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DNA bridges in anaphase

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Contents

Abstract

Chromosome missegregation poses a non-reversible threat to genome stability, leading to chromosomal abnormalities that can have severe consequences for cellular function. Accurate chromosome segregation is important for maintaining chromosome integrity, ensuring that daughter cells inherit a complete set of chromosomes. Errors during this process can arise from various defects. In this master thesis, I focus on unresolved DNA entanglements that stretch into bridge-like structures in anaphase and can interfere with proper sister chromatid segregation. These events might lead to chromosomal instability, a hallmark of cancer. Uncovering how cells detect and resolve such structures in anaphase is crucial for understanding the mechanisms responsible for chromosome missegregation. A key protein binding to these structures is PICH, a DNA translocase with a binding preference for stretched DNA. PICH acts as a recruiting platform for other proteins that also contribute to the resolution of anaphase DNA bridges. I analyzed PICH binding to anaphase bridges in various cell lines. By analyzing key parameters, such as length, bridge number per cell, bridge-staining properties with conventional DNA dyes, colocalizing proteins, and irradiation effects, I determined that 1) anaphase bridges are transient structures mostly arising from centromeric regions of chromosome, 2) their prevalence decreases as anaphase progresses, 3) PICH-coated bridge-like structures may already form in metaphase, 4) CIP2A, a protein that binds TOPBP1, localizes to a subset of ultrafine DNA bridges. These findings highlight the complexity of anaphase DNA bridge formation and resolution during chromosome segregation and provide a valuable resource for further experiments on genome stability during mitosis.

Key words: Chromosome segregation, anaphase bridges, PICH protein

Zusammenfassung

Die Fehlverteilung von Chromosomen stellt eine nicht umkehrbare Bedrohung für die Stabilität des Genoms dar und führt zu chromosomalen Abweichungen, die schwerwiegende Folgen für die Zellfunktion haben können. Eine genaue Chromosomenverteilung ist wichtig für die Aufrechterhaltung der Chromosomenintegrität und stellt sicher, dass Tochterzellen einen vollständigen Chromosomensatz erben. Fehler in diesem Prozess können durch verschiedene Defekte entstehen. In dieser Masterarbeit konzentriere ich mich auf ungelöste DNA-Verschlingungen, die sich in der Anaphase zu brückenartigen Strukturen ausdehnen und die ordnungsgemäße Trennung der Schwesterchromatiden stören können. Diese Ereignisse könnten zu chromosomaler Instabilität führen, einem Kennzeichen von Krebs. Zu verstehen, wie Zellen solche Strukturen in der Anaphase erkennen und auflösen, ist entscheidend, um die Mechanismen zu verstehen, die für die Fehlverteilung von Chromosomen verantwortlich sind. Ein Schlüsselprotein, das an diese Strukturen bindet, ist PICH, eine DNA-Translokase mit einer Bindungspräferenz für gedehnte DNA. PICH wirkt als Rekrutierungsplattform für andere Proteine, die ebenfalls zur Auflösung von DNA-Brücken in der Anaphase beitragen. Ich habe die PICH-Bindung an Anaphase-Brücken in verschiedenen Zelllinien analysiert. Durch die Analyse wichtiger Parameter wie Länge, Brückenzahl pro Zelle, Färbeeigenschaften der Brücken mit herkömmlichen DNA-Farbstoffen, mitlokalisierte Proteine und Bestrahlungseffekte stellte ich fest, dass 1) Anaphase-Brücken vorübergehende Strukturen sind, die meist aus den zentromeren Bereichen der Chromosomen entstehen, 2) ihre Häufigkeit mit fortschreitender Anaphase abnimmt, 3) PICH-beschichtete brückenartige Strukturen möglicherweise bereits in der Metaphase entstehen, 4) CIP2A, ein Protein, das an TOPBP1 bindet, sich an einem Teil der ultrafeinen DNA-Brücken lokalisiert. Diese Ergebnisse verdeutlichen die Komplexität der Bildung und Auflösung von DNA-Brücken in der Anaphase während der Chromosomenverteilung und liefern eine wertvolle Grundlage für weitere Experimente zur Genomstabilität während der Mitose.

Schlüsselwörter: Chromosomenverteilung, Anaphase-Brücken, PICH-Protein

1 INTRODUCTION

1.1 DNA threads pose a problem for the cell

1.1.1 Accurate chromosome segregation is crucial for maintaining genome integrity

The ultimate goal of every life form is to ensure the successful transmission of its genetic material to the next generation. A somatic cell cycle consists of interphase and mitosis. During mitosis, sister chromatids are segregated into two daughter cells, ensuring that each cell inherits one complete set of chromosomes. Faithful chromosome segregation is critical for maintaining genomic stability. This stability relies on three key processes: the faithful duplication of DNA, the effective repair of DNA damage, and the equal distribution of replicated genomes to daughter cells. Among these, the proper distribution of duplicated genomes during the final stages of cell division—anaphase and cytokinesis—is particularly crucial. Errors in this stage lead to genomic changes that persist beyond cell division, potentially giving rise to cells with harmful genomic content. Problems with chromosome segregation can often be identified by the presence of anaphase bridges, a hallmark of genomic instability. Anaphase bridges are structures that emerge during anaphase when DNA or chromatin fails to segregate properly, resulting in DNA lagging or tethered between two dividing cells (Bizard & Hickson, 2018). Failure to remove these bridges can result in cell cycle arrest or cytokinesis failure. In cases where chromatin bridges are improperly resolved, it can lead to chromosome fragmentation, micronucleus formation, chromothripsis, or genomic instability (Hong et al., 2021). Multiple mechanisms, discussed in the next section, facilitate the resolution of anaphase bridges arising from DNA catenation or recombination intermediates. If there is a persisting potential mitotic defect such as persistent chromatin bridge, there are kinases which can delay abscission. Failure to delay abscission increases the likelihood of chromosome breakage during cell division. This highlights that cells possess mechanisms to postpone cytokinesis when chromosome segregation is impaired. Such mechanism provides additional time for resolving chromatin bridges, ensuring genomic stability (Norden et al., 2006). Ultrafine bridges (UFBs), another type of anaphase bridges, are resolved through the coordinated action of helicases and

topoisomerases, which act together on the DNA bridge to help resolve the intertwined DNA strands (Chan & Hickson, 2011). However, the mechanisms for complete resolution of UFBs remains unanswered.

1.2 Anaphase bridges and their origins

Formation of abnormal chromosomal structures during anaphase was already described around the turn of the 20th century by cytologists such as David von Hansemann and Theodor Boveri. Around 80 years ago, Barbara McClintock demonstrated that dicentric chromosomes formed by the fusion of two chromosomes, can initiate a "breakage-fusion-bridge" cycle. During anaphase, these dicentric chromosomes are pulled to opposite poles, forming chromatin bridges that are prone to random breakage. In subsequent cell cycles, the broken chromosome ends may fuse again, perpetuating a recurrent cycle of bridge formation and breakage (McClintock, 1942). In addition to dicentric chromosome fusions, DNA replication and repair processes also produce DNA entanglements that link sister chromatids, termed ultrafine bridges (UFBs) (Fernández-Casañas & Chan, 2018).

Therefore, anaphase bridges are broadly separated into two categories: chromatin bridges and ultrafine bridges. Chromatin bridges, which can be visualized with conventional DNA dyes, represent chromatinized DNA structures that can be visualized during chromosome segregation by DAPI staining. In contrast, UFBs escaped detection for a long time because they cannot be stained with such dyes but can be seen using antibodies against specific proteins that localize on these structures (Finardi et al., 2020). Early work on UFBs demonstrated that 1) ultrafine bridges are significantly more abundant and can be observed in the majority of anaphase cells, while chromatin bridges are more rare and that 2) the frequency of UFBs decreases as anaphase progresses and they are largely absent by telophase, indicating UFBs are resolved during anaphase itself (Baumann et al., 2007; K.-L. Chan et al., 2007).

1.2.1 Centromeric UFBs

As mentioned before, UFBs may arise from DNA replication or repair processes. These includes DNA catenanes, unresolved recombination intermediates and late replication intermediates (LRI) (Bizard & Hickson, 2018). DNA catenanes are naturally occuring topological linkages between sister chromatids, consisting of dsDNA, mostly removed

during G2 and early on during mitosis by type II topoisomerases (Cuvier & Hirano, 2003). Because DNA is a double helix, any process that involves separating the two strands such as replication, transcription, or recombination naturally creates topological stress, resulting in the accumulation of supercoiled DNA. As replication or transcription progresses, DNA helicases move ahead of the replication fork, unwinding the double helix and pushing the twists of the helix forward. This activity generates positive supercoiling in front of the fork and negative supercoiling behind it due to the underwinding of DNA. Topological tension caused by positive supercoiling has to be relaxed, otherwise it can hinder helicase activity and stall the replication fork. Axial rotation alone is not enough to relieve all supercoils generated during replication. To solve these topological challenges, cells rely on essential enzymes called topoisomerases (Pommier et al., 2016). These are classified into two types based on their mechanism: Type I topoisomerases (TOP1 and TOP3) cut one DNA strand to allow the other strand to pass through before resealing it, while Type II topoisomerases (TOP2) cut both strands, enabling another double-stranded segment to pass through (Pommier et al., 2016). During mitosis, catenanes between sister chromatids are most frequently retained at centromeric regions (Baumann et al., 2007; K.-L. Chan et al., 2007). UFBs that arise from centromeres, termed centromeric UFBs (**C-UFBs**), are the most common type of UFB and are believed to be a result of topological linkages of double-stranded DNA (dsDNA) catenanes (Baumann et al., 2007; K.-L. Chan et al., 2007). Their number increase upon the inhibition of TOP2A (Baumann et al., 2007; K.-L. Chan et al., 2007). TOP2A is believed to be play a central role also because it was shown to catalyze decatenation of dsDNA (Waizenegger et al., 2000). TOP2A is enriched at centromeres in mitosis, indicating that some residual DNA linkages may persist into mitosis. The majority of catenaned DNA molecules can likely be resolved by TOP2A under normal conditions. However, it is unclear if TOP2A is as effective at resolving all catenated structures on centromeres and peri-centromeres where cohesion between sister chromatids is maintained prior to anaphase. Moreover, decatenation activity or binding of TOP2A may be reduced when centromeres are under tension following bi-orientation and during chromosome segregation.

1.2.2 Other UFBs

Late-replication intermediates (LRIs) are another source of DNA linkages, which arise from unreplicated regions, where the two complementary strands are still annealed to each other. LRIs connects sister chromatids through their under-replicated DNA region, as opposed to dsDNA catenanes that links them topologically. The resolution or cleavage of such structures might result in DNA breaks or single-stranded DNA (ssDNA) regions since DNA replication has not been finished, which can pose a threat to genomic integrity.

These are mostly formed at regions difficult to replicate known as common fragile sites (CFSs) (K. L. Chan et al., 2009). Replication of CFSs are often left incomplete, especially after replication stress (Lukas et al., 2011). UFBs associated with CFSs are closely related to FANCD2-FANCI protein complex, appearing at the termini of a UFBs, and therefore termed as fragile-site UFBs, or **FS-UFBs** (K. L. Chan et al., 2009). Telomeric regions are also prone to form LRIs because of their repetitive DNA sequences, which in turn may stall replication forks (Barefield & Karlseder, 2012). Telomeric UFBs, or **T-UFBs**, increase in number if replication of telomeric regions is impaired. This was shown by depletion of Werner helicase, an important enzyme that contributes to the resolution of G-quadruplexes (Barefield & Karlseder, 2012). These are not normally observed in unperturbed cells but increase in number after over-expression of TRF2, component of the Shelterin complex which protects telomeres. Their formation is proposed to be caused by replication stalling that generates replication intermediates, mimicking the effects of replication stress (Nera et al., 2015).

Unresolved DNA intermediates may also result from homologous recombination (HR), which have been suggested to produce UFBs that impair proper chromosome segregation (Sarbajna & West, 2014). This claim was validated by two independent studies, which identified a novel category of **HR-UFBs** (Y. W. Chan & West, 2018; Tiwari et al., 2018). HR is DNA repair pathway that exploits the genetic information from one sister chromatid to correct lesions on second sister chromatid and can be utilized to repair DSBs (Prado, 2018). HR-UFBs arise during recombinational repair of DSBs, where the DNA joint molecules, such as Holliday junctions, are formed between the recombining DNA strands, which covalently links them (Wyatt & West, 2014). To differentiate these bridges from other UFB types, they were termed HR-UFBs, as they are

distinct from FS-UFBs by not binding FANCD2 foci, indicative of LRIs. Inhibition of HR machinery via BRCA1 or RAD51 depletion suppressed the formation of FANCD2-negative UFBs, further supporting the distinction between HR-UFBs and FS-UFBs. If these intermediates are not resolved prior to anaphase, they can result in chromosomal missegregation (Garner et al., 2013; Wyatt et al., 2013).

The structural diversity of UFBs is evident from studies using chemical treatments that alter UFBs frequencies at specific loci. For example, C-UFBs persist when sister chromatid disjunction is inhibited using the Topoisomerase II inhibitor ICRF-193, particularly in PICH-deficient cells (Nielsen et al., 2015). FS-UFBs and T-UFBs are induced by replication stress, such as treatment with the polymerase inhibitor aphidicolin (Barefield & Karlseder, 2012). Moreover, introducing bacterial HJ resolvases into resolvase-deficient cells reduced the formation of HR-UFBs (Y. W. Chan & West, 2018). Anaphase bridges can be observed even in the absence of stressors, such as treatment with replication inhibitors, and in untransformed cells arising mostly from centromeres (K. L. Chan et al., 2009; K.-L. Chan et al., 2007).

1.3 PICH is a DNA translocase specific to ultrafine bridges

1.3.1 PICH is a main player in anaphase bridge detection

In 2007, two research group independently reported that Plk1-interacting checkpoint helicase (PICH) and a well-known BLM protein comprising Bloom's syndrome helicase, exhibit a remarkable localization on thread-like structures connecting chromatin masses during anaphase in human cells (Baumann et al., 2007; K.-L. Chan et al., 2007). At the same time, bromodeoxyuridine (BrdU) confirmed that these thin threads are composed of DNA (K.-L. Chan et al., 2007). These observations mark the beginning of studies into these previously non-recognized DNA linkages between two disjoined sister chromatids. PICH and BLM remains the best described UFB-associated proteins binding to UFBs.

The Plk1-interacting checkpoint helicase (PICH) belongs to the SNF2 family type of ATPases and was first identified as a direct interactor of Plk1, a key mitotic kinase, through screen for Plk1-binding proteins (Baumann et al., 2007). Plk1 in fact phosphorylates PICH, which is mediated by CDK1, regulating its activity during mitosis (Baumann et al., 2007). PICH contains domains characteristic for SNF2-class helicases,

including DEXH and HELICc motifs, as well as TPR domains at its N- and C-termini, which facilitate protein-protein interactions (Baumann et al., 2007; Flaus, 2006). Although PICH localizes in the cytoplasm during interphase, it re-localizes to centromeres/kinetochores during mitosis, after nuclear envelope breakdown (Baumann et al., 2007). Most notably, PICH decorates thin UFB structures between sister chromatids during anaphase. Initially, PICH was proposed to function as a spindle assembly checkpoint activator. This role was later shown to be an off-target effect of PICH small interfering RNA (siRNA) oligos on the expression of MAD2 checkpoint protein (Hübner et al., 2010). Despite this, consistent evidence shows that PICH is essential for chromosome organization, architecture and segregation (Baumann et al., 2007; Kaulich et al., 2012; Nielsen et al., 2015). PICH knockdown causes embryonic lethality in mice which is proposed to be caused by increased DNA damage produced during chromosome segregation (Albers et al., 2018). PICH was also shown to remodel nucleosome positions, suggesting PICH acts as a nucleosome-remodeler protein (Ke et al., 2011). However, it was not possible to reproduce this activity by another research group (Biebricher et al., 2013). More recent study from 2018 again proposed that PICH may act as a force-dependent nucleosome remodeler (Spakman, 2022). They observed that in absence of tension, PICH is not able to access regions between neighboring nucleosomes, but when forces of >3pN are applied, PICH is observed to bind the linker DNA regions (Spakman, 2022). These findings are consistent with the previously proposed model suggesting that PICH is recruited to UFBs only after mitotic spindle forces disrupt nucleosome-nucleosome interactions (Biebricher et al., 2013). Furthermore, they could observe that PICH can facilitate the inner-turn nucleosome unwrapping at tensions of >3pN and after nucleosome unwrapping, collision between nucleosomes and PICH translocating on DNA may results in histone sliding along the DNA (Spakman, 2022). PICH key ability to translocate along DNA in an ATP-dependent manner was shown more than 10 years ago. While PICH lacks helicase activity, it functions as a DNA-dependent ATPase and a translocase with a remarkable high binding affinity for dsDNA under tension (Biebricher et al., 2013; Sarlós et al., 2018). Compared to other translocases, PICH exhibits slower and less processive movement, as well as spontaneous directional reversals during translocation. Its high binding affinity for DNA under tension may explain why PICH can specifically associate with the underlying structure of UFBs but not with the rest of the genome (Biebricher et

al., 2013). This also suggest that PICH may be the primary sensor of UFBs. Supporting this idea, PICH depletion completely abolished recruitment of other known UFB-binding proteins discussed below.

1.3.2 BLM and TOP3A

Another key UFB-binding protein is BLM helicase. BLM belongs to the highly conserved and important family of RecQ helicases, known to be required for maintenance of genome integrity (Chu & Hickson, 2009). In BLM knockout cells DNA replication defects (Bachrati & Hickson, 2008) and increased HR activities are observed, leading to elevated sister chromatid exchanges and spontaneous chromosome breaks (Bachrati & Hickson, 2003). Furthermore, increased formation of anaphase bridges and UFBs has been observed in the absence of BLM (K.-L. Chan et al., 2007). BLM interacts with several proteins critical for genome stability, including TOP3A (Wu et al., 2000), RM1 and RM2 (Singh et al., 2008; Xu et al., 2008), RPA (Brosh et al., 2000), and Fanconi anemia proteins (Meetei et al., 2003). BLM, TOP3A, RM1 and RM2 together form a complex named BTRR complex. BTRR has a role in resolution of DNA entanglements, which may explain its role in chromosome segregation. Recruitment of BTRR complex to UFBs (Ke et al., 2011; Kong et al., 2024) is PICH-dependent and possibly mediated by interaction of C-terminus of PICH and BLM (Ke et al., 2011; Kong et al., 2024). However, how BLM and PICH resolve UFBs remains unknown. PICH was shown to stimulate TOP2A decatenation activity in vitro, but this did surprisingly not require PICH translocation activity (Nielsen et al., 2015). It is therefore not known if PICH and BLM serve as an activators of TOP2A or promote alternative decatenation mechanisms in mitosis.

TOP3A is a type IA topoisomerase, which catalyze cleavage and subsequent re-joining of ssDNA to relieve the torsional stress introduced during strand separation by helicases (Vos et al., 2011). Since TOP3A can act on ssDNA as a decatenase (Wu & Hickson, n.d.; Yang et al., 2010), as well as resolve dsDNA catenanes or LRI in the presence of BLM (Sarlós et al., 2018), their interaction is thought to be important for UFB resolution. It is also believed that BLM unwinds UFBs during anaphase, with subsequent loading of RPA, which presence indicates formation of ssDNA. When BLM is absent, RPA presence is abolished (Y. W. Chan et al., 2018; Hengeveld et al., 2015). Since ssDNA is thought to be more fragile than dsDNA, it is possible that the creation of ssDNA regions on UFBs may

allow for efficient cleavage or rupture. However, it is unknown if RPA-coated ssDNA structures are as fragile as their 'naked' forms. It is thought that RPA may trigger a recruitment of other downstream factors or nucleases, which would aid in the resolution UFBs. Another study proposed that loading of RPA to ssDNA protects UFBs from breaking (Sarlós et al., 2018)

1.3.3 Other UFB-binding factors

In addition to PICH and BLM, other factors were identified to recognize UFBs. This includes RIF1 (Hengeveld et al., 2015) and TOPBP1 (Broderick et al., 2015). TOPBP1 (DNA Topoisomerase 2-binding protein 1) is well-known protein required for efficient initiation of DNA replication and DNA damage response (Mäkiniemi et al., 2001). Due to its localization to centrosomes, it has been suggested that TOPBP1 functions during mitotic progression (Bang et al., 2011). In anaphase, TOPBP1 displays UFB-like localization (Germann et al., 2014). TOPBP1 seems to coat only a subset of UFBs and a specific short sub-regions of them, while its localization was shown to be independent of PICH and BLM (Broderick et al., 2015). TOPBP1 is a large protein that recruits multiple additional factors. TOPBP1 foci appear on chromatin before formation of UFBs in anaphase, indicating that it may work as a marker of damaged regions. This suggests that TOPBP1 may operate earlier in mitosis than other UFB-related factors. TOPBP1 was shown to recruit SLX4, a key component for endonuclease assembly (Pedersen et al., 2015). This could help cleave entangled DNA strands on chromosomes. The 7th and 8th BRCT domains of TOPBP1 have been shown to be necessary for interaction with TOP2A, suggesting that TOPBP1 may recruit TOP2A to UFBs for DNA decatenation in mitosis (Broderick et al., 2015). Despite the fact that their interaction was detected in vitro, TOP2A signal is always very weak along the length of UFBs, appearing as a punctuate foci (Broderick et al., 2015), Therefore, if TOPBP1 does recruit TOP2A, they probably do not interact directly.

RIF1 was first identified as a telomere-associated protein (Hardy et al., 1992) and later found to play roles in repairing DNA breaks (Silverman et al., 2004), replication control (Yamazaki et al., 2013), and potentially in resolving UFBs during mitosis (Hengeveld et al., 2015). RIF1 has been shown to work with BLM helicase to recover stalled replication forks (Xu et al., 2010), but is dependent on PICH (and not on BLM) for its recruitment to

UFBs (Hengeveld et al., 2015). RIF1 interacts with both C- and N- terminus of PICH in vitro. Nevertheless, RIF1's loading onto UFBs was not shown to be eliminated by deletions of C- and N- terminus in vivo (Hengeveld et al., 2015). It is unclear if PICH translocase activity or direct protein-protein interactions are needed to recruit RIF1. Alternatively, RIF1 may be recruited to UFBs through downstream PICH binding partners such as TOP3A and RMI1/RMI2.

Fanconi Anemia (FA) proteins, including FANCD2, FANCI, and FANCM, were first identified through studies of the genetic disorder FA (Datta & Brosh, 2019). These proteins are crucial for DNA repair and responding to replication stress. During S phase, they work with factors like the BTR complex to repair DNA lesions that stall replication forks, while the FANCD2-FANCI complex also protects these forks from degradation (Schlacher et al., 2012). Some FA proteins also localize to anaphase bridges, and FA-deficient cells show increased bridging, suggesting a role in resolving DNA entanglements in mitosis (K. L. Chan & Hickson, 2009; Naim & Rosselli, 2009). FANCD2-FANCI foci mark UFBs at fragile sites, believed to arise from incomplete replication, and their presence is elevated under replication stress (K. L. Chan et al., 2009; Naim & Rosselli, 2009). FANCD2-FANCI complexes are not found on UFBs formed by inhibition of TOP2A or recombination pathway (Y. W. Chan & West, 2018; Tiwari et al., 2018). FANCM may contribute to the resolution of DNA bridges directly at later stages of the cell cycle, as it possess a DNA translocate activity (Gari et al., 2008). Taken together, this indicates that FANCD2-FANCI specifically marks anaphase brides originating from LRIs.

1.3.4 Cohesin may interfere with sister chromatids separation

Accurate chromosome segregation requires the proper resolution of DNA entanglements, but also requires sister chromatid cohesion, which is mediated by cohesin. Cohesin is multi-protein complex, composed of different core subunits, SMC1, SMC3, RAD21/SCC1 and SA1/2, which are thought to enclasp DNA strands as a ring and hold them together until they segregate (Haering et al., 2008). During prophase, cohesin is removed from chromosome arms by WAPL protein (Kueng et al., 2006), opening the SMC3-SCC1 interface (Peters et al., 2008), and then at metaphase cohesin is removed from centomeres by the protease separase, opening the cohesin ring by cleavage of Scc1 (Uhlmann et al., 1999; Waizenegger et al., 2000).

Cohesin complexes promote the formation of TOP2-dependent DNA catenanes and inhibit their resolution through decatenation. In vitro experiments demonstrated that cohesin facilitates the catenation of DNA plasmids when TOP2A is present (Losada & Hirano, 2001). The first evidence that cohesin bound to DNA inhibits decatenation in vivo came from studies in yeast using DNA plasmids. Catenanes are specifically maintained at centromeric regions and are resolved by TOP2A only after centromeric cohesin is cleaved before the onset of anaphase (L. H.-C. Wang et al., 2010). A fraction of cohesin remains bound to centromeres during mitosis, maintaining cohesion at these critical regions until all chromosomes achieve biorientation on the mitotic spindle. Separase is the key player in the final sister chromatid separation (Hauf et al., 2001; Uhlmann et al., 1999). In cells where Wapl is depleted, cohesin release from the chromosome arms is inhibited, and the cohesin complexes persist along chromosome arms until anaphase. At this stage, separase takes over, cleaving centromeric cohesin at the metaphase-to-anaphase transition (Kueng et al., 2006). The notion that cohesin hinders the removal of DNA linkages is further supported by findings that depletion of WAPL protein, a factor that weakens cohesin binding, leads to increased formation of anaphase bridges (Haarhuis et al., 2013). Staining for PICH revealed a marked increase in ultrafine DNA bridges, from 14% in control cells to 25% in WAPL-depleted cells (Haarhuis et al., 2013). DAPI-positive chromatin bridges were also detected in WAPL-depleted cells (Haarhuis et al., 2013; Tedeschi et al., 2013). A study in *Drosophila* showed that heterochromatic regions ectopically placed on chromosome arms with high cohesin levels starting from S phase can stretch during anaphase, even after cohesin is cleaved (Oliveira et al., 2014). This suggests that cohesin that is present before anaphase can interfere with the separation of sister chromatids by physically restricting them, rather than affecting TOP2 function or preventing its access to DNA. These constraints may promote the formation of catenanes by TOP2A instead of their resolution, a model further supported by evidence that artificially tethering DNA plasmids also increases catenation (Sen et al., 2016).

Regardless of their origin, if UFBs are not resolved before cytokinesis, they can lead to major chromosomal rearrangements, which are hallmark of cancer. These rearrangements likely result from DSBs, formed when the bridge eventually breaks. Even a single unresolved bridge can results in increasing amount of gross genome

rearrangements, as shown by experiments (Umbreit et al., 2020). But how would chromatin bridges break? In addition to the direct mechanical breakage of chromatin bridges by actomyosin contractile forces, recent studies have revealed that nucleases also play a role in processing bridge DNA (Maciejowski et al., 2015). Recent research has demonstrated that nucleases contribute to the processing of DNA bridges. Cells with chromatin bridges formed through telomeric fusions were shown to persist into the following G1 phase without being ruptured by mechanical forces (Maciejowski et al., 2015). Instead, they continue to stretch and are then processed by the TREX1 exonuclease, which acts on single-stranded breaks in order to generate RPA-ssDNA (Maciejowski et al., 2015, 2020). If ssDNA regions overlap, this induces DSBs and bridges may be resolved (Maciejowski et al., 2015). Another nuclease recently proposed in resolution of chromatin bridges is ANKLE1 (Hong et al., 2021; Jiang et al., 2023). First, a mechanism for chromatin bridge resolution that involves LEM-3 nuclease cleavage in *C.elegans* embryos was shown (Hong et al., 2018). LEM-3 localizes to the midbody, positioning itself at the center of chromatin bridge during cytokinesis (Hong et al., 2018). LEM-3 has its human ortholog, ANKLE1, which is also directed to the midbody during cytokinesis (Jiang et al., 2023). ANKLE1 processes chromatin bridges generated by DNA-damaging agents to prevent the emergence of G1-specific 53BP1 nuclear bodies, which mark sites of DNA damage (Jiang et al., 2023). Studies shown that ANKLE1 can cleave also Holliday junctions (Freeman et al., 2023), a type DNA entanglement that induce formation of UFBs (Wyatt & West, 2014). It is proposed that ANKLE1 introduces nicks in the bridge DNA near the midbody, facilitating further processing by TREX1 to produce extensive ssDNA. This model is supported by findings showing that deletion of ANKLE1 partially reduces the presence of TREX1-dependent, RPA-coated ssDNA in chromatin bridges, indicating that ANKLE1 may act as a priming nuclease for TREX1 (Jiang et al., 2023)

1.4 Characterization of other UFB-binding proteins

1.4.1 TOPBP1 and CIP2A work together in mitosis

Mitotic cells that face DNA damage can either attempt to repair it during mitosis or stabilize it until a phase of the cell cycle that is more suitable for repair. Intriguingly, cells opt for stabilization rather than immediate repair of double-strand breaks (DSBs), setting

the stage for repair during subsequent phases after mitosis (Giunta et al., 2010). One key player in this process is MDC1, which facilitates the recruitment of TOPBP1 to sites of DNA damage (Leimbacher et al., 2019). Data from (Laine et al., 2021) indicated that CIP2A may interact with TOPBP1, which was identified among the 10 co-dependent DNA damage response (DDR) genes with the most functional similarity to CIP2A. Later on, CIP2A was identified as a new component in this response (De Marco Zompit et al., 2022). This study was the first to describe CIP2A as a TOPBP1-interacting factor at DSB sites during mitosis. CIP2A binds to a specific sequence region between BRCT5 and BRCT6 in TOPBP1, which was later shown by proximity ligation assays, further confirming that CIP2A and TOPBP1 physically interact at DSB sites on mitotic chromosomes. CIP2A behaves differently depending on the cell cycle stage. In undamaged cells during interphase, it localizes to the cytoplasm but later accumulates at centrosomes together with TOPBP1 during mitosis (De Marco Zompit et al., 2022; J.-S. Kim et al., 2013). While undetectable at DSB sites during interphase, CIP2A becomes enriched at foci on mitotic chromosomes upon irradiation, where it colocalizes with TOPBP1 at γH2AX-marked DSB sites, but could not be found on centromeric regions (De Marco Zompit et al., 2022). Functional studies revealed that depletion of CIP2A results in the loss of TOPBP1 foci in irradiated RPE1 cells, and vice versa, TOPBP1 depletion leads to the disappearance of CIP2A foci. This interdependence between the two proteins suggests a crucial partnership in mitotic DSB repair (De Marco Zompit et al., 2022). The absence of CIP2A has consequences for cellular stability. Cells lacking CIP2A struggle to repair DSBs during mitosis, leading to the formation of micronuclei containing acentric chromosome fragments and ultimately results in chromosome instability (De Marco Zompit et al., 2022).

1.4.2 TOPBP1 goes to ultrafine bridges

Using an independent yeast two-hybrid screen, TOPBP1 was identified as a human topoisomerase-II-binding protein, interacting with the C-terminal domain of TOP2A (Yamane et al., 1997). Structurally, TOPBP1 shares similarities with proteins such as RAD4 and XRCC1, known for their roles in repairing DNA damage caused by ionizing and ultraviolet radiation (Yamane et al., 1997). These findings suggested that TOPBP1 may function as a regulator of DNA strand breaks caused by the catalytic activity of

topoisomerase II. TOPBP1 is recruited to stalled replication forks following replication stress, implicating its involvement in rescuing stalled forks (Mäkiniemi et al., 2001). Studies explored the interaction between TOPBP1 and ultrafine bridges (UFBs), specifically centromeric UFBs (C-UFBs), which arise from unresolved DNA catenations requiring TOP2A for resolution (Broderick et al., 2015). Inhibiting TOP2A using agents such as ICRF-193 significantly increased the frequency of TOPBP1-associated UFBs during anaphase and telophase, while treatment with the replication inhibitor aphidicolin had no similar effect (Broderick et al., 2015). This suggests that TOPBP1 is specifically recruited to UFBs originating from TOP2A inhibition rather than replication stress. Moreover, TOPBP1 depletion led to an accumulation of C-UFBs, further supporting its role in UFB resolution (Broderick et al., 2015). The colocalization of TOP2A and TOPBP1 on C-UFBs indicates that TOPBP1 may facilitate TOP2A recruitment to these structures, promoting their resolution. Findings proposed a model in which TOPBP1 acts as a mediator, stabilizing UFBs and recruiting TOP2A to resolve DNA catenations before chromosomal segregation (Broderick et al., 2015). Intriguingly, TOPBP1 often localizes to a narrower region of PICH-coated UFBs, persisting even after PICH dissociates during late telophase (Germann et al., 2014). Time-lapse imaging revealed that TOPBP1 colocalizes with PICH on UFBs in early anaphase and promotes their elongation while inhibiting the formation of chromatin bridges (Broderick et al., 2015). Replication stress, such as that induced by aphidicolin, enhances TOPBP1-PICH colocalization and results in the stabilization of UFBs (Broderick et al., 2015). In yeast, Dpb11 (the TOPBP1 homolog) plays a dual role. First, it prevents chromatin bridge formation and second, it stabilizes UFBs to ensure proper chromosomal segregation (Germann et al., 2014). Simultaneous depletion of Dpb11 and failure of NoCut checkpoint leads to increased genomic instability, further emphasizing its critical role (Germann et al., 2014). In summary, TOPBP1 emerges as a multifunctional protein with roles in replication, DNA damage response, and UFB resolution. It promotes the elongation of these bridges while suppressing the formation of chromatin bridges (Broderick et al., 2015). TOPBP1 has been observed to colocalize with PICH and RPA at a subset of ultrafine bridges, while its depletion leads to an accumulation of chromatin bridges and at the same time simultaneously reduces the frequency of long ultrafine bridges (Germann et al., 2014).

1.4.3 CIP2A has a role in mitotic progression

CIP2A is a human oncoprotein known for its role in stabilizing c-Myc in human malignancies (Junttila et al., 2007). Initially identified in the early 2000s as a 90 kDa protein overexpressed in human gastric cancer (Hoo et al., 2002), CIP2A was later named "Cancerous Inhibitor of PP2A" due to its direct interaction with PP2A. By inhibiting PP2A activity toward c-Myc, CIP2A stabilizes c-Myc, thereby promoting malignant cell growth (J.-S. Kim et al., 2013). While originally thought to be predominantly cytoplasmic, a weak nuclear signal was also detected. In paper from 2013, CIP2A was shown to localize to the cytoplasm during interphase (Kim et al., 2013). As cells transitioned from the S phase to the G2-M phase, CIP2A signal became concentrated in the pericentrosomal region and later localized at spindle poles during mitosis, suggesting that CIP2A localization is closely tied to cell cycle progression (Kim et al., 2013). Depletion of CIP2A resulted in significant mitotic defects, including the formation of multipolar spindles, misaligned chromosomes, and lagging chromosomes, highlighting CIP2A's critical role in ensuring proper mitotic progression (Kim et al., 2013). In irradiated U2OS interphase cells treated with leptomycin B, CIP2A formed nuclear foci that colocalized with TOPBP1, implying that spatial separation of CIP2A and TOPBP1 outside of mitosis may prevent complex formation between them (De Marco Zompit et al., 2022). There is also evidence of an active transport mechanism directing CIP2A to the nucleus prior to mitosis. In cancer cells, CIP2A was shown to interact with the endoplasmic reticulum protein LRRC59, with both proteins accumulating at the nuclear envelope during G1/S and subsequently entering the nucleus at G2/M, where CIP2A formed its characteristic foci (Pallai et al., 2015). Interestingly, depletion of LRRC59 disrupted nuclear CIP2A foci formation at G2/M and to overall reduction of both nuclear and cytoplasmic CIP2A levels, suggesting a general decrease in CIP2A abundance, which could account for the loss of foci (Pallai et al., 2015). Therefore, nuclear envelope breakdown during prophase might be sufficient to enable CIP2A migration to mitotic chromatin. CIP2A is known to form dimers and is thought to oligomerize through its coiled-coil domain (Trivedi et al., 2023; J. Wang et al., 2017). TOPBP1 also has the ability to oligomerize and form condensates, raising the possibility that interactions between higher order assemblies of CIP2A and TOPBP1 may underlie the formation of filamentous structures (Frattini et al., 2021; A. Kim et al., 2020). Such assemblies could be critical for maintaining genome integrity, as deletion of the

CIP2A coiled-coil domain eliminated CIP2A foci in mitotic cells and significantly reduced viability in BRCA2−/− cells (Adam et al., 2021). Another research group proposed that these higher order structures are tethers that potentially stabilize broken chromosomes until next G1 phase (Leimbacher et al., 2019).

1.5 Aim of the study

The aim of this study is to characterize fundamental properties of anaphase DNA bridges in a variety of human cell lines. This includes their shape, prevalence, and protein colocalization patterns. In the first chapter, I describe how I characterized different features of anaphase bridges in various mid-anaphase cells based on PICH staining, including their length, prevalence and origin, as well as the effect of irradiation. In chapter two, I describe how I gained insights on anaphase bridges formation at the beginning of anaphase in HeLa cells with normal and increased levels of sister chromatid cohesion. In chapter three, I discuss proteins that colocalize with PICH on anaphase bridges, focusing on TOPBP1 and CIP2A, factors that are known for their cooperation at double-strand break sites in mitosis.

2 RESULTS

2.1 Characterization of anaphase bridges

2.1.1 Every second mid/late-anaphase cell is PICH-bridge positive

I set out to generate a dataset of untreated, asynchronously cycling anaphase cells to see how prevalent DNA bridges are in HeLa, U2OS, DLD1, U251, SH-SY5Y and RPE1 cells

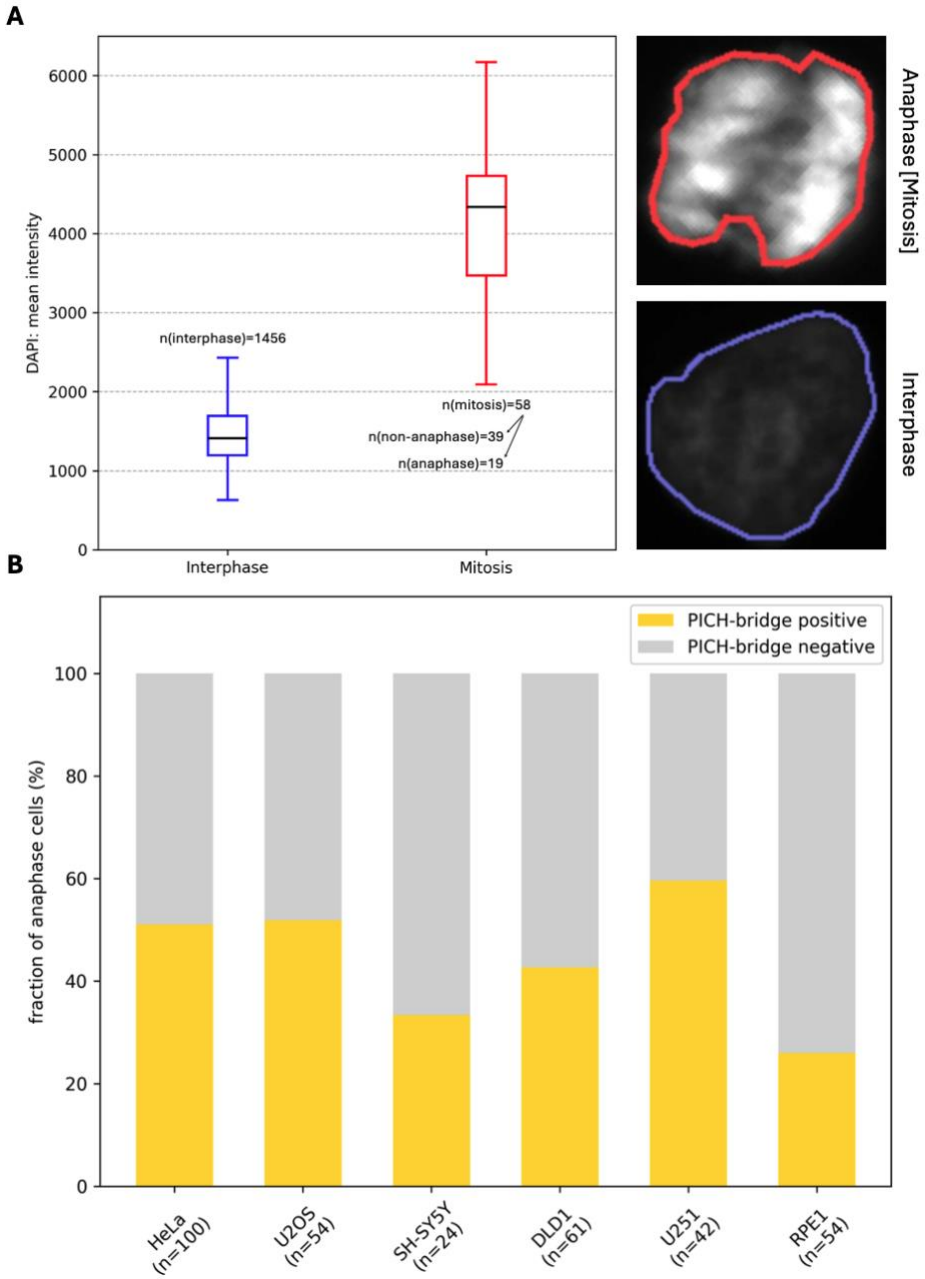


Figure 1. Prevalence of PICH-bridge positive anaphase cells. A) Quantification of mitotic and anaphase index based on DAPI mean intensity signal in untreated, asynchronous HeLa cells. Boxplots indicate median values (black lines), the 25%-75% interquartile range (IQR)(box), and have whiskers at 1.5x IQR. N indicates the number of cells analyzed. **B)** Prevalence of PICH-bridge positive and PICH-bridge negative anaphase cells in different cell lines. Number of identified anaphase cells is indicated.

without exposure to chemical inhibitors or other perturbations. First of all, it is worth to mention that the mitotic index is usually around 3% in asynchronously cycling cells. Consistently, the anaphase index is much lower. I confirmed this HeLa cells. They have a mitotic index of almost 3%, and an anaphase index slightly above 1% (Figure 1A). These numbers are very low, implying that one needs to inspect approximately 1000 cells to find 10 anaphase cells in untreated, asynchronously cycling cells. I recorded every anaphase cell, regardless of being PICH-bridge positive or negative. With this unbiased approach, I addressed how often PICH-bridge positive cells could be observed. Our quantification implies that every second mid/late-anaphase cell is PICH-bridge positive when it comes to cancerous cell lines. In a non-cancerous cell line, this number appeared to decrease to every fourth cell being PICH-bridge positive (Figure 1B). Some cell lines like U251 or U2OS are showing higher prevalence of PICH-bridge positive cells, while in RPE1, there is a lower proportion of PICH-bridge positive cells, suggesting variability in PICH-bridge formation among different cell lines. This could indicate cell line specific differences in chromosome segregation dynamics.

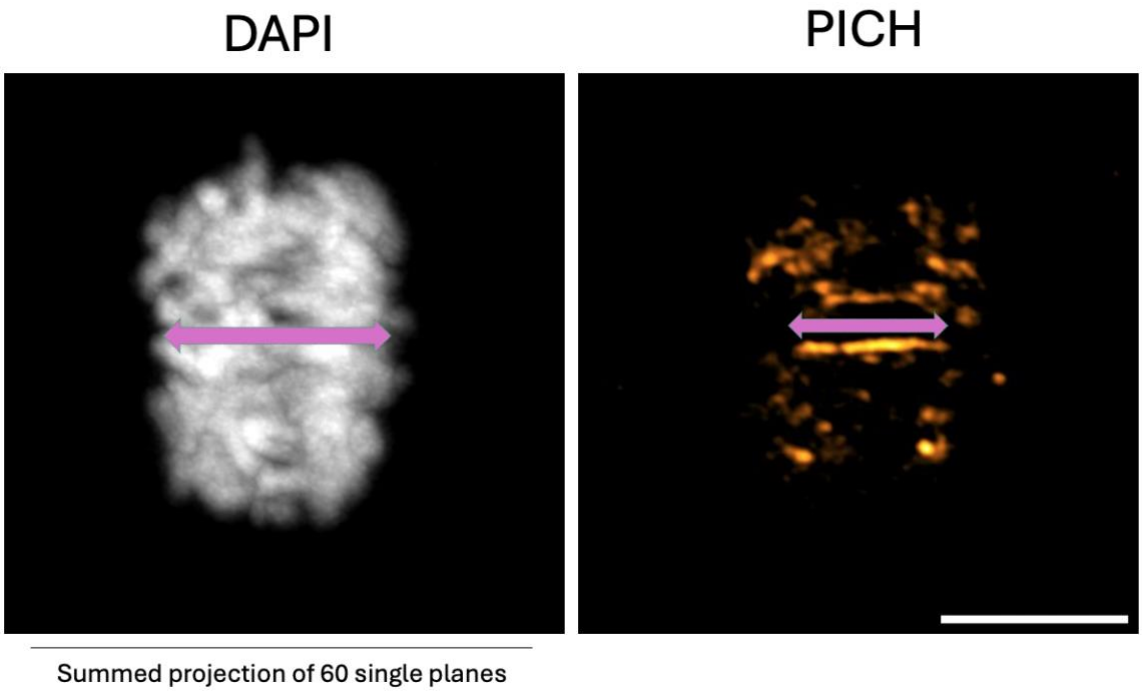


Figure 2. Measurement examples. Purple arrows indicate the progression of chromosome segregation (left panel) and the length of PICH-positive bridges (right panel). Scale bar 5µm.

To quantify anaphase bridges in our recorded collection of cells, the DAPI channel was used to visualize DNA and the progression of chromosome segregation was determined manually by measuring the distance between two separating chromosome masses along the longest axis of the cell, measured in summed projection (Figure 2). Similar measurements were used to determine the length of PICH-positive bridges (Figure 2).

2.1.2 Anaphase bridges are transient structures

Next, I investigated whether PICH-positive bridges are transient structures, decreasing in number as anaphase progresses, as shown previously in (Baumann et al., 2007). Most

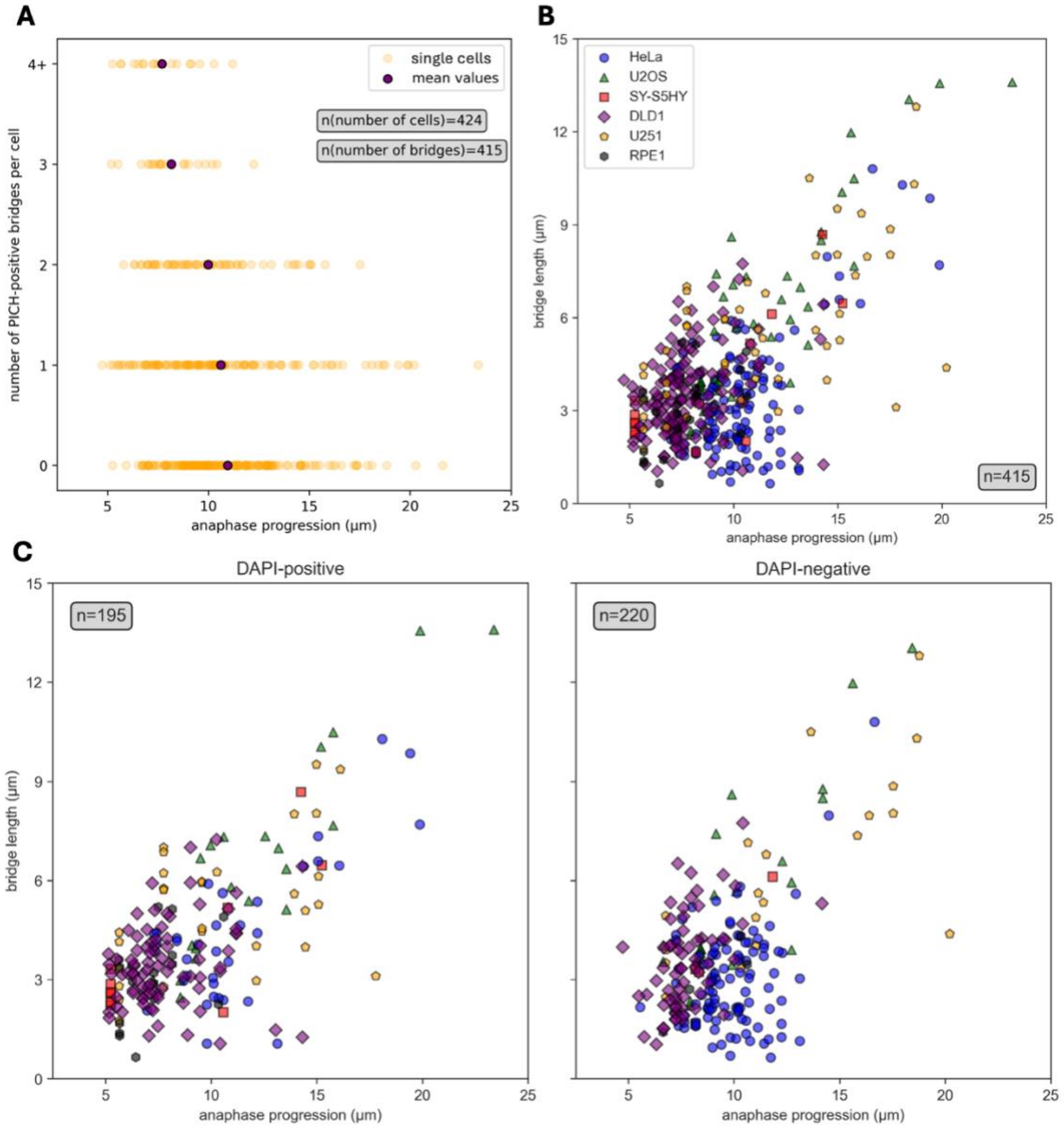


Figure 3. Characterization of PICH-positive bridges in anaphase progression. A) Yellow dots represent 424 individual cells (from all analyzed cell lines). These are divided in five categories based on the number of PICH bridges per cell. Purple dots indicate the mean value per category. **B)** The length of 415 PICH-positive bridges is plotted against anaphase progression. Different symbols indicate different cell lines. **C)** Data as in panel B, but separated for DAPI-positive (left) and DAPI-negative (right).

of the cells in this dataset have zero, one or two bridges. Notably, cells with one or two PICH-positive bridges tend to appear later in anaphase, whereas cells with four PICH-

positive bridges are more frequently observed during mid-anaphase. Our results indicate that the number of PICH-positive bridges per cell is highest at earlier stages of anaphase and decreases with increasing anaphase progression (Figure 3A). Although the trend is not perfectly linear, this is consistent with PICH-positive bridges being transient structures.

I also included anaphase cells without PICH bridges in this analysis. While some PICH-bridge negative cells appear in early anaphases, they predominantly cluster around mid-to-late anaphase and become more frequent later in anaphase, further supporting the notion that anaphase bridges are transient structures timely resolved during early-mid anaphase which should not persist until late anaphase (Figure 3A).

Next, I analyzed the lengths of individual PICH-positive bridges and correlated them to anaphase progression. In both cancerous and non-cancerous cell lines, a trend of PICH-coated bridges being relative short (around 2-5μm) during mid-anaphase, and if persisting, getting progressively longer (beyond 8μm) during late anaphase/telophase can be observed (Figure 3B).

2.1.3 Ultrafine bridges are three times more prevalent than chromatin bridges in HeLa cells

The trend described in the previous chapter is most clearly observed on U2OS and U251 cells, where it is clear that in case PICH-positive bridges persist until late anaphase, they

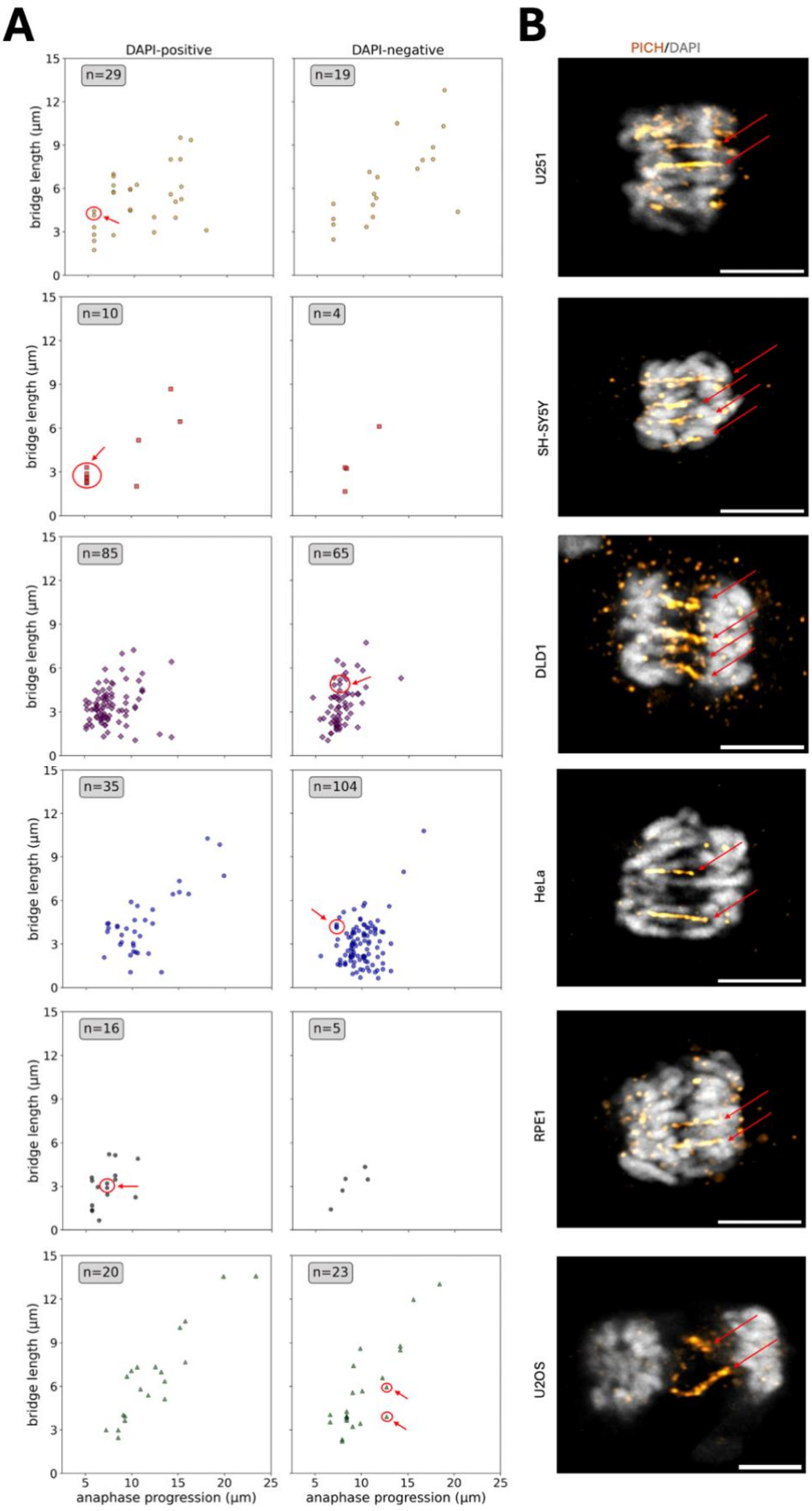

A
DAPI-positive
DAPI-negative
n=29
n=19
n=10
n=4
n=85
n=65
n=35
n=104
n=16
n=5
n=20
n=23
bridge length (µm)
anaphase progression (µm)
B
PICH/DAPI
U251
SH-SY5Y
DLD1
HeLa
RPE1
U2OS

Figure 4. Distribution of anaphase bridges in different cell lines. A) Distribution of chromatin (DAPI positive) and ultrafine (DAPI negative) bridges plotted against anaphase progression for each cell line. Number of PICH-positive bridges is indicated. Red circles indicate bridges from the same cell that are shown in panel B. **B)** Selected examples of anaphase cells with bridges from the different cell lines. Only single planes from a z-stack are shown here. Red arrows indicate PICH-positive bridges, corresponding to the red circles in panel A. Scale bar 5µm.

progressively increase in length (Figure 3C). This indicates that as stretching forces are pulling sister chromatids towards opposite poles, the underlying structures of PICH-coated bridges probably undergo mechanical stretching, elongating over time if not resolved properly.

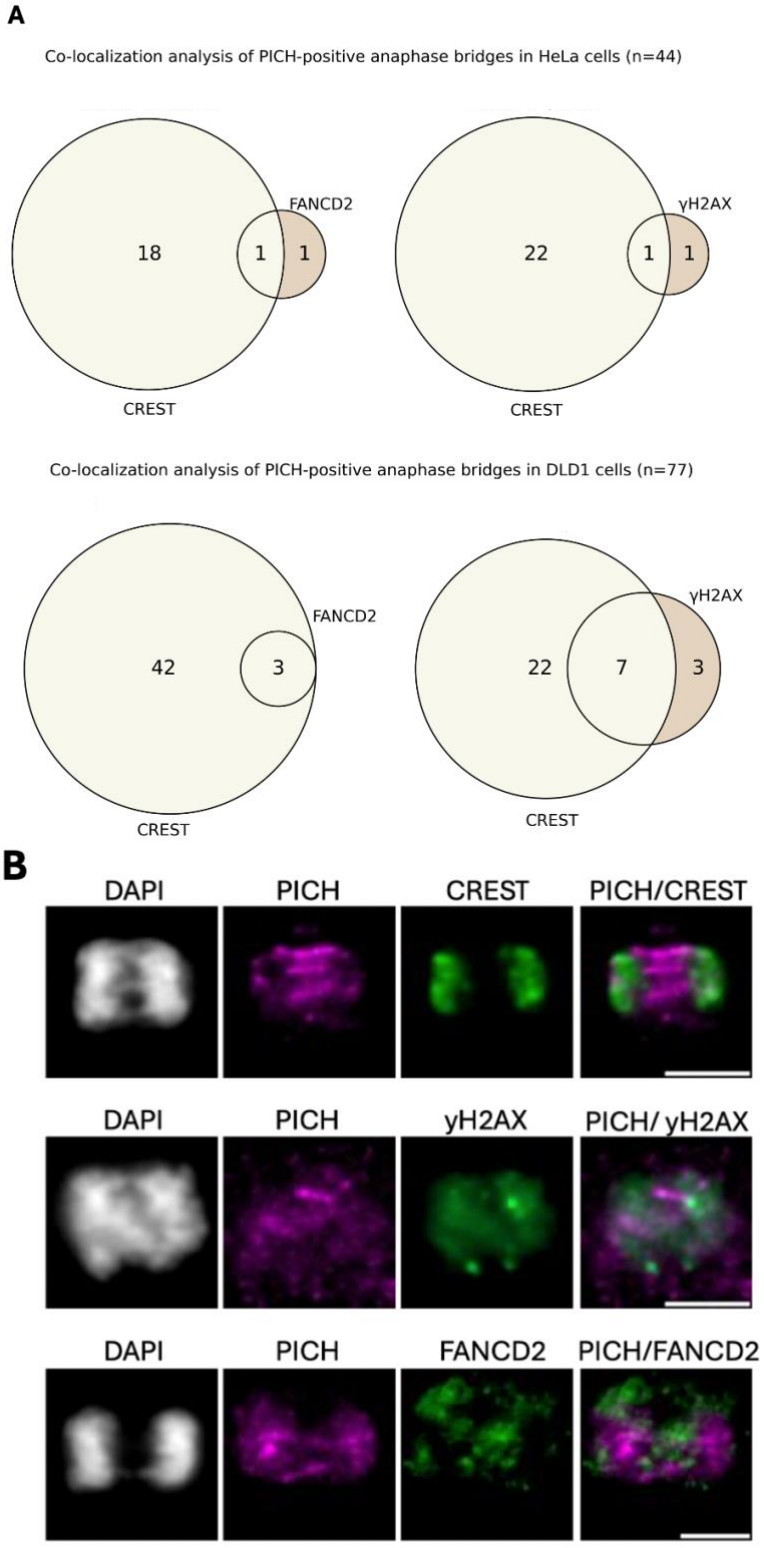
Furthermore, the prevalence of PICH-positive bridges decreases significantly towards telophase, with most PICH-positive bridges clustering around a separation distance of 10 µm. (Figure 3C).

Interestingly, in DLD1 and HeLa cells, the PICH-bridge length distribution is clustered in the lower range of anaphase progression, indicating that PICH-positive bridges might be more efficiently resolved at early stages of anaphase, when compared to other cell types (Figure 4A). This variation between different cell lines may indicate differences in DNA repair mechanisms affecting the resolution of anaphase bridges.

Lastly, I examined whether PICH-positive bridges are rather DAPI-positive or DAPI-negative. In most cell lines analyzed, PICH-positive bridges were most of the time still chromatinized (DAPI-positive). By contrast, in HeLa cells, most anaphase DNA bridges were DAPI-negative, and thus, by definition, ultrafine bridges (Figure 4A). This suggest that the chromatin composition of persistent bridges may vary across cell types, potentially reflecting differences in underlying molecular architectures and resolution mechanisms of such structures.

2.1.4 Anaphase bridges are mostly arising from centromeric region

To further investigate the origin of anaphase bridges, I analyzed untreated, asynchronously cycling HeLa and DLD1 cells. Quantification of the total number of PICH



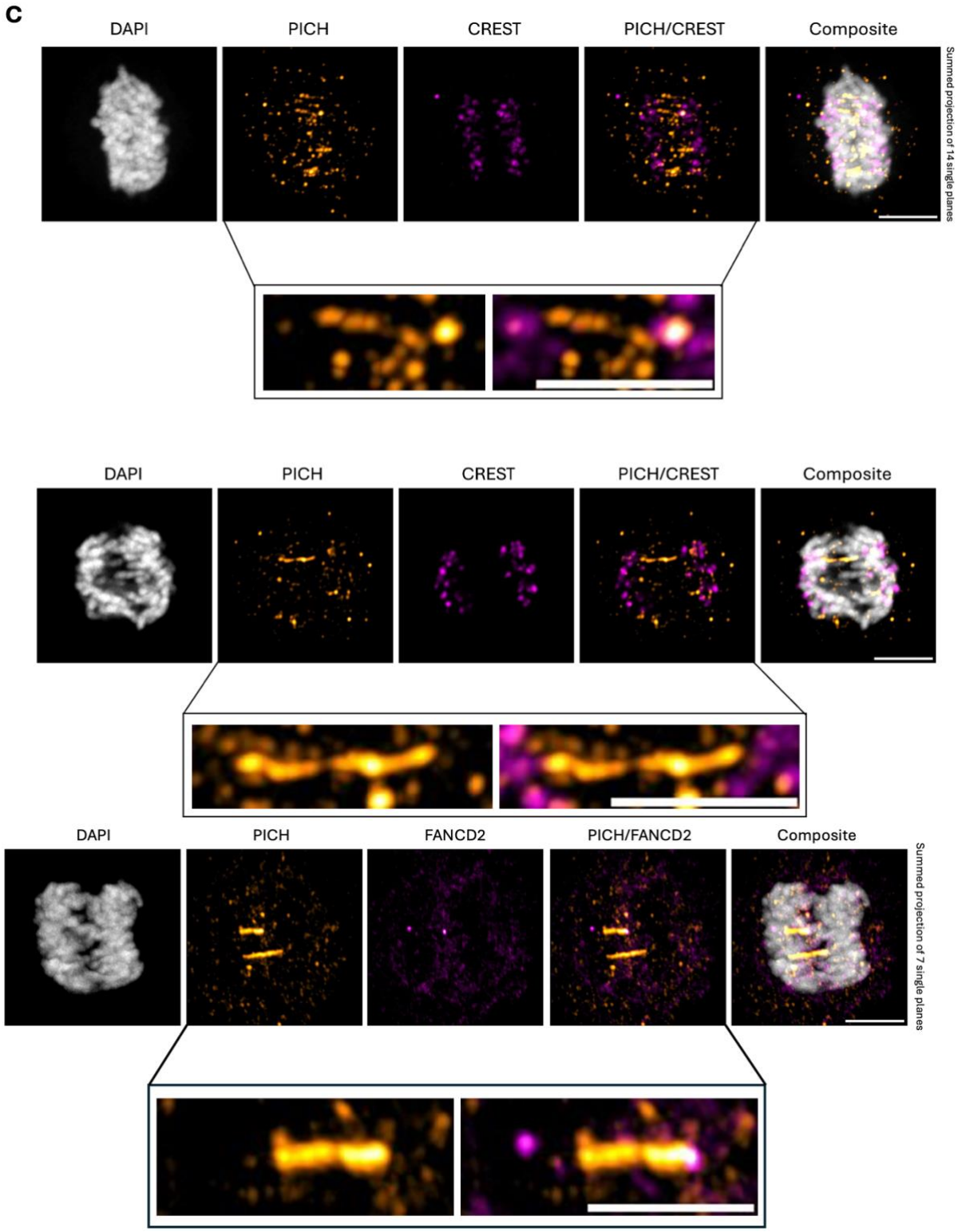


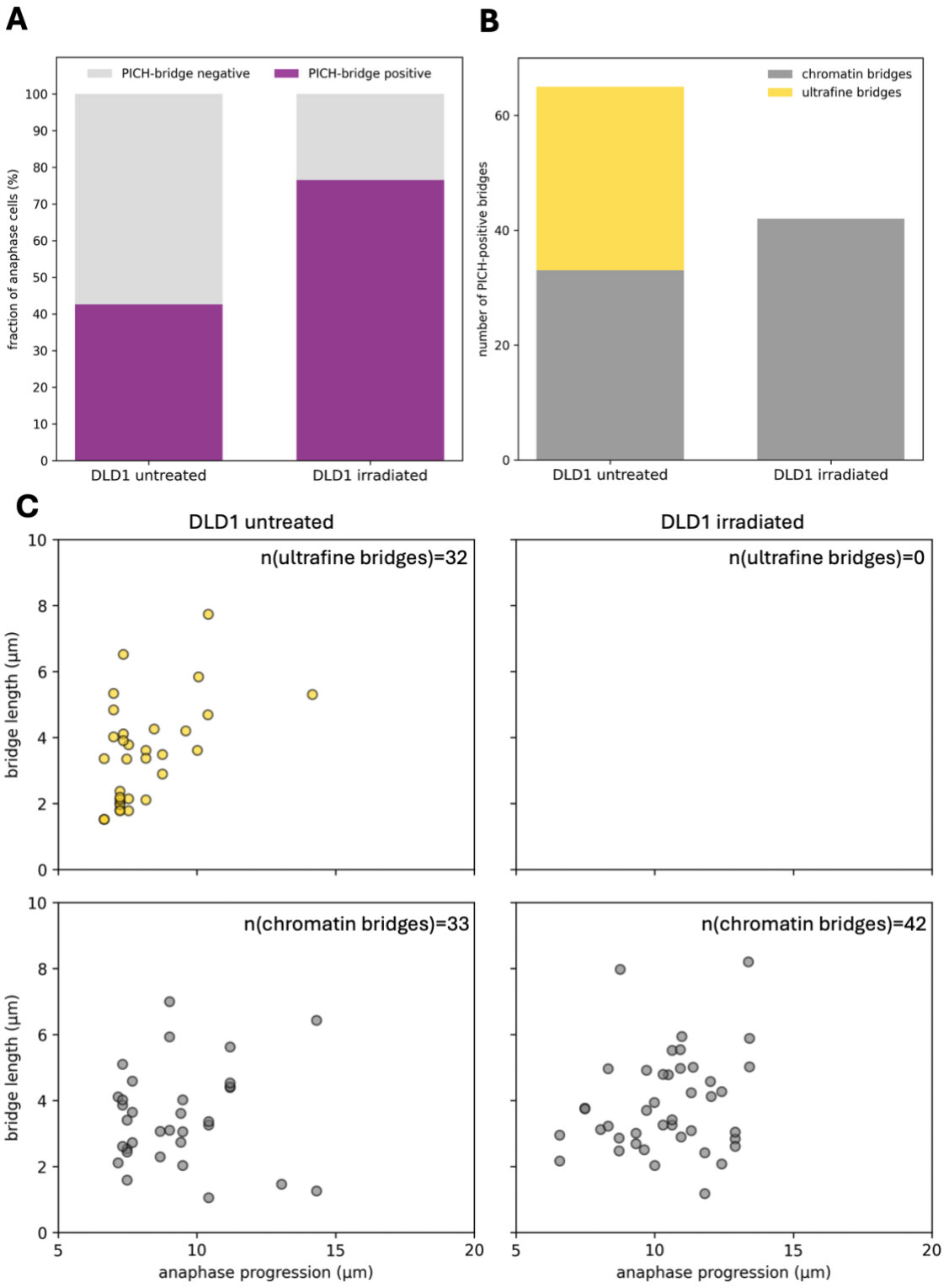
Figure 5. Origin of anaphase bridges. A) Quantification of PICH-positive bridges based on CREST, γH2AX and FANCD2 colocalization staining in HeLa and DLD1 cells. **B)** DLD1 cells with PICH-positive bridges and CREST, γH2AX, or FANCD2 co-staining recorded on Zeiss Axio Imager M2 (Perutz 5th floor). Scale bar 5µm. **C**) DLD1 cells with PICH-positive bridges and CREST, γH2AX, or FANCD2 co-staining recorded on a Zeiss confocal LSM 980 with Airyscan mode (Perutz BioOptics). Scale bar 5µm, magnified images scale bar 3µm.

positive bridges indicates that majority of them originates from centromeric regions, as staining with CREST revealed. Only a very small fraction of anaphase bridges exhibited an alternative origin, marked by staining for FANCD2 and γH2AX (Figure 5A). Images illustrate PICH-positive bridges arising from CREST-positive centromeric regions, γH2AX-positive regions and FANCD2-positive regions (Figure 5B). Images where higher resolution could be reached are shown as well (Figure 5C). These findings suggest that centromere-associated bridges may constitute the primary source of anaphase bridges, while bridges of a different origin are less frequently detected in unperturbed HeLa and DLD1 cells.

There is a strong limitation in the images presented in Figure 5B, which were acquired using a standard fluorescence microscope with limited resolution. As a result, determining the colocalization of PICH with FANCD2 or γH2AX was challenging. In contrast, the confocal images shown in Figure 5C provide higher resolution and clarity, visualizing the PICH-positive bridges and their origin from CREST-positive centromeric regions.

2.1.5 Irradiation increases the prevalence of chromatin bridges

To determine whether irradiation influences PICH-positive bridges in our cells, I analyzed DLD1 cells. Upon irradiation, the number of PICH-positive bridge cells increased from 43% to 77%. A notable effect of irradiation was observed in chromatinization status of PICH-positive bridges. Upon irradiation, all PICH-positive bridges found within irradiated cells became chromatinized (DAPI-positive), while in untreated cells, a significant subset of PICH-positive bridges was ultrafine (DAPI-negative) (Figure 6B). There is no clear trend observed between untreated and irradiated chromatin bridges regarding their length in cells with various degrees of anaphase progression (Figure 6C, 6D).


A
PICH-bridge negative
PICH-bridge positive
fraction of anaphase cells (%)
DLD1 untreated
DLD1 irradiated
B
chromatin bridges
ultrafine bridges
number of PICH-positive bridges
DLD1 untreated
DLD1 irradiated
C
DLD1 untreated
DLD1 irradiated
n(ultrafine bridges)=32
n(ultrafine bridges)=0
n(chromatin bridges)=33
n(chromatin bridges)=42
bridge length (µm)
anaphase progression (µm)

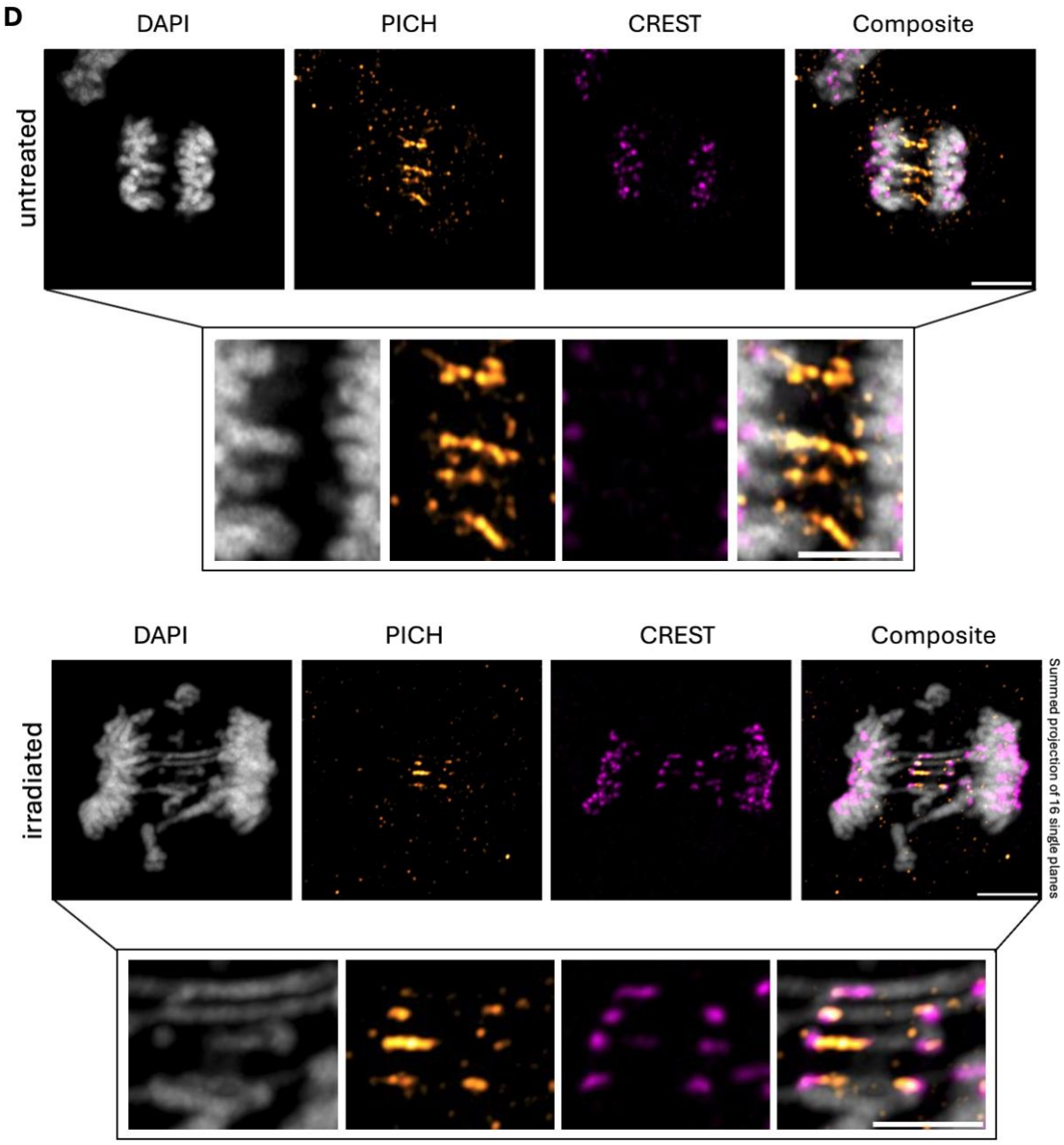


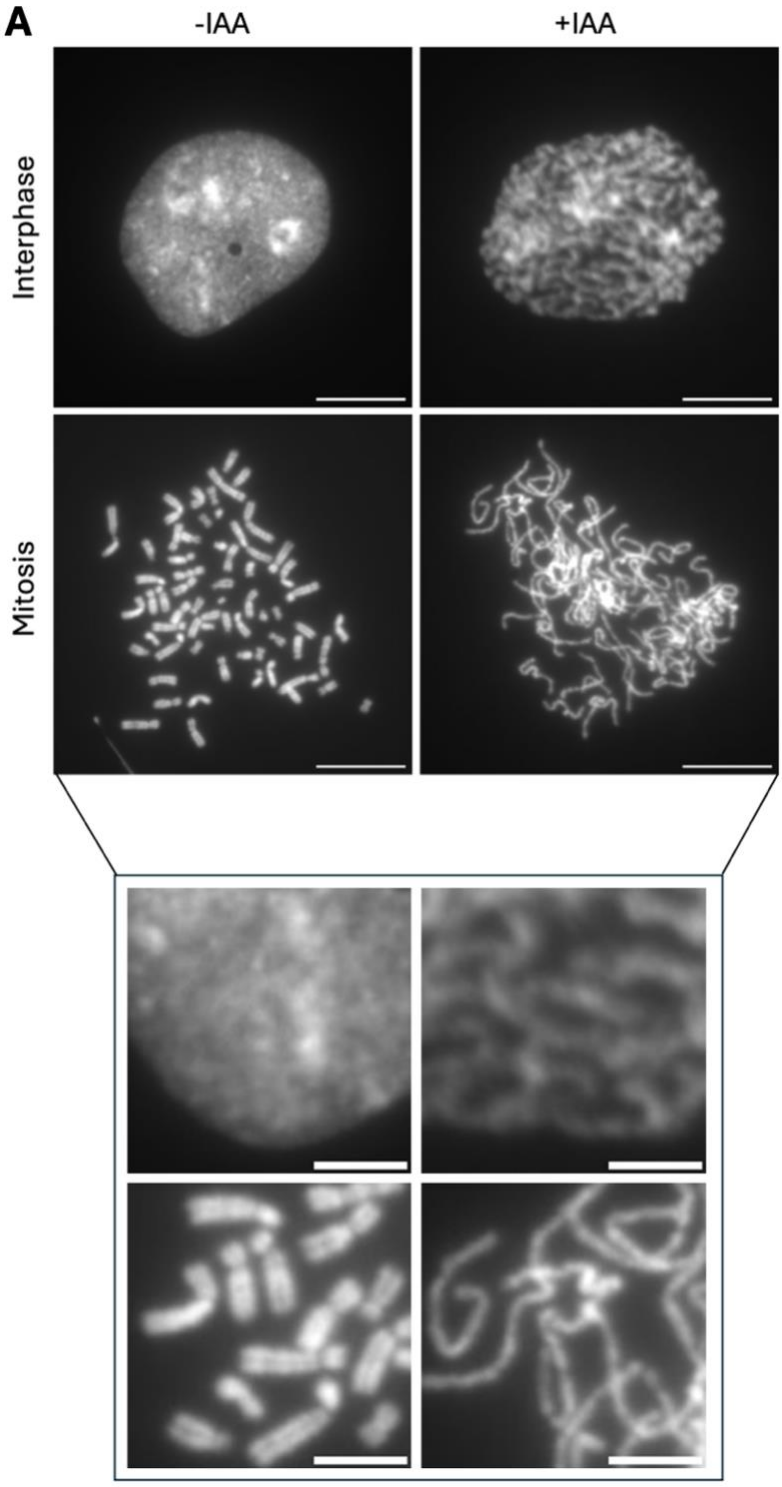
Figure 6. Irradiation effects on DLD1 cells and on anaphase bridges. **A**) Irradiation influence the number of PICH-bridge positive cells. **B**) Irradiation influence on the number of chromatin bridges and ultrafine bridges. **C)** Effects of irradiation on distribution and prevalence of ultrafine PICH-positive bridges (yellow) and chromatin PICH-positive bridges (grey) in untreated versus irradiated cells showed in panel 6B. **D)** Untreated versus irradiated (summed projection) images of DLD1 cell. Scale bar 5μm, magnified images scale bar 3μm.

2.2 WAPL depletion as an indicator of early anaphase

To further investigate the key parameters of PICH-bridge positive cells, I examined HeLa Scc1-eGFP WAPL^{AID} cells in which after addition of auxin (IAA), WAPL is degraded and, consequently, cohesin remains bound along chromosome arms until the onset of anaphase. It is challenging to determine if fixed cells are in early anaphase or in metaphase using DNA staining methods. However, upon WAPL depletion, the absence of cohesin on chromosome arms marks cells that have already entered anaphase, even if the sister chromatids did just or barely or not even start to segregate significantly . This helped me to capture cells in very early anaphase and to characterize anaphase bridges in them. Importantly, based on previous work, WAPL depletion was reported to increase the number of cells with PICH-positive ultrafine bridges from 14% to 25% (Haarhuis et al., 2013).

2.2.1 Auxin treatment abrogated cohesin removal on mitotic chromosomes in HeLa Scc1-eGFP WAPL^{AID} cells

First, to detect and confirm the depletion phenotype, chromosome spreads were performed. Images of WT and depleted interphase and mitotic cells demonstrate the efficient depletion of WAPL protein in our cells (Figure 7A). In this case, chromosome



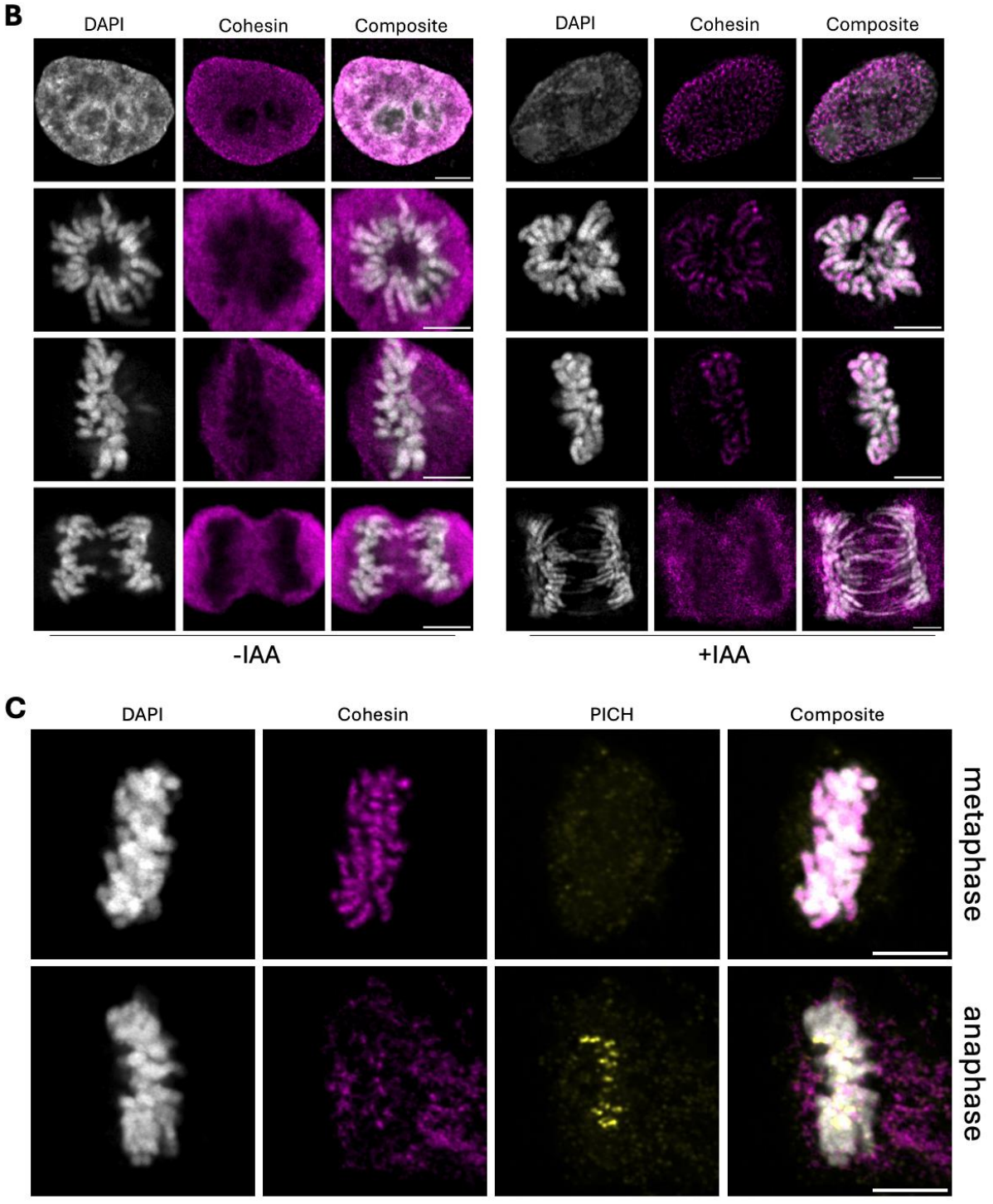


Figure 7. Effects of WAPL depletion on structure and cohesin localization in HeLa Scc1-eGFP WAPl^{AID} cells. A) Chromosome spreads of HeLa Scc1-eGFP WAPL^{AID} cells that were or were not exposed to auxin (IAA). DNA was stained with DAPI. Scale bars 15 µm and 5 µm. **B)** Representative images showing cohesin localization in interphase and mitosis HeLa Scc1-eGFP WAPL^{AID} cells with and without IAA treatment. Scale bar 5 µm. **C)** Representative images showing the localization of cohesin (magenta) and PICH (yellow) in HeLa Scc1-eGFP WAPL^{AID} following IAA treament. DNA is stained with DAPI (grey). Top row shows a metaphase cell, while the bottom row shows a cell in early anaphase. Scale bar 5 µm.

spreads in cells after addition of IAA are showing elongated structure of chromosomes without their characteristic X-shape appearance (Figure 7A). By looking at cohesin the effects of WAPL-depletion on the prophase pathway are evident (Figure 7B). Using the absence of cohesin as a tool to study cells in early anaphase, I then inspected PICH signals. PICH signal is bright in early anaphase cells, allowing the clear visualization of individual PICH-positive bridges (Figure 7C). In contrast, in metaphase cells, PICH signals appear weaker and diffused, with no PICH-positive bridges detectable. This observation suggests that PICH is either not recruited or not yet functionally engaged with chromosomal structures during metaphase and accumulates on bridges upon the initiation of anaphase.

2.2.2 The number of PICH-positive bridges goes up to 8 per cell in early anaphase

Anaphase bridges quantified from early anaphases are showing lengths of bridges that are very short at the anaphase onset, clustering mostly in the lower range of anaphase progression (Figure 8A). This aligns with previously observed suggestion that PICH-positive bridges might be more efficiently resolved at earlier stages of anaphase. The number of PICH-positive bridges is decreasing as anaphase progresses (Figure 8B), indicating again that PICH-positive bridges are most probably transient structures subjected to stretching forces.

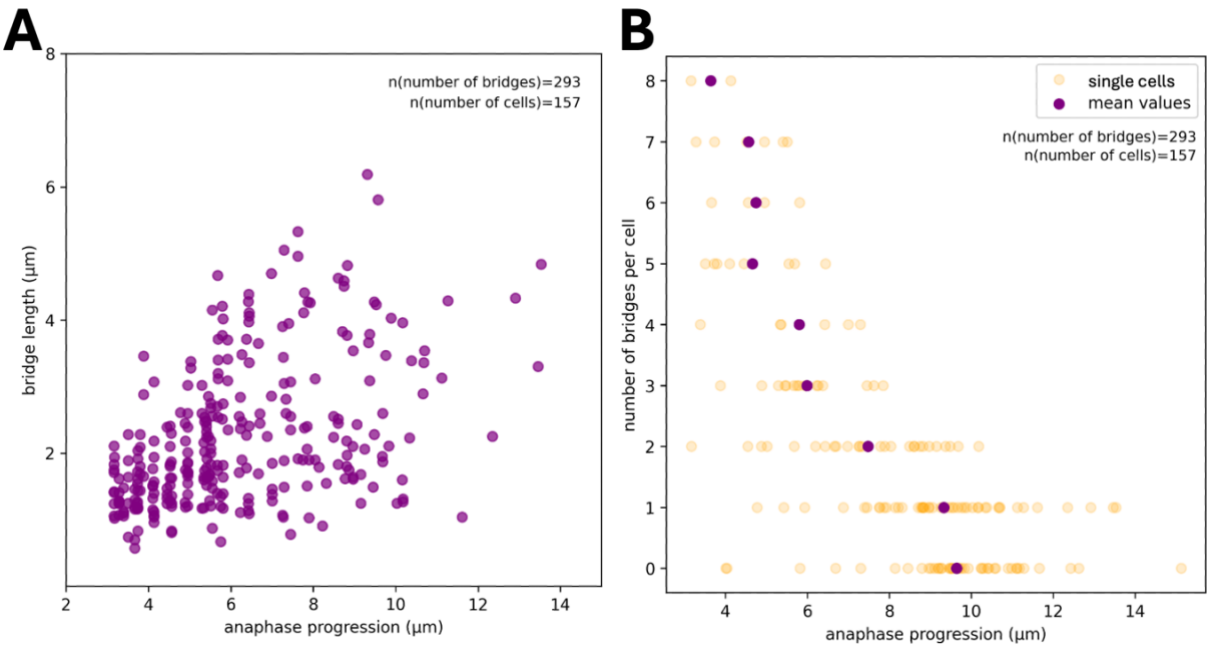


Figure 8. Effects of WAPL depletion on number and length of PICH-bridges in HeLa Scc1-eGFP WAPL^{AID} cells. A) The length of 293 PICH-positive bridges is plotted against anaphase progression. **B)** Yellow dots represent 157 individual HeLa Scc1-eGFP WAPL^{AID} cells. These are divided into 8 categories based on the number of PICH-positive bridges per cell. Purple dots indicate the mean value per category.

2.2.3 WAPL depletion might buy cells more time

By identifying early anaphases in HeLa Scc1-eGFP WAPL^{AID} cells, I was able to extend our quantification of number of PICH-positive bridges per cell. In this comparison, I observed

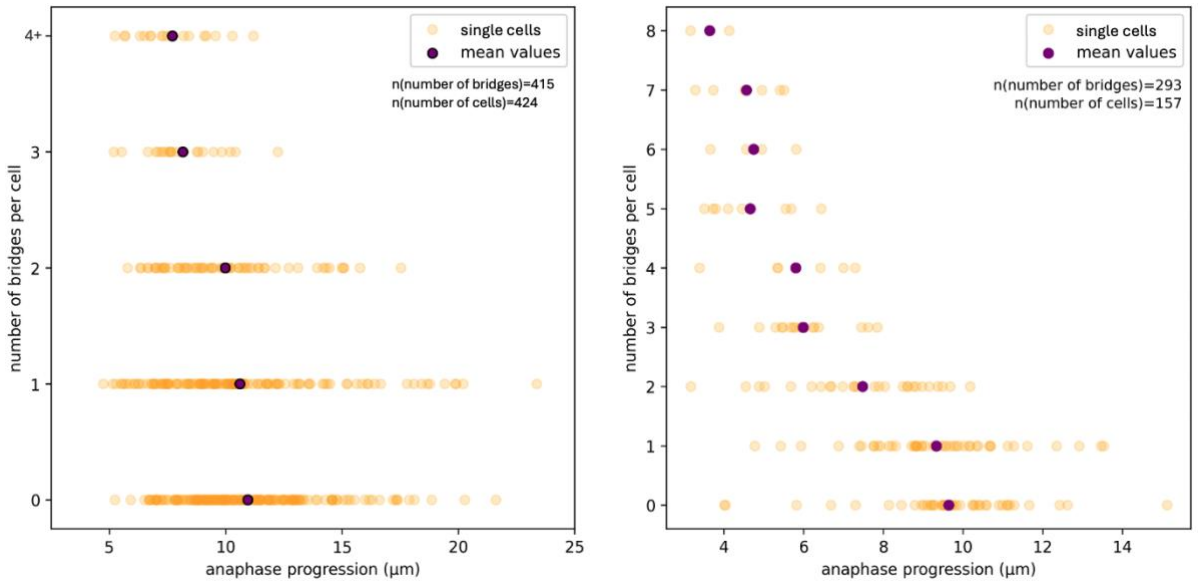


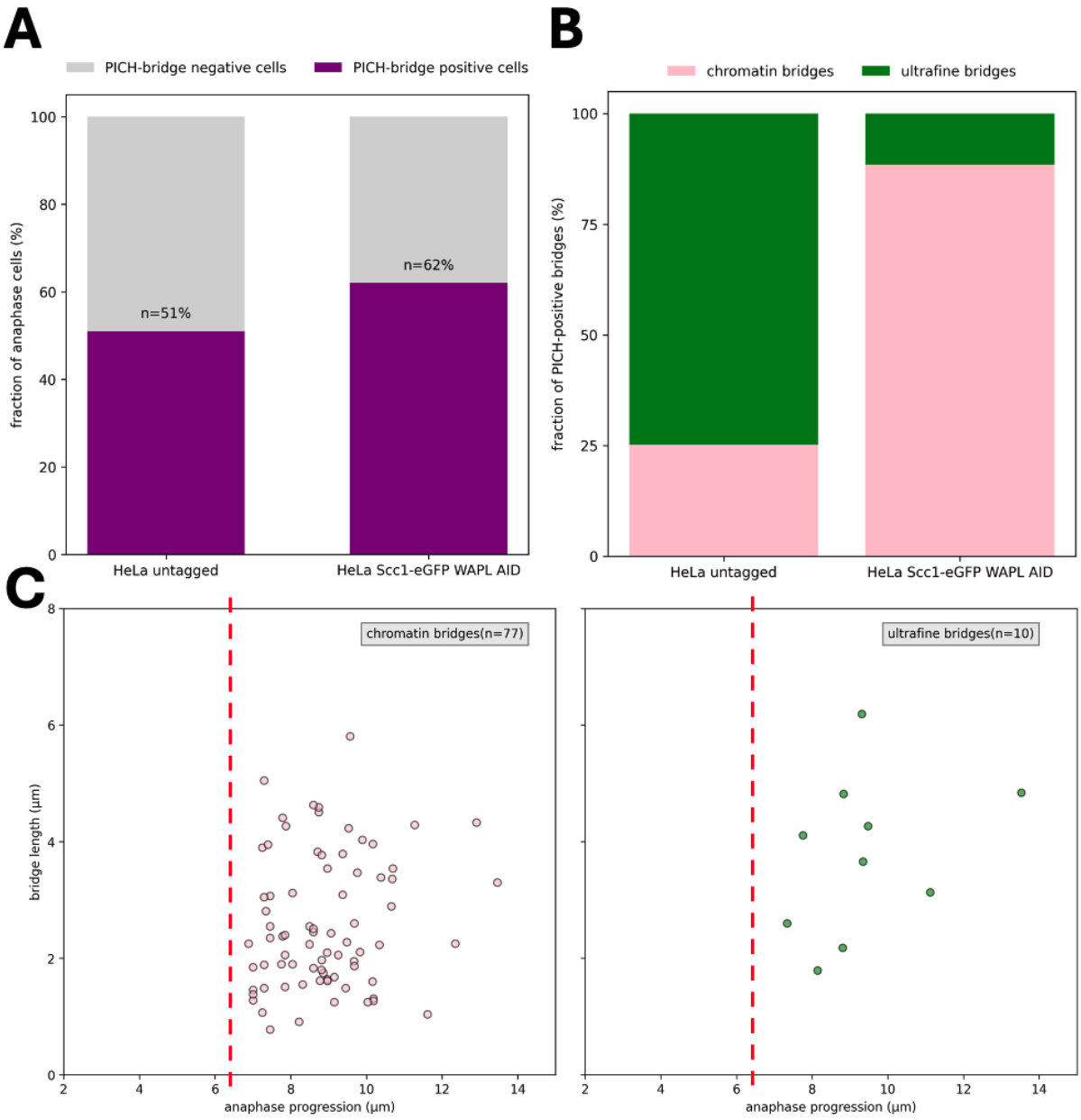
Figure 9. Side-by-side comparison of PICH-bridge positive cells in HeLa and HeLa Scc1-eGFP WAPL^{AID} cells. Figure 9 combines data already shown in Figure 3A and Figure 8B to provide a direct comparison of the number of PICH-positive bridges per cell in HeLa untagged (left, Figure 3A) and HeLa Scc1-eGFP WAPL^{AID} (right, Figure 8B). Yellow dots represent individual HeLa or HeLa Scc1-eGFP WAPL^{AID} cells, while purple dots indicate the mean value per category.

that in HeLa untagged cells, the mean separation distance for cells exhibiting 4 bridges per anaphase cell is approximately 8 µm. In contrast, in HeLa Scc1-eGFP WAPL^{AID} cells, the mean chromosome segregation progression in cells with 4 bridges cell is around 6 µm. At a separation distance of around 8 µm, HeLa Scc1-eGFP WAPL^{AID} cells typically exhibit only 2 PICH-positive bridges per cell (Figure 9). This might be attributed to the prolonged presence of cohesin on chromosomes arms and centromeres, potentially leading to an extended metaphase and anaphase. The extended duration of these

phases could provide the cell with additional time to resolve anaphase bridges, leading to a lower frequency of anaphase bridges at later stages of chromosome separation.

2.2.4 WAPL depletion increases the number of chromatin bridges

In my hands, WAPL depletion resulted in an increase in PICH-bridge positive cells, rising from 51% to 62% (Figure 10A), correlating with previously shown increase in number of ultrafine bridges or catenanes identified through PICH staining (Haarhuis et al., 2013). Next, I assessed whether PICH-positive anaphase bridges from HeLa Scc1-eGFP WAPL^{AID} cells were chromatinized (DAPI-positive) or ultrafine (DAPI-negative). The majority of observed anaphase bridges became chromatinized following IAA treatment



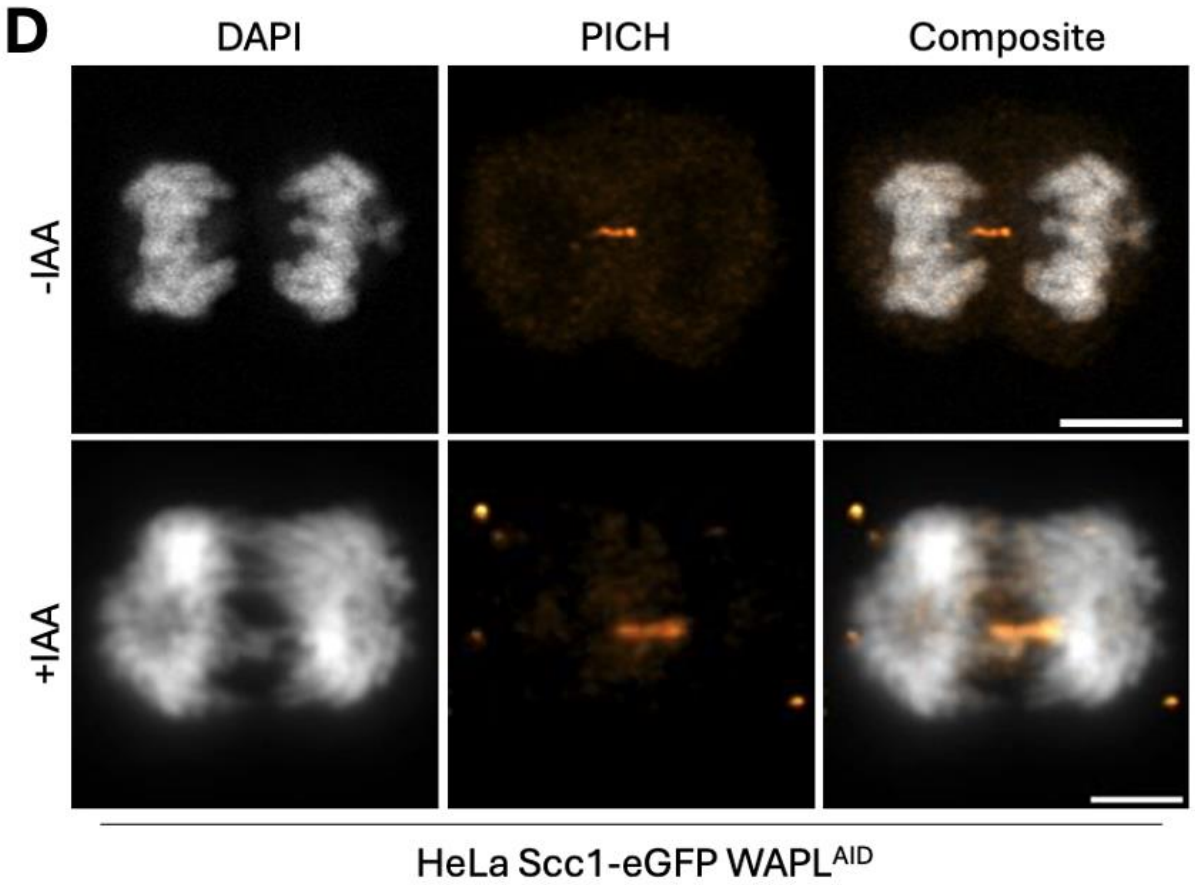


Figure 10. Effects of WAPL depletion on number of PICH-bridge positive cells and on bridge type in HeLa Scc1-eGFP WAPL^{AID} cells. A) Effects of WAPL depletion on number of PICH-bridge positive cells in and HeLa Scc1-eGFP WAPL^{AID} after auxin (IAA) treatment compared to untreated HeLa. **B)** Effects of WAPL depletion on prevalence of chromatin bridges (pink) and ultrafine bridges (green) after IAA treatment in HeLa Scc1-eGFP WAPL^{AID} cells compared to untreated HeLa. **C)** Effects of WAPL depletion on distribution of chromatin bridges (pink) and ultrafine bridges (green) in HeLa Scc1-eGFP WAPL^{AID} cells upon IAA treatment showed in panel 10B. The red dashed line indicates the threshold for correct distinction between chromatin bridges and ultrafine bridges in HeLa Scc1-eGFP WAPL^{AID} after IAA treatment. Below this threshold, the distinction between chromatin bridges and ultrafine bridges could not be reliably determined. **D)** Images of HeLa Scc1-eGFP WAPL^{AID} cells with and without IAA treatment, stained for PICH (gold) and DNA (grey). The anaphase progression measured for both examples is approximately 10 µm. Scale bar 5 µm.

(Figure 10B). When compared to HeLa untagged, the proportion of chromatin bridges had increased three-fold, suggesting that late cohesin removal may influence chromatinization status of anaphase bridges. Comparison of chromatinization status was feasible only in cells where the anaphase progression exceeded approximately 6

μm. In cells where chromosomal masses are separated by less than 6 μm, the proximity of the segregating genomes make it challenging to distinguish whether a PICH-positive bridge should be scored as a chromatin bridge or as an ultrafine bridge. For this reason, only a subset of anaphase cells was analyzed for their chromatinization status (Figure 10C).

2.2.5 PICH-positive bridges at the anaphase onset

Further analysis of early anaphase cells, as described in Figure 7, revealed an interesting pattern of PICH-positive bridges frequently colocalizing with centromeric cohesin signals in the middle of a PICH-positive bridge. This suggests that arm cohesin is by this time cleaved-off, appearing as a diffused cytoplasmic signal. Co-staining with CREST

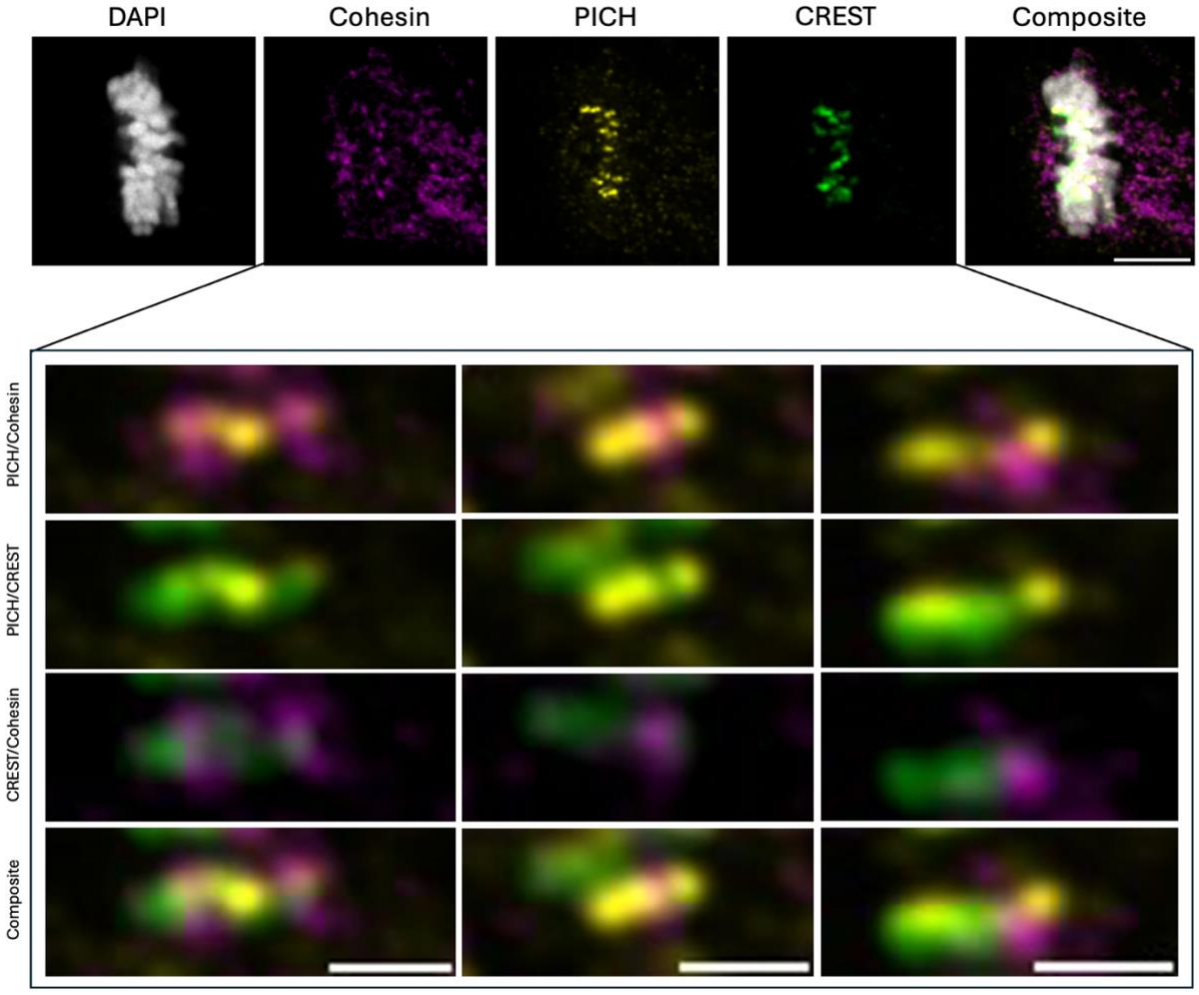
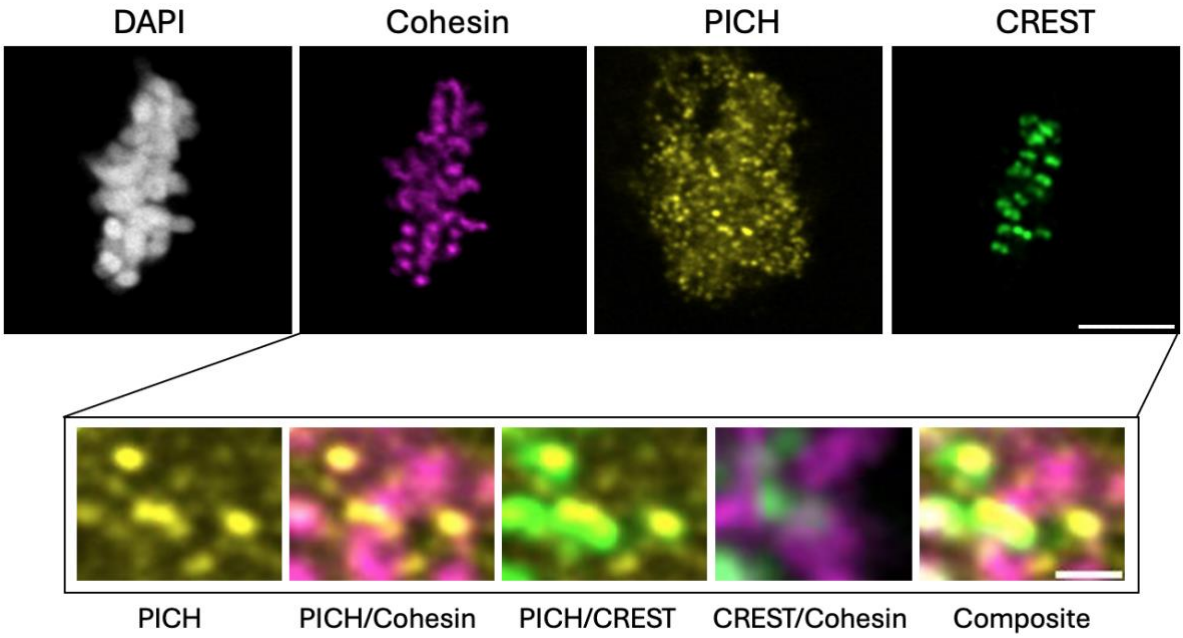


Figure 11. Early anaphase PICH-positive bridges on single planes. A close-up look onto the same cell from Figure 7C with addition of CREST (green) signal. Figure is showing three PICH-positive elongated signals overlapping with CREST (green) and cohesin (magenta). Scale bar 5 μm, magnified images scale bar 1 μm.

suggests that these bridges originate from centromeric regions. Presence of cohesin in the center of PICH-positive bridges likely represents centromeric cohesin that is about to be cleaved by separase. If there is a persistent cohesin at centromeric region or other regions of chromosome at the anaphase onset, cohesion may still link the dividing sister chromatids, potentially explaining the increased incidence of chromatin bridges (Figure 10B-D) that is observed upon IAA treatment in HeLa Scc1-eGFP WAPL^{AID} cells.

2.2.6 PICH-positive bridges are probably appearing already during metaphase

In Figure 7C, I compare the PICH signal in fixed cells that reside in metaphase and in very early anaphase. PICH signal seems diffused in metaphase cell and clustered on bridge-like structures only at anaphase onset. Interestingly, if we change signal intensities of



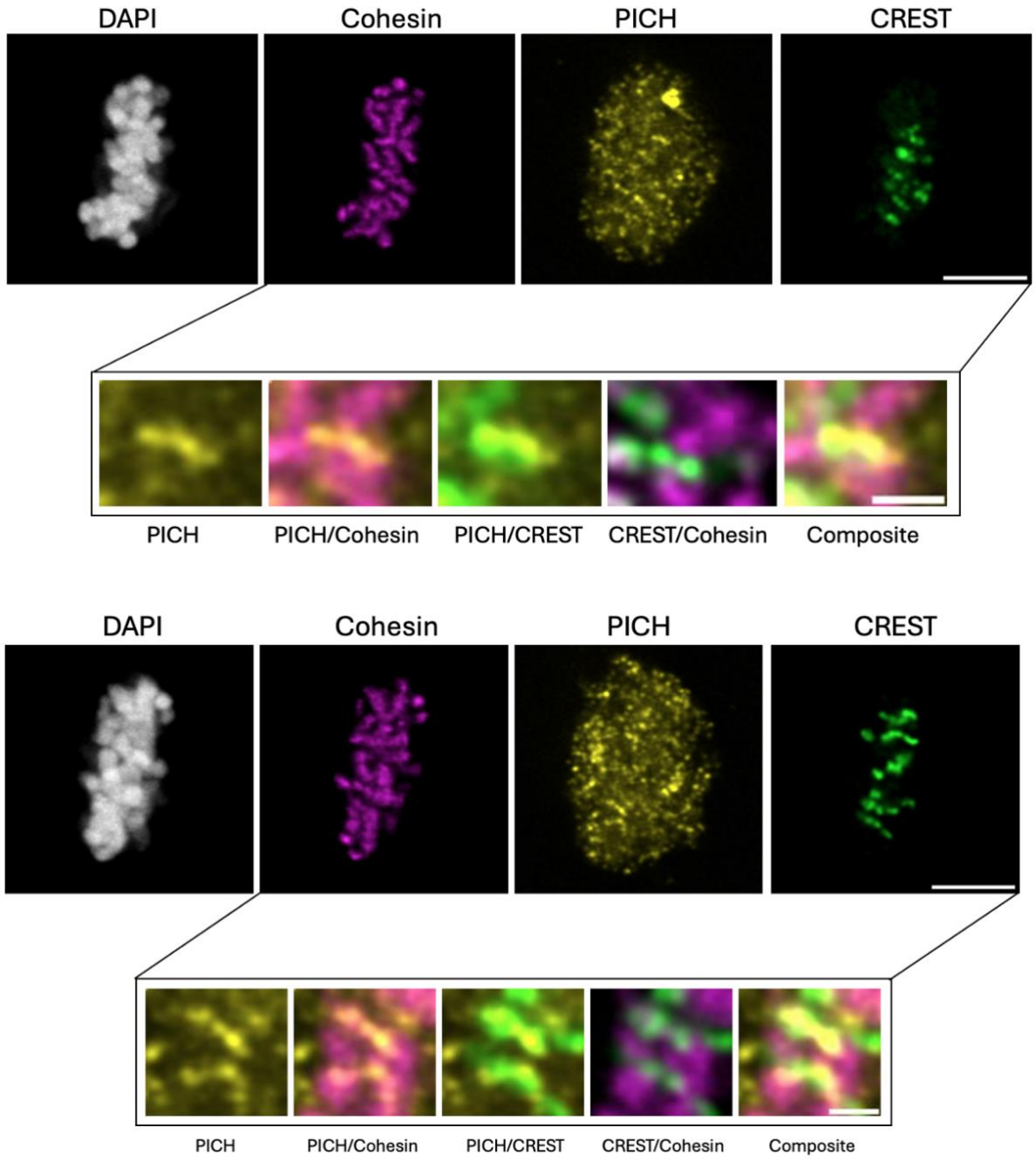


Figure 12. Enhanced PICH signal on metaphase plate. Representative images of a metaphase cell showing 3 different single planes of CREST (green), cohesion (magenta) and PICH (yellow) in HeLa Scc1-eGFP WAPL^{AID} cells upon IAA treatment. DNA is stained with DAPI. Scale bar 5 µm, magnified images scale bar 3 µm.

PICH signal in metaphase cell (to an extent that signals in anaphase cell are saturated), PICH signal in metaphase cell resembles an elongated anaphase bridge-like structure, overlapping with a CREST signal (Figure 12). The presence of such structures on a metaphase plate suggests that PICH-positive bridges may form earlier than previously

assumed, possibly originating from centromeric region based on CREST signal (Figure 12).

2.3 CIP2A is potentially contributing to resolution of ultrafine bridges

I inspected proteins that are recruited to PICH-positive ultrafine bridges. TOPBP1 is a well-characterized DNA repair and checkpoint regulator, recruited to ultrafine bridges. TOPBP1's presence on UFBs could signal replication stress and delay anaphase progression to prevent chromosomal missegregation. If ultrafine bridges persist beyond critical point, checkpoint proteins may stall mitotic exit to allow proper bridge resolution, maintaining chromosome integrity.

Another potential regulator of ultrafine bridges resolution is CIP2A, known inhibitor of PP2A, a phosphatase that promotes mitotic progression. From recent studies, we know CIP2A and TOPBP1 are dependent on each other for recruitment to DNA double-strand breaks during mitosis (De Marco Zompit et al., 2022), raising the possibility that these two proteins function together on ultrafine bridges as a part of a checkpoint mechanism to prevent chromosome missegregation. Because of that, I looked at these two proteins throughout the cell cycle itself, during both interphase and mitosis. Next, I tested whether CIP2A localizes to ultrafine bridges along PICH.

2.3.1 TOPBP1 and CIP2A colocalize in mitosis

I analysed 217 interphase cells and localized TOPBP1 foci in 82 of them. CIP2A colocalized with TOPBP1 in 15 out of 82 TOPBP1-positive cells, which makes almost 20% of interphase cells being positive for both signals in asynchronous, non-treated population of HeLa cells. This indicates that during interphase, TOPBP1 is probably

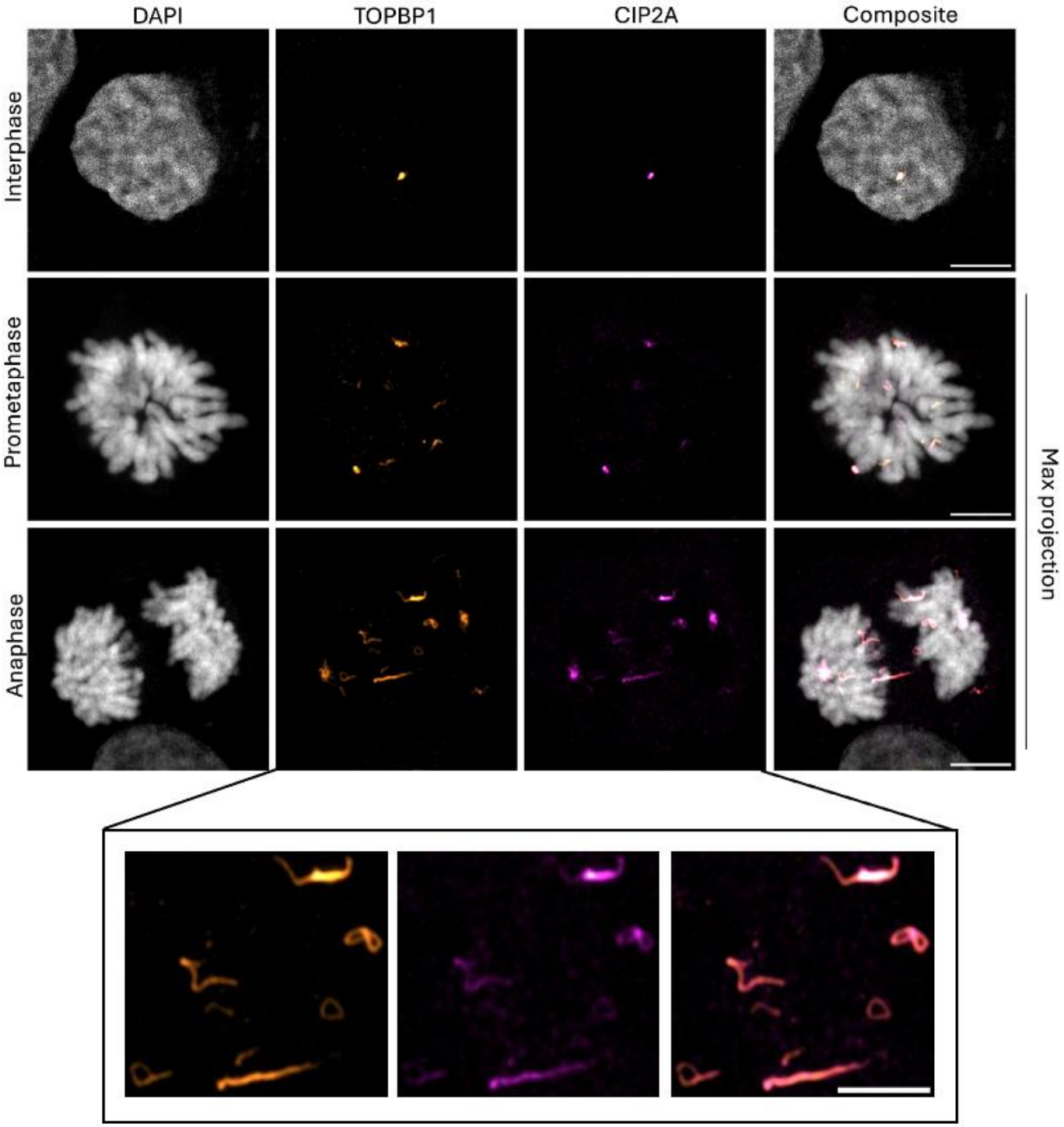


Figure 13. TOPBP1 and CIP2A characterization during mitosis. Representative image showing the localization of TOPBP1 and CIP2A in HeLa cells during interphase, prometaphase and anaphase. DNA is stained with DAPI. Scale bar 5 μm, magnified images scale bar 3 μm.

recruited to sites of DNA damage in interphase independently of CIP2A, as CIP2A is often not recruited at all. CIP2A therefore did not interact with TOPBP1 signal in interphase cells in all observed cases, which correlates with our current understanding of how CIP2A works. CIP2A localizes to cytoplasm under normal conditions and only upon treatment with various inhibitors, CIP2A has been observed to colocalize with TOPBP1 in nucleus (Ummethum et al., 2023). However, in mitosis, based on quantification of prometaphase cells, TOPBP1 was seen colocalized with CIP2A in 100% of cases. This correlates with (De Marco Zompit et al., 2022), where they show their dependency. In 4 out of 21 anaphase cells, anaphase bridges could be observed with TOPBP1 and CIP2A signals colocalizing in between dividing chromosomal masses (Figure 13). The recruitment of TOPBP1 to anaphase bridges was already shown by (Germann et al., 2014), but the recruitment of CIP2A along with TOPBP1 to anaphase bridges has not been described (Figure 13).

2.3.2 TOPBP1 localization to ultrafine bridges

Next, I looked at TOPBP1 signal during anaphase in relation to PICH. TOPBP1 localized to anaphase bridges in 23% of all HeLa cells that were analyzed for TOPBP1-bridge signal (Figure 14). TOPBP1-bridges were localizing to PICH-positive bridges mostly coating only a part of a PICH-positive bridge. However, TOPBP1-bridge signal was also observed in cells not positive for PICH-bridge signal. Examples of TOPBP1-bridge signal coating a part of PICH-positive bridge are shown in Figure 15.

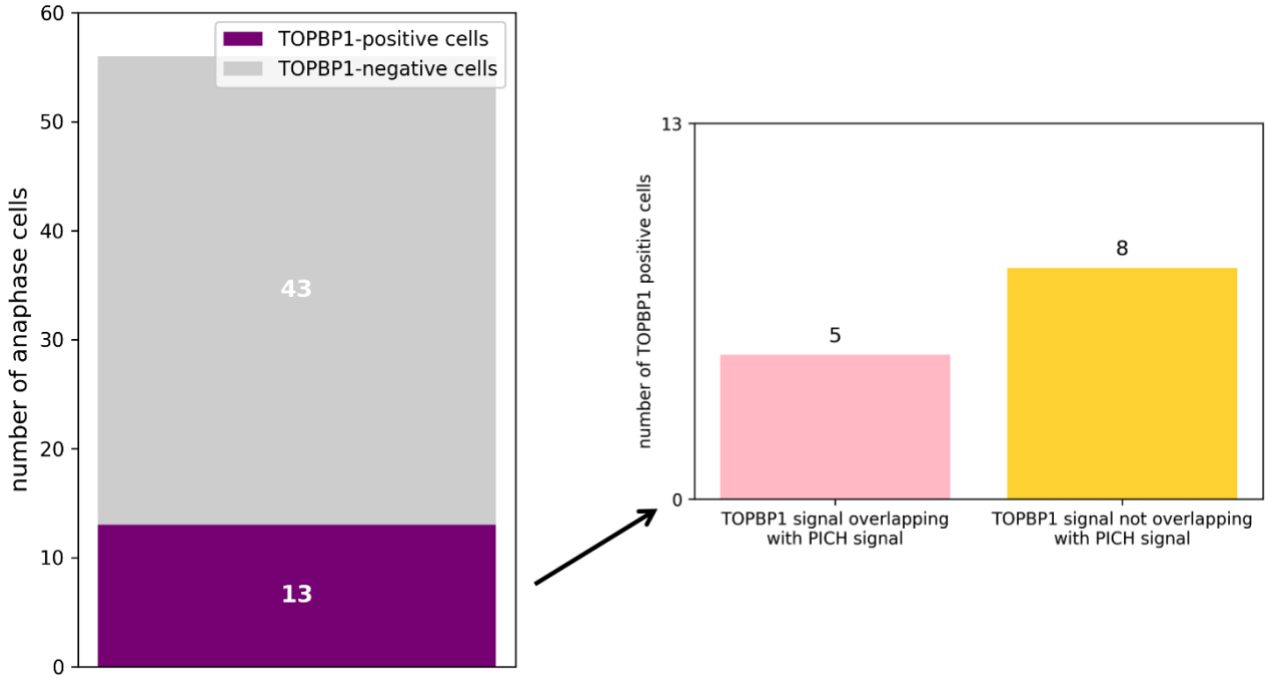


Figure 14. Prevalence of TOPBP1 on anaphase bridges. Left panel shows the total number of HeLa cells analyzed for TOPBP1-bridge signal (n=56), while the right panel provides a breakdown of TOPBP1-bridge positive cells relative to PICH signal.

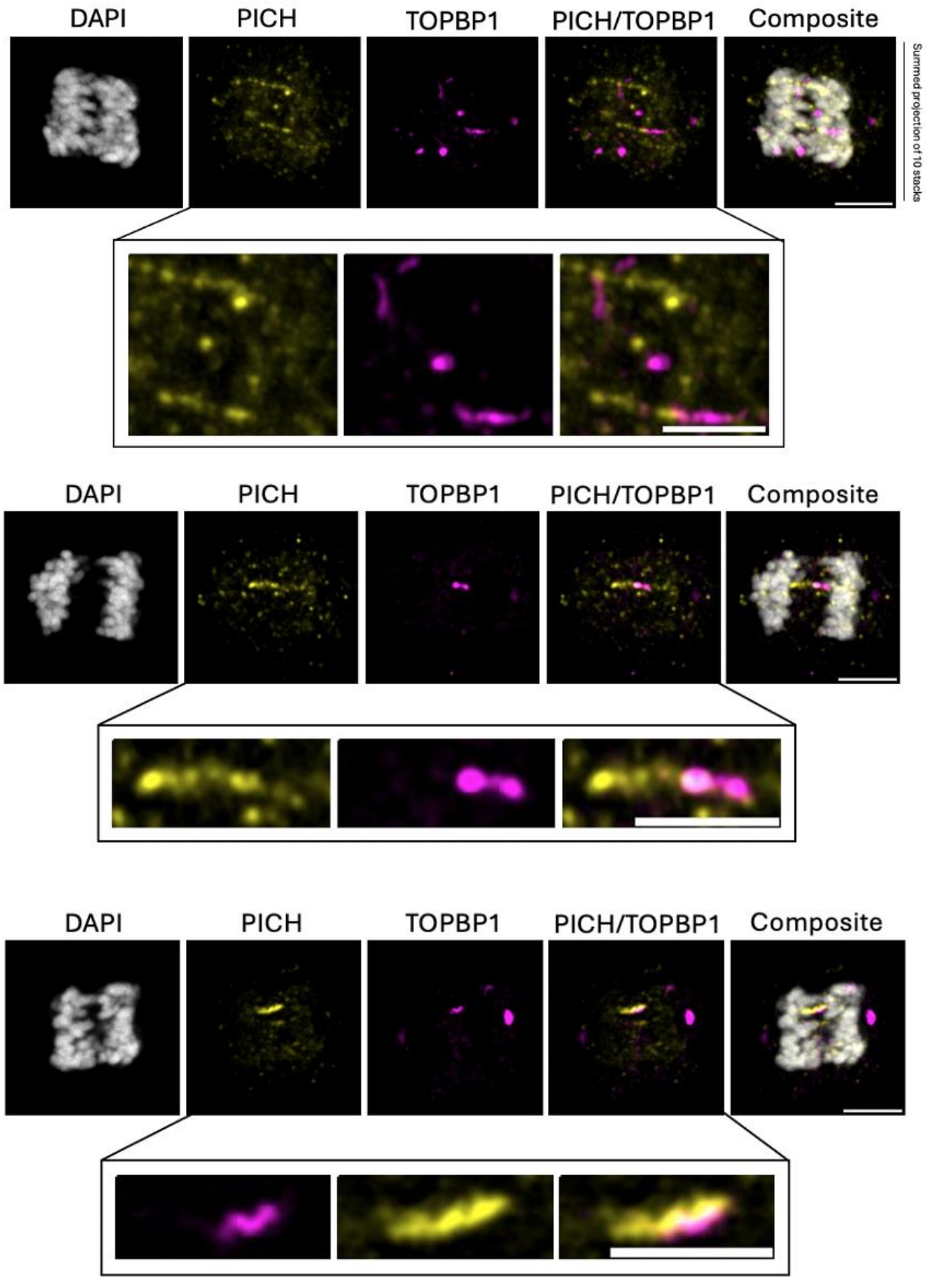

DAPI
PICH
TOPBP1
PICH/TOPBP1
Composite
Summed projection of 10 stacks
DAPI
PICH
TOPBP1
PICH/TOPBP1
Composite
DAPI
PICH
TOPBP1
PICH/TOPBP1
Composite

Figure 15. TOPBP1 localization to anaphase bridges. Representative images of PICH and TOPBP1 signals localizing on anaphase bridges. Scale bar 5 μm, magnified images scale bar 3 μm.

First, it is important to consider that these observations are made in fixed cells, capturing a single moment in time. The overlay of PICH and TOPBP1 signals reveals partial colocalization, but not complete overlap. This could be explained by several factors. One possibility is that TOPBP1 coats only a specific region of PICH-positive bridge, rather than uniformly covering its entire length. However, since we are working with fixed samples, we cannot determine whether TOPBP1 was dynamically coating the entire PICH-positive bridge moments before fixation or not. It is also possible that TOPBP1 localizes selectively to regions of active DNA processing along the UFB, marking a resolution site from which the PICH-positive bridge gradually disappears. This would suggest a spatially regulated function of TOPBP1 in ultrafine bridges processing and resolution.

2.3.3 CIP2A is a novel ultrafine bridges-binding protein

Tracking TOPBP1 localization during anaphase prompted further investigation into the localization of CIP2A in relation to PICH-positive bridges in anaphase. Given the observation of TOPBP1 and CIP2A colocalization on bridge-like structures during anaphase (Figure 13), I sought to determine whether CIP2A exhibits a similar localization pattern with PICH like TOPBP1 does. Indeed, CIP2A was observed to localize to PICH-positive bridges,but only in a small number of cases (Figure 16). Again, we need to consider that these observations are made on fixed samples, and provide only limited insight into the dynamic behavior of UFB-binding proteins.

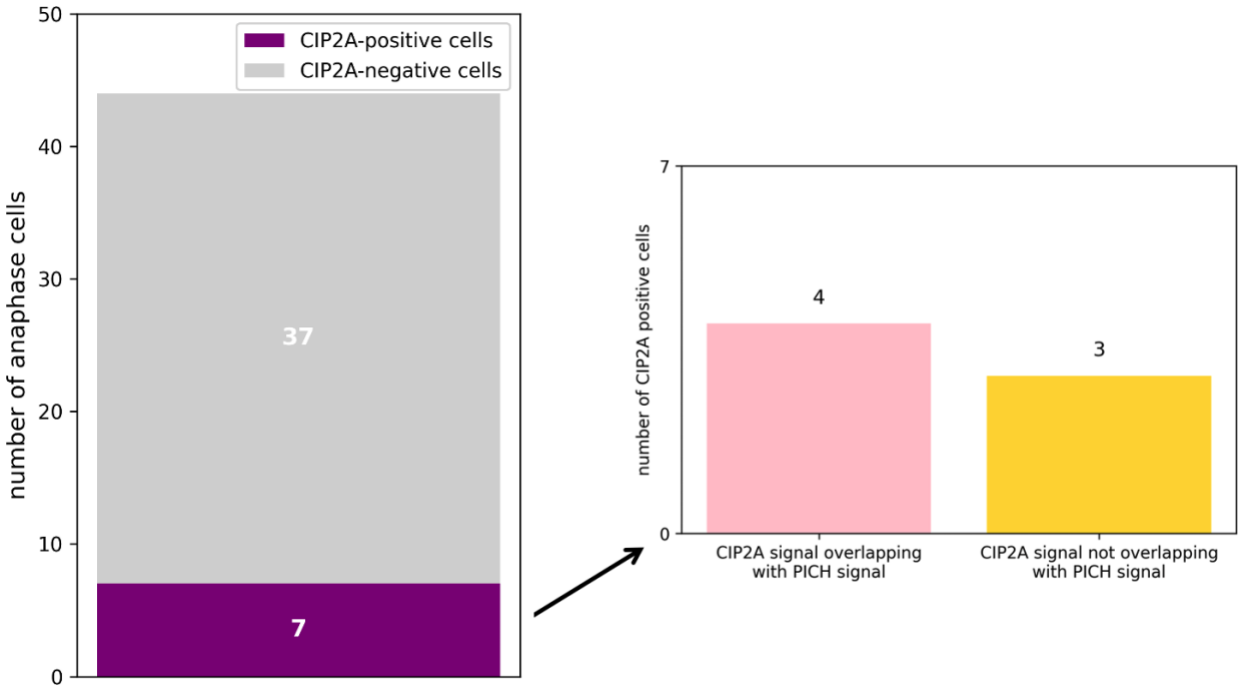


Figure 16. Prevalence of CIP2A anaphase bridges. Left panel shows the total number of HeLa cells analyzed for CIP2A signal (n=44), and the right panel provides a breakdown of CIP2A-bridge positive cells relative to PICH signal.

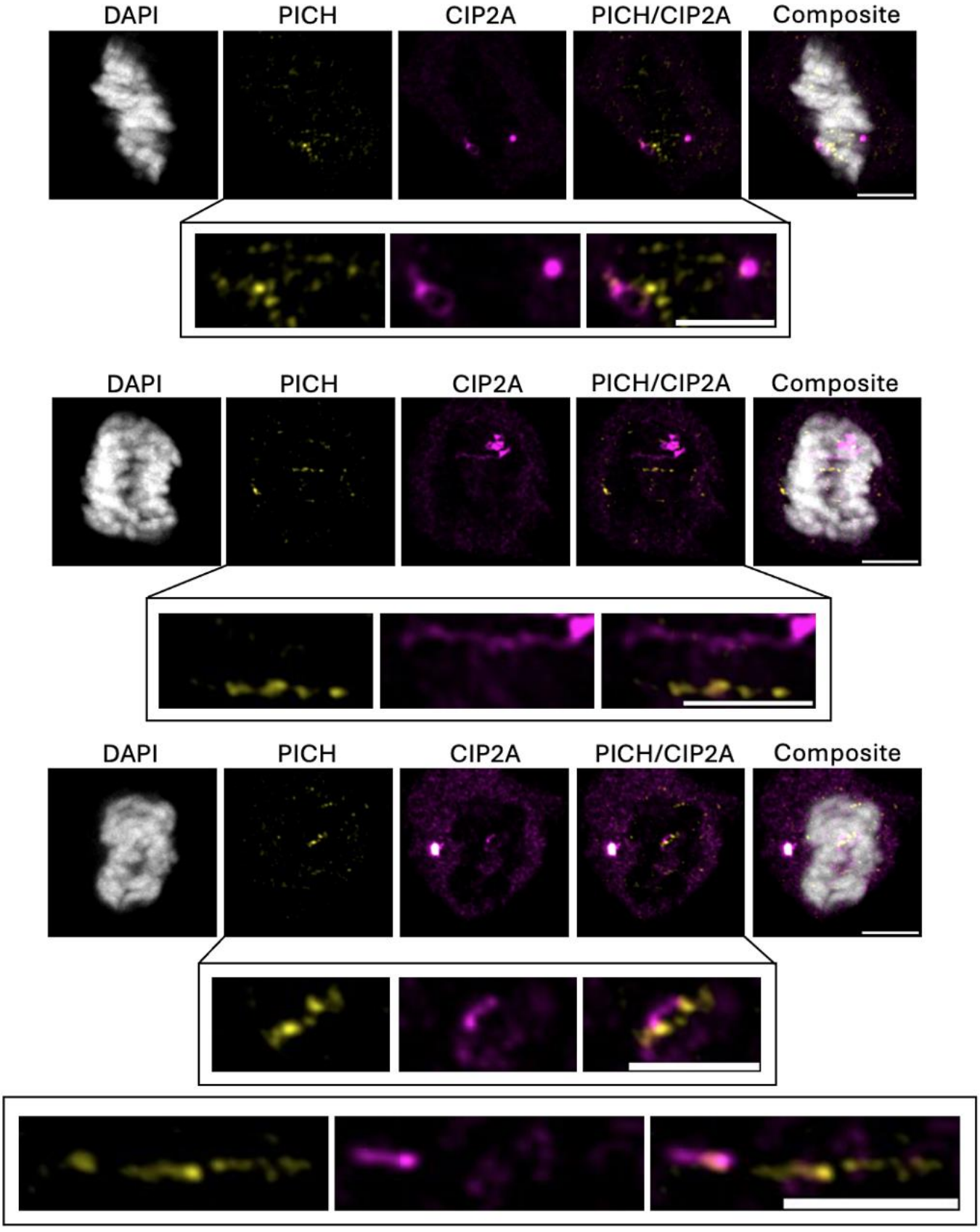

DAPI
PICH
CIP2A
PICH/CIP2A
Composite
DAPI
PICH
CIP2A
PICH/CIP2A
Composite
DAPI
PICH
CIP2A
PICH/CIP2A
Composite

Figure 17. CIP2A localization to anaphase bridges. Representative images of PICH and CIP2A localization on anaphase bridges. Scale bar 5µm, magnified images scale bar 3µm.

In the merged PICH and CIP2A representatives (Figure 17), the CIP2A signal appears to coat only a portion of the PICH-positive bridge rather than spanning its entire length, as seen before for TOPBP1. CIP2A also localizes as distinct punctate foci, often positioned at flanking regions of the PICH-positive bridge, but in some cases, it is completely absent from the bridge itself. This pattern could reflect a dynamic resolution process, where CIP2A was previously associated with a now-resolved PICH-positive bridge but is no longer detectable at the moment of fixation. Alternatively, CIP2A may selectively mark regions requiring active processing, whereas PICH remains bound along the entire length of the ultrafine bridge due to the persistent mechanical tension. This suggests that CIP2A and PICH may function at different stages or regions of UFB resolution, with PICH stabilizing the bridge and CIP2A potentially regulating its processing.

3 DISCUSSION

3.1 Anaphase bridges are dynamic structures

Our mechanistic understanding of anaphase DNA bridge formation and resolution is incomplete. Over the course of this master thesis, I aimed to gain insights on frequency, length, and persistence of these structures during anaphase. A range of proteins localize to ultrafine bridges, with PICH being the earliest and most bridge-specific factor. Given its strong association with anaphase bridges, PICH was examined as a key factor in addressing these questions. I also focused on TOPBP1 and its binding partner CIP2A during anaphase.

Anaphase is generally the shortest stage of the cell cycle, underscoring the importance of the cell to be fully prepared for segregating its genetic material accurately. In asynchronously cycling cells, the mitotic index is typically around 3%, meaning the proportion of cells in anaphase is even lower. In case of HeLa cells, the anaphase index was calculated to be slightly above 1% (Figure 1A), emphasizing the challenging part of identifying anaphase cells among hundreds of cells under the microscope. Given that anaphase typically lasts only about 5 to 10 minutes, the probability of capturing cells at early anaphase or anaphase onset is very low.

The entire characterization of PICH-positive bridges in this study was conducted in asynchronously cycling non-treated cells, which posed additional challenges, but also created a unique dataset. This approach allowed for the observation of chromosomal segregation defects occurring in unperturbed conditions, offering insights into how cells manage anaphase bridges without external disruptions.

The data characterizing PICH-positive bridges in this study aligns with previous key findings and reinforce existing knowledge on anaphase bridge dynamics. Early studies on anaphase bridges (Baumann et al., 2007) suggested that bridges are transient structures appearing at the anaphase onset, while progressively disappearing as cell shifts towards telophase. The data presented in this thesis, collected across various cell lines, supports this notion. Cells exhibit a higher number of bridges per cell at anaphase onset, followed by a gradual decrease in bridge number per cell as anaphase progresses (Figure 3A). This trend reflects the transient nature of anaphase bridges, where PICH translocase, helicases and topoisomerases are recruited to facilitate their resolution,

ensuring that cells enter cytokinesis without persisting chromosomal entanglements that could otherwise impair genome integrity in subsequent stages of the cell cycle.

One of the fundamental and quite simple question I sought to address was the frequency of PICH-positive bridges in both cancerous and non-cancerous cell lines. In cancer cell lines, the probability of observing a PICH-coated DNA bridge in a mid-anaphase cell is approximately 50% (Figure 1B). In the non-cancerous cell line that I investigated, RPE1, the frequency is lower, with one in four mid-anaphase cells exhibiting a PICH-positive bridge (Figure 1B). Despite these differences, I conclude that PICH-coated anaphase bridges are frequently seen in all cell lines investigated, likely reflecting topological constraints that are common during chromosome segregation.

Although I have only looked at fixed cells during the course of this master thesis, I examined the relationship between the length of PICH-coated bridges and anaphase progression. A clear trend emerged: cells in early anaphase tend to have shorter PICH-positive bridges, whereas cells later in anaphase -if PICH-positive bridges persisted- typically exhibit longer bridges (Figure 3B). Although not suprising, this raises a question of whether longer bridges represent structures that resolve later by chance, or that the underlying structures itself is different from earlier resolved bridges.

Examples of long PICH-positive bridges in early anaphase and short PICH-positive bridges in cells with advanced chromosome segregation are observed across different cell lines analyzed (Figure 3B). These ‘outliers’ in the dataset could be of interest and two are shown in Figure 18) The long bridges in early anaphase suggest that PICH-coated bridges do -at least under some circumstances- not slow down the segregation of the sister chromatids that they link. It is also interesting to ask why short bridges in late anaphase cells do not elongate and why their flanking regions do not contain PICH. Addressing this requires more insight into the ultrastructure of the bridge and the flanking regions of the bridge. Since bridge resolution is a dynamic process, the apparent

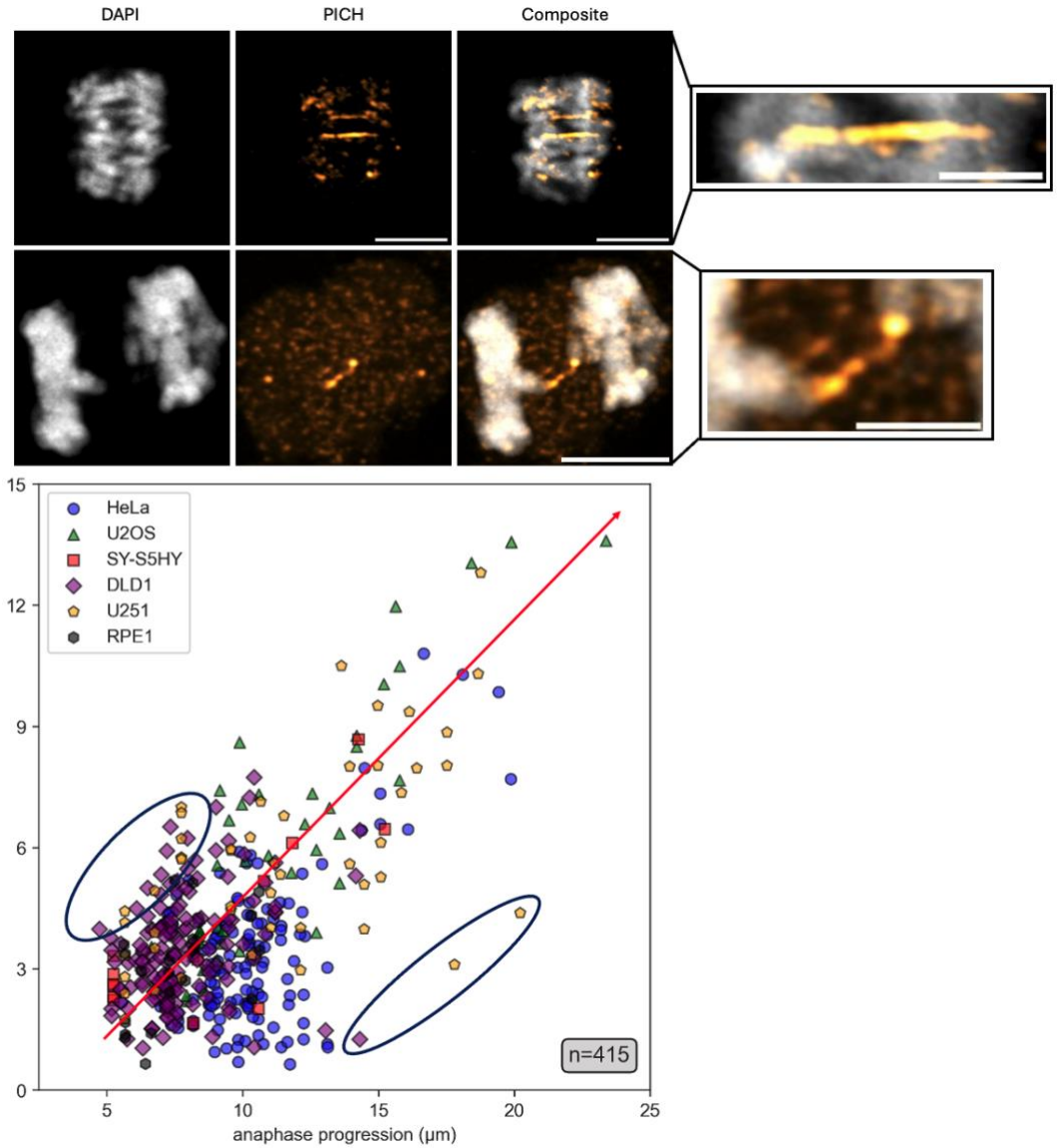


Figure 18. Examples of outliers. Previously shown quantification from Figure 3B and its possible explanation for observing outliers (black circles) relative to the linear trend observed (red arrow). Early anaphase cell (upper image) shows an anaphase cell of a short separation distance with a long PICH-positive bridge, while the image below shows an anaphase cell of a longer separation distance with a long PICH-positive bridge**.** Scale bar 5 µm, magnified image scale bar 2 µm.

structure of PICH-coated bridge highly depends on the timing of cell fixation during anaphase progression. Another possibility is that what appears to be a short PICH-positive bridge may in fact be only a remnant of a long anaphase bridge, which had begun to resolve just before fixation. In the future, it will thus be important to observe the formation and disappearance of anaphase DNA bridges in live-cell imaging. It is also important to study the fate of PICH-positive bridges that persist until late anaphase and

may lead to cytokinesis failure, cell cycle arrest, or micronuclei formation. A possible approach to investigate this further involves treating cells with for example Topoisomerase II inhibitor, known to increase the frequency of anaphase bridges. By coupling this treatment with live-cell imaging, we could track the progression of cells with persistent PICH-positive bridges and determine whether they successfully complete division, undergo mitotic failure or develop micronuclei. This would provide valuable insights into the cellular consequences of unresolved anaphase bridges.

A key aspect of this study was to determine whether anaphase bridges in our dataset could be classified as chromatin bridges (DAPI-positive) or ultrafine bridges (DAPI-negative). Chromatinized PICH-positive bridges can be easily identified by DAPI staining, however, distinguishing between these two bridge types is challenging in early anaphase, when chromosomal masses are still in close proximity. In DLD1, U2OS, U251, SY-S5HY, RPE1, the number of cells with chromatin bridges was higher or similar to the number of cells with ultrafine bridges (Figure 4). HeLa cells are a notable exception: they displayed more frequently ultrafine bridges compared to chromatin bridges (Figure 4). This highlights potential cell line specific mechanisms governing anaphase bridge formation and resolution. It is unclear what this observation has to do with the extreme aneuploidy of HeLa cells or other factors, such as cell-line specific timing of anaphase onset or centromere structure.

Next, I sought to determine the genomic origin of PICH-positive bridges. PICH-positive bridges were co-stained with CREST, FANCD2 and γH2AX (Figure 5). In asynchronously cycling non-treated cells, vast majority of identified bridges were classified as centromeric in origin, as revealed by CREST staining (Figure 5A). These bridges do not have FANCD2 and γH2AX foci on flanking regions of PICH-positive bridges, which are known markers of replication stress and DNA damage. The observation that majority of anaphase bridges originate from centromeric regions, while only a subset arises from homologous recombination intermediates or incomplete DNA replication, may be attributed to the persistence of catenation at centromeres until anaphase onset.

To gain further insights into anaphase bridges dynamics, DNA damage was induced by irradiation, which significantly increased the number of PICH-coated bridges. Following irradiation, the number of PICH-bridge positive cells in mid/late anaphase increased from 43% to 77% (Figure 6A), suggesting that DNA damage either promotes the formation

of anaphase bridges or prolongs their persistence during anaphase. When comparing lengths of PICH-positive bridges in untreated DLD1 cells versus irradiated DLD1 cells in relation to anaphase progression, no significant differences were observed (Figure 6C). PICH-positive bridges continued to follow the previously described pattern, where they are typically short in early anaphase and increase in length if they persist throughout chromosome segregation. This suggests that irradiation does not alter the overall dynamics of bridges during chromosome segregation. However, an interesting observation was that following irradiation, all anaphase bridges became chromatinized (Figure 6B). In untreated DLD1 cells, nearly half of all anaphase bridges were scored as ultrafine bridges. The increased number of cell positive for PICH-bridge may be explained by increased incidence of double-strand breaks that irradiation induces, as well as increased prevalence of chromatin bridges. Staining for gammaH2ax could reveal if these bridges are arising from regions with increased DSBs, but was not conducted.

3.2 Formation of anaphase bridges may initiate already at metaphase with early PICH recruitment

As previously discussed, identifying PICH-positive bridges in cells that have just initiated anaphase poses a challenge. One of the primary reasons for this difficulty is that early anaphase cells can appear morphologically similar to metaphase cells, having their chromosomal masses aligned on metaphase plate. This makes it difficult to investigate the immediate events following anaphase onset. To overcome this limitation and reliably identify early anaphase cells, we utilized the HeLa Scc1-eGFP WAPL^{AID} cell line. In this system, depletion of WAPL protein via auxin treatment prevents arm cohesin removal until anaphase onset. Once released and not detectable anymore on chromosomes, it helps us indicate the anaphase onset. This approach helped record and quantify PICH-positive bridges in very early anaphase cells, thereby extending our characterization of PICH-positive bridges with insights on early moments of formation and resolution of them.

The mitotic chromosome structure of the WAPL^{AID} cells following auxin treatment differs significantly from that of untagged cells. On metaphase spreads, chromosomes appear highly elongated with remaining arm cohesion, as predicted for WAPL depleted cells

(Figure 7A). This phenotype arises due to the prolonged retention of cohesin on chromosome arms, preventing cohesin to be removed during prophase and therefore preventing sister chromatid arms to individualize. Confocal images of HeLa Scc1-eGFP WAPL^{AID} cells before and after addition of auxin were recorded to show the prolonged retention of cohesin on chromosomes in mitosis. Cohesin retained at chromosomal arms up until metaphase and signal diffused during anaphase (Figure 7B). Better visualization of cohesin behaviour in HeLa Scc1-eGFP WAPL^{AID} cells can be observed on a close-up representative image of metaphase and anaphase cell (Figure 7C), where cohesin stays strongly associated with chromatin along metaphase cell, while rapidly diffusing at anaphase onset, indicating that chromosomal masses either initiated or are ready to initiate chromosomal segregation.

The analysis of PICH-positive bridge lengths in early anaphases confirms the previously observed trend: in the initial stages of anaphase, PICH-positive bridges are short at anaphase onset, often measuring less than 1 μm, and progressively elongate, reaching up to 6 μm at later stages of anaphase progression (Figure 8A). Additionally, the number of PICH-positive bridges per cell peaks at anaphase onset, with some cells exhibiting up to eight PICH-positive bridges per cell, following a gradual decrease towards the end of anaphase (Figure 8B).

In HeLa Scc1-eGFP WAPL^{AID} cells, anaphase bridges tend to resolve at shorter chromosomal separation distances compared to untagged HeLa cells (Figure 9). This shift suggests that bridge resolution may occur earlier during anaphase in HeLa Scc1-eGFP WAPL^{AID} cells. One possible explanation is the altered dynamics of cohesin removal due to WAPL depletion, which may prolong the duration of metaphase and early anaphase onset. The extended duration of these stages could allow more time for resolving chromosomal bridges, resulting in fewer bridges persisting at later stages of chromosome segregation.

A key difference observed in HeLa Scc1-eGFP WAPL^{AID} treated with auxin is the increase in PICH-bridge positive cells from 51% to 62% (Figure 10A), as well as the twofold increase in chromatin bridges compared to HeLa untagged cells (Figure 10B). Increased prevalence PICH-bridge positive cell and chromatin bridges may result from residual cohesin along chromosome arms, likely due to unresolved catenation. These findings

highlights the potential role of cohesin and topological constrains in anaphase bridges resolution.

This study proposes that the formation of anaphase bridges and recruitment of PICH on them may initiate prior to anaphase onset, potentially when chromosomes are fully aligned at metaphase plate (Figure 12). Such early recruitment of PICH to bridge-like structures likely serves as a proactive mechanism to help resolve early DNA entanglements that could otherwise persist into anaphase and cause problems. This early engagement would be advantageous for the cell, as anaphase represents the shortest phase of the cell cycle, typically lasting only a few minutes. Consequently, resolving problematic DNA structures prior to anaphase onset might be beneficial to prevent chromosomal instability or cytokinesis failure. Another possibility is that PICH associate with anaphase-like bridges already forming on the metaphase plane due to pre-existing localization of PICH to centromeres. PICH on centromeres may recognize DNA entanglements before anaphase onset due to increased tension on centromeres from microtubule-kinetochore attachment. Increased centromeric tension would therefore attract PICH to early bridge-like structures and therefore aid to the recruitment of additional proteins at the anaphase onset for efficient resolution.

One more explanation might be that indeed, potential anaphase bridges are already forming during metaphase and PICH is recruited to these structures, however, because the cell itself does not anticipate segregation problems at metaphase, only a minimal amount of PICH is recruited to resolve them. As the cell transitions further and bridges persist, PICH recruitment intensifies, leading to a brighter signal on anaphase onset. The increased signal may reflect the need of recruitment of PICH and therefore potentially also other factors that help resolve anaphase bridges to prevent genomic instability.

3.3 CIP2A localizes to anaphase bridges

Finally, I sought to investigate the dynamics of other UFB-associated proteins. TOPBP1 has been previously shown to bind ultrafine bridges, whereas the CIP2A contribution to ultrafine bridges formation and resolution remains unknown. The interaction of TOPBP1 and CIP2A was previously characterized in the context of DNA damage response during interphase, however, their role in chromosome segregation remains largely unexplored.

TOPBP1 and CIP2A signals colocalized in all mitotic cells, providing additional evidence that their role is cell-cycle dependent. Figure 13 illustrates their colocalization and interaction throughout different stages of the cell cycle. Using immunofluorescence microscopy, distinct subcellular distributions of TOPBP1 and CIP2A could be observed, suggesting potential stage-specific functions of these proteins in chromosomal segregation and genome stability. During interphase, both TOPBP1 and CIP2A localize to nucleus, appearing as distinct nuclear foci. However, as cell transitions into mitosis, their distribution undergoes a significant shift. Both proteins display increased association with chromatin, especially on chromosome arms, where they form filamentous structures. Their presence at these sites might indicate a role in the repair of DNA damage foci, further supporting their function in preventing genomic instability during mitosis.

During anaphase, in a subset of cells, TOPBP1 and CIP2A colocalize in between segregating chromosomes (Figure 13), likely on anaphase bridges. This colocalization, highlighted in the magnified region, suggests a potential functional interplay between TOPBP1 and CIP2A in chromosome segregation and mitotic exit. Their presence on anaphase bridges may indicate a role in stabilizing mitotic structures and ensuring the proper resolution of chromosomal linkages, thereby contributing to genomic integrity and successful chromosome segregation.

An interesting and striking property of TOPBP1-CIP2A signals in mitotic cells is the appearance of single or double-circled structures. Double or single-circled structures could arise from incomplete DNA repair (preprint de Haan et al., 2025). After screening hundreds of PICH-positive cells, I was unable to detect PICH localizing on such patterns, nor did I observe PICH colocalizing with TOPBP1 or CIP2A on these structures. This supports that TOPBP1 and CIP2A have also ultrafine-bridge independent roles during mitosis. Unlike anaphase bridges, which experience mechanical tension, these circular structures may represent DNA entanglements that are not under high tension but still require repair for correct chromosome segregation. This would explain why I never saw PICH overlapping with such structures, as PICH has a preference for high-tension areas.

The observed localization of TOPBP1 aligns with previous studies identifying TOPBP1 as one of the key proteins localizing to ultrafine bridges. Quantification revealed that approximately 23% of all anaphase cells quantified for TOPBP1 signal display a

detectable TOPBP1-positive bridge (Figure 14), where TOPBP1-positive bridges mostly coat only a part of PICH-positive bridge (Figure 15). One possible explanation for this partial recruitment might be that TOPBP1 localizes only to a subset of ultrafine bridges, especially those that require additional processing for their resolution. It is important to consider that TOPBP1 binding to ultrafine bridges is transient, which may limit its detection of ultrafine bridges in fixed cells. TOPBP1 might be required only at a specific stage of bridge resolution, after which it dissociates rapidly. This dynamic localization suggests a potential interaction with other repair or remodeling factors before its release. Further investigation using live-cell imaging would be necessary to determine the precise kinetics and regulation of TOPBP1 recruitment and dissociation during anaphase bridge resolution.

Although CIP2A has been shown to have an important role in mitotic progression, its recruitment to anaphase bridges was not shown yet. Analysis of CIP2A localization in anaphase HeLa cells revealed similar patterns of association with PICH-positive ultrafine bridges as TOPBP1 does, suggesting a potential role for CIP2A in ultrafine bridges resolution. Specifically, CIP2A was observed to partially coat PICH-positive bridges or localize to flanking regions adjacent to the bridges (Figure 17). These patterns were detected in approximately 20% of analyzed HeLa cells (Figure 16), a frequency comparable to that observed for TOPBP1 localization on anaphase bridges. Given that TOPBP1 has been shown to interact with Topoisomerase II (TOP2A), it is tempting to speculate whether CIP2A improve or stabilize this interaction, thereby influencing the efficiency of ultrafine bridges resolution. One of the interesting questions arising is whether the CIP2A localization to ultrafine bridges is modulated by TOP2A activity. Since TOP2A inhibition has been shown to increase the recruitment of TOPBP1 to ultrafine bridges, it would be interesting to investigate whether CIP2A follows a similar pattern. If so, this would suggest a role for CIP2A in the regulation of topoisomerase-mediated bridge resolution, potentially functioning as a stabilizing factor in this pathway. Similarly to TOPBP1, CIP2A may be selectively recruited to regions that require active enzymatic processing. PICH may stabilize the ultrafine bridge, maintaining its structure under mechanical stress, while CIP2A could regulate downstream processing events, potentially coordinating the recruitment or activity of enzymes such as topoisomerases or helicases. Given CIP2A's known role in chromosomal instability in cancer, its

involvement in ultrafine bridges resolution may represent a novel mechanism by which CIP2A contributes to genome maintenance, warranting further investigation into its functional interactions with established UFB-associated proteins such as TOPBP1, BLM and PICH. Our findings suggest a novel role of CIP2A in UFB resolution, with potential implications for genome stability and faithful chromosome segregation.

3.4 Limitations of study

Several limitations of this master thesis must be acknowledged, as they may potentially influence the interpretation of the results. The most significant limitation is that all data were generated from fixed samples. While immunofluorescence on fixed cells provide valuable spatial information, it limits the ability to capture the dynamic and transient nature of anaphase bridges. Live-cell imaging approaches could help overcome this limitation by allowing real time observations of proteins dynamics during anaphase bridge formation and resolution. Another technical limitation is the use of conventional fluorescent microscope that limites the detailed study of structures such as ultrafine anaphase bridges. Considering the very thin structure of ultrafine bridges, imaging with super-resolution microscope could significantly improve the quantitative and qualitative analysis, especially during early anaphase. Additionally, this master thesis is constrained by limited sample size due to relying on asynchronous, non-treated cell populations. This approach naturally reduced the number of cells captured in anaphase, yielding a dataset of a few hundred anaphase cells. While this may introduce variability in my quantification, having a population of asynchronously cycling non-treated anaphase cells serves as a strength of the study. By analyzing them, we demonstrated that anaphase bridges formation occur under physiological conditions.

4 CONCLUSION

The findings presented in this thesis advance our current understanding of PICH on ultrafine bridges during anaphase. Investigation of the genomic origins of ultrafine bridges revealed that the majority of arise from centromeric regions, while a smaller subset was associated γH2AX and FANCD2 staining. Interestingly, irradiation induced a marked increase in chromatinized anaphase bridges, further supporting the role of replication stress and DNA damage in bridge formation.

Our observations suggest the early anaphase bridges and early recruitment of PICH to them may begin in metaphase, potentially serving as a proactive mechanisms to detect DNA entanglements before anaphase onset. Such early PICH-recognizing bridges events would be advantageous, as it would allow cells recognize potential segregation barriers prior to the time-constrained anaphase.

This thesis also highlights the dynamic recruitment of TOPBP1 and CIP2A during anaphase. While TOPBP1 has been shown to associate with ultrafine bridges 10 years ago, our observations reveal that CIP2A localizes to ultrafine bridges as well, suggesting a potential role during chromosome segregation.

Despite the limitations of fixed-cell analysis and sample size, this work may provide a foundation for future studies exploring ultrafine bridges. Further investigation using live-cell systems and super-resolution microscopy will be essential to explore the transient and dynamic nature of UFB-binding proteins. Understanding this process is important, as defective bridge resolution is linked to genomic instability.

5 MATERIALS AND METHODS

5.1 Antibodies for fluorescence-based immunostaining

ANTIBODIES	SPECIES	SOURCE	IDENTIFIER	DILUTION
anti-ERCC6L	Mouse	Abnova	Cat #3F12-2B10	1:100
anti-TOPBP1	Rabbit	Abcam	Cat #ab2402	1:1000
anti-CIP2A	Mouse	Santa Cruz	Cat #Sc-80659	1:500
anti-GFP	Rabbit	Thermofisher	Cat #A-11122	1:1000
Crest	Human			1:2500
anti-FANCD2	Mouse	Santa Cruz	Cat #sc-20022	1:1000
anti-γH2AX	Rabbit	Abcam	Cat #ab81299	1:1000
Anti-rabbit Alexa Fluor 448	Donkey	ThermoFisher	Cat #A10037	1:1000
Anti-mouse Alexa Fluor 555	Donkey	ThermoFisher	Cat #A-21206	1:1000

5.2 Cell culture

HeLa cells and HeLa-WAPL^{AID}-Scc1-eGFP and U2OS cells were grown in Dulbecco's Modified Eagle Medium high-glucose supplied with 10% (v/v) FBS and 1% (v/v) PenStrep. DLD1 cells wer cultured in RPMI medium supplied with 10% (v/v) FBS and 1% (v/v) PenStrep. RPE1 cells and SH-SY5Y cells were grown in Dulbecco's Modified Eagle Medium F-12 , while RPE1 cells were supplied with 10% (v/V) FBS and SH-SY5Y were supplied with 15% (v/v) FBS and 1% (v/v) PenStrep. U251 cells were grown in Dulbecco's Modified Eagle Medium supplied with 10% (v/v) FCS and 1% (v/v) PenStrep. All cell lines were cultured in a humidified incubator at 37°C, with 5% CO_2. We thank the Peters lab (IMP) for providing various HeLa cell line, the Slade lab (Max Perutz Labs) for providing us with U251 cells.

5.3 Immunofluorescence and microscopy

Cells on the coverslip were fixed with 4% PFA (Sigma-Aldrich, diluted in 1x PBS of pH=7,4), for 10 minutes, followed by three times 1x PBS wash. Fixed cells were permeabilized using 0.3% Triton X-100 diluted in 1x PBS for 5 minutes, followed by incubation with blocking buffer (3% BSA and 10% FBS diluted in 1x PBS) for 1 hour. Cells were incubated with primary antibodies diluted in blocking buffer overnight at 4°C and then washed with 1x PBS three times. Secondary antibodies (diluted in blocking buffer) were used for 1 hour at room temperature in the dark. After that, for DNA staining and mounting ProLong Diamond Antifade Mountant with DAPI (ThermoFisher) was used.

Coverslips were stored at 4°C overnight. Images were acquired using Zeiss LSM 980 with Airyscan 2 detector, equipped with Plan-Apochromat 63x/1.4 oil immersion objective or Zeiss Axio Imager M2 and ZEN software. Z-stack images were projected using summed projection into one image using FIJI (z-step 0.13um).

5.4 Chromosome spreads

HeLa WAPL^{AID} Scc1-eGFP cells were grown in 10cm dishes and treated with 10uM of CKD1 inhibitor RO-3306 overnight. One dish was treated with 500uM of Auxin (IAA) for 16 hours and the other dish served as a control without addition of IAA. Dishes were washed three times with PBS and cells were scraped off. 2ml were taken and transferred into a falcon tube and 5ml of tap water was added. Samples were incubated for 10 minutes at room temperature. Carnoy's fixative (1:3) was made fresh prior to use and 7ml of fixative was added to hypotonically treated cells. Cells were spun down for 5 minutes at 250rcf. Most of the supernatant was removed and only a little bit was left at the bottom. Solution was resuspended by tapping against the tube, and 10ml of Carnoy's fixative was added again. Cells were spun down for 5 minutes at 250rcf. Process was repeated two more times. Suspension was kept in approximately 2ml of Carnoy's fixative. 100ul were taken and spread on dry slide from approximately suitable distance. After spreading samples on slide and drying out, ProLong Diamond Antifade Mountant with DAPI (Invitrogen) was added onto slides. After approximately one hour, it was possible to inspect these slides under the microscope.

5.5 Image analysis

5.5.1 Fiji analysis

Images were quantified using Fiji software (Schindelin et al., 2012). To properly quantify cells in our collection, the DAPI channel was used to visualize chromatin and the distance was measured by drawing a freehand straight line between two separating chromosome masses along the longest axis of the cell center, measured in summed projection. Same approach was used for measuring anaphase bridges, but their length was measured on single z-planes, to avoid overlapping of anaphase bridges in summed projection.

5.5.2 QuPath analysis

To accurately detect and distinguish interphase and mitotic cells, “Positive cell detection” analysis was chosen in QuPath software (Bankhead et al., 2017). Several mitotic cells were analyzed for mean DAPI intensity and based on that, treshold for DAPI channel was set to 2000. Cells above DAPI mean intensity of 2000 were segmented into red (mitotic) category, whereas cells below DAPI mean intensity were segmented into blue (interphase) category.

5.6 Chromatin bridges and ultrafine bridge distinction

To score PICH-positive bridges for being chromatin bridges, PICH-positive structures would have to be overlapping with DAPI signal, to be evaluated as chromatin bridges. Otherwise, if no DAPI signal detected along PICH-positive bridge, bridges would be scored as ultrafine bridges.

5.7 Irradiation

U251 underwent treatment with 1x 8Gy and were fixed 24 hours after this treatment. I am thankful to Luca Lippert for performing irradiation experiments.

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6.1 List of Figures