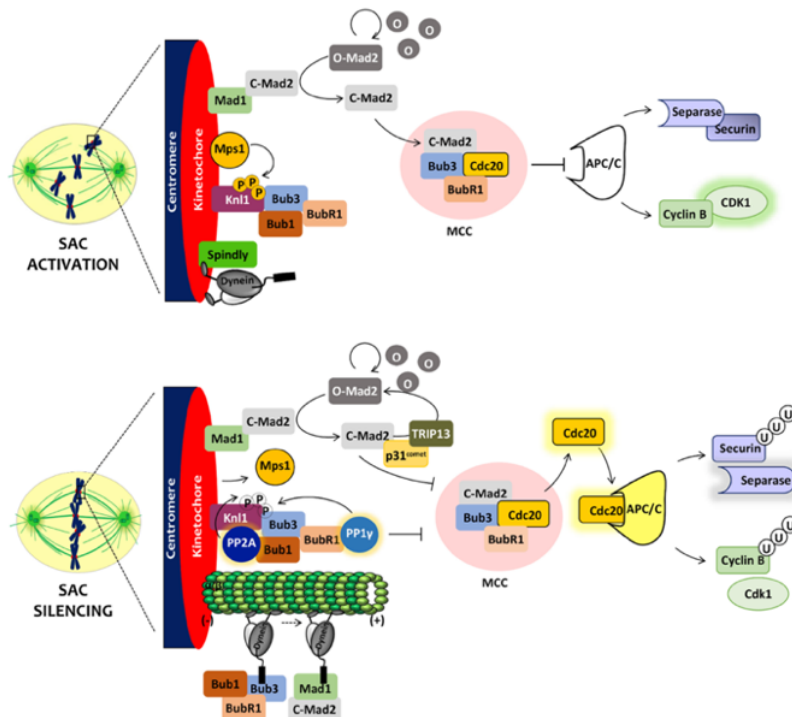


Figure 14. The spindle assembly checkpoint regulation. SAC prevents anaphase until all chromosomes are properly attached, then silences to activate APC/C and trigger mitotic exit. From (Henriques et al., 2019).

2. Errors in kinetochore-microtubule dynamics

The proper segregation of chromosomes depends on the accurate attachment of spindle microtubules to kinetochores, as already explained. This interaction is highly dynamic as MTs constantly undergo cycles of polymerization and depolymerization until they achieve the correct orientation (amphitelic attachment). However, not all attachment errors are equally well detected by the SAC. In merotelic attachment, and because this arrangement maintains tension at the kinetochore, it often escapes SAC surveillance. If not corrected, merotelic attachments can result in chromosomes lagging during anaphase, leading to mis-segregation (Fig. 13) (Gegan et al., 2011; Ye et al., 2019). In some cases, the lagging chromosome fails to reintegrate into the main nucleus after cell division and instead becomes encapsulated in a separate micronucleus, a structure strongly associated with genomic instability (Thompson & Compton, 2011).

The correction of merotelic attachments relies on the dynamic instability of kinetochore-microtubule interactions. In many cancer cells, however, these attachments become hyper-stabilized, meaning

that microtubules are less likely to detach once bound. While this increased stability might appear advantageous, it paradoxically reduces the cell's ability to repair faulty attachments. As a result, erroneous connections persist, leading to recurrent lagging chromosomes, micronuclei formation and ultimately chromosomal instability (Fig. 15)

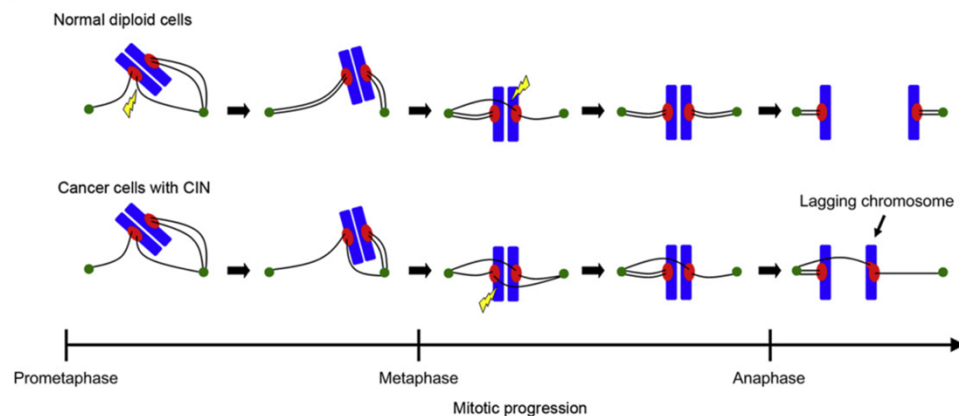


Figure 15. Hyperstable kinetochore-microtubule attachments promote chromosome missegregation in CIN cells. In normal cells, kinetochore-microtubule attachments are frequently released, allowing error correction before anaphase. In cancer cells with chromosomal instability, these attachments are overly stable, causing erroneous connections to persist and leading to lagging chromosomes during anaphase. Adapted from (Bakhoum et al., 2009).

This mechanism highlights why kinetochore-microtubule dynamics are not only essential for faithful segregation in normal cells but also a

critical contributor to aneuploidy and genome instability in cancer when their regulation is altered.

3. Cohesion loss between sister chromatids

The accurate segregation of chromosomes during mitosis relies on the cohesion that tightly links sister chromatids from the time they are duplicated (S-phase) until they are ready to separate (anaphase). This cohesion is mediated by cohesin complexes that act as a molecular “glue” holding the chromatids together. The cohesin complexes are only cleaved once every chromosome has achieved proper attachment and the SAC has been satisfied. This regulation ensures that chromatids are not pulled apart too early, which would inevitably lead to mis-segregation and aneuploidy (Nasmyth & Haering, 2009; Peters et al., 2008). Such premature cleavage of cohesins is most prominently observed during the maturation of oocytes, particularly in females of advanced maternal age. This phenomenon is considered one of the key explanations for the increased incidence of aneuploidy in the offspring of older mothers (Chiang et al., 2010; Lister et al., 2010).

Beyond reproduction, cohesion defects are also relevant in somatic cells and cancer biology. Subtle weakening of cohesion, through mutations and reduced expression of cohesion regulators can destabilize segregation during mitosis and contribute to chromosomal instability. Although not as well characterized as in oocytes, this mechanism provides yet another pathway by which cells can accumulate aneuploid karyotypes, ultimately fueling tumor evolution (De Koninck & Losada, 2016; Di Nardo et al., 2022).

4. Centrosome amplification and dysfunction

Centrosomes are the primary microtubule-organizing centers in animal cells, and they play an essential role in forming a bipolar spindle that ensures equal chromosome segregation during mitosis. Each cell normally duplicates its centrosome once per cell cycle, producing exactly two centrosomes that define the two spindle poles. When this process is disrupted, however, cells can end up with supernumerary centrosomes, a phenomenon frequently observed in cancer (Godinho, 2014; Mittal et al., 2021). Additional centrosomes can arise from failed

cytokinesis, cell fusion or errors in centrosome duplication (Kalkan et al., 2022) . Cells with supernumerary centrosomes tend to form multipolar spindles, which cause chaotic chromosome segregation and usually produce non-viable progeny. To survive, cancer cells often cluster extra centrosomes into two poles, creating a pseudo-bipolar spindle. This strategy permits division but increases the risk of erroneous attachments, especially merotelic ones, thereby fueling chromosomal instability (Ganem et al., 2009; Kiermaier et al., 2024). Even when centrosome number is normal, abnormal timing of centrosome separation can disrupt spindle geometry. Both premature and delayed separation promote faulty attachments and chromosome mis-segregation (Nam et al., 2015).

5. Cellular Stresses

While many causes of aneuploidy stem directly from defects in the mitotic machinery, a wide range of cellular stresses can also compromise the fidelity of chromosome segregation. These stresses

interfere with processes upstream or parallel to mitosis, creating conditions that favor chromosomal instability (Giam & Rancati, 2015).

One major contributor is DNA replication stress. If cells enter mitosis before replication is fully and properly completed, unresolved DNA structures can persist between sister chromatids. These manifest as DNA bridges during anaphase, which often break or mis-segregate, thereby generating chromosome abnormalities and fueling chromosomal instability (Giam & Rancati, 2015).

Another source of stress is oxidative stress, caused by an excess of reactive oxygen species (ROS). ROS can weaken sister chromatid cohesion and promote premature chromatid separation. In mitosis, this loss of cohesion increases the probability of mis-attachments and erroneous segregation (Perkins et al., 2016; van Schie & de Lange, 2021).

Proteotoxic stress also slows proliferation and perturbs mitosis, thereby increasing the chance of chromosome mis-segregation. Numerical chromosome changes shift gene and protein dosage on a large scale, overloading protein quality-control systems that are unable to fully restore balance (Pavelka et al., 2010). This

imbalance induces endoplasmic reticulum (ER) stress and activates the unfolded protein response (UPR). In cells with CIN and aneuploidy, the excess or misfolded proteins overwhelm the ER folding capacity, leading to activation of the UPR. Sustained UPR activation can cause cell-cycle arrest or if damage is irreparable, trigger apoptosis (Almanza et al., 2019).

A last example is mechanical stress from the cellular environment that can influence chromosome segregation. When cells grow in conditions of strong physical confinement that alter their shape, the geometry of spindle assembly is disturbed. This distorted spindle architecture increases the frequency of incorrect kinetochore-microtubule attachments and ultimately contributes to chromosomal instability (Bastianello et al., 2024; Lancaster et al., 2013).

B. Aneuploidy as a hallmark of cancer

Although aneuploidy generally reduces cellular fitness and slows proliferation in normal contexts, it is strikingly prevalent in cancer. Large-scale genomic studies show that nearly 90% of human tumors

harbor some form of aneuploidy (Taylor, Shih, Ha, Gao, Meyerson, et al., 2018). This paradox, how a process typically detrimental to normal cells becomes so common in malignant ones, remains an active area of research. Current evidence suggests that cancer cells develop mechanisms to tolerate and even exploit the stresses associated with aneuploidy. For example, loss of p53 function allows cells with abnormal karyotypes to bypass growth arrest or apoptosis (M. Li et al., 2010), while tetraploidy provides a genomic “buffer” that increases tolerance for additional chromosomal changes (Fig. 16) (Bielski et al., 2018).

In the following subsections, we will examine how aneuploidy contributes to tumor initiation, fuels progression and influences treatment response.

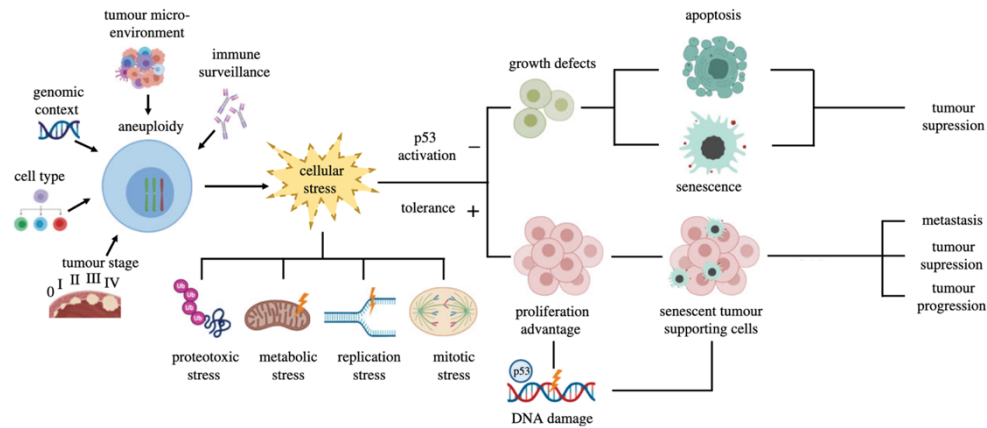


Figure 16. Context-dependent effects of aneuploidy in cancer. Aneuploidy induces multiple forms of cellular stress that activate checkpoint pathways such as p53. In cells with intact stress responses, this leads to growth arrest, apoptosis or senescence and tumor suppression. In contrast, stress-tolerant aneuploid cells can maintain proliferation despite DNA damage, thereby contributing to tumor progression and metastasis. Adapted from (Zhou et al., 2020).

1. Early steps in tumor initiation

Several mechanisms have been proposed to explain how aneuploidy can act as an initiating force in cancer. One important route is through loss of heterozygosity. When a cell already carries one mutated allele of a tumor suppressor gene, aneuploidy may remove the remaining normal allele, thus completing the “second hit” required for tumorigenesis. An example is found in colonic tumors, where cells lost the chromosome 18 copy carrying the normal *Apc* (a tumor suppressor

gene) allele and simultaneously gained an extra copy containing the mutant *Apc* allele (Baker & van Deursen, 2010). A second mechanism involves gene dosage imbalances. Gains or losses of specific chromosomes alter the copy number of multiple oncogenes and tumor suppressors at once. Aneuploidy is thought to promote tumour initiation and progression by allowing cells to proliferate despite chromosomal imbalances (Soto et al., 2017).

By fostering genomic instability, it may also accelerate the accumulation of mutations, promoting intratumoral heterogeneity and providing adaptive advantages under adverse conditions such as nutrient deprivation, growth factor withdrawal or exposure to cytotoxic agents like chemotherapy and radiotherapy (A. J. X. Lee et al., 2011). In GBM, for instance, chromosome 7 gain (which amplifies the oncogene *EGFR*) and chromosome 10 loss (which reduces the tumor suppressor gene *PTEN*) are frequent early events and provide strong growth advantages to affected cells (Körber et al., 2019). Conversely, pushing chromosomal imbalance beyond tolerable limits can trigger cell death

and exert a tumour-suppressive effect (Silk et al., 2013; Zasadil et al., 2016).

The observation of aneuploidy in precancerous lesions, such as lung carcinoma in situ, suggests that chromosomal imbalances may arise very early in the transformation process and contribute directly to malignant initiation (V. H. Teixeira et al., 2019). The effects of aneuploidy depend on the degree of CIN and the tissue type. Low or moderate levels of CIN may generate aneuploid cells that survive long enough to accumulate further oncogenic changes, promoting transformation. In contrast, very high levels of CIN can be toxic, preventing clonal expansion of pre-neoplastic cells (Zhou et al., 2020).

2. Advanced steps in tumor progression

Although aneuploidy can be detected during tumorigenesis, it is generally more frequent in advanced cancers, pointing to an important role in tumor progression. For example, in breast cancer, metastatic lesions tend to harbor a higher burden of aneuploidy compared with the primary tumor (Bakhoun et al., 2018). In clear-cell renal cell carcinoma,

recurrent loss of chromosomes 9p and 14q is strongly linked to metastatic disease, indicating that certain chromosomal imbalances can actively drive tumor spread (Turajlic et al., 2018). For instance, in colon cancer cells, gain of chromosome 5 induced a more invasive phenotype by suppressing epithelial gene expression while upregulating metalloproteinases, enzymes that promote tissue invasion (Vasudevan et al., 2020). Such findings indicate that selected chromosomal alterations can directly drive metastatic behavior rather than only occurring as byproducts of tumor evolution.

Chromosomal instability may also facilitate metastasis by creating a constant pool of karyotypic diversity. This heterogeneity increases the likelihood that some cancer cells will acquire traits that allow them to overcome the selective pressures of the metastatic cascade (Bakhoum et al., 2018). Aneuploidy and CIN create genetic diversity that enables cancer cells to adapt to environmental constraints sustaining tumor evolution.

C. Influence on resistance mechanisms

CIN and aneuploidy are frequently linked to poor treatment outcomes in many cancer types, but their relationship with therapy response is complex (Ben-David & Amon, 2020).

On one hand, aneuploidy can generate extensive genetic heterogeneity within tumors, increasing the likelihood that cells will survive therapeutic pressure allowing resistance. Consistently, cancers harboring CIN show greater resistance to a variety of treatments, with resistance often tied to the selection and enrichment of karyotypes that provide a survival advantage (Comaills & Castellano-Pozo, 2023; Ippolito et al., 2021). Aneuploid cells may also resist treatment through cell cycle alterations. Because they typically proliferate more slowly (Zerbib et al., 2024), they can be less sensitive to drugs that target rapidly dividing cells, such as cisplatin (Replogle et al., 2020).

On the other hand, certain therapies exploit CIN by pushing chromosomal segregation errors beyond tolerable limits. For example, treatments such as paclitaxel (Zasadil et al., 2014), which perturbs

microtubule dynamics, or irradiation (Bakhoum et al., 2015), which induces segregation defects, can further elevate chromosome mis-segregation. In cancer cells that already exhibit high CIN, this additional burden can surpass tolerable thresholds, triggering lethal stress and sensitizing them to therapy rather than promoting resistance.

Overall, the impact of CIN and aneuploidy on therapy response depends on context: chronic CIN often promotes resistance, whereas acute, therapy-induced bursts of CIN can sensitize tumors to treatment. Understanding the timing, intensity and cellular context of CIN and aneuploidy will be critical for exploiting these processes therapeutically.

CHAPTER 3

DNA Damage Response and Cell Cycle Regulation

Cells invariably suffer DNA damage arising from exogenous insults (radiation, chemical agents) and endogenous sources (replication errors, oxidative stress). To maintain genomic integrity, cells activate the DNA Damage Response (DDR), a network of sensing, signaling and effector mechanisms that detect lesions, pause the cell cycle and either initiate repair or, when damage is excessive, trigger apoptosis (Carusillo & Mussolino, 2020a). Among the repair systems are multiple pathways specialized for different lesion types, each critical to preventing mutagenesis. Aneuploidy is strongly linked to DNA damage, since errors in chromosome segregation often create lagging chromosomes or micronuclei that are prone to breakage and faulty replication. These events can generate complex rearrangements and activate p53 pathways that induce cell cycle arrest in a normal setting (Janssen et al., 2011; J. Zhu et al., 2018). In this chapter, we will first describe how DDR detects and signals DNA damage, then review the major repair pathways and their implications in cancer, examine the

interplay between aneuploidy and DNA damage and finally, explore how checkpoints regulate cell cycle progression (Fig. 17).

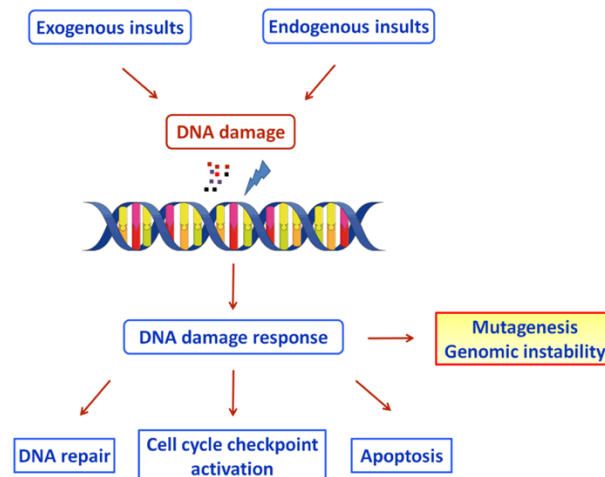


Figure 17. Overview of the DNA damage response (DDR). Exogenous and endogenous insults generate DNA damage, which activate the DDR. This signaling network coordinates DNA repair, cell cycle checkpoint and apoptosis to safeguard genome integrity. From (Papanikolaou et al., 2022).

A. The DNA damage response (DDR)

The DDR is a highly conserved surveillance network that preserves genomic stability by coupling the recognition of DNA lesions to downstream cellular programs (Jackson & Bartek, 2009). At the core of the DDR, there are sensors that detect structural abnormalities in DNA and initiate signaling cascades. Through lesion-specific cascades, the DDR mobilizes repair factors and enforces cell-cycle checkpoints and,

when damage exceeds repair capacity, activates apoptosis or senescence. In doing so, the DDR maintains the fidelity of genetic information over time and limits the mutagenesis and chromosomal instability that drive cancer (Carusillo & Mussolino, 2020).

1. Sensors and signaling

The initiation of the DDR depends on specialized sensor complexes that recognize abnormal DNA structures and recruit upstream kinases to the site of damage.

Single-strand breaks (SSBs) generated during replication stress is rapidly coated by replication protein A (RPA), which facilitates the recruitment of the kinase ATR and its partner ATRIP. In parallel, the KU70/80 heterodimer detects DNA ends and recruits DNA-PKcs, forming the DNA-PK holoenzyme that functions both as a sensor and as a signaling hub in the context of non-homologous end joining (NHEJ) kind of repair (Blackford & Jackson, 2017).

Double-strand breaks (DSBs) can arise when unrepaired SSBs occur in close proximity or are encountered by the replication machinery, converting them into more lethal DNA lesions. DSBs are detected

primarily by the MRE11-RAD50-NBS1 (MRN) complex, which serves as a platform for the recruitment and activation of ATM, initiating a phosphorylation cascade at the lesion (Ciccia & Elledge, 2010) (Fig. 18). Importantly, the organization of these signaling cascades ensures that even limited DNA damage is rapidly detected and translated into a robust cellular response that maintains genomic integrity.

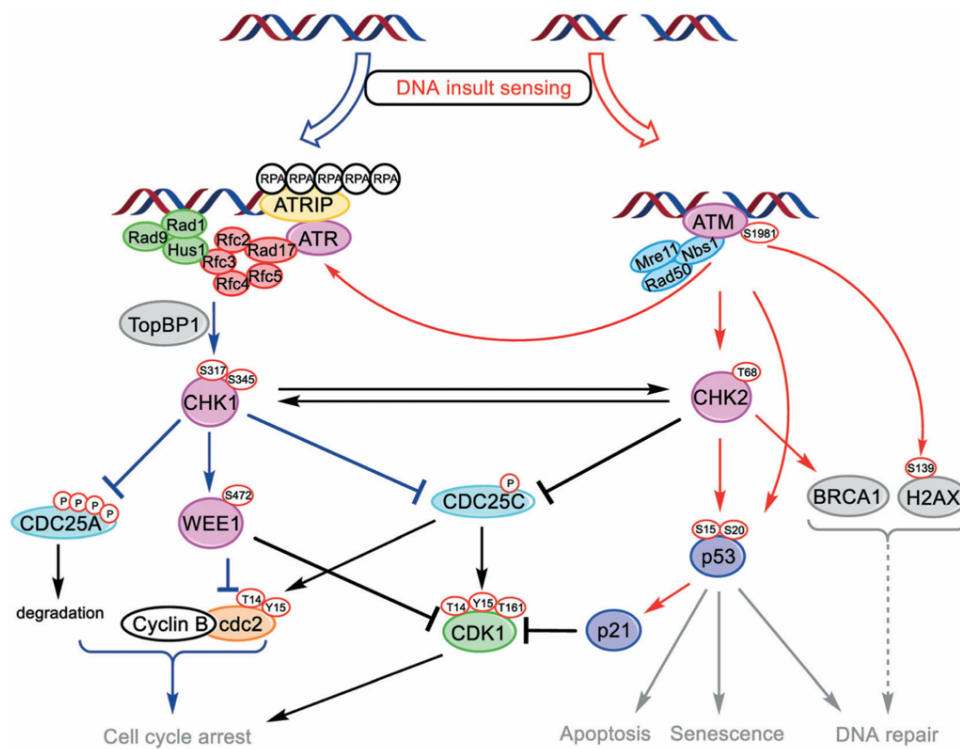


Figure 18. DDR signaling cascade. ATM and ATR kinases activate CHK1/CHK2 and phosphorylate mediators such as H2AX and BRCA1, leading to cell-cycle arrest, DNA repair, apoptosis or senescence. Adapted from (Ronco et al., 2016).

2. Transducers and effectors

Once activated, the apical kinases ATM, ATR and DNA-PKcs phosphorylate a wide range of substrates, including the histone variant H2AX (γ H2AX), which spreads around the lesion and creates a permissive chromatin environment for mediator proteins such as MDC1, 53BP1 and BRCA1 to assemble at damage sites (Ciccio & Elledge, 2010).

These mediators amplify the initial signal and form foci that concentrate DNA repair factors at the site of damage, thereby ensuring efficient recognition and processing of the lesion. A key step in the phosphorylation network involves the checkpoint kinases CHK1 and CHK2, which are phosphorylated by ATR and ATM, respectively, and act as central transducers to relay signals from the damage site to downstream effectors (Ciccio & Elledge, 2010; Jackson & Bartek, 2009). Through inhibition of CDC25 phosphatases, CHK1/CHK2 suppress cyclin-dependent kinase (CDK) activity, thereby enforcing cell-cycle arrest at both the G1/S and G2/M transitions.

At the effector level, the tumor suppressor p53 plays a pivotal role in integrating DDR signals. Activated p53 induces transcriptional programs

that promote DNA repair, transient cell-cycle arrest or in the case where damage is irreparable, the initiation of apoptosis (Roos & Kaina, 2006).

These responses are highly context-dependent: limited DNA damage typically triggers temporary arrest to allow repair and subsequent recovery, whereas extensive or persistent damage results in terminal outcomes that eliminate genetically compromised cells. Thus, DDR effectors represent the decision-making branch of the pathway, linking DNA damage sensing to adaptive or terminal fates, thereby maintaining genome stability and serving as a critical barrier to tumorigenesis (Fig. 16).

B. DNA repair

To preserve genome integrity, cells rely on DNA repair systems that continuously monitor and resolve a wide variety of lesions. These mechanisms are highly conserved and operate with remarkable precision, ensuring that both small base modifications and large-scale chromosomal breaks can be faithfully corrected. Efficient repair is essential to maintain cellular function and prevent malignant transformation, whereas impaired repair promotes the accumulation of

mutations and chromosomal rearrangements that fuel tumorigenesis (Clarke & Mostoslavsky, 2022).

1. Repair pathways

Multiple repair pathways have evolved to address DNA lesions. DSBs represent one of the most cytotoxic forms of DNA damage and are repaired mainly through NHEJ and homologous recombination (HR). NHEJ, mediated by the KU70/80 heterodimer and DNA-PKcs, directly ligates broken DNA ends and is active throughout the cell cycle, but its error-prone nature often results in insertions or deletions at the repair site (Chang et al., 2017). In contrast, HR provides high-fidelity repair by using the sister chromatid as a template but is restricted to the S and G2 phases when a homologous sequence is available. HR involves complex machinery, including RAD51, BRCA1/2 and the MRN complex (Ciccica & Elledge, 2010).

Other specialized repair pathways resolve less severe but more frequent lesions. Base excision repair (BER) recognizes single-base modifications, such as oxidative or alkylation damage, through DNA

glycosylases that removes damaged bases, followed by gap filling by DNA polymerase β and ligation (Dianov, 2011; Hoeijmakers, 2001). Nucleotide excision repair (NER) eliminates helix-distorting DNA lesions through damage recognition, local unwinding by TFIIH, dual incision flanking the lesion, and gap filling by DNA polymerases (Zachary et al., 2025). Mismatch repair (MMR) is responsible for fixing small errors that occur during DNA replication, mainly base-base mismatches and insertion/deletion loops. The system works by detecting the error using MutS and MutL homologs, removing the faulty DNA stretch, and resynthesizing the correct sequence (G.-M. Li, 2008). Collectively, these pathways provide overlapping and complementary safeguards, ensuring that genomic information is maintained with high fidelity (Fig 19).

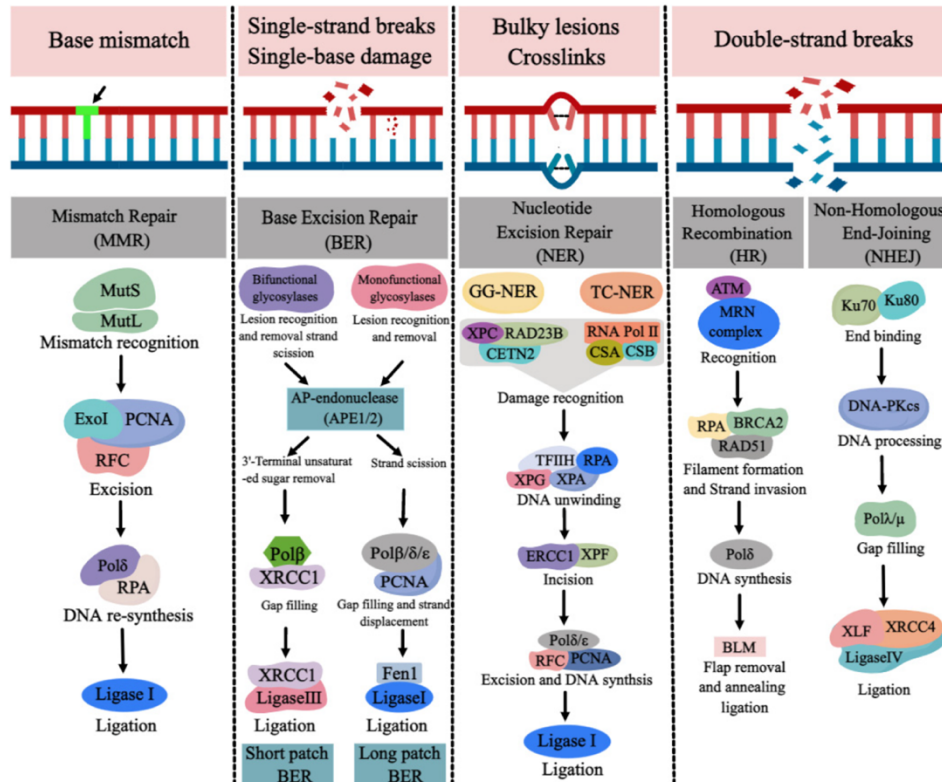


Figure 19. Major DNA repair pathways. Different types of DNA damage are corrected by distinct repair mechanisms: base mismatches by mismatch repair (MMR), single-base damage and single-strand breaks by base excision repair (BER), bulky adducts and crosslinks by nucleotide excision repair (NER), and double-strand breaks by homologous recombination (HR) or non-homologous end joining (NHEJ). From Scholarly Community Encyclopedia.

2. Implication in cancer

Defects in DNA repair systems are a major source of genomic instability and a defining feature of cancer. NHEJ acts as both a guardian and a disruptor in cancer, suppressing tumorigenesis by repairing DNA

double-strand breaks but, when deregulated or acting in the absence of other repair pathways, promoting genomic instability and carcinogenesis (Perry et al., 2017).

Inherited mutations in HR factors, particularly BRCA1 and BRCA2, underlie hereditary breast and ovarian cancers, illustrating how defective high-fidelity repair accelerates mutagenesis (Raimundo et al., 2021).

In cancer, BER defects foster mutagenesis and tumorigenesis with many tumors upregulating BER to survive oxidative and therapy-induced damage. Accordingly, pharmacologic BER inhibition can overwhelm repair capacity, enhance radio/chemosensitivity or be synthetically lethal (Grundy & Parsons, 2020).

Defects in NER cause disorders like Xeroderma Pigmentosum, characterized by extreme UV sensitivity and early-onset skin cancer, highlighting the protective role of this pathway against environmental carcinogens (Schärer, 2013).

When MMR is defective, these mistakes accumulate across the genome, leading to microsatellite instability and a higher risk of cancers such as colorectal and endometrial tumors (Haraldsdottir et al., 2014).

Impaired repair pathways contribute to ongoing tumor heterogeneity, fostering adaptation under selective pressures. Thus, DNA repair plays a dual role in cancer: its failure drives tumorigenesis, but it also provides powerful leverage points for therapy.

C. Crosstalk between Aneuploidy and DNA Damage

Aneuploidy is a hallmark of many human cancers and functions both as a cause and a consequence of genomic instability. While it often originates from chromosome segregation errors during mitosis, the resulting karyotypic imbalance imposes metabolic and replicative stress that exacerbates DNA damage and fuels further instability. In this way, aneuploidy and DNA damage are not independent events but part of a reinforcing cycle, where chromosomal missegregation produces

structural lesions, while unbalanced genomes generate replication stress that sustains chronic DDR activation (J. Zhu et al., 2018).

A central link in this cycle lies in the process of mitosis, where accurate chromosome segregation is critical to genome stability. When this process fails, errors in chromosome distribution not only alter chromosome number but also create direct structural threats to DNA integrity. For example, lagging chromosomes, which typically arise from merotelic attachments, frequently evade correction by the SAC and persist into anaphase. As cells progress through division, these lagging chromosomes are pulled in opposite directions, exposing them to mechanical stretching forces that can physically shear chromatin fibers and generate DSBs (Bakhoum et al., 2009, 2018; Janssen et al., 2011).

The consequences of mitotic errors extend even further into cytokinesis, where chromatin bridges form between daughter cells. These bridges, unable to be properly resolved, are especially fragile. They can be broken by mechanical tension or membrane scission, ultimately generating chromosome fragments and DSBs that

are transmitted to progeny cells (Hayashi & Karlseder, 2013; Petsalaki & Zachos, 2021).

As a result, missegregated chromosomes become not only a numerical aberration but also a structural source of genome instability, thereby amplifying the cycle between aneuploidy and DNA damage (Fig. 20).

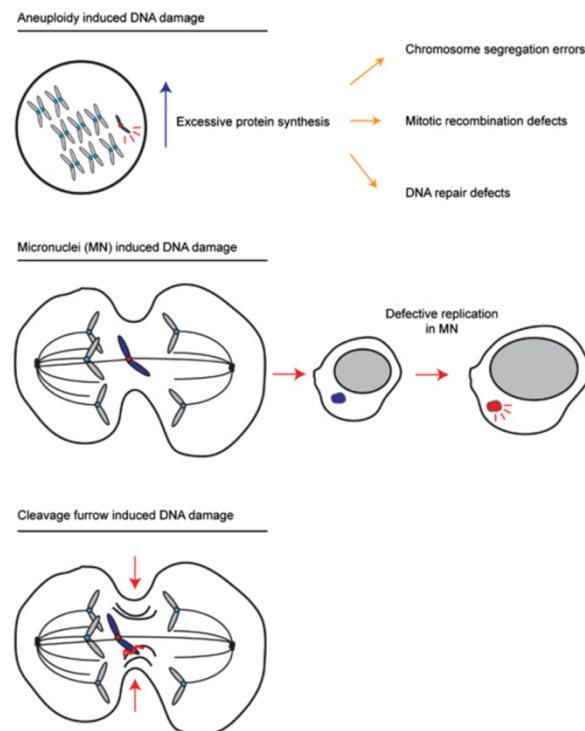


Figure 20. Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. Aneuploidy-associated stresses disrupt chromosome segregation and repair, while missegregated chromosomes form micronuclei with defective replication. Chromatin bridges can also break during cytokinesis. These events convert segregation errors into structural DNA damage. Adapted from (Janssen & Medema, 2013).

D. Cell Cycle Checkpoints

Cell cycle checkpoints are surveillance mechanisms that ensure the orderly progression of the cell cycle and protect genome integrity under conditions of stress. These control systems monitor the status of DNA replication, chromosome attachment to the spindle and centrosome integrity, delaying cell cycle transitions until problems are resolved. Checkpoints act as a critical barrier to the propagation of DNA damage and chromosomal instability, and their malfunction is a hallmark of many cancers (Song et al., 2024).

1. G2/M regulation

Beyond its canonical role in pausing mitotic entry after DNA damage, the G2/M checkpoint is a highly dynamic control system that balances the need for repair with the necessity of proliferation. Once repair is achieved, cells must actively recover from checkpoint arrest by reactivating CDK1-cyclin B complexes, a process regulated by phosphatases such as CDC25 (Ciccia & Elledge, 2010; Syljuåsen et al., 2006). In contrast, if DNA lesions persist for too long, cells may

undergo checkpoint adaptation, where they slip into mitosis despite unrepaired damage (mitotic slippage), often resulting in chromosome missegregation and further genomic instability (Kalsbeek & Golsteyn, 2017; Sinha et al., 2019).

In cancer, deregulation of G2/M control frequently reflects this altered balance. Tumor cells often bypass sustained arrest by attenuating p53 signaling, enabling proliferation despite persistent DNA lesions. This not only fosters chromosomal instability but also impacts therapy responses, as G2/M checkpoint defects can sensitize cells to agents that induce replication stress while conferring resistance to others (Fig. 21) (Jung et al., 2021; Smith et al., 2020).

Thus, the G2/M checkpoint is not only a barrier to mitotic entry but also a flexible decision-making node whose deregulation in cancer shifts the balance toward genome instability.

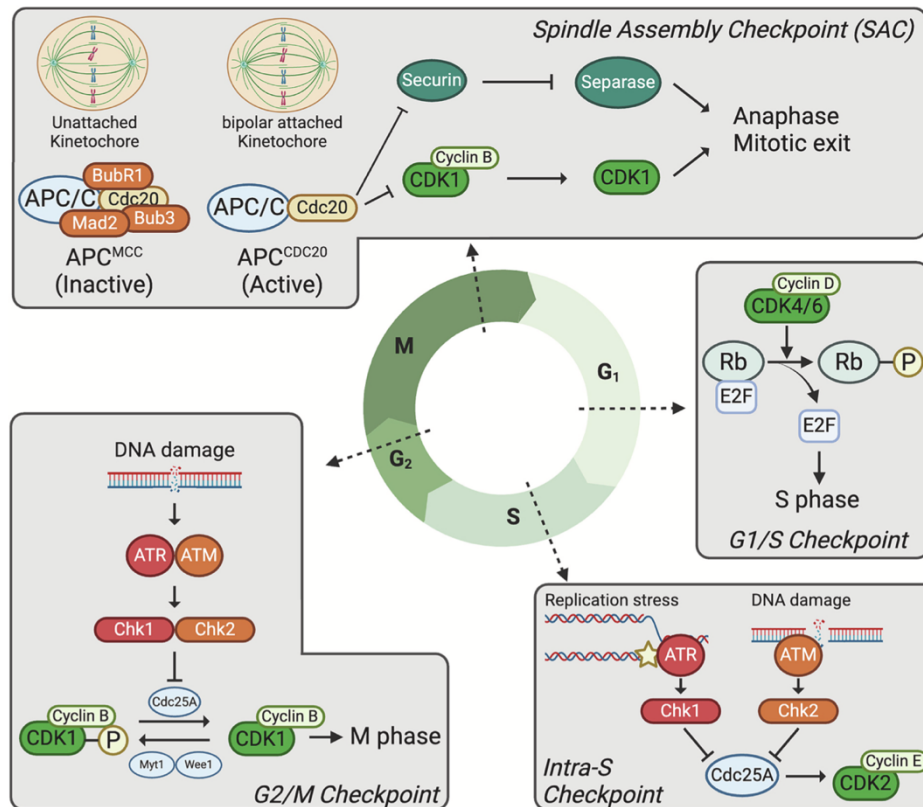


Figure 21. Overview of major cell-cycle checkpoints. Diagram summarizing the G₁/S, intra-S, G₂/M, and spindle assembly checkpoints. These pathways coordinate CDK activity, DNA repair and chromosome attachment to ensure proper DNA replication and accurate chromosome segregation before cell-cycle progression. Adapted from (Lang & Yang, 2025).

2. Other cell cycle checkpoints

In addition to the G₂/M transition, several other checkpoints play essential roles in guarding genome integrity and are frequently disrupted in cancer.

The G1/S checkpoint is critical for preventing replication of damaged DNA. Activated primarily through the p53/p21 axis, it inhibits CDK2 activity, thereby delaying entry into S phase until damage is repaired (Visconti et al., 2016). Inactivation of this checkpoint is a defining feature of many cancers, most notably through p53 mutations or retinoblastoma (RB) pathway disruption, allowing cells to replicate damaged genomes unchecked (Fig. 21) (Al Bitar & Gali-Muhtasib, 2019).

The intra-S checkpoint functions during DNA replication to stabilize replication forks when lesions or replication stress is detected. This checkpoint is largely mediated by ATR and CHK1, ensuring replication is coordinated with repair. Its impairment leads to fork collapse and accumulation of DSBs, driving chromosomal instability and therapy resistance (Fig. 21) (Iyer & Rhind, 2017; Smith et al., 2020).

Finally, the SAC, explained earlier, operates later in mitosis to ensure that chromosomes are properly attached to spindle microtubules before segregation. Defects in SAC components such as MAD2 and BUBR1 weaken the fidelity of chromosome segregation, promoting aneuploidy and tumor evolution (Musacchio, 2015).

CHAPTER 4

Glioblastoma

A. General overview

GBM is the most frequent and aggressive malignant primary brain tumor in adults and represents the prototypical high-grade glioma in neuro-oncology (Ostrom et al., 2019). Originating from glial cells, GBM is characterized by diffuse infiltration into surrounding brain parenchyma, remarkable cellular and molecular heterogeneity and resistance to therapy, which together account for its poor prognosis (Rabah et al., 2023).

For decades, GBM was defined primarily by histological criteria such as hypercellularity, nuclear atypia, necrosis and microvascular proliferation (Fig. 22) (Louis et al., 2016).

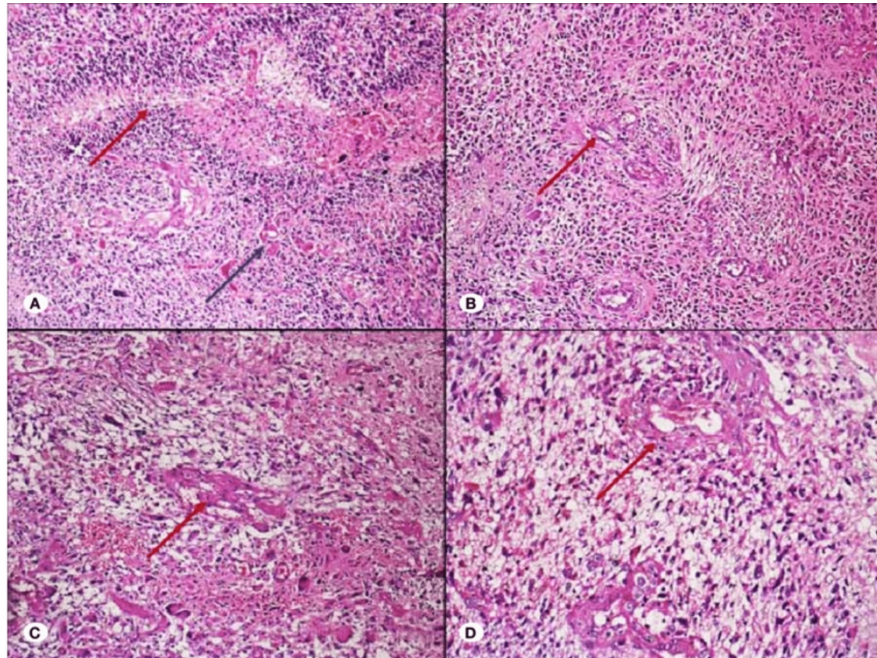


Figure 22. Histopathological features of glioblastoma. Glioblastoma is an aggressive tumor composed of pleomorphic astrocytic cells with marked nuclear atypia and mitosis. A) Palisading necrosis (red arrow) and microvascular proliferation (blue arrow) are important characteristic features of GBM (arrow) (x10). B) Marked nuclear atypia and classical type vascular proliferation (arrow) (x10). C, D) Bizarre vascular pattern with vascular clusters, vascular garlands and glomeruloid vascular formations (arrows) (C:x20, D:x40) (Hematoxylin eosin stain). Adapted from (Tastekin et al., 2016).

However, extensive molecular research has revealed that these tumors encompass distinct biological entities with divergent clinical courses (Brat et al., 2020). This has led to a shift from morphology-based classification toward integrated histo-molecular diagnosis, culminating in the 2021 WHO classification that redefined GBM strictly as an IDH-

wildtype astrocytic tumor in adults, namely high-grade glioma (HGG) (Louis et al., 2021). This update reflects the central role of molecular features in guiding diagnosis, prognosis and treatment, and it emphasizes GBM as a distinct and coherent biological entity.

1. WHO classification

The classification of GBM has undergone substantial refinement as molecular profiling has reshaped the understanding of diffuse gliomas. Historically, GBM was subdivided into IDH-wildtype or “primary GBM,” arising de novo in older patients with a poor prognosis, and IDH-mutant, or “secondary GBM,” developing from lower-grade gliomas in younger patients with a more favorable outcome (Louis et al., 2016). While both groups shared similar histological features, studies demonstrated that they were biologically distinct, with divergent genetics, clinical behavior and therapeutic responses. These findings led to the recommendations of the cIMPACT-NOW consortium, which recognized that IDH-mutant diffuse astrocytomas should not be considered glioblastomas regardless of grade (Brat et al., 2018, 2020).

The WHO CNS5 classification (2021) of gliomas introduced an integrated molecular framework for adult-type diffuse gliomas, grouping them into three main diagnostic entities according to IDH mutation status and 1p/19q codeletion: Astrocytoma: IDH-mutant, Oligodendroglioma: IDH-mutant and 1p/19q-codeleted, and Glioblastoma: IDH-wildtype. Within IDH-mutant gliomas, ATRX loss is a defining molecular feature of astrocytomas and is mutually exclusive with 1p/19q codeletion, thereby helping to distinguish astrocytoma, IDH-mutant from oligodendroglioma, IDH-mutant. Within this scheme, GBM is strictly defined as an IDH-wildtype astrocytic tumor in adults, diagnosed either by the presence of classical histological hallmarks such as microvascular proliferation and necrosis or by the identification of defining molecular alterations such as EGFR amplification (Fig. 23) (Brat et al., 2018; Louis et al., 2021). This integrated approach ensures that GBM is recognized based on its biological signature rather than morphology alone, enabling more consistent prognostication and therapeutic planning.

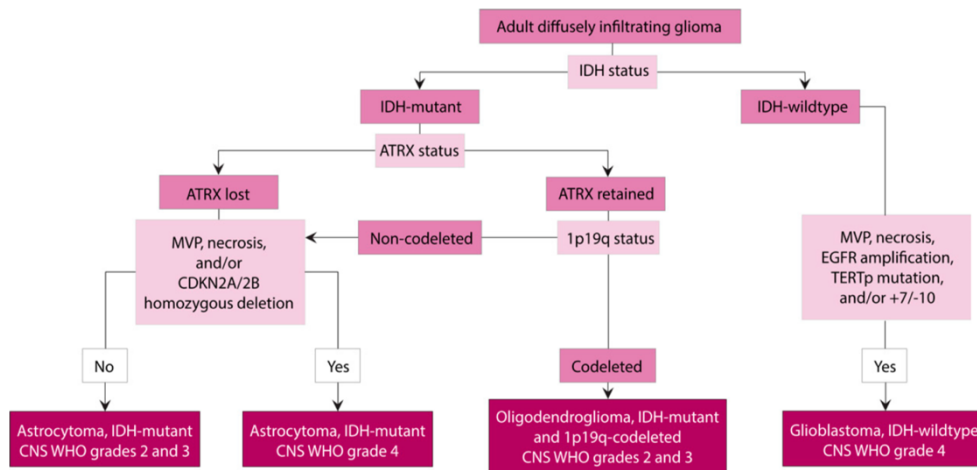


Figure 23. Diagnostic algorithm of adult diffusely infiltrating gliomas with integration of morphologic and molecular characteristics. Once a diffusely infiltrating glioma is identified on H&E stained sections, the tumour must be assessed for the presence of an *IDH1/2* mutation. In IDH-mutant gliomas, ATRX is lost in tumour cells, this is consistent with astrocytoma. Additionally, grade 4 tumours are identified by the presence of microvascular proliferation (MVP), necrosis, and/or *CDKN2A/2B* homozygous deletion. If ATRX nuclear expression is retained, an assessment of 1p19q status should be pursued. If whole arms of chromosomes 1p and 19q are codeleted, the diagnosis is oligodendroglioma, IDH-mutant and 1p19q-codeleted. In non-codeleted tumours, the final diagnosis is astrocytoma, IDH-mutant. In IDH-wildtype diffusely infiltrating gliomas, the presence of MVP, necrosis, *EGFR* amplification, *TERT* promoter (*TERTp*) mutation, and/or combined whole chromosome 7 gain and whole chromosome 10 loss (+7/-10) defines a glioblastoma, IDH-wildtype. Adapted from (Martin et al., 2023).

2. Epidemiology and prognosis

Glioblastoma accounts for approximately 15% of all primary central nervous system (CNS) tumors and nearly 50% of malignant CNS tumors

(Ostrom et al., 2019). Its incidence in Belgium corresponds to around 6.72 per 100 000 person/year and leading to about 15,000 deaths per year in Europe (Pinson et al., 2023). Although rare inherited cancer predisposition syndromes may lead to GBM, the majority of cases are sporadic. Established risk factors are limited, with head irradiation increasing incidence, whereas epidemiological data suggest that allergies and atopic conditions reduce risk (Ostrom et al., 2019; Z. Pan et al., 2025).

Clinically, GBM typically presents with neurological symptoms reflecting its infiltrative growth pattern and tumor location, most commonly in the cerebral hemispheres. Patients may present with new-onset seizures, focal neurological deficits such as aphasia or hemiparesis, cognitive or personality changes, or symptoms of raised intracranial pressure including persistent headache, nausea and vomiting (Oronsky et al., 2021; Sipos et al., 2025). Prognosis remains dismal: it is a dreaded cancer with a median survival of only 12-15 months and virtually all patients suffer fatal relapse following re-growth of the tumour (Koshy et al., 2012). Prognostic factors include patient

age, performance status, extent of surgical resection and MGMT promoter methylation status. But even in favorable groups, recurrence is nearly universal, and five-year survival rates remain below 10% (Fekete et al., 2023; Ostrom et al., 2019). These sobering statistics illustrate the biological aggressiveness of GBM and the urgent need for more effective therapies.

B. Molecular and cellular features

1. Molecular and genetic drivers

GBM is driven by recurrent disruptions of a limited number of pathways that together define its malignant behavior. Large-scale genomic studies, including the TCGA project, identified core signaling axes as nearly universally affected: the RTK/PI3K pathway, the p53 pathway and the RB pathway (Fig. 24) ("Cancer Genome Atlas Research Network", 2008).

The RTK pathway's most alarming alterations is the amplification and mutation of EGFR occurring in ~40% of cases, often combined with PTEN

loss or PI3K mutations, sustaining proliferative and survival signaling. p53 pathway alterations were detected in about 85% of GBMs, most often through CDKN2A loss, *p53* mutations or deletions or MDM family amplification. These changes were largely mutually exclusive.

Similarly, nearly 80% of tumors showed disruption of the RB pathway, either through CDKN2A deletion, CDK4/6 amplification or direct RB1 mutation/deletion (Brennan et al., 2013). In addition to EGFR amplification, molecular hallmark alterations such as TERT promoter mutations and the combined whole chromosome 7 gain and whole chromosome 10 loss (+7/-10) pattern occur in the majority of IDH-wild-type GBMs, providing early oncogenic events and are associated with shorter patient survival (Brat et al., 2018; Stichel et al., 2018). These clonal mutations in GBM highlight the therapeutic importance of molecular characterization.

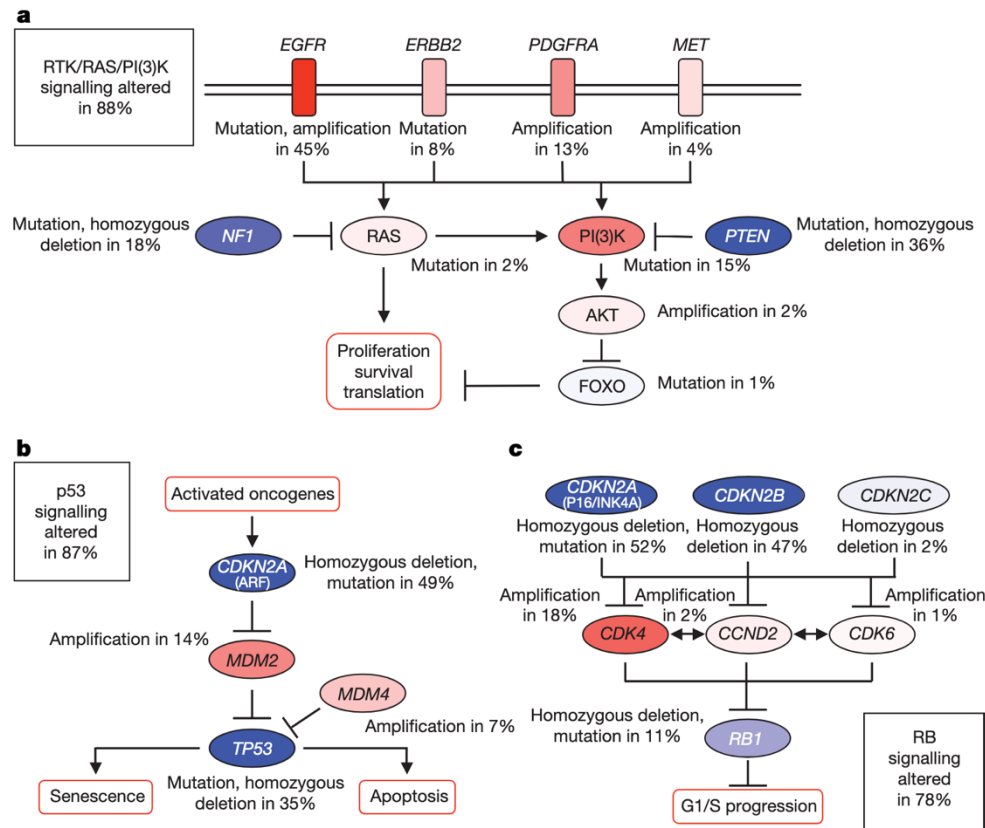


Figure 24. Core signaling pathways altered in glioblastoma. Summary of the major genetic alterations affecting (a) RTK/RAS/PI3K signaling, (b) the p53 pathway, and (c) the RB cell-cycle regulatory pathway. Red indicates activating genetic alterations, with frequently altered genes showing deeper shades of red. Conversely, blue indicates inactivating alterations, with darker shades corresponding to a higher percentage of alteration. For each altered component of a particular pathway, the nature of the alteration and the percentage of tumours affected are indicated. Boxes contain the final percentages of glioblastomas with alterations in at least one known component gene of the designated pathway. Adapted from (The Cancer Genome Atlas Research Network, 2008).

2. Epigenetic alterations

Epigenetic dysregulation is a hallmark of GBM, with the most clinically relevant alteration being O⁶-methylguanine-DNA methyltransferase (MGMT) promoter methylation. Present in approximately 45% of newly diagnosed cases, this modification silences the MGMT DNA repair enzyme, thereby reducing the capacity to reverse alkyl adducts on guanine and increasing tumor sensitivity to the alkylating agent temozolomide (TMZ), which remains the standard chemotherapy in GBM since 2005 (Hegi et al., 2005). As such, MGMT promoter methylation is firmly established as a predictive and prognostic biomarker of treatment response. Beyond this, the use of DNA methylation profiling has become an important diagnostic tool in the classification of glioblastoma and other CNS tumors. Genome-wide methylation signatures generate reproducible molecular fingerprints that remain stable across samples, providing higher diagnostic precision than histology alone. The development of classifiers such as the molecular neuropathology (MNP) system has demonstrated high accuracy in distinguishing CNS tumor entities, establishing methylation

profiling as an essential component of modern neuro-oncology diagnostics (Bertero et al., 2024; Capper et al., 2018). More broadly, the presence of DNA repair defects in GBM highlights the therapeutic potential of exploiting epigenetically driven vulnerabilities.

3. Tumor microenvironment

The GBM tumor microenvironment (TME) is profoundly influenced by both tumor-intrinsic and stromal factors that sustain malignancy and therapy resistance. The amoeboid phenotype of GBM, which was observed in DIAPH3-depleted tumor cells, is accompanied by hyperactivation of the EGFR pathway through EGFR accumulation in endosomes (Hager et al., 2012), destabilization of the nuclear membrane (Reis-Sobreiro et al., 2018), and the release of extracellular vesicles known as oncosomes (Di Vizio et al., 2009). These vesicles actively remodel the microenvironment by inhibiting the proliferation of immune cells while enhancing the growth of adjacent tumor cells, thereby promoting tumor progression (J. Kim et al., 2014).

A central component of the TME are tumor-associated macrophages (TAMs) and resident microglia, which can comprise up to half of the tumor mass. Instead of increasing anti-tumor immunity, they are reprogrammed into immunosuppressive states that secrete cytokines, growth factors and matrix-remodeling enzymes, thereby enhancing invasion and angiogenesis (Andersen et al., 2021; Khan et al., n.d.). Another major stromal contributor is the population of cancer-associated fibroblasts (CAFs), which remodel the extracellular stroma through the secretion of protumorigenic molecules, thereby sustaining tumor aggressiveness and therapy resistance (Ermakov et al., 2022; C. Pan et al., 2025). Interestingly, evidence from cutaneous squamous cell carcinoma suggests that DIAPH3 can promote the transformation of normal fibroblasts into CAFs by stimulating filopodia assembly and inducing CAF-related gene expression through inhibition of nuclear p53 activity. This suggests a mechanistic connection DIAPH3-driven actin remodelling, stromal activation and GBM progression (Cangkrama et al., 2020).

C. Therapeutical strategies

The current standard-of-care for newly diagnosed IDH-wildtype GBM has been established through international consensus and remains unchanged since its reaffirmation in 2020 by the Society for Neuro-Oncology and the European Association of Neuro-Oncology (Wen et al., 2020). It consists of maximal safe surgical resection, followed by radiotherapy with concomitant and adjuvant TMZ (Fig. 22). In addition, the use of Tumor Treating Fields (TTF) in combination with maintenance TMZ has been recognized as an evidence-based treatment option, providing a survival benefit in eligible patients (Melhem et al., 2022). However, since the introduction of the Stupp protocol nearly two decades ago, no new therapy has succeeded in significantly improving patient outcomes. Numerous strategies, targeting tyrosine kinase pathways, DNA repair mechanisms or immune checkpoints, have failed to demonstrate efficacy in phase III clinical trials (Dewdney et al., 2023). This persistent lack of progress underscores the urgent need to better

understand the mechanisms driving GBM aggressiveness and therapeutic resistance.

1. Standard-of-care

Current management of GBM begins with microsurgical resection, which serves both diagnostic and therapeutic purposes. Tumor tissue obtained during surgery allows for precise pathological and molecular classification, while the main goal is to achieve maximal safe resection without compromising neurological function or quality of life (Lara-Velazquez et al., 2017). The extent of removal depends on tumor location and the patient's overall condition: in eloquent regions or frail patients, stereotactic or open biopsy may be preferred, whereas maximal resection is pursued in operable cases (Singh et al., 2024). Following surgery, patients typically receive radiotherapy (RT) (60 Gy in 30 fractions) with concomitant daily TMZ, followed by six cycles of adjuvant TMZ, as established by the Stupp protocol (Fig . 25). The Stupp protocol relies on surgical debulking, the alkylating agent TMZ and RT (Stupp et al., 2005). The cytotoxicity of TMZ is mainly caused by the

methylation of the O6 position of guanine, which creates the methyl adduct O6-meG (Strobel et al., 2019). This results in a continuous cycle of DNA base mismatch repair with eventual strand breaks, leading to cellular apoptosis.

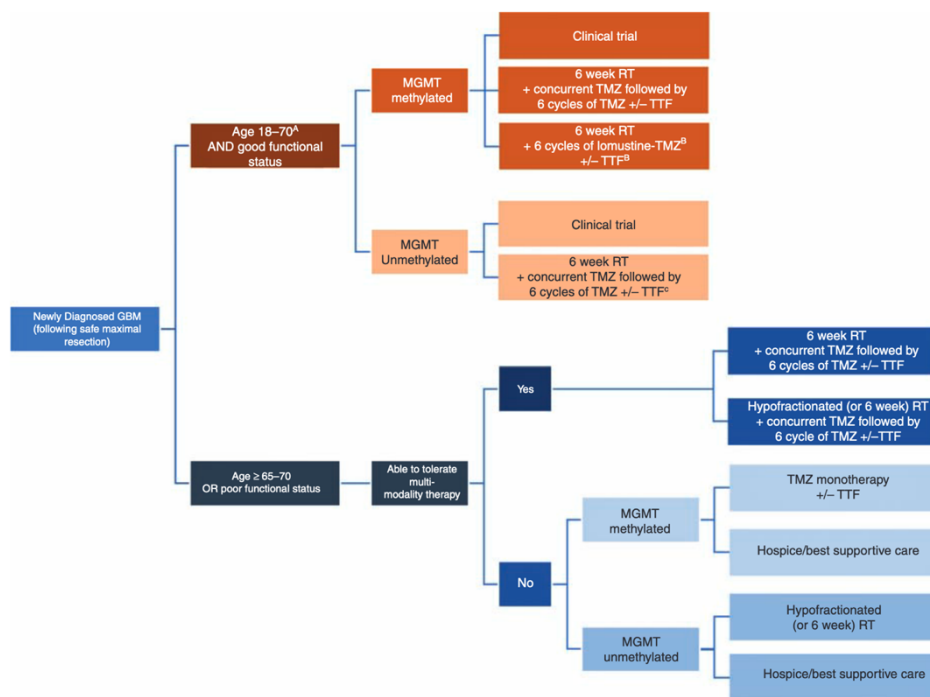


Figure 25. Standard-of-care for newly diagnosed glioblastoma. Decision tree illustrating recommended therapies based on patient age, functional status and MGMT promoter methylation, including combinations of radiotherapy, temozolomide, tumor-treating fields, clinical trials or supportive care. Adapted from (Wen et al., 2020).

Ultimately, the principal biological effects of TMZ are to kill GBM cells or stop their proliferation by inducing DNA damage beyond the cellular capacity for repair. In addition, TTF has emerged as an adjuvant option in some centers. This device applies alternating electric fields through scalp electrodes worn for ≥ 18 hours per day, disrupting mitotic spindle formation and promoting apoptosis in dividing cells (Strobel et al., 2019). When combined with maintenance TMZ after the Stupp regimen, TTF improved overall survival by nearly 6.7 months in clinical trials, supporting its use as a complementary therapy in selected patients (Stupp et al., 2017).

2. Management of recurrence

The management of recurrent GBM remains one of the greatest challenges in neuro-oncology, as no available therapy has consistently demonstrated a meaningful improvement in overall survival. Prognosis at recurrence is generally dismal, with therapeutic decisions needing to be individualized according to patient performance status, tumor location, prior treatments and patient preference (El-Khayat & Arafat, 2021; Vaz-Salgado et al., 2023).

Standard options such as re-operation or re-irradiation may be considered in carefully selected patients, though benefits are limited. Systemic therapies have also yielded modest outcomes: regorafenib, an oral multi-kinase inhibitor, showed encouraging activity in a large real-world cohort, yet survival gains remained modest and heterogeneous across patients (Lombardi et al., 2021). Immunotherapeutic strategies, including immune checkpoint inhibitors, vaccines and cell-based therapies, have generated considerable interest, but to date their efficacy in recurrent GBM has been hampered by profound tumor heterogeneity and an immunosuppressive microenvironment (Ng et al., 2024).

In light of these limitations, clinical trial enrollment is strongly encouraged at recurrence, as it remains the most promising avenue for patients to access innovative therapies while contributing to the development of future treatment strategies. This stands in sharp contrast with newly diagnosed GBM, where a well-defined standard-of-care combining surgery, radiotherapy and TMZ has been firmly

established, underscoring the lack of comparable consensus for the recurrent setting.

In elderly patients with glioblastoma, particularly those aged ≥ 70 years or with a low performance status (KPS < 70), management should be carefully individualized. When patients cannot tolerate aggressive therapy, best supportive care (BSC) (named also Palliative Care) remains an appropriate and compassionate option, prioritizing comfort, symptom control and preservation of quality of life. For patients who can tolerate multimodal therapy, a short-course radiotherapy regimen combined with three weeks of TMZ, followed by six adjuvant cycles, has demonstrated clinical benefit (Perry et al., 2017). In more fragile individuals, hypofractionated radiotherapy or exclusive BSC may provide the most balanced approach, minimizing treatment burden while aligning care with patient goals (Mazarakis et al., 2024).

3. Challenges and future perspectives

Despite incremental progress, GBM remains an incurable malignancy, and multiple biological and clinical barriers limit the

effectiveness of current therapies. The tumor displays heterogeneity and plasticity, driven by genomic instability and an adaptive tumor microenvironment, which together enable rapid escape from therapeutic pressure. In addition, the blood-brain barrier continues to restrict drug delivery, while the immunosuppressive milieu hampers effective anti-tumor immunity. Immunotherapeutic approaches have shown only modest efficacy so far largely due to TME-mediated immune evasion (Y. Liu et al., 2024). Innovative strategies to bypass or modulate the blood-brain barrier are under development, aiming to improve drug delivery (Jimenez-Macias et al., 2025). Promising avenues for recurrent GBM include the use of multi-omics and DNA methylation profiling to guide personalized therapy (Wu et al., 2024).

As explained earlier, targeting intrinsic cellular vulnerabilities in GBM such as the disruption of the SAC by using inhibitors of mitotic kinases such as Aurora kinases and PLK1 have shown promise in preclinical glioma models (Lang & Yang, 2025). Similarly, modulation of the DDR offers a means to overcome treatment resistance. Agents targeting ATR or ATM can potentiate the effects of radiotherapy and TMZ

by preventing efficient DNA repair (Ferri et al., 2020). Finally, tumor aneuploidy is gaining a lot of interest as a potential therapeutic vulnerability, with recent work suggesting that aneuploidy-associated stress pathways may sensitize GBM cells to immune checkpoint blockade (Spurr & Pitroda, 2024). Ultimately, meaningful advances will depend on biomarker-driven trial designs, earlier detection of resistance and therapeutic approaches that not only extend survival but also preserve neurological function and quality of life.

AIM OF THE THESIS

DIAPH3 is needed to regulate actin and microtubule dynamics during cell division (Bartolini et al., 2008). It localizes to the centrosome and contributes to mitotic spindle organization and kinetochore-MT interactions. Loss of DIAPH3 destabilizes spindle polarity, induces multipolar divisions and promotes aneuploidy in daughter cells.

Previous work in mouse models has demonstrated that DIAPH3 is essential for correct karyokinesis in neural progenitors. Its loss disrupts mitotic fidelity, resulting in chromosomal instability, aneuploidy and progenitor cell death, which in turn leads to abnormal cortical development (Damiani et al., 2016; Lau et al., n.d.). These findings established DIAPH3 as a crucial regulator of chromosome segregation in the developing brain and suggested that its deficiency could contribute to tumorigenesis.

Errors in mitosis often trigger mitotic catastrophe and lead to cell death, thereby limiting the proliferation of defective cells (Sazonova et al., 2021). However, when apoptosis or checkpoint surveillance is compromised, aneuploid cells may escape elimination (Weiss et al., 2022). Depending on the cellular context and the extent of chromosomal

imbalance, aneuploidy can exert dual effects on tumorigenesis. Low to moderate levels of aneuploidy may enhance adaptability and promote tumor initiation and progression, whereas excessive chromosomal instability can overwhelm cellular homeostasis, leading to proteotoxic stress, mitotic arrest, or cell death. This duality role of aneuploidy has long been recognized in cancer biology: while aneuploidy is a hallmark of many highly proliferative cancers and is frequently associated with poor prognosis, disease progression, metastasis and therapeutic resistance (Danielsen et al., 2017), it can also act as a barrier to tumor growth when genomic instability exceeds tolerable thresholds (Hosea et al., 2024a).

GBM, the most common and aggressive primary brain tumor in adults, is strongly characterized by chromosomal instability and extensive aneuploidy (Ostrom et al., 2019). Recurrent chromosomal alterations are central features of this malignancy contributing to its aggressive phenotype (Vargas-Rondón et al., 2017). Given that DIAPH3 deficiency favors aneuploidy in neural stem cells (Lau et al., 2021), we

hypothesized that its loss in GBM cells could contribute to aneuploidy aggressiveness and drive DNA damage responses.

To explore this, we first performed a bioinformatics study, published as “*DIAPH3 is upregulated in high-grade gliomas and linked to chromosomal instability.*” in the journal of Neuro-Oncology Advances. Using TCGA and single-cell RNA-seq datasets, we demonstrated that *DIAPH3* expression is specifically increased in grade 4 gliomas. Although its prognostic power did not surpass the 2021 WHO CNS5 classification, *DIAPH3* was predominantly expressed in mitotic cells and strongly co-expressed with genes regulating mitotic progression and chromosome segregation. We also identified potential transcriptional regulators linked to proliferation and tumor progression. Importantly, we confirmed in GBM cell lines that *DIAPH3* is upregulated during mitosis and that its knockdown increases aneuploidy.

Building on these findings, we carried out experimental investigations in human GBM cell culture models. We used U251 cells, which harbor a mutated *p53*, and U87 cells, which retain wild-type *p53*. Using siRNA targeted against *DIAPH3*, we knocked down our protein of

interest and analyzed aneuploidy, DNA damage induction, DDR activation, cell cycle progression and cell death. To confirm specificity, we also performed knockdown of p53 alone and the combined inhibition of both p53 and DIAPH3, and re-examined the induction of aneuploidy and DDR activation, particularly in U87 cells with functional *p53*. We further explored whether DIAPH3 suppression influences responses to TMZ and radiotherapy, with an emphasis on cell cycle regulation. Finally, to validate these phenotypes pharmacologically, we inhibited formin activity using SMIFH2, and repeated the aneuploidy and DDR experiments under these conditions.

Together, these complementary approaches integrate clinical bioinformatics with experimental validation in cell culture, allowing us to define the contribution of DIAPH3 to chromosomal instability, DNA damage responses and, to some extent, therapeutic resistance in GBM. The objectives of this thesis were to:

- Integrate bioinformatics evidence to establish the mechanistic relevance of DIAPH3 in HGGs.

- Assess the impact of DIAPH3 loss on aneuploidy in GBM cell models.
- Investigate the induction of DNA damage and activation of DDR pathways upon DIAPH3 knockdown.
- Analyze the effects of DIAPH3 depletion on cell cycle progression and cell death.
- Confirm the role of DIAPH3 in *p53* wild-type cells (U87) by selectively removing *p53* alone or in combination with DIAPH3.
- Evaluate the influence of DIAPH3 suppression on cell cycle regulation using TMZ and radiotherapy.
- Confirm the specificity of DIAPH3-dependent phenotypes by pharmacological inhibition with SMIFH2.

By combining bioinformatics evidence with experimental studies in these well-characterized GBM cell models, this thesis aims to clarify the role of DIAPH3 in maintaining genome integrity. These findings will provide new mechanistic insights into how DIAPH3 safeguards mitotic fidelity and whether its deregulation creates vulnerabilities that can be exploited for therapeutic benefit in GBM.

MATERIALS AND METHODS

Datasets

Expression and methylation data were obtained from The Genotype-Tissue Expression (GTEx) Portal, The Cancer Genome Atlas (TCGA) Program, and the datasets GSE182109 and GSE123678 from the NCBI-GEO database. TCGA-LGG and TCGA-GBM samples were reclassified according to the 5th edition of the WHO Classification of Tumours of the Central Nervous System (WHO CNS5) (Louis et al., 2021). The table used for this reclassification is publicly available as Supplementary Table S2 in the following publication: <https://www.mdpi.com/article/10.3390/ijms24010157/s1> (Zakharova et al., 2022).

Software and computing

All data analyses were performed using R software version 4.4.1 (2024-06-14) and GraphPad Prism version 10.4.0. Some of the steps involved in processing the single cell RNA sequencing (scRNA-seq) data from dataset GSE182109, which required large amounts of computing

resources, were carried out using R software version 4.2.0 (2022-04-22) running on a high-performance computing cluster with 1,536 cores (30 AMD Epyc Naples and 32 Intel Sandy Bridge compute nodes). The R codes were written with the help of ChatGPT-4o (OpenAI, San Francisco, CA, USA).

Comparison of DIAPH3 expression in normal and tumor tissues

To compare DIAPH3 expression data in GTEx normal tissue and TCGA tumor tissue, expression and phenotypic data, including tissue type, were retrieved from the UCSC Xena platform (gtex_RSEM_Hugo_norm_count.gz and TcgaTargetGtex_RSEM_Hugo_norm_count.gz datasets) with the UCSCXenaTools package (S. Wang & Liu, 2019).

Extraction of expression and clinical data from TCGA

Transcriptomic and clinical data from TCGA-LGG and TCGA-GBM cohorts were accessed via the TCGAbiolinks R package. Gene

expression data were queried, downloaded, and prepared through a standardized workflow, including preprocessing based on correlation filtering, normalization using the "gcContent" method, and quantile-based filtering (Colaprico et al., 2016; Silva et al., 2016). Clinical data were separately retrieved for each project, combined, and filtered to include only primary tumor samples.

Survival analysis

Survival data were matched to DIAPH3 expression levels. For each subgroup – all gliomas combined, low-grade, and high-grade gliomas – samples were dichotomized into high- and low-expression groups based on the median DIAPH3 expression specific to that subgroup. Kaplan-Meier survival curves and associated statistical analyses were then performed using GraphPad Prism.

Gene ontology (GO) enrichment

GO enrichment analyses were conducted to identify Biological Processes (BP), Cellular Components (CC), and Molecular Functions (MF) associated with genes showing significant Pearson correlation with DIAPH3 expression in the TCGA-LGG and TCGA-GBM cohorts. GO enrichment analysis was performed using the gseGO function from the clusterProfiler package, with a minimum gene set size of 100, a maximum of 500, and a p-value cutoff of 0.05 (Xu et al., 2024). Only positively regulated pathways were retained for further analysis.

Analysis of scRNA-seq data

The scRNA-seq data from the GSE182109 study were analyzed to identify cell type-specific expression patterns and perform GO enrichment analysis. Raw expression data from 10X Genomics were processed for each sample using the Seurat R package, with cells filtered based on mitochondrial content (>25%) and gene feature counts (200-9000) (Hao et al., 2024). Predicted cell types were annotated using

the scATOMIC R package and cell cycle phases were scored using predefined S and G2/M gene sets (Nofech-Mozes et al., 2023). To infer the CNV status of individual cells, we employed the CNV mode of scATOMIC, which utilizes CopyKAT for CNV inference. This approach allowed to classify cells as either diploid or aneuploid based on their inferred CNV profiles. The processed data were normalized, variable features identified and scaled to regress out cell cycle effects. All samples were integrated into a single Seurat object, and dimensionality reduction (PCA and UMAP) and clustering (resolution = 0.1) were performed. Analysis of GO enrichment was carried out as described above.

Analysis of *DIAPH3* promoter methylation

DNA methylation profiles from TCGA-LGG and TCGA-GBM cohorts were analyzed using data from the Illumina HumanMethylation450k array to investigate methylation patterns in a genomic region spanning *DIAPH3* on chromosome 13. Data Annotation information for the 450k platform was obtained using the IlluminaHumanMethylation450kanno.ilmn12.hg19 package. Probes

located within a 1 kb region upstream of *DIAPH3* were identified. Methylation beta-values for these probes were retrieved from the GDC portal using the TCGAbiolinks R package and filtered to include only primary tumor samples.

Similarly, DNA methylation data and clinical data from the GSE123678 dataset were retrieved from the NCBI-GEO database. Annotation and filtering steps mirrored those used for the TCGA data.

TF binding motif analysis

TF binding motif analysis was performed on genes significantly correlated with *DIAPH3* expression (Pearson correlation > 0.5) using the RcisTarget R package (Aibar et al., 2017). Motif rankings were retrieved from the hg38_10kbp_up_10kbp_down_full_tx_v10_clust database, and enrichment analysis was conducted to identify TF motifs with a normalized enrichment score (NES) > 3. Position Weight Matrices (PWMs) for the identified TFs were obtained from the JASPAR2022 database, and their binding sites were mapped onto the putative *DIAPH3* promoter region (~1000 bp upstream of the transcription start site, TSS)

using the searchSeq function from the TFBSTools package (Tan & Lenhard, 2016). Conservation scores for this region were imported from the UCSC hg38 phastCons20way track and used to assess the evolutionary conservation of TF binding sites.

ChIP-seq-based identification of TF binding sites

To identify TFs binding to the putative *DIAPH3* promoter region, we analyzed ChIP-seq peak data from the ReMap2022 database. TF-specific BED files were obtained and processed using the R packages GenomicRanges, and rtracklayer. Each BED file was imported as a GRanges object and annotated with the corresponding TF name. All entries were concatenated into a single GRanges object and filtered for overlaps with a ± 1000 bp window downstream of the *DIAPH3* TSS. Only peaks with a ReMap score above 50 and detected in selected cell lines (K-562, Hep-G2, HEK293, or U266B1) were retained. When multiple peaks were found for the same TF, only the highest-scoring peak was kept.

Cell Culture

U251-MG glioblastoma cells (*harboring the p53^{R273H} mutation, leading to impairment of p53 binding to canonical response elements, resulting in a loss of tumor-suppressive activity. Mutant p53 can acquire oncogenic gain-of-function properties thereby rewiring transcriptional programs that promote proliferation and malignant progression (X. Chen et al., 2022)*) and U87-MG glioblastoma cells (wild-type p53) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Both cell lines were authenticated by Multiplexion GmbH (Friedrichshafen, Germany) using the Multiplex Cell Authentication assay. The analyses confirmed a 100% identity with their respective reference profiles and excluded cross-contamination with other human cell lines.

Cells Transfection

U251 and U87 cells were transfected for 72 hours with a pool of four siRNAs targeting DIAPH3 (ON-TARGETplus Human DIAPH3 siRNA SMARTpool, Dharmacon), using DharmaFECT 1 transfection reagent (Dharmacon), following the manufacturer's instructions. A non-targeting siRNA pool (ON-TARGETplus Non-targeting Control siRNA, Dharmacon) was used as a negative control.

Immunoblot

Cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 1 mM EDTA). Equal amounts of protein were resolved on 4–20% Mini-PROTEAN TGX precast gels (Bio-Rad) and transferred to PVDF membranes. Membranes were blocked with 5% non-fat dry milk or bovine serum albumin (BSA) in TBS-T (TBS with 0.1% Tween-20), then incubated overnight at 4 °C with the following primary antibodies diluted in blocking buffer: rabbit

polyclonal anti-DIAPH3 (Abcam, #AB245660; 1:2500), rabbit

monoclonal anti-GAPDH

(Cell Signaling Technology, #2118; 1:10000) and rabbit anti-p53 (Cell Signaling Technology, #9282, 1:1000). After washing, membranes were incubated for 1 hour at room temperature with HRP-conjugated goat anti-rabbit secondary antibody (Dako). Signal was detected using enhanced chemiluminescence (Fusion Pulse system), and band intensities were quantified using the “Gel Analyzer” tool in Fiji (ImageJ).

Isolation of mitotic and interphase U251-MG cells

To isolate mitotic cells, U251-MG GBM cells were cultured under standard conditions and subjected to a single mechanical shake-off. Culture flasks were firmly tapped to selectively detach rounded mitotic cells, which were immediately collected from the supernatant. Adherent cells remaining attached to the culture surface (predominantly interphase cells) were then harvested by gentle scraping in cold PBS.

Both mitotic and adherent cell fractions were lysed separately and processed for protein extraction followed by immunoblot analysis.

Metaphase spread

Cells were treated with 0.05 µg/mL colcemid for 3 hours to arrest cells in metaphase. They were then collected by centrifugation at 1,000 rpm for 5 minutes, incubated in hypotonic solution (0.065M KCl in distilled water), and fixed in methanol:acetic acid (3:1). Metaphase spreads were prepared by dropping the fixed cell suspension onto clean microscope slides. Slides were stained with Wright stain diluted in phosphate buffer and examined under a 100× oil immersion objective using an Axiovert 200 M microscope (Zeiss, Germany).

Cell Death

Apoptosis was assessed by analyzing mitochondrial membrane potential and cell membrane integrity using DiOC₆ (3,3'-dihexyloxacarbocyanine iodide) and Propidium Iodide (PI) staining,

respectively. Cells were harvested, washed twice with PBS, and resuspended in DMEM media containing 40 nM DiOC₆ and 5 µg/mL PI. The samples were incubated at 37°C for 30 minutes in the dark. Following incubation, cells were immediately analyzed using a BD FACS Canto II flow cytometer. Data were analyzed using FlowJo software. Mitochondrial depolarization (loss of DiOC₆ signal) and membrane permeability (PI positivity) were used as indicators of early and late apoptosis, respectively.

Immunofluorescence

To detect the subcellular localization of γH2AX and RAD51, cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes, followed by permeabilization with a buffer (PBS containing 0.5% Triton X-100). After washing, cells were blocked with blocking buffer (ThermoFisher) for 1 hour at room temperature to prevent nonspecific binding. Primary antibodies were applied as follow: rabbit monoclonal anti-p-histone H2AX (Cell Signaling Technology, #9718, 1:400) and rabbit monoclonal anti-RAD51 (Cell Signaling Technology, #65653,

1:500) and incubated overnight at 4 °C. The following day, cells were washed three times with Washing buffer (Cytoskeleton) and subsequently incubated with appropriate fluorescent secondary antibodies (Invitrogen) for 1 hour at room temperature in the dark. Nuclear DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were acquired using a Axiovert 200M Zeiss inverted fluorescence microscope. Data were quantified by analyzing 50 nuclei per trial (n = 3) for each condition, totaling 150 nuclei per condition.

Single-cell gel electrophoresis (comet) assay

The comet assay was performed to evaluate DNA strand breaks in U87 and U251 cells using the Comet Assay Kit (Abcam, #ab238544), following the manufacturer's instructions. Cells were transfected with either siRNA Control or siRNA DIAPH3 for 48 hours. Cells were then harvested and washed with ice-cold PBS. Cell suspensions were mixed with low-melting-point agarose at 37 °C, and 75 µL of the mixture was immediately

pipetted onto comet slides. Once the agarose solidified, slides were immersed in prechilled lysis solution and then in alkaline solution to allow DNA unwinding. Electrophoresis was carried out at 1 V/cm for 20 minutes under alkaline conditions. After electrophoresis, slides were washed, dried and stained with the DNA dye provided in the kit. Images were captured using a fluorescence microscope, and comet tail length was measured using OpenComet plugin in ImageJ software to quantify DNA damage. For each condition, comet tail length was measured for 50 cells per replicate (n = 3), totaling 150 comets per condition.

Real-time qPCR

Total RNA was purified using Trizol method and RNA expression was evaluated in U251 and U87 cells for DIAPH3. RT-qPCR was performed using 5 µL of cDNA, 12.5 µL of SyberGreen Mix (BioRad, Temse, Belgium) and 250 µM of primers (Fw: AGAAGCGCCCCAAGTTTCAT, Rv: CAGGTGGGAAGTGGAGGTC) in a total reaction volume of 25 µL. The reaction was initiated at 95°C for 3 min and was followed by 40 cycles of

denaturation at 95°C for 10 s, annealing at 60°C for 1 min and extension at 72°C for 10 s. The data were recorded on a DNA Engine Opticon RT-qPCR Detection System (BioRad), and the cycle threshold (Ct) values for each reaction were determined using analytical software from the same manufacturer. To normalize the data, Gapdh gene was used as a housekeeping gene.

Cell Cycle

Cells were incubated with EdU for 1h 30min before harvest. In this case, EdU was detected using Click-iT® Plus EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (C10632, Thermo Fisher Scientific) following the manufacturer's instructions and FxCycle Violet stain (Molecular Probes, # F10347) was used for DNA staining. The staining procedure was performed according to the manufacturing protocol. Cell cycle distribution was analyzed by flow cytometry (BD FACS Canto II) and FlowJo software.

Irradiation

U251 and U87 cells were seeded in 6-well plates and transfected with either control siRNA or siRNA targeting DIAPH3. 24 hours after transfection, the cells were exposed to ionizing radiation at doses of 0 Gy (control), 4 Gy, 8 Gy or 12 Gy. Irradiation was performed at room temperature using an IBL 637 (^{137}Cs) gamma irradiator (Cisbio International, France). Following irradiation, the cells were incubated for an additional 48 hours, after which they were fixed, and EdU and Fx cycle analysis was carried out to assess cell cycle distribution.

Reagents

SMIFH2 (Sigma-Aldrich, #S4826) and Temozolomide (TMZ) (Sigma-Aldrich, #T2577) were both dissolved in pure DMSO to prepare stock solutions of 25 μM and 50 mM, respectively. Both were equipped and wrapped in a brown centrifugal tube for protection against light and stored at $-20\text{ }^{\circ}\text{C}$.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA), except for Levene's tests, which were conducted in R (version 4.4.1; R Foundation for Statistical Computing, Vienna, Austria). The specific statistical tests applied, the number of biological replicates, and the corresponding p-values are indicated in each figure legend. A p-value < 0.05 was considered statistically significant.

Code availability

All codes are available at https://github.com/NTajeddine/Jabbour_et_al_2025.

RESULTS

DIAPH3 is overexpressed in high-grade gliomas and transcriptionally regulated by proliferation-linked factors

Expression data from the GTEx project show that *DIAPH3* expression in normal tissues is highest in bone marrow and testis (Fig. 26A). In contrast, expression is low in tissues such as muscle where there is little cell division. Based on expression data from GTEx and TCGA project, *DIAPH3* expression is significantly higher in tumour tissues compared to normal tissues (Fig. 26B). As *DIAPH3* expression is similar in grade 4 astrocytomas and GBM, these samples were grouped under the name CNS5-HGG (high-grade gliomas) (Fig. S1). Grade 2 and grade 3 gliomas are grouped together as CNS5-LGG (low-grade gliomas). The expression of *DIAPH3* is similar in normal brain tissue and CNS5-LGG samples. However, it increases significantly in CNS5-HGG samples (Fig. 26C). Mutations in the *DIAPH3* gene are very rare (less than 1%) in TCGA samples from gliomas.

Using the UCSC Genome Browser, we identified a region of high TFs binding site density in the ReMap Density track, extending up to 1,000 base pairs upstream of the *DIAPH3* transcription start site (TSS).

Based on this observation, we focused our analyses on this promoter-proximal region to assess DNA methylation patterns and to identify candidate TFs potentially involved in the regulation of *DIAPH3* expression.

Analysis of TCGA glioma samples revealed that this region is consistently hypomethylated, with no correlation observed between its methylation levels and *DIAPH3* expression (Fig. S2A). We confirmed this finding using data from the GSE123678 dataset, which includes both normal brain and glioma samples (Court et al., 2019). Again, methylation was very low and did not differ significantly between normal and tumor samples (Fig. S2B).

We next applied the RcisTarget package to identify TFs that may regulate genes co-expressed with *DIAPH3* in TCGA gliomas samples. We then searched for binding motifs of these candidate TFs within the 1,000 bp region upstream of the *DIAPH3* TSS (Fig. 26D). Several TFs identified in this region are known regulators of the cell cycle, including TFDP1, SP2, E2F6, E2F8, and members of the NF-Y complex. Their presence suggests that *DIAPH3* expression may be tightly coupled to cell cycle

control. In addition, TFs such as HOXA2, HOXB2, FOXI1, and IRF6, which are involved in developmental and differentiation processes, were also identified.

Finally, we interrogated the ReMap2022 database to determine whether binding of these transcription factors to the upstream region of DIAPH3 has been experimentally demonstrated by ChIP-seq. The ChIP-seq data indicate transcription factor occupancy of the 1,000 bp region upstream of the DIAPH3 transcription start site (TSS) by TFDP1, SP2, E2F8, and members of the NF-Y family, consistent with their potential involvement in the transcriptional regulation of DIAPH3, although these data do not distinguish between direct DNA binding and indirect association through protein–protein interactions (Fig. 26E).

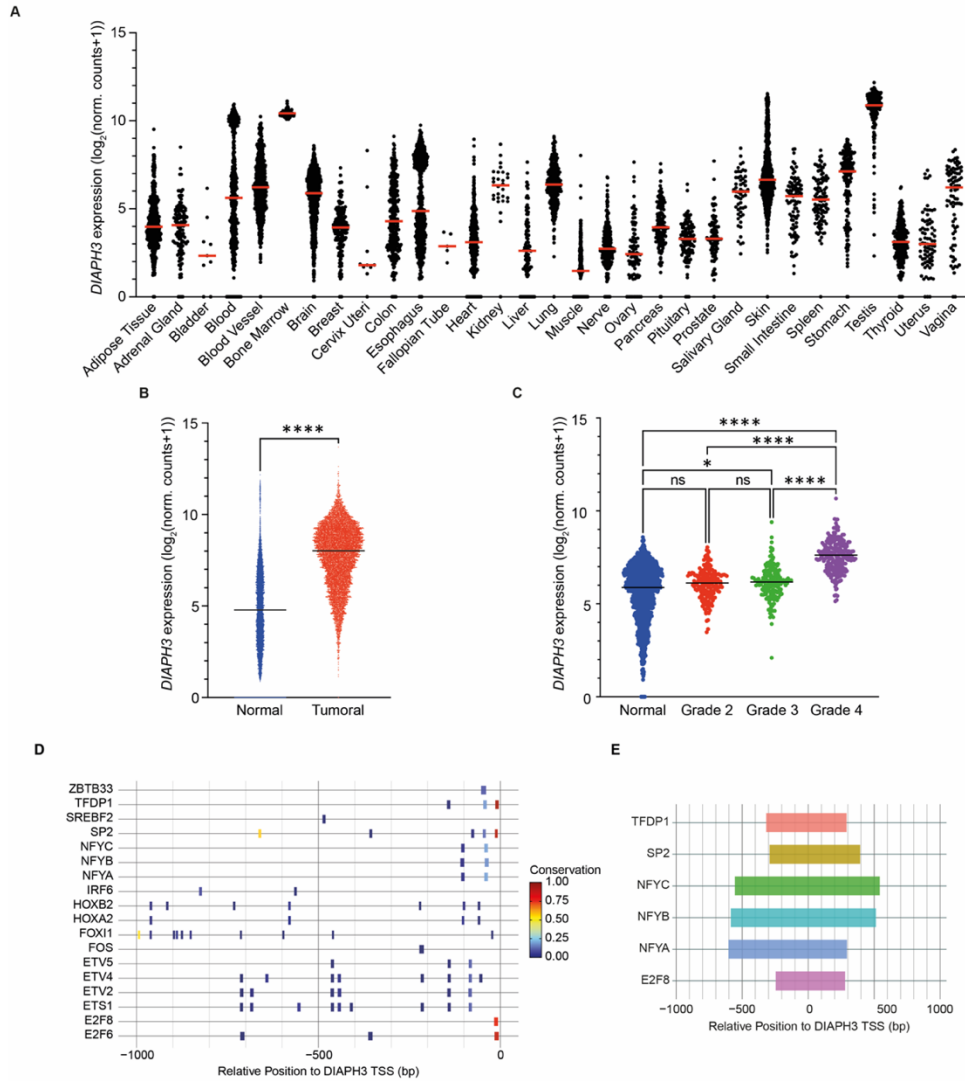


Figure 26. A. *DIAPH3* expression in healthy tissue samples from The GTEx Portal. Each point represents one sample, and the bars represent the median expression. **B.** Expression of *DIAPH3* in healthy tissue samples from the GTEx and in tumor tissue samples from TCGA. Mann-Whitney test was used to determine statistical significance. (**** $p < 0.0001$). **C.** Expression of *DIAPH3* in normal brain tissue samples from the GTEx and gliomas from the TCGA reclassified as grade 2, 3 or 4 according to the WHO CNS5 classification. A Kruskal-Wallis One-Way ANOVA was performed (* $p < 0.05$; **** $p < 0.0001$). **D.**

TF binding motifs identified in the putative promoter region of *DIAPH3* and their conservation scores. The relative positions of the motifs are shown on the x-axis, with their distance from the TSS, while the corresponding TFs are listed on the y-axis. Rectangles represent the positions of the motifs, colored according to their mean conservation scores (low to high conservation: dark blue to dark red). **E.** ChIP-seq-validated TF binding sites within 1,000 bp upstream of the *DIAPH3* transcription start site (TSS), based on ReMap2022 data. Only high-confidence peaks (score > 50) detected in selected cell lines (K-562, Hep-G2, HEK293, U266B1) are shown. For each TF, the most confident binding site overlapping the region is displayed.

Prognostic impact of *DIAPH3* expression in TCGA gliomas stratified by WHO CNS5 classification

In the TCGA data, we analysed the association of *DIAPH3* with survival in patients with CNS5-LGG or CNS5-HGG. High *DIAPH3* expression, defined as above the cohort median, is associated with a poor prognosis when considering all gliomas, with median survival decreasing from 107 months in the *DIAPH3* low group to 22 months in the *DIAPH3* high group (Fig. 27A). However, this association is no longer observed when CNS5-LGG and CNS5-HGG samples are analysed separately (Fig. 27B and 27C, respectively). This indicates that *DIAPH3* expression does not provide additional prognostic value beyond the CNS5 classification as a prognostic marker.

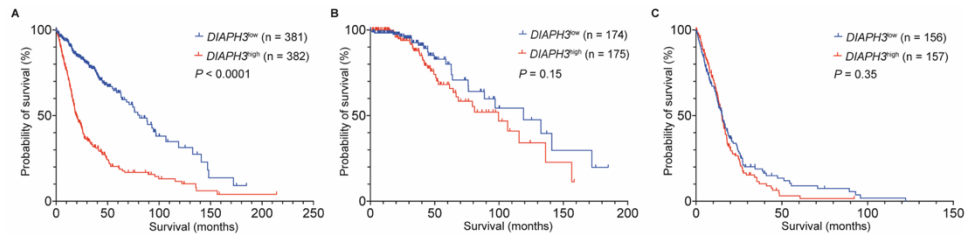


Figure 27. A. Kaplan–Meier survival curves for overall survival in glioma patients from TCGA, stratified by *DIAPH3* expression level (*log-rank test*). Data were censored at the time of last follow-up. **B.** Same analysis as in A, restricted to TCGA glioma samples reclassified as grade 2 or 3 according to the WHO CNS5 classification (referred to as CNS5-LGG). **C.** Same analysis as in A, restricted to TCGA glioma samples reclassified as grade 4 according to the WHO CNS5 classification (referred to as CNS5-HGG).

Proteins involved in karyokinesis, post-translational modifications and the formation of the extracellular matrix show an expression profile like that of *DIAPH3*

To determine the role of *DIAPH3* in gliomas, we compiled a list of genes whose expression is similar to that of *DIAPH3* in TCGA gliomas patients. We then studied the processes in which these genes were involved by performing gene enrichment analyses in the GO knowledgebase. Genes expressed in a similar way to *DIAPH3* are involved in biological processes relating to mitosis and, in particular, DNA replication, karyokinesis and chromosome segregation (Fig. 28A).

These include the *CENPE*, *KIF18A*, *KIF23* and *SKA3* genes (Fig. 28B). *BORA*, *BUB1* and *PLK1* which encode proteins that ensure and control the attachment of kinetochores to microtubules during metaphase, also show an expression profile similar to that of *DIAPH3*. As expected, the cellular components that are over-represented in the list of genes that have similar expression to *DIAPH3* in gliomas are related to chromosomes, centromeric regions and the mitotic spindle (Fig. 28C). More surprisingly, a group of genes encoding proteins associated with the endoplasmic reticulum were also found. These proteins include glycosyltransferases (*DDOST*, *POGLUT3*, *RPN2*) and disulphide isomerases (*P4HB*, *PDIA4*, *PDIA6*) (Fig. 28D).

Genes expressed as *DIAPH3* were enriched for molecular functions associated with isomerase, glycosyl- and hexosyltransferase activities and DNA binding and DNA modification (Fig. 28E). This is consistent with the biological processes and cellular components identified previously. A significant number of genes encoding proteins known to be constituents of the extracellular matrix also show similar expression to *DIAPH3*. Of these, those with the highest correlation with

DIAPH3 expression were *COL21A1*, *EFEMP2*, *HSPG2*, *LAMB1* and *LAMC1* (Fig. 28F).

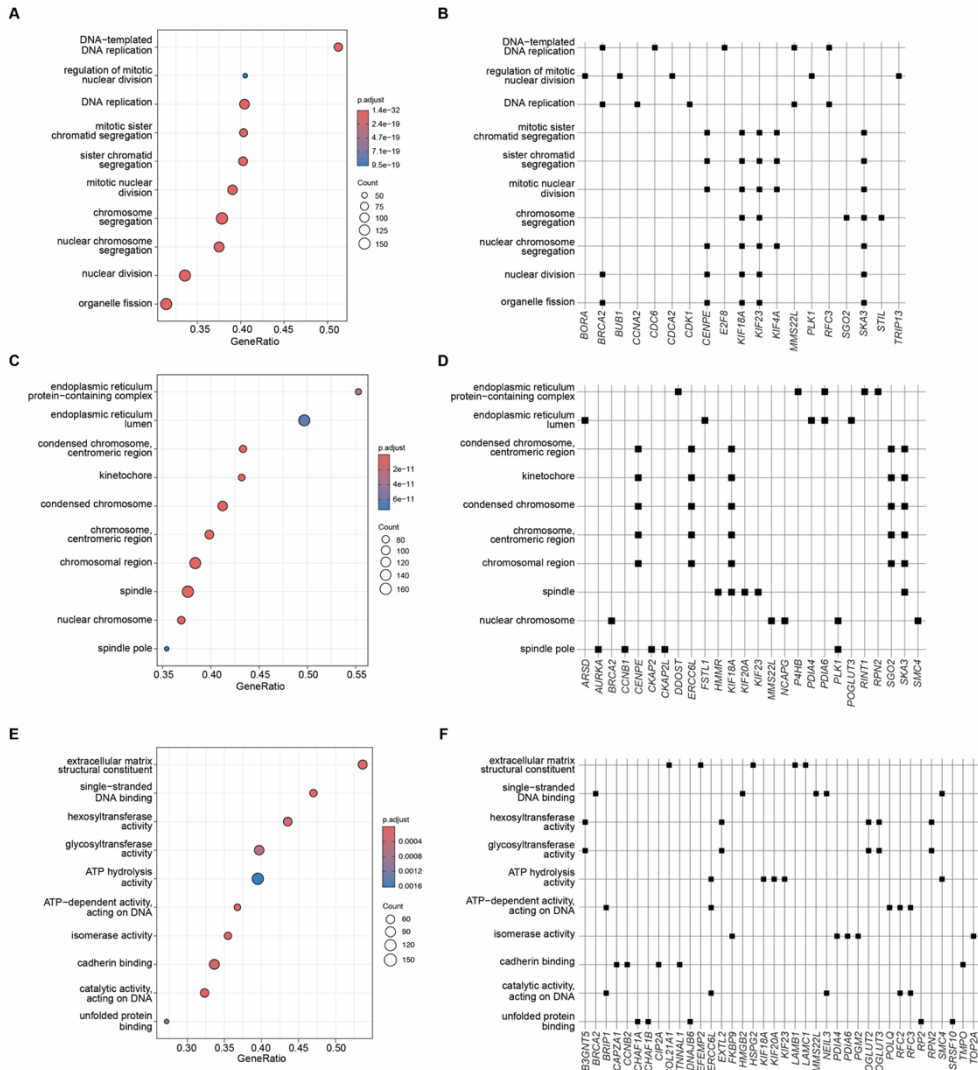


Figure 28. A. GO enrichment analyses showing the top 10 biological processes enriched in genes positively correlated with *DIAPH3* expression in glioma patients from TCGA. Each dot represents a biological process, with the size of the dot indicating the number of genes involved in the pathway (GeneRatio), and the color corresponding to the adjusted p-value. Pathways are sorted by GeneRatio, highlighting the most significant and enriched biological processes in glioma samples. **B.** Top 5 genes contributing to the enrichment of the top 10 activated biological processes. The figure highlights the top 5 genes most correlated with *Diaph3* expression that contribute to the enrichment of the top 10 positively activated biological processes identified in the gene set enrichment analysis. Rows represent the biological processes and columns represent the most correlated genes. Each tile indicates the involvement of a gene in a specific biological process, emphasizing their contribution to pathway activation in glioma samples. **C.** The same as in A, but for the cellular components. **D.** The same as in B, but for the cellular components. **E.** The same as in A, but the molecular functions. **F.** The same as in B, but for the molecular functions.

DIAPH3 is mainly expressed in dividing GBM cells

To study how *DIAPH3* was expressed within the cells that constitute GBM tumor masses, we analyzed expression in scRNA-seq data. To do this, we used the GSE182109 study which sequenced a total of 201,986 cells from 44 samples from 18 GBM patients (Abdelfattah et al., 2022). *DIAPH3* was expressed in ~6% of all cells (Fig. 29A). Approximately 10% of gliomas cells expressed *DIAPH3*. *DIAPH3* was also expressed in stromal cells (~14%) and, to a much lesser extent, in blood cells (~2%).

In GBM cells, *DIAPH3* expression was clearly predominant in cells undergoing mitosis, with 30% of G2/M cells expressing *DIAPH3* as compared with only 3% of G0/G1 cells (Fig. 29B).

As with the TCGA samples, we compiled a list of genes whose expression was similar to that of *DIAPH3* (Supplementary Table 2). However, we limited this analysis to GBM cells in G2/M phase (~6000 cells). We then performed GO enrichment on this list. For all three ontologies, we found processes associated with mitosis and, more specifically, with chromosome segregation (Fig. 29C, D and E).

Contrary to what was observed in the TCGA bulk RNAseq data, the expression of glycosyltransferases, disulfide isomerases and extracellular matrix components was not associated with that of *DIAPH3* in GBM cells in the GSE182109 study.

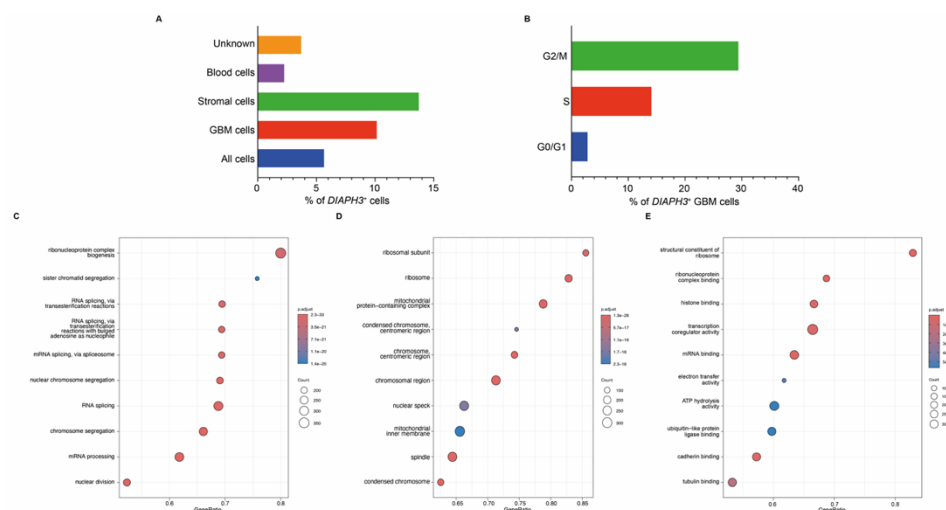


Figure 29. A. Percentage of cells expressing *DIAPH3* by cell type in the GSE182109 scRNA-seq study. **B.** Percentage of GBM cells expressing *DIAPH3* by cell cycle phase. **C.** GO enrichment analyses showing the top 10 biological processes enriched in genes positively correlated with *DIAPH3* expression in GBM cells in the G2/M phase. **D.** The same as in A, but for cellular components. **E.** The same as in A, but for molecular functions.

Aneuploidy is correlated with *DIAPH3* expression

Since our own experimental data show that *DIAPH3* is involved in SAC function and GO enrichment data suggest that *DIAPH3* is involved in correct chromosome segregation during mitosis, we analyzed aneuploidy in TCGA glioma samples as a function of *DIAPH3* expression. Surprisingly, the aneuploidy score was significantly higher in samples where *DIAPH3* expression was higher than the median (Fig. 30). Similar

results were obtained for other genes known to be involved in SAC (*BUB1*, *BUB1B*, *MAD2L2*). Only *MAD1L1* expression was inversely correlated with the aneuploidy score.

We also analyzed *DIAPH3* expression in GBM cells from dataset GSE182109. Chi-squared analysis showed that *DIAPH3* was expressed more frequently in aneuploid cells than in diploid cells (Fig. S3).

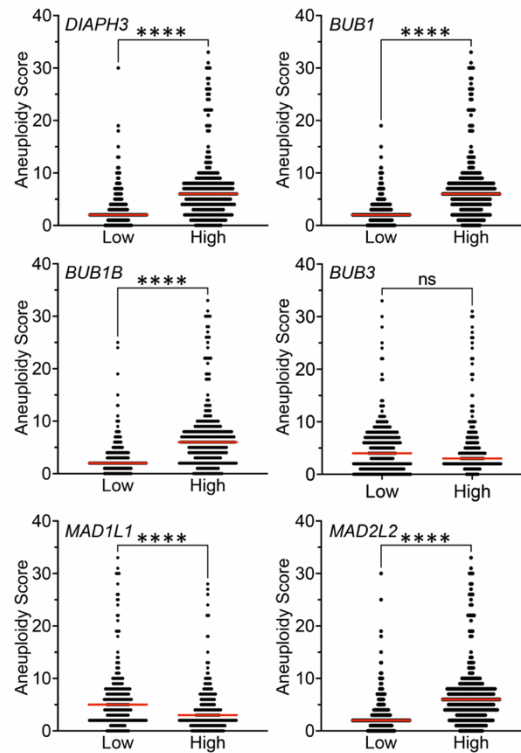


Figure 30. Aneuploidy scores in glioma samples from TCGA as a function of the expression of *DIAPH3* and genes encoding proteins involved in SAC. The cut-off value used to determine low and high gene expression is the median expression of the gene in all the samples.

DIAPH3 knockdown induces aneuploidy in p53-mutated GBM cells

To complement our bioinformatic analyses of DIAPH3 expression in glioma patient datasets, we performed in vitro experiments using human GBM cells. In U251-MG cells, a well-characterized GBM cell line, we observed that DIAPH3 is highly expressed and that its expression increases during mitosis (Fig. 31A and 31B).

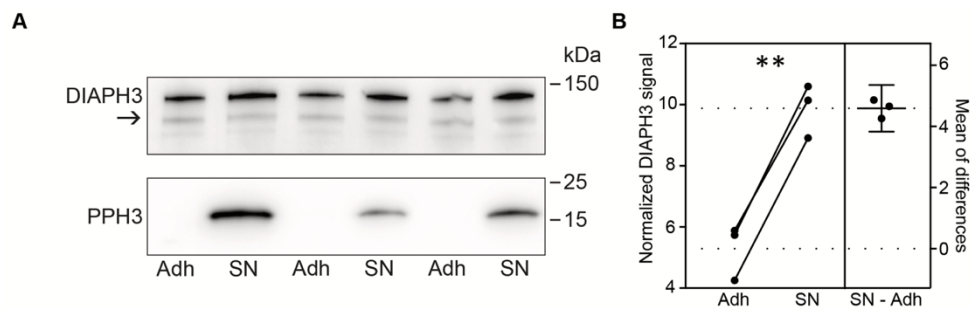


Figure 31. A. Immunoblot analysis of DIAPH3 expression in adherent cells (Adh) and in cells collected from the supernatant (SN) following mechanical detachment by tapping the culture flasks, a method used to enrich for mitotic cells. A non-specific band (indicated by an arrow) serves as a loading control. Expression of phospho-histone H3 (PPH3) confirms the enrichment of mitotic cells in the supernatant fraction. **B.** Quantification of DIAPH3 expression normalized to the non-specific loading control. Data are presented as an estimation plot showing paired individual measurements, the mean difference, and its 95% confidence interval ($p = 0.0015$; *paired t-test*).

To investigate whether changes in *DIAPH3* expression are merely correlated with aneuploidy or may actively contribute to it, we transiently

depleted DIAPH3 in two GBM cell lines by a pool of 4 siRNAs targeting specific sequences of DIAPH3 mRNA. We showed by immunoblot and by RT-qPCR that DIAPH3 expression was drastically reduced in both cell lines 72 hours post-transfection (Fig. 32A and 32B). We counted the number of chromosomes in ~100 metaphase spreads in each cell line. The standard deviation of the mean chromosome number was significantly higher after repression of DIAPH3 in U251, a p53-mutated GBM cell lines (Fig. 32C and 32D). This reflects an exacerbation of aneuploidy following repression of DIAPH3. However, this effect was not present in U87 cells which express a WT form of p53 (Fig. 32E and 32F).

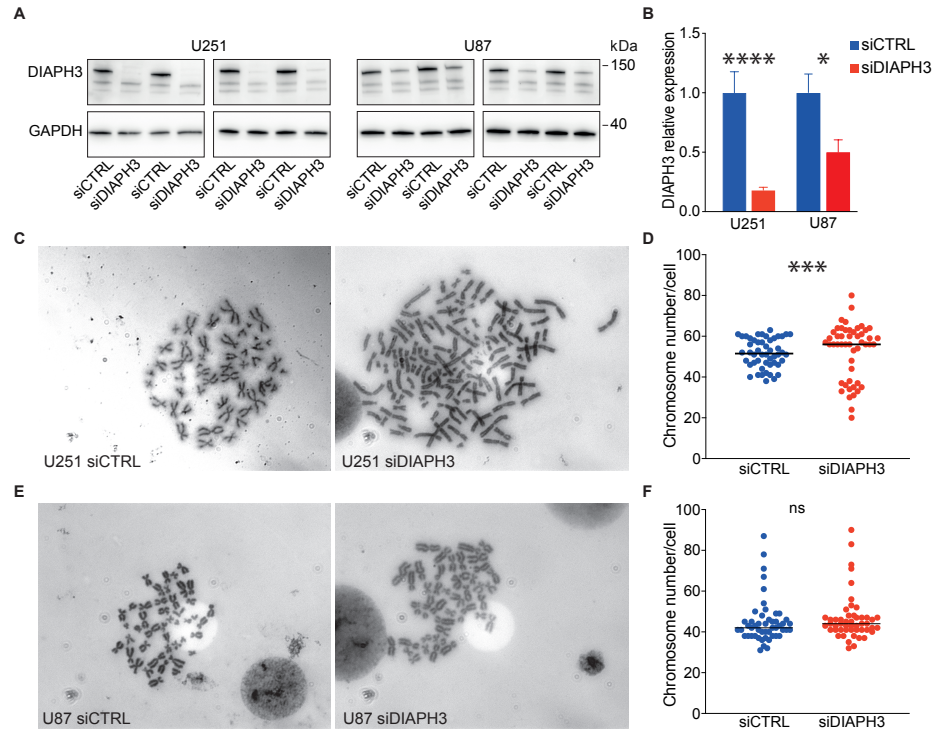


Figure 32. A. Immunoblot confirming efficient DIAPH3 knockdown in U251 and U87 cells transfected with a pool of four siRNAs targeting DIAPH3 mRNA (siDIAPH3), compared with non-targeting control siRNA (siCTRL). GAPDH served as a loading control. **B.** Quantification of DIAPH3 mRNA expression by RT-qPCR under the same conditions. Bars represent mean $\pm$ SEM from three independent experiments. Mann–Whitney test: **** $p < 0.0001$; * $p < 0.05$. **C, E.** Representative metaphase spreads illustrating chromosome counts in U251 (C) and U87 (E) cells after transfection with siCTRL or siDIAPH3. Cells were arrested in metaphase with colcemid for 3 h prior to chromosome spreading. **(D, F)** Scatter plots showing the number of chromosomes per cell in U251 (D) and U87 (F) cells under the indicated conditions. Each dot represents a single cell; horizontal bars denote median values. Levene’s test was used to assess differences in variance between groups, based on the rationale that increased aneuploidy manifests as broader chromosome number distributions rather than shifts in central tendency. $n = 50$ cells per condition, pooled from at least three independent experiments. *** $p < 0.001$; ns, not significant.

DIAPH3 depletion induced DNA lesions in p53-mutated cells

We next measured DNA strand breaks by using single-cell gel electrophoresis (also known as comet assay) that determines the amount of DNA lesions (Fig. 33). Interestingly, DIAPH3 repression induced *per se* a low but significant increase in DNA breaks in U251 cells (Fig. 33A and 33B). In contrast, DIAPH3-siRNA dramatically decreased the level of DNA breaks in U87 cells (Fig. 33C and 33D).

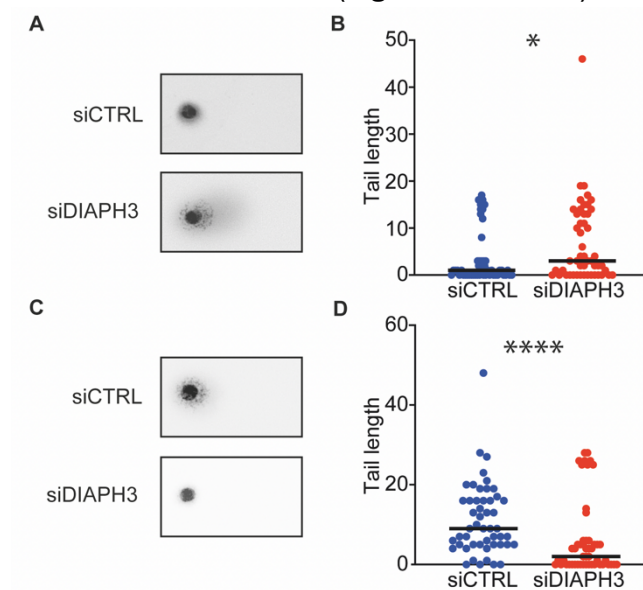


Figure 33. A, C. Representative fluorescence micrographs of comet assays showing DNA strand breaks in U251 (A) and U87 (C) cells 48 h after transfection with siCTRL or siDIAPH3. **B, D.** Quantification of comet tail length (μm) from 50 nuclei per condition, analyzed in three independent experiments using the OpenComet plugin in ImageJ. Each dot represents an individual nucleus; horizontal bars indicate median values. Statistical analysis was performed using the Mann–Whitney test (**** $p < 0.0001$; * $p < 0.05$).

In line with those observation, DIAPH3 depletion in U251 cells induced DDR, as assessed by the increase of γ H2AX foci/cell, a marker of DDR activation (Fig. 34A and 34B). This effect was abolished in U87 cells (Fig. 34C and 34D).

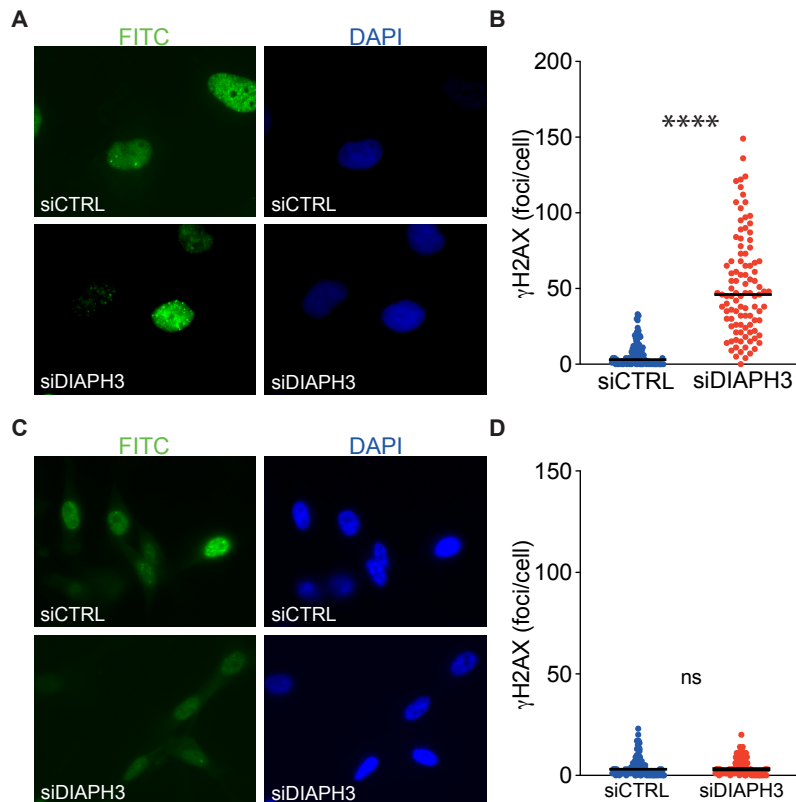


Figure 34. A, C. Representative immunofluorescence images showing γ H2AX nuclear foci (green) in U251 (A) and U87 (C) cells transfected with siCTRL or siDIAPH3. Nuclei were counterstained with DAPI (blue) and mounted with ProLong Gold antifade reagent. **B, D.** Quantification of γ H2AX foci per nucleus from ≥ 100 nuclei per condition, pooled from three independent experiments. Each dot represents a single nucleus; horizontal bars indicate median values. Statistical analysis was performed using the Mann–Whitney test (**** $p < 0.0001$; ns, not significant).

Given that RAD51 foci formation reflects the recruitment of the homologous recombination machinery to DNA double-strand breaks, we next assessed RAD51 foci in both cell lines to evaluate whether DIAPH3 depletion modulates homologous recombination repair. In U251 cells, DIAPH3 knockdown markedly increased the number of RAD51 foci per cell (Fig. 35A and 35B), whereas this effect was not observed in U87 cells (Fig. 35C and 35D). These results are consistent with our previous observations that DDR activation upon DIAPH3 repression is only detected in p53-mutated cells.

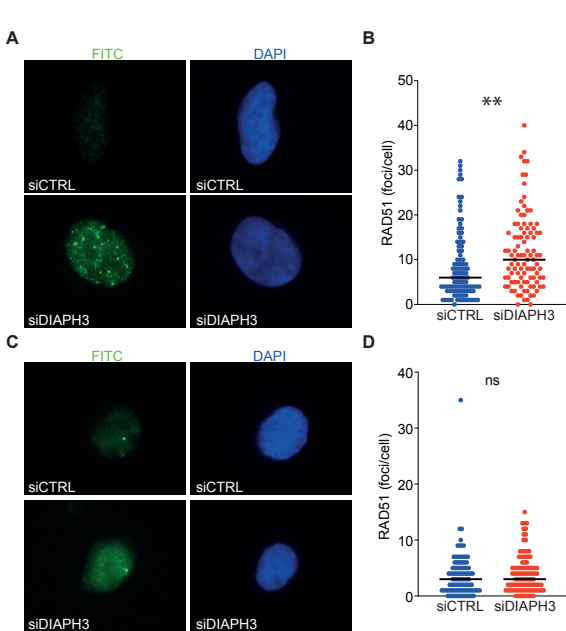


Figure 35. A, C.

Representative immunofluorescence images showing RAD51 nuclear foci (green) in U251 (A) and U87 (C) cells transfected with siCTRL or siDIAPH3. Nuclei were counterstained with DAPI (blue) and mounted with ProLong Gold antifade reagent. **B, D.** Quantification of RAD51 foci per nucleus from ≥ 100 nuclei per condition, pooled from three independent experiments. Each dot represents an individual nucleus; horizontal bars indicate median values.

Statistical analysis was performed using the Mann-Whitney test (** $p < 0.01$; ns, not significant).

Repression of DIAPH3 altered cell viability and cell cycle progression

We next investigated the impact of DIAPH3 repression on cell viability and cell cycle distribution. In U251 cells, DIAPH3 knockdown reduced cell death, as assessed by the dissipation of mitochondrial membrane potential detected with the fluorescent probe DiOC₆(3) (Fig. 36A). In contrast, DIAPH3 repression increased cell death in U87 cells (Fig. 36B). Regarding cell cycle progression, DIAPH3 knockdown induced a G2/M arrest in both cell lines (Fig. 36C and 36D). Moreover, DIAPH3 repression increased the proportion of U87 cells in the sub-G1 fraction and the proportion of hypertetraploid cells in U251. These findings are consistent with the notion that DIAPH3 repression promotes cell death in U87 cells while favoring aneuploidy in U251 cells.

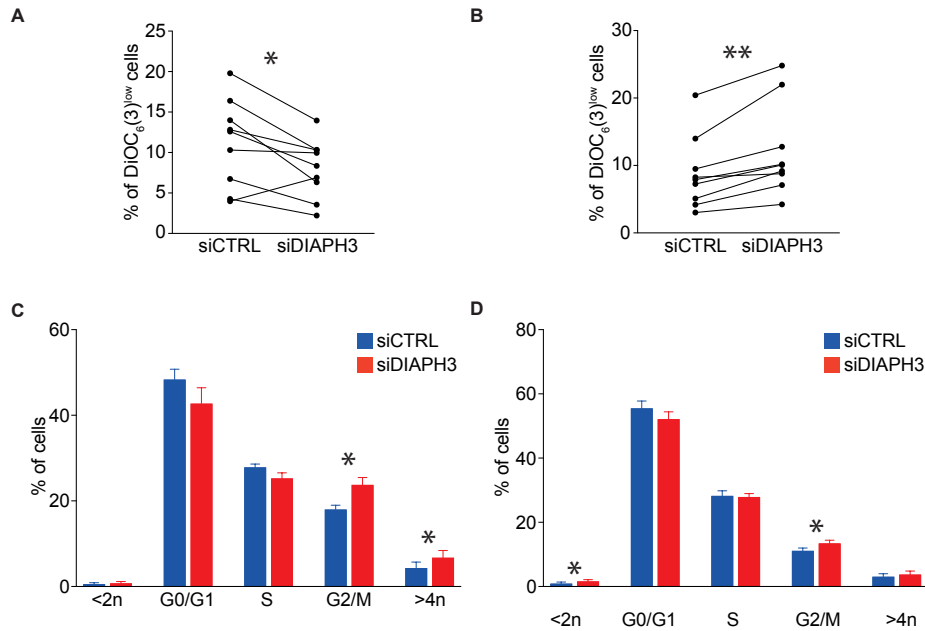


Figure 36. A, B. Quantification of mitochondrial membrane potential in U251 (A) and U87 (B) cells following siRNA-mediated DIAPH3 depletion. Cells were incubated with 40 nM DiOC₆(3) for 30 min, and fluorescence intensity was analyzed by flow cytometry using FlowJo software. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test (** $p < 0.01$; * $p < 0.05$). **C, D.** Cell-cycle distribution of U251 (C) and U87 (D) cells after transfection with siCTRL or siDIAPH3. Cells were pulse-labeled with EdU for 1.5 h before fixation, and total DNA content was stained with FxCycle Violet. Data represent mean $\pm$ SEM from three independent experiments. Statistical analysis was performed using the Mann–Whitney test (* $p < 0.05$).

Repression of p53 in U87 reproduced the effects of DIAPH3 depletion in U251 cells

Our results revealed marked differences in aneuploidy and DNA damage induced by DIAPH3 repression between U87 and U251 cells. To

determine whether these differences were attributable to p53 function, we repressed p53 in U87 cells. Efficient reduction of p53 protein levels after siRNA transfection was confirmed by immunoblotting (Fig. 37A). Combined repression of p53 and DIAPH3 in these cells recapitulated the effects observed after DIAPH3 knockdown in U251 cells. Accordingly, siRNA against DIAPH3 increased aneuploidy in p53-depleted U87 cells (Fig. 37B). Similarly, DNA damage, as detected by γ H2AX staining, was further amplified upon concomitant p53 and DIAPH3 depletion (Fig. 37C).

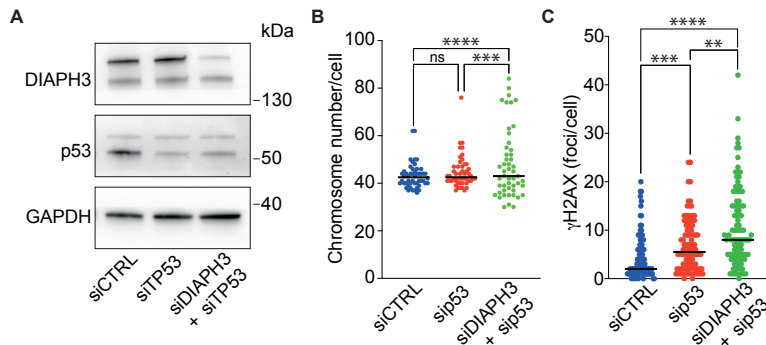


Figure 37. A. Immunoblot showing efficient knockdown of DIAPH3 and p53 proteins in U87 cells transfected with siRNAs targeting DIAPH3 (siDIAPH3), p53 (siTP53), or both. GAPDH was used as a loading control. **B.** Quantification of chromosome number per cell following metaphase spread analysis under the indicated siRNA conditions. Each dot represents an individual cell; horizontal bars indicate median values. Statistical analysis was performed using Levene's test (**** $p < 0.0001$; *** $p < 0.001$; ns, not significant). **C.** Quantification of γ H2AX foci per nucleus from ≥ 100 nuclei per condition, pooled from three independent experiments. Each dot represents one nucleus; horizontal bars indicate median values. Statistical analysis was performed using one-way ANOVA (**** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$).

Pharmacological inhibition of DIAPH3 recapitulated the effects of DIAPH3 siRNA

We next sought to determine whether pharmacological inhibition of DIAPH3 would phenocopy the effects of DIAPH3 knockdown. To this end, we treated U251 and U87 cells with SMIFH2, a small-molecule inhibitor that blocks formin activity and has been reported to inhibit DIAPH family proteins. Consistent with our siRNA data, SMIFH2 treatment strongly increased aneuploidy in U251 cells but had no detectable effect on aneuploidy in U87 cells (Fig. 38A and 38B). In contrast, SMIFH2 enhanced activation of the DNA damage response in both cell lines, as evidenced by a significant rise in the number of γ H2AX foci per cell (Fig. 38C and 38D). Thus, pharmacological inhibition of DIAPH3 largely recapitulates the effects of DIAPH3 silencing on chromosomal stability in U251 cells, while also inducing a broader DDR activation detectable in both cell types.

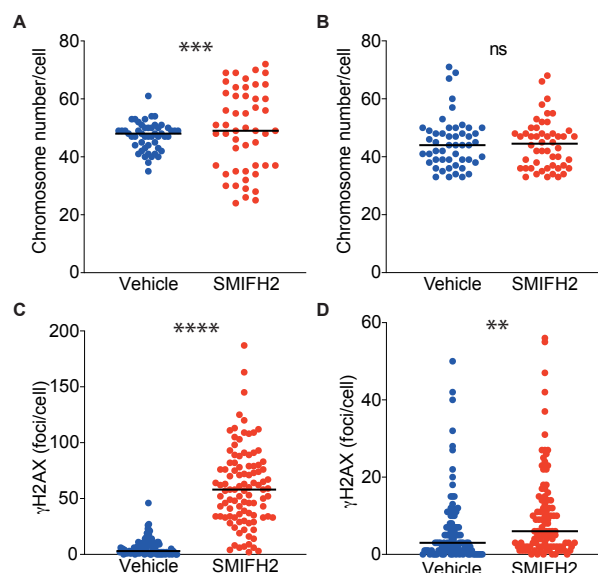


Figure 38. A, B. Quantification of chromosome number per cell following metaphase spread analysis in U251 (A) and U87 (B) cells treated with SMIFH2 or vehicle (DMSO) for 24 hours. Each dot represents an individual cell; horizontal bars indicate median values. Statistical comparison was performed using Levene's test to assess differences in variance rather than central tendency (** $p < 0.001$; ns, not significant). **C, D.** Quantification of γH2AX foci per nucleus in U251 (C) and U87 (D) cells after SMIFH2 treatment. Data were collected from ≥ 100 nuclei per condition, pooled from three independent experiments. Each dot represents a single nucleus; horizontal bars denote median values. Statistical analysis was performed using the Mann–Whitney test (**** $p < 0.0001$; ** $p < 0.01$).

Interaction effects between DIAPH3 knockdown and treatments

Because TMZ and RT are combined in the Stupp protocol for glioblastoma treatment, we next examined whether DIAPH3 repression

could alter the response of GBM cells to these therapies. Two-way ANOVA revealed that, in the presence of TMZ, the only significant effect was an increase in cell death, reflected by a higher subG1 fraction, in U87 cells. No significant effect of TMZ was observed on G2/M arrest in either cell line, and no interaction between TMZ and DIAPH3 knockdown was detected (Fig. 39).

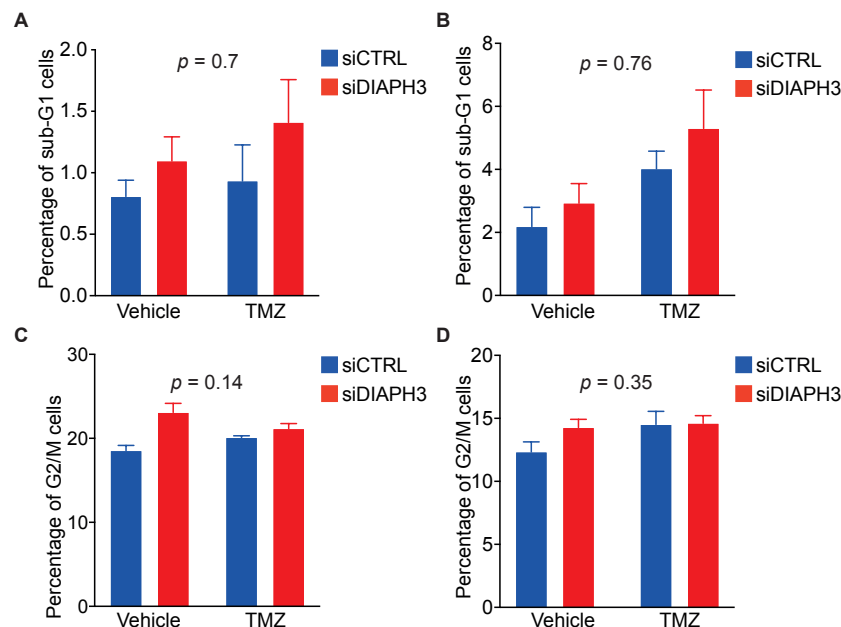


Figure 39. A,B. U251 and U87 cells were transfected with a non-targeting control siRNA (siCTRL) or a pool of siRNAs targeting DIAPH3 (siDIAPH3) for 48 hours, followed by treatment with TMZ (100 μ M) or vehicle (DMSO) for an additional 48 hours. (A, B) Quantification of sub-G1 populations in U251 (A) and U87 (B) cells. **C, D.** Quantification of G2/M-phase populations in U251 (C) and U87 (D) cells under the same conditions. All data represent mean $\pm$ SEM from three independent experiments. Statistical analysis was performed using two-way ANOVA; the *p*-values shown on each graph correspond to the interaction term between treatment (TMZ vs DMSO) and siRNA (siDIAPH3 vs siCTRL).

In contrast, RT markedly induced both G2/M arrest and cell death in U251 and U87 cells. Notably, DIAPH3 knockdown significantly potentiated these radiation-induced effects only in U251 cells (Fig. 40A and 40C), whereas no interaction between RT and DIAPH3 repression was observed in U87 cells (Fig. 40B and 40D). Altogether, these findings indicate that DIAPH3 repression enhances the sensitivity of p53-mutated GBM cells to irradiation, but not that of p53-WT cells.

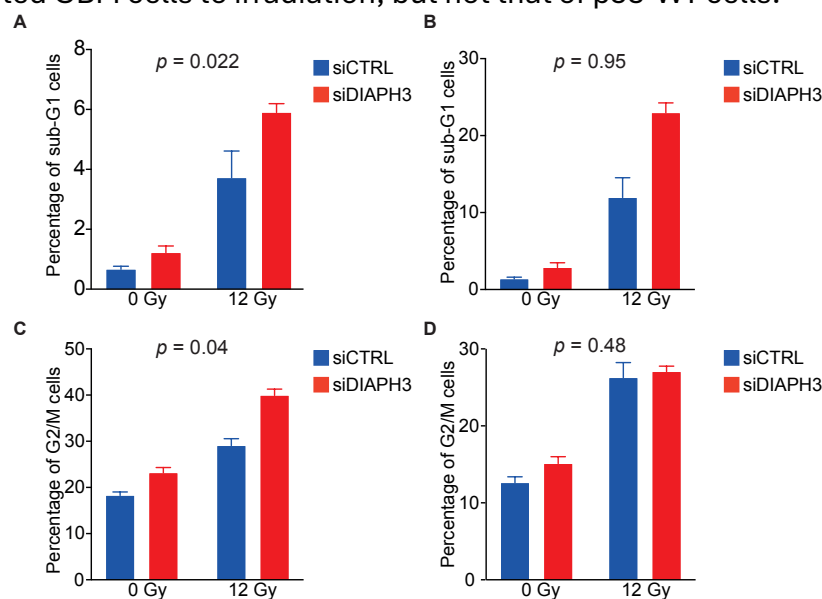


Figure 40. A,B. U251 and U87 cells were transfected with siCTRL or siDIAPH3 for 48 hours, then exposed to ionizing radiation (12 Gy) or left unirradiated (0 Gy). (A, B) Quantification of sub-G1 populations in U251 (A) and U87 (B) cells 48 hours after irradiation. **C, D.** Quantification of G2/M-phase populations in U251 (C) and U87 (D) cells under the same conditions. All data represent mean $\pm$ SEM from three independent experiments. Statistical analysis was performed using two-way ANOVA; the p-values shown on each graph correspond to the interaction between irradiation (12 Gy vs 0 Gy) and siRNA (siDIAPH3 vs siCTRL).

DISCUSSION

Bioinformatics Insights into DIAPH3 Expression and its Clinical Relevance in Glioma

The most striking finding of this bioinformatics analysis is that high DIAPH3 expression is specifically associated with high-grade gliomas, as its expression levels are comparable between normal brain tissue and low-grade gliomas. Interestingly, using an optimal DIAPH3 expression cutoff of 6.7 ($\log_2(\text{normalized counts} + 1)$) determined by ROC analysis, we achieved a sensitivity of 85.7% and a specificity of 80.2% in distinguishing CNS5-HGG from CNS5-LGG. These results suggest that DIAPH3 could represent a useful biomarker for differentiating low-grade from high-grade gliomas. Moreover, because its expression appears to be largely restricted to high-grade gliomas, DIAPH3 may also represent a potential therapeutic target whose selective expression pattern could offer opportunities for tumor-specific intervention.

These findings appear to contrast with our previous clinical study, in which high DIAPH3 expression was associated with improved overall survival. This apparent discrepancy is likely explained by major

differences in study design and clinical context, as the previous analysis was performed on a relatively small cohort (73 patients) and was restricted to a specific subgroup of GBM patients, notably those with MGMT-methylated tumors. In this biologically and clinically more homogeneous setting, DIAPH3 expression may reflect distinct tumor dependencies or treatment responses, whereas the present bioinformatic analysis explores DIAPH3 expression across large, heterogeneous glioma cohorts and primarily captures its association with tumor grade, mitotic activity and chromosomal instability rather than direct prognostic value.

One possible explanation for the selective overexpression of *DIAPH3* in high-grade gliomas is its association with *IDH* status. Indeed, the vast majority of gliomas classified as grade 4 according to the WHO CNS5 criteria are *IDH*-wild type. *IDH* mutation induces the formation of D-2-hydroxyglutarate, a competitive inhibitor of TET, which is an activator of DNA demethylation (Han et al., 2020). *IDH* mutation is therefore associated with DNA hypermethylation and a better prognosis, notably in gliomas. It could therefore be hypothesized that if DIAPH3 is more

highly expressed in *IDH*-wild type gliomas, it is because its promoter is demethylated. However, our analyses show that the *DIAPH3* promoter is only slightly methylated and that its methylation level does not change significantly between normal brain tissue and gliomas.

Since *DIAPH3* is involved in the normal functioning of the spindle assembly checkpoint, the fact that it is expressed in high-grade gliomas, and therefore associated with a poor prognosis, may seem surprising. One might expect that reducing *DIAPH3* expression by altering the SAC would increase aneuploidy and reduce tumour aggressiveness. However, this is not the case. On the contrary, the more *DIAPH3* is expressed, the higher the aneuploidy score in gliomas. The same observation is made for the expression of other genes coding for proteins whose role in SAC is well established. An obvious explanation for this apparent contradiction is that overexpression of certain SAC proteins in tumor cells may be a consequence rather than a cause of SAC failure. It is also plausible that both overexpression and underexpression of these proteins disrupt the delicate balance required for proper kinetochore function and accurate chromosome segregation, thereby increasing the

risk of aneuploidy. Indeed, both overexpression and underexpression of SAC proteins disturb mitotic fidelity (Hosea et al., 2024b). Our *in vitro* findings indicate that repression of DIAPH3 increases aneuploidy in GBM cells. This suggests that changes in DIAPH3 expression not only correlate with aneuploidy but may also cause it.

It should be noted that the frequency of *DIAPH3* mutation in cancers is very low (less than 2% of all TCGA samples) and there is no difference between the aneuploidy scores of samples with wild-type or mutated *DIAPH3*. It can therefore be concluded that neither low *DIAPH3* expression nor *DIAPH3* mutation is the cause of increased aneuploidy in tumor cells.

The significance of aneuploidy in cancer cells is complex and not fully understood (Sdeor et al., 2024). Aneuploidy is often found in cancer cells, where it is thought to favor cancer initiation or progression (Sansregret & Swanton, 2017). The ability of cancer cells to resist senescence inducers or proapoptotic signals is thought to allow aneuploid cells to survive and proliferate (Soto et al., 2017). By promoting genomic instability, aneuploidy may also facilitate genomic

mutations leading to tumor heterogeneity and conferring phenotypic adaptations to cancer cells in the absence of growth factors or nutrients or in the presence of cytotoxic insults such as chemotherapy or radiation (A. J. X. Lee et al., 2011). In contrast, exacerbating aneuploidy in cancer cells by destabilizing the SAC may lead to tumor suppression through the induction of cell death (Silk et al., 2013; Zasadil et al., 2014). Aneuploidy may also favor the induction of mitotic catastrophe by drugs that interfere with chromosome segregation (Janssen et al., 2011; Kops et al., 2004).

The key pathway consistently enriched in all GO analyses, regardless of dataset (TCGA or scRNA-seq) or ontology, is cell division, in particular mitotic progression and chromosome segregation. Genes positively correlated with *DIAPH3* expression are associated with mitotic nuclear division, sister chromatid segregation and DNA replication. This strongly suggests that the observations made on the role of *DIAPH3* in SAC function in cell and mouse models can be extrapolated to glioma patients. This also explains why *DIAPH3* is mainly

expressed in tissues where mitotic activity is intense, such as bone marrow, testis and cancers.

Our analysis revealed that several TFs – TFDP1, E2F8, SP2, and the NF-Y complex – bind to the putative *DIAPH3* promoter region, as confirmed by ChIP-seq data from the ReMap2022 database. All are known regulators of cell cycle genes and have established roles in cancer. TFDP1 forms heterodimers with E2F TFs to regulate genes involved in the G1/S transition and mitotic progression. Both TFDP1 and E2F8 have been linked to increased proliferation and poor prognosis in several malignancies, including GBM (Ju et al., 2025; D. Y. Lee et al., 2023; X. Lu et al., 2016). SP2, although less studied, belongs to SP protein family, which is involved in the regulation of cell proliferation, differentiation and apoptosis (Safe, 2023). It is associated with a poor prognosis in hepatocellular carcinoma (Y. Zhu et al., 2020). The NF-Y complex regulates genes involved in DNA replication and mitosis. Overexpression of NF-Y – mostly *NFYA* – is oncogenic and decreases sensitivity to anti-neoplastic drugs (Dolfini et al., 2024). Together, these findings support the hypothesis that *DIAPH3* is integrated into a

transcriptional network controlling mitosis and cell proliferation – pathways commonly hijacked in cancer – and suggest a functional role for DIAPH3 in glioma progression and chromosomal instability (Fig. 41).

A fundamental limitation of retrospective studies on clinical data is their inability to establish causal relationships, as they can only reveal correlations. Nevertheless, the strength of the present bioinformatic analysis lies in its strong indication that the effects of DIAPH3 on proper chromosome segregation, previously observed in cellular and animal models, may also extend to glioma patients.

Our bioinformatics analyses reveal a coherent picture in which DIAPH3 emerges as a key player associated with mitotic regulation, chromosomal segregation and glioma progression. However, while these findings provide strong correlative evidence and valuable clinical insights, they do not by themselves establish causality. To address this limitation and to elucidate the functional contribution of DIAPH3 to genomic stability and tumor behavior, we investigated its mechanistic role using in vitro experimental models of glioblastoma.

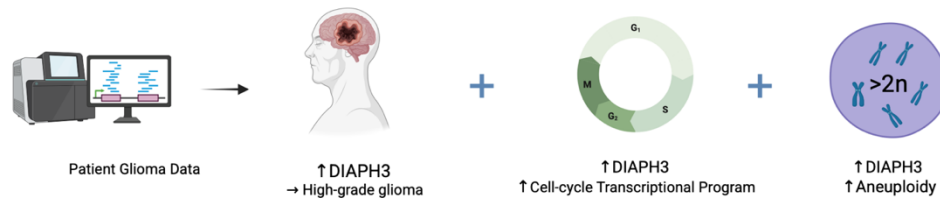


Figure 41. Bioinformatic framework linking DIAPH3 overexpression to glioma malignancy and chromosomal instability. Integrative analyses of patient glioma datasets reveal that DIAPH3 is selectively overexpressed in high-grade gliomas, but not in normal brain or low-grade tumors, enabling robust discrimination between CNS5-HGG and CNS5-LGG. Elevated DIAPH3 expression is associated with activation of a cell-cycle and mitosis-related transcriptional program, enrichment of genes involved in chromosome segregation, and increased aneuploidy. Created using BioRender.

Experimental Evidence for DIAPH3 as a Regulator of Genome Stability in Glioblastoma

Building on the bioinformatics evidence linking DIAPH3 to mitotic activity and glioma aggressiveness, we next examined its functional role through experimental studies. These analyses aimed to clarify how DIAPH3 modulation affects chromosomal stability, DNA damage response and GBM cell fate in different p53 contexts. Our study identifies DIAPH3 as a key regulator of chromosomal stability and DDR in GBM, with effects that are strongly dependent on the cell lines used.

We show that DIAPH3 repression induces aneuploidy and activates DDR pathways in p53-mutated GBM cells, whereas it triggers cell-cycle arrest and a comparatively modest impact on cell death. Although one possible explanation could be that chromosome mis-segregation engages a p53-dependent checkpoint capable of eliminating unstable cells, our data indicate that overt cell death is limited in U87 cells. Therefore, p53 activation may instead slow proliferation, promote transient arrest or selectively restrict the propagation of the most unstable cells rather than inducing robust cell

death. Conversely, in p53-mutated cells, which lack this surveillance, mitotic errors accumulate and give rise to viable but aneuploid progeny. Consistent with this hypothesis, silencing p53 in U87 cells led to the emergence of aneuploid cell populations, thereby recapitulating the phenotype observed in p53-mutated U251 cells.

It is worth noting, however, that in the absence of functional p53, DIAPH3 repression led to a pronounced cell-cycle arrest at the G2/M transition. It may seem paradoxical that inhibition of a protein likely involved in the SAC results in a prolongation of mitosis, given that the primary function of the SAC is to delay anaphase until all chromosomes are properly attached to the spindle. Nevertheless, our observations are consistent with previous studies showing that inhibition of canonical SAC regulators such as BUB1, PLK1 or AURKB can slow mitotic progression and induce G2/M arrest (Choi et al., 2015; Gelb et al., 2025; Park et al., 2019).

Incidentally, both over- and under-expression of SAC components have been reported to increase aneuploidy (Hosea et al., 2024). This is likely because SAC proteins function within stoichiometric

multiprotein complexes in which both excess and deficit of individual partners disturb the delicate balance required for proper kinetochore-microtubule attachment and accurate chromosome segregation. Given the central role of the SAC in maintaining chromosomal fidelity, it is not surprising that perturbing SAC components promotes aneuploidy (Hosea et al., 2024). DIAPH3 inhibition induces aneuploidy in normal cells, whereas our findings demonstrate that DIAPH3 repression can further exacerbate chromosomal instability in cancer cells, which are already intrinsically aneuploid.

Several studies have shown that SAC defects activate the ATM/Chk2-dependent DDR, reflecting a *bona fide* DSB response triggered by the rupture of mis-segregating chromosomes during cytokinesis (Janssen et al., 2011; Shi et al., 2019; Zhou et al., 2020). Interestingly, DDR activation can in turn promote further chromosomal instability (J. Zhu et al., 2018). In this way, aneuploidy and DNA damage are not independent phenomena but part of a self-reinforcing cycle, in which chromosome mis-segregation generates structural DNA lesions,

while an unbalanced genome induces replication stress that perpetuates chronic DDR activation.

Consistently, DIAPH3 repression markedly increased DNA strand breaks and activated the DDR in p53-mutated U251 cells, as evidenced by comet assays and the elevated number of γ H2AX foci per nucleus. These effects became apparent in U87 cells only after p53 depletion, supporting the idea that p53-WT cells respond to DIAPH3 loss primarily by enforcing cell-cycle control that eliminates chromosomally unstable cells before they can complete mitosis and produce progeny carrying DNA damage. In line with this, the formation of RAD51 foci, a well-established marker of homologous recombination and DNA repair, was strongly induced in U251 cells following DIAPH3 repression but not in U87 cells. This indicates that DNA repair pathways are effectively engaged in p53-deficient cells that survive mitotic errors, whereas they remain inactive in p53-proficient cells that prevent the proliferation of severely damaged cells. These observations are consistent with previous studies showing that disruption of the SAC induces cell-cycle arrest or cell death in a p53-dependent manner (Ohashi et al., 2015; Soto

et al., 2017). Importantly, our results indicate that in U87 cells DIAPH3 repression does not lead to substantial cell death, and additional work will be required to delineate how p53 constrains genomic instability without strongly inducing cell death.

Although the increase in cell death observed upon DIAPH3 repression in p53-WT U87 cells was modest, it may nonetheless be biologically meaningful. Alternatively, the reduction in proliferative capacity and accumulation of cells at G2/M may themselves be sufficient to limit the expansion of unstable clones, independent of overt apoptotic elimination. It is also plausible that other mechanisms, such as mitotic slippage, p53-mediated senescence-like states or selective retention of more stable cells within the population, could also contribute and warrant further investigation. Further studies are needed to clarify these possibilities.

SMIFH2 was initially identified as a broad-spectrum inhibitor of FH2 domains through a chemical screen for compounds that block formin-mediated actin polymerization (Rizvi et al., 2009). It prevents actin nucleation and filament elongation across multiple species,

making it a valuable but non-selective research tool. In our study, SMIFH2 reproduced the effects of DIAPH3 repression in p53-mutated U251 cells by inducing pronounced aneuploidy and activating the DDR. In p53-WT U87 cells, SMIFH2 also triggered DNA damage, albeit to a much lesser extent, suggesting that its effects may either be stronger than those achieved by DIAPH3 silencing or partly result from inhibition of other formins. This interpretation is consistent with its known lack of specificity toward individual formin isoforms. Given its broad activity, the clinical use of SMIFH2 is not feasible. Therefore, if DIAPH3 proves to be a promising therapeutic target in GBM the development of highly selective DIAPH3 inhibitors will be required to enable targeted therapeutic modulation without interfering with other formin-dependent processes.

In our experimental conditions, TMZ had minimal effects on cell death and cell-cycle distribution in both GBM cell lines. Moreover, DIAPH3 repression did not modify the cellular response to TMZ, indicating that loss of DIAPH3 does not enhance TMZ sensitivity. In contrast, ionizing radiation markedly induced both G2/M arrest and cell

death in U87 and U251 cells, and DIAPH3 repression significantly potentiated these effects in U251 cells. This radiosensitizing effect was not observed in p53-WT U87 cells, suggesting that DIAPH3 loss selectively increases radiation sensitivity in p53-mutated GBM cells.

The mechanisms underlying the distinct influence of DIAPH3 repression on the cellular responses to TMZ and radiation remain unclear but may reflect the fundamentally different types of DNA damage and repair pathways engaged by these treatments. In particular, ionizing radiation has been shown to directly promote chromosome missegregation during mitosis, leading to the formation of micronuclei that amplify genome damage through subsequent chromosomal fragmentation (Bakhoun et al., 2015). Consistently, experimental suppression of whole chromosome missegregation was reported to reduce secondary chromosomal defects and significantly improve the survival of irradiated cells. It is therefore plausible that by enhancing genomic instability, DIAPH3 repression targets a specific vulnerability of cancer cells, particularly GBM, thereby increasing their susceptibility to radiation-induced cytotoxicity (Fig. 42).

However, because GBM treatment in patients relies on the combined administration of RT and TMZ, additional experiments evaluating the consequences of DIAPH3 knockdown under concomitant RT-TMZ exposure will be required to determine whether these interactions persist in clinically relevant conditions. Furthermore, our findings should be validated in patient-derived glioma models, such as primary GBM cultures, as these systems more faithfully recapitulate the intratumoral heterogeneity, genetic context, and treatment-resistant subpopulations found in vivo. Using such models would allow us to determine whether the effects of DIAPH3 repression observed in established cell lines translate to the diverse cellular states present in patient tumors, thereby strengthening the clinical relevance of DIAPH3 as a potential therapeutic target.

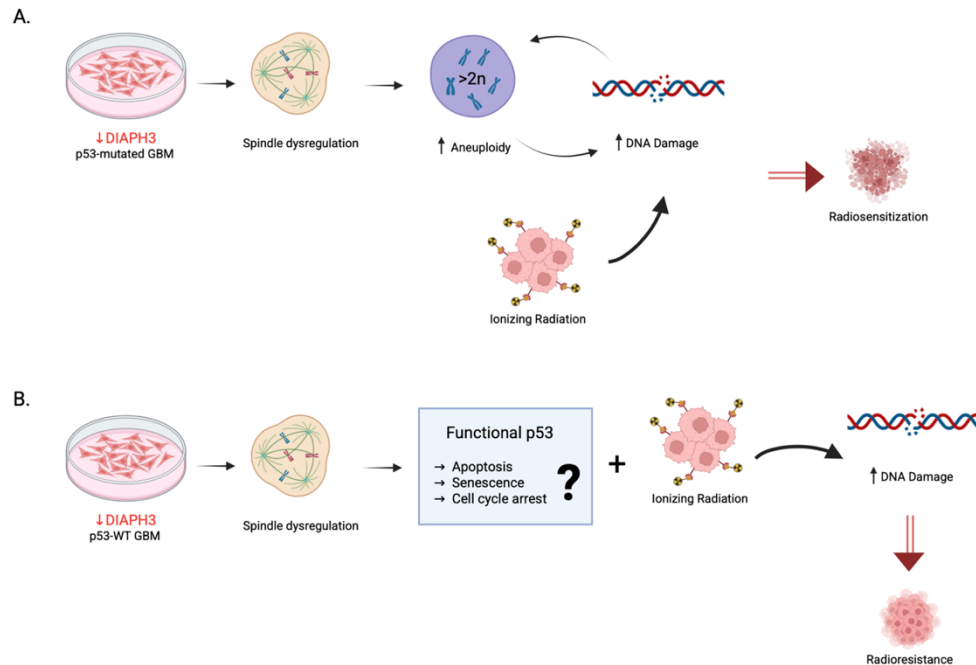


Figure 42. p53-dependent consequences of DIAPH3 repression on chromosomal stability, DNA damage response, and radiosensitivity in glioblastoma. **A.** In p53-mutated GBM cells, DIAPH3 repression induces spindle dysregulation, leading to chromosome mis-segregation and aneuploidy, which in turn triggers DNA damage and chronic DDR activation. The accumulation of viable but genomically unstable cells enhances susceptibility to ionizing radiation, resulting in radiosensitization. **B.** In p53-wild-type GBM cells, DIAPH3 repression also perturbs spindle function, but functional p53 signaling could promote cell-cycle arrest, senescence or limited apoptosis, thereby restricting the propagation of aneuploid cells. As a consequence, DNA damage accumulation is constrained and no radiosensitization is observed. Created using BioRender.

CONCLUSIONS AND FUTURE PERSPECTIVES

This work provides a comprehensive investigation into the role of DIAPH3 in glioma biology, bridging large-scale bioinformatics analyses with functional experimental validation. we have uncovered a coherent narrative in which DIAPH3 emerges as a key regulator of chromosomal stability, mitotic fidelity and cellular response to DNA damage.

Our bioinformatics analyses across multiple datasets reveal that DIAPH3 expression is selectively elevated in high-grade gliomas, while remaining low in normal brain and low-grade gliomas. This differential expression pattern suggests that DIAPH3 activation is a hallmark of malignant transformation within the glioma spectrum. Functional enrichment analyses consistently pointed toward mitotic progression, sister chromatid segregation, and DNA replication as the most significantly associated pathways, highlighting the contribution of DIAPH3 to cell-cycle control and genomic integrity.

Complementing these computational insights, our experimental studies demonstrated that DIAPH3 repression profoundly alters the fate of glioblastoma cells, with effects dictated by p53 status. Promoting

aneuploidy, DDR activation and radiosensitivity in p53-mutated cells, effects not seen in p53-wild-type cells. This dual outcome underscores the context-dependent role of DIAPH3 in maintaining genomic stability: protective in normal or p53-intact cells, yet permissive to instability in the p53-deficient tumor context. These results identify DIAPH3 as a potential therapeutic target to enhance the efficacy of radiotherapy in aggressive gliomas and support the development of selective DIAPH3 inhibitors as modulators of mitotic fidelity in cancer.

Overall, this work lays the foundation for future research exploring the nuclear functions of DIAPH3. As an actin-binding protein with the capacity to shuttle between the cytoplasm and nucleus via the importin α/β pathway (Miki et al., 2009), DIAPH3 may extend its regulatory influence beyond cytoskeletal organization and mitotic spindle dynamics. Increasing evidence supports the existence of nuclear actin polymerization as a key mechanism controlling chromatin architecture, DNA Damage control and DNA repair processes (Ulferts et al., 2024). Given DIAPH3's established involvement in chromosome segregation and DNA damage response, it is plausible that

its nuclear localization contributes to actin-dependent chromatin remodeling or repair foci formation. Investigating the conditions that trigger DIAPH3 nuclear import, such as DNA damage, replication stress or mitotic exit, could reveal a previously unrecognized layer of regulation linking actin dynamics to genome maintenance. Elucidating this nuclear role would not only expand our understanding of DIAPH3's multifunctionality but also uncover new conceptual connections between cytoskeletal dynamics and nuclear genome stability in glioma biology.

SUPPLEMENTARY DATA

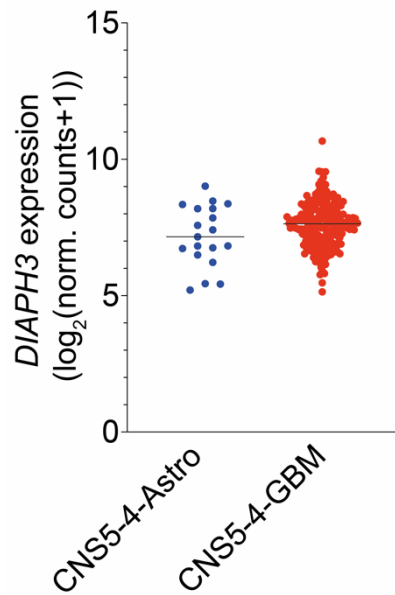


Fig. S1. *DIAPH3* expression in grade 4 astrocytomas and GBM classified according to the 2021 WHO CNS5 classification. Each point represents one sample, and the bars represent the median expression.

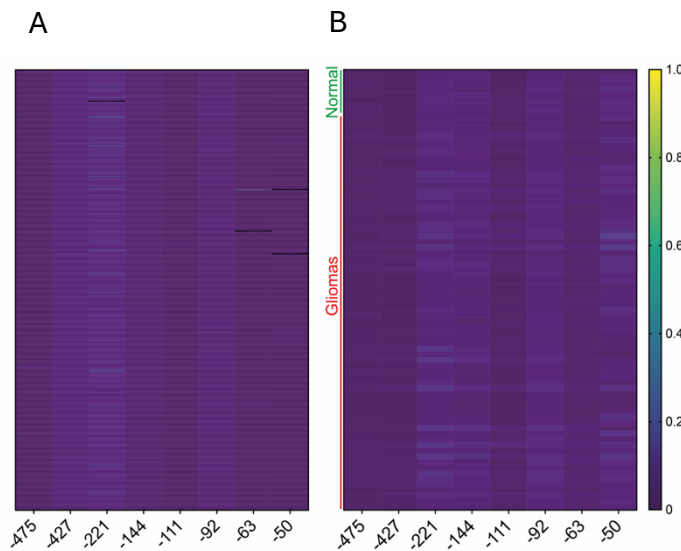


Fig. S2. A Methylation of the *DIAPH3* promoter in TCGA glioma samples, expressed as beta-values by relative position to the *DIAPH3* TSS. **B** The same as in A, but for the normal, glioma samples from the GSE123678 dataset.

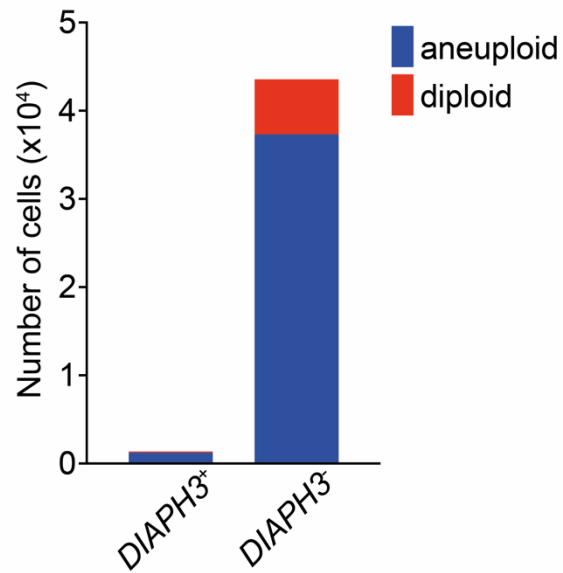


Fig. S3. *DIAPH3* expression in diploid and aneuploid GBM cells from the dataset GSE182109. Contingency analysis by Fisher's exact test, $p < 0.0001$

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