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**DISSECTING THE ROLE OF EXTRACHROMOSOMAL
HISTONE H2B IN CYTOKINESIS**

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ABSTRACT

Histones are constitutive components of nucleosomes, the basic units of DNA packaging in eukaryotes and their post-translational modifications regulate chromatin structure. Among different modifications, specific phosphorylation of the N-terminal tail of H2B, at S14 in vertebrate and S10 in yeast, has been linked to DNA damage response (DDR) and chromatin condensation in apoptosis and meiosis. In addition to chromatin-related functions, a few extrachromosomal activities of specific histones have been described. Recently, our laboratory demonstrated that histone H2B localizes at the midbody, the intercellular bridge connecting two daughter cells during cytokinesis. The cytokinetic specific localization of histone H2B is independent of the presence of chromosome bridges at the cleavage plan, indicating a distinct role from chromatin organization of histone H2B. At the midbody, H2B is phosphorylated at S14 (H2B-S14^P) by homeodomain interacting protein kinase 2 (HIPK2), a kinase involved in DDR and development, whose activity is required for cytokinesis and prevention of tetraploidization. In HIPK2-defective cells, expression of a phosphomimetic H2B-S14D mutant is sufficient to overcome cytokinesis defects and rescue cell division and proliferation. This unexpected finding uncovers new role of extrachromosomal histone H2B in cell division. However, the function exerted by histone H2B in cytokinesis is unknown.

Here we show that histone H2B-S14^P localizes in specific areas, during different steps of cell division, from metaphase to telophase. Furthermore, in late telophase, at the abscission onset, H2B-S14^P show an asymmetric distribution probably due to a specific sub-structural localization. Moreover, we show that the midbody localization of histone H2B is independent of both DNA and RNA. In addition, microscopic studies and live-cell imaging of HeLa cells depleted for histone H2B revealed that histone H2B is necessary for faithful cytokinesis. Indeed, depletion of H2B results in prevention of cell cleavage, accumulation of the connections between daughter cells with the formation of long intercellular bridges (LIBs), and abscission defects and delay. H2B depletion results in tubulin disorganization, presumably linked to LIBs formation and the stretching conditions triggered by abscission defects, thus supporting a role for histone H2B in cytokinesis. Furthermore, in HeLa cells, H2B depletion do not affect the spatio-temporal localization of proteins at the cleavage

furrow (*e.g.*, Aurora B, INCENP, PRC1 and PLK1). In the subsequent stage, the midbody formation, PLK1, PRC1, Aurora B, Survivin, INCENP, ECT2, MKLP1 and HIPK2, are present at the midbody and follow the tubulin structures. In contrast, severe defects of localization and organization are observed for the abscission factors ALIX, ESCRT-III subunit CHMP4B, and Spastin, while the localization of Citron kinase is not affected. By in vitro biochemical assays, we show that H2B directly interacts with CHMP4B (Charged Multivesicular Body Protein 4B), a core subunit of the ESCRT-III (Endosomal Sorting Complex Required for Transport-III)-dependent filaments, recently identified as cortical constriction helices required for abscission.

Overall, our data indicate that histone H2B is an important regulator of the final step of cell division and reveal a novel function of extrachromosomal histone H2B for a faithful abscission.

INTRODUCTION

1. The cell cycle

Cell growth and division follow a series of events known as the cell cycle. During cell cycle, chromosomes are duplicated and one copy of each duplicated chromosome segregates to each of two daughter cells. In eukaryotes, the cell cycle is divided into four different phases: gap 1 (G1), synthesis (S), gap 2 (G2) and mitosis (M) (**Fig 1**). The first three phases are collectively named interphases (I). During G1 phase, a cell confirms that internal and external conditions are favorable for a new round of DNA synthesis and division, and commits itself to the process. S phase comprises the duplication of chromosomes. A new copy of each chromosome is synthesized and the two identical DNA molecules are called sister chromatids. G2 phase represents further growth and preparation for entry into mitosis. M phase culminates with the generation of two identical daughter cells by portioning the chromatids to opposite poles of the cell so that each daughter cell receives a copy of each chromosome. An additional phase (G0) refers to a quiescent state in which the cell remains metabolically active, but no longer proliferates unless appropriate extracellular signals are received. Progression through the cell cycle is controlled biochemically by a network of molecular signal and stringent checkpoint that ensure the cell meets the requirements for passage into the next phase (Kops et al., 2005).

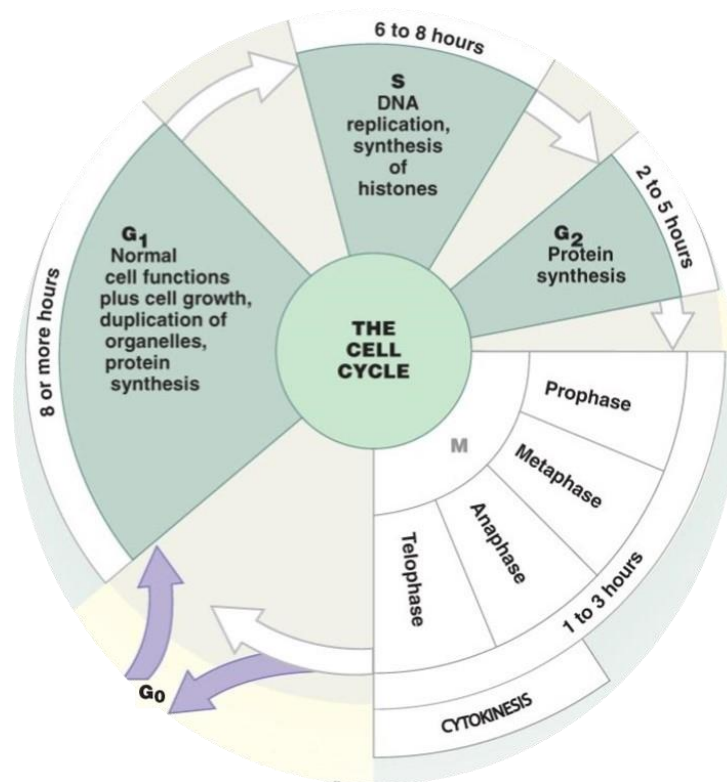


Figure 1. The eukaryotic cell cycle.

The cell cycle consists of four distinct phases: G1 phase, S phase (synthesis), G2 phase (all collectively known as interphase) and M phase (mitosis). M phase is itself composed of two tightly coupled processes: mitosis, in which the chromosomes are divided between the two daughter cells, and cytokinesis, in which the cytoplasm divides in half forming two distinct cells (modified by *Enderson Education inc* 2011).

1.1 Different stages of Mitosis

Mitosis can be subdivided into five morphologically distinct phases: Prophase, Prometaphase, Metaphase, Anaphase, Telophase and Cytokinesis (**Fig. 2**).

At the mitosis onset, animal cells show the most drastic alterations in cytoskeletal organization. During prophase, the largest phase of mitosis, after nuclear envelope breaks down (NEBD), adherent cultured cells become rounded. The centrosomes, which have also been duplicated during S phase, separate and migrate to opposite poles of the nucleus. The microtubule cytoskeleton is re-organized to assemble the bipolar mitotic spindle (a dynamic structure capable of aligning pairs of sister chromatids, sensing chromosome misalignment, and generating forces to segregate chromatids). Chromosomes condense and sister chromatids produced by DNA replication during S phases are captured by microtubule. In prometaphase, held together by a structure known as the centromeres, sister chromatids migrate to the

equatorial plane in the center of the cell known as the metaphase plate. At the metaphase, chromosomes align themselves along the metaphase plate. During anaphase sister chromatids separate and move to opposite poles of the mitotic apparatus, or spindle, segregating one of the two sister chromatids to each daughter cell. Mitosis end with telophase during which the nuclear envelope re-forms around the segregated chromosomes as they decondense, the spindle disassemble and physical division of the cytoplasm, called cytokinesis, occurs.

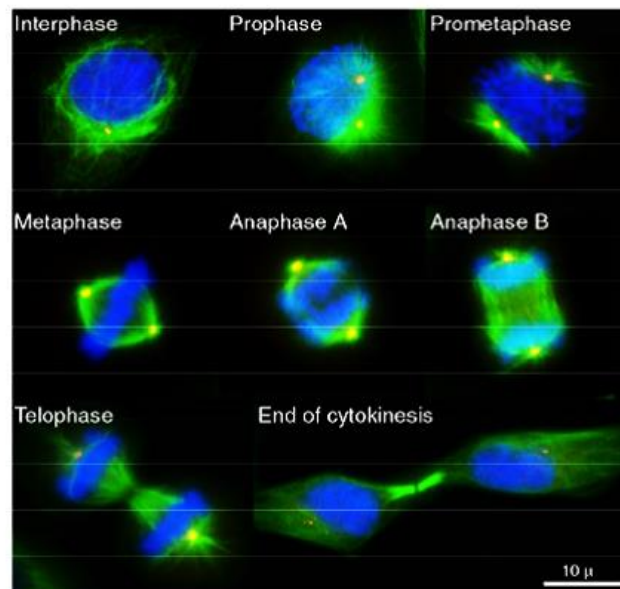


Figure 2. The different stages of M phase.

The immunofluorescence images illustrate HeLa cells in different cell cycle phases. The mitotic spindle is shown in green (α -Tubulin), the centrosomes in red (γ -Tubulin) and DNA in blue (DAPI staining).

1.2 Cytokinesis, the final stage of cell division

Cytokinesis is the final stage of the cell cycle through which a single cell divides into two daughter cells while partitioning its cellular content. The process begins at the onset of anaphase when the metaphase plane is specified and finishes when the sister cells are physically separated (GloTzer et al., 2005; Barr and Gurneberg, 2007). Cytokinesis is a highly regulated process, requiring an intricate interplay between cytoskeletal, chromosomal, and cell cycle regulatory pathways (Fededa and Gerlich 2012). Failure to complete cytokinesis has been proposed to promote tumourigenesis by leading to tetraploidy and ensuing chromosomal instability (Fujiwara et al., 2005; Caldwell et al., 2007).

Faithful inheritance of the genome requires tight temporal coordination of cytokinesis with chromosome segregation. This is achieved by a common molecular cue, the activation of the E3 ubiquitin ligase anaphase-promoting complex (APC), which initiates both chromosome segregation and cytokinetic furrow ingression. The APC triggers chromosome segregation by targeting securin, an inhibitor of the protease separase that destroys the cohesive link between sister chromatids, for proteasome-mediated destruction. Simultaneous targeting of the cyclin-dependent kinase 1 (CDK1) coactivator cyclin B for degradation leads to CDK1 inactivation, resulting in dephosphorylation of many CDK1 substrates by the counteracting phosphatases, which promotes cytokinetic furrow ingression and mitotic exit.

The cytokinesis can be divided into four stages:

- **Stage I: *Positioning the Division Plane and Initiating Cytokinesis***
- **Stage II: *Ingression of the Cleavage Furrow***
- **Stage III: *Formation of the Midbody***
- **Stage IV: *Abscission***

Each stages is dependent on the proper execution of the prior stage, and thus interference with any stage may result in cytokinesis failure (**Fig. 3**) (Normand and King, 2010; Miezwa and Gerlich, 2014).

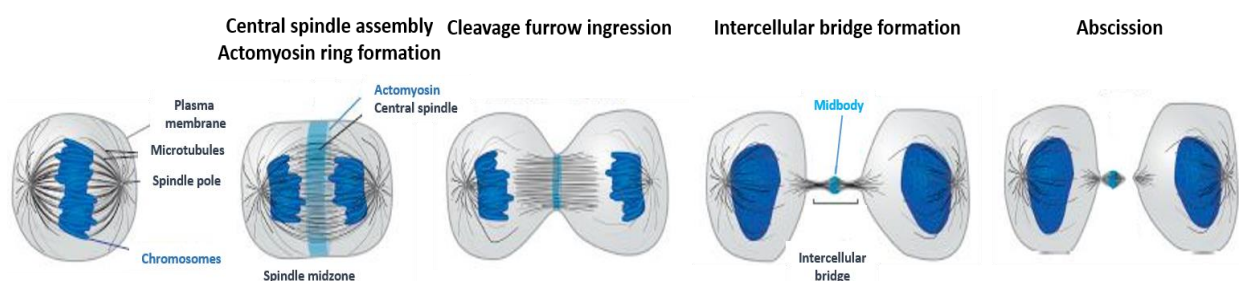


Figure 3. Stages of cytokinesis.

Cytokinesis initiates during anaphase when the two sets of sister chromatids segregate toward opposing spindle poles. Microtubules of the mitotic spindle then reorganize to form the central spindle. Contraction of the actomyosin ring ingresses the attached cell cortex to form the cleavage furrow. Completion of actomyosin ring contraction results in the formation of the intercellular bridge, which contains the midbody at its center. Abscission proceeds by disassembly of the microtubules adjacent to the midbody and membrane fission to physically separate the nascent daughter cells (modified by Mierwa and Gerlich, 2014).

The **first stage of cytokinesis** is characterized by a microtubules reorganization of the mitotic spindle that mediates division plane specification. Three populations of microtubules have been implicated in the regulation of cytokinesis. *Equatorial astral microtubules*, which emanate from the spindle pole to the site of cleavage, may be stabilized in the equatorial cortical region and deliver positive signals that stimulate formation and contraction of the cleavage furrow (Canman et al., 2003). *Polar astral microtubules*, which emanate from the spindle pole to sites away from the site of the furrow, may help position the cleavage furrow by inhibiting cortical contractility, perhaps by spatially biasing the pattern of myosin recruitment (Burgess et al., 2005). *Central spindle microtubules*, which form an overlapping network between the spindle poles following anaphase, send positive signals that become especially important during later steps of cytokinesis (Bringmann et al., 2005).

The *reorganization of mitotic spindle* is mediated by different protein. A key factor in the central spindle assembly is the microtubule-bundling protein regulator of cytokinesis 1 (PRC1), which binds the microtubules once CDK1 inhibitory phosphorylation has been removed at the anaphase onset (Mollinari et al., 2002). CDK1 also inhibits microtubules bundling by phosphorylating centralspindlin complex, which consists of two subunits of the kinesin-6 motor, also known as mitotic kinesin-like protein 1 (MKLP1), and the Rho-family GTPase-activating protein, Male germ cell Rac GTPase-activating protein (MgcRacGAP), respectively (Mishima et al., 2002). Central spindle assembly requires a third essential component, the chromosomal passenger complex (CPC) comprising the Aurora B kinase, the inner centromere protein (INCENP), borealin, and survivin. The CPC relocates from centromeres to the spindle center at the anaphase onset, depending on the removal of a CDK1 phosphorylation from the INCENP subunit and directly contribute to microtubule bundling (Carmena et al., 2012). In animal cells, spindle reorganization and *cleavage plane specification* is regulated by the small GTPase Ras homolog family member A (RhoA), which accumulates at the site of the future furrow. Similar to other GTPase, RhoA is regulated by guanine-nucleotide exchange factors (GEFs) and GAP and GTPase activating proteins (GAPs). An important activator of RhoA is the GEF Epithelial cell transforming sequence 2 (ECT2), which targets to the central spindle by binding the phosphorylated subunit MgcRacGAP of centralspindlin. This latest interaction is mediated by a Polo Kinase 1 (PLK1), which localizes at the centrosomes (spindle poles) and thereafter relocates at midzone where acts as positive regulator of cytokinesis by phosphorylating MgcRacGAP (Petronczi et al., 2007).

In the **second stage of cytokinesis**, RhoA leads to recruitment and activation of effectors that organize the *furrow* and stimulate its ingression. In particular, once at the equatorial plate, activated RhoA promotes actomyosin ring assembly and contraction through two regulatory pathways. On the one hand, RhoA mediates actin polymerization through activation of formins that stimulate nucleation of unbranched actin filaments. On the other hand, RhoA indirectly activates a motor protein myosin II by activating both myosin light chain kinase Rho-associated protein kinase (ROCK) and Citron kinase (Severson et al., 2002).

The **third stage of cytokinesis** is characterized by formation of the *midbody* and stabilization of the cytokinetic furrow. As furrow ingression progresses, the constricting furrow compacts the midzone microtubule array and forms an intercellular bridge, or midbody, that is 1-2 microns in diameter. The midbody is a tightly packed antiparallel microtubule bridge that transiently connects the two daughter cells at the end of cytokinesis. These microtubules overlap at the midbody, which also contains an electron-dense matrix of unknown composition. During the formation of the intercellular bridge, more than 100 different proteins relocate from central spindle to distinct domains at the midbody (**Fig. 4**) (Skop et al., 2004). PRC1 remain associated with the microtubules of the central overlapping zone called “dark zone” in reference to its antibody-excluding characteristic, whereas the CPC proteins localize at microtubules adjacent to the midbody termed “flanking zone”. Several actomyosin ring components, including anillin, septins, Citron kinase, and RhoA, localize to a ring surrounding the midbody in a region called “bulge zone” (Hu et al., 2012).

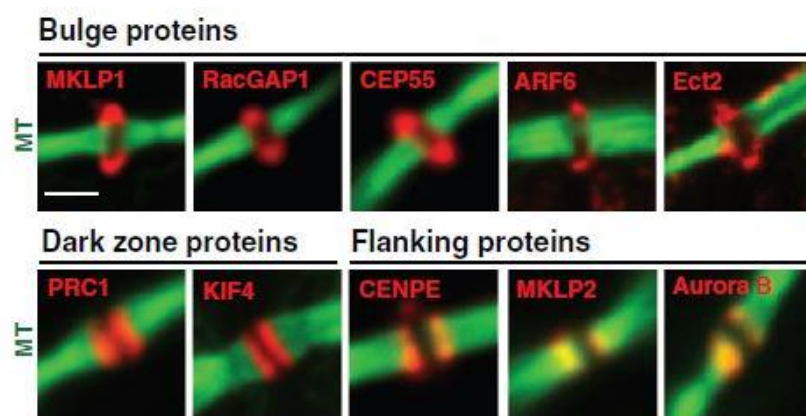


Figure 4. Distinct domain of the midbody.

Midbody proteins were categorized into three groups according to their localizations on the midbody in immunofluorescence. (green microtubule; red different proteins) (Modified by Hu et al.,2012).

The midbody serves as a platform to orchestrate the cytoskeleton rearrangements, plasma membrane remodeling, and recruitment of the functional complexes needed for abscission, the last steps of cytokinesis. Mass spectrometry analyses have shown that midbodies contain not only proteins related to the cytoskeleton, but also factors involved in other pathways, such as lipid raft and vesicle trafficking (Skop et al., 2004). Indeed, plasma membrane of the midbody is enriched by specific lipids. Phosphoinositides and their phosphorylated derivatives play a particularly important role in cytokinesis. Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) accumulates at the equatorial cortex when the cleavage furrow ingresses. This specifies the localization of various cytokinesis proteins such as anillin, septin, RhoA, and MgcRacGAP (Echard, 2012; Emoto et al., 2005). Phosphatidylinositol 3,4,5-triphosphate (PtdIns3P) accumulates close to the midbody where it binds FYVE domain-containing centrosomal protein (FYVE-CENT) and tetratricopeptide repeat domain 19 (TTC19), two factors that mediate accumulation of the abscission factor charged multivesicular protein 4B (CHMP4B) subunit of the endosomal sorting complex required for transport-III (ESCRT-III) (Sagona et al., 2010). Endosomal vesicles also contribute to cytokinesis. At the midbody, upon complete ingression of the cleavage furrow, endosomal vesicles containing ras-related protein in brain 11 (Rab11) and Rab11-family of interacting protein-3 (FIP3) mediate depolymerization of cortical actin filaments of the actomyosin ring through the delivery of p50RhoGAP. Other than their function in remodeling actin filaments, Rab11/FIP3 vesicles also contribute to a gradual narrowing of the intercellular bridge, which precedes the formation of rippled constriction containing ESCRT-III of 17 nm filaments (Guizetti et al., 2011; Schiel et al., 2012).

Furrowing actomyosin ring disassembly, the midbody is stabilized by scaffold proteins such as anillin and septins. In particular, cortical anchorage of the intercellular bridge beyond the central midbody region depends on anillin, which localizes at the midbody, as well as at adjacent regions. Anillin binds to PtdIns(4,5)P₂ through its C-term pleckstrin homology (PH) domain (Liu et al., 2012) and links to underlying septin filaments (Kechad et al., 2012). Anillin is recruited and maintained at the intercellular bridge through binding to Citron kinase. Citron kinase further contributes to the stability of the midbody by binding to MKLP1 and another microtubule-associated kinesin, kinesin family member 14 (KIF14). Those kinesins interact with PRC1 at the central midbody (Gruneberg et al., 2006). Thus, the stability of the intercellular bridges depends on multiple factors that tether the midbody and the adjacent regions to plasma membrane.

In the **fourth and last step of cytokinesis** called *abscission*, the two daughter cells are physically separated. A wide variety of proteins involved in vesicle and protein trafficking, membrane scission, and cytoskeleton rearrangement are required for abscission, suggesting that the final stage of cytokinesis is just as complex as earlier stages (**Fig. 5**).

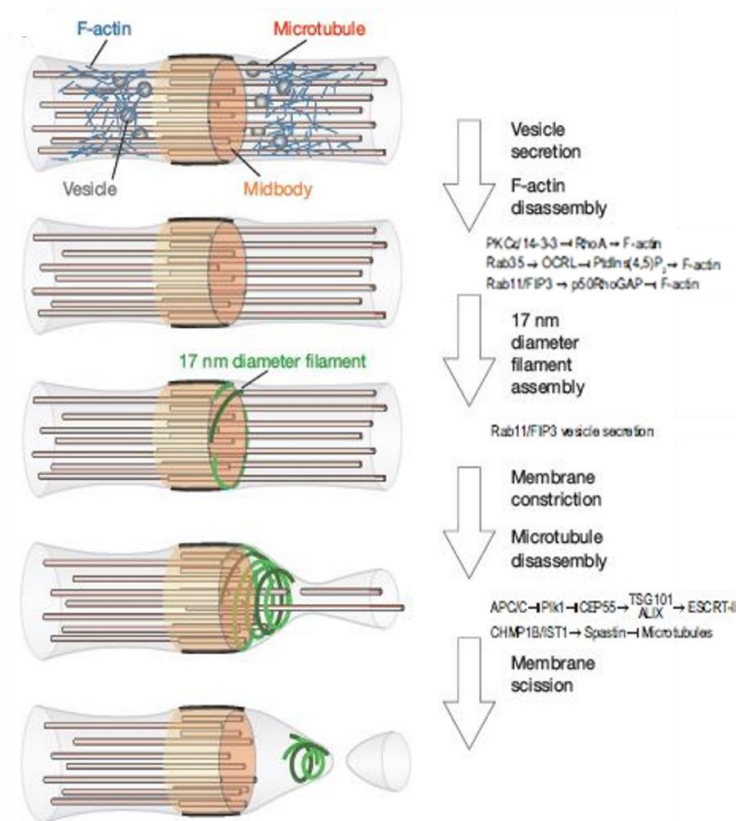


Figure 5. Schematics of intercellular bridge maturation.

Complete ingression of the cleavage furrow is followed by disassembly of cortical F-actin. Fusion of vesicles correlates with gradual narrowing of the intercellular bridge on both sides of the midbody. Abscission proceeds by assembly and constriction of 17 nm filaments adjacent to the midbody and simultaneous disassembly of the microtubules lateral to the midbody (modified by Fededa and Gerlich, 2012; Miezwa and Gerlich, 2014).

1.3 Abscission, the final steps of cytokinesis

During *abscission*, the final steps of cytokinesis, the intercellular bridge the connects the two daughter cells is severed adjacent to the midbody. Three models of cell separation have been proposed (**Fig. 6**).

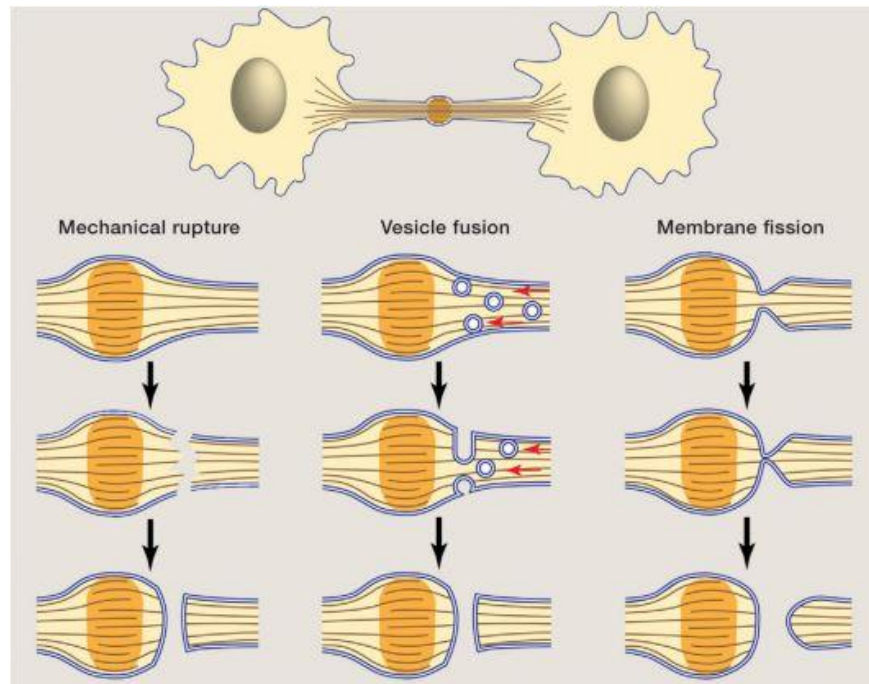


Figure 6. Schematic representation for Abscission models.

The mechanical rupture model (left) posits that as daughter cells move away from one another, the intercellular bridge is torn. The vesicle fusion model (center) proposes that vesicles delivered to the midbody fuse with each other and with the plasma membrane to separate the daughter cells. The membrane fission model (right) proposes that the midbody membrane is acutely constricted, and then sealed and severed (modified by Elia et al., 2013).

The first abscission model proposed was the mechanical rupture model. It was suggested that the connection between the two daughter cells is stretched and eventually broken by mechanical rupture as the daughter cells pull in opposite direction. Mullins proposed that a wound healing mechanism seals the membrane opening. Evidence that cell adhesion molecules, such as integrins, and cellular wound healing component are required for successful cytokinesis is some cells support this model (Kanada et al., 2005; La Flamme et al., 2008). However, because the abscission occurs also in nonadherent cells (*e.g.*, leukocytes and lymphocytes) and nonmotile cells that do not migrate away from one another under physiological condition, researchers began to look for alternate mechanism.

A second model of abscission proposes that vesicle may fuse with the plasma membrane before abscission to establish separating membranes from within the intercellular bridge. Vesicle from Golgi-secretory pathway and from recycling endosome accumulate at the intercellular bridge together with several vesicles targeting and tethering factors, including

centriolin, the exocyst complex, Rab5, Rab11, v- and t-soluble N-ethylmaleimide-sensitive factor activating protein receptor (v- and t-SNARE). These factors were all shown to be required for abscission (Fielding et al., 2005; Gromley et al., 2005; Kouranti et al., 2006). However, electron microscopy studies showed that vesicle disappear from the intercellular bridge before abscission. Thus the failure in abscission upon depletion of these factors, however, seems more likely to result from perturbed intercellular bridge maturation rather than defects during the final abscission stage (Guizetti et al., 2011).

The third model proposes that midbody membrane is acutely constricted and then severed. This model indicates the involvement of the endosomal sorting complex required for transport (ESCRT) machinery that mediate the final membrane scission event and separates the daughter cells. The ESCRT-III machinery mediates topologically equivalent constriction and scission events during both multivesicular body formation (MVB) and retrovirus budding from plasma membrane (McCullough et al., 2013). Once the midbody has thinned, about half of its initial width, a group of proteins component of the ESCRT machinery localize at the midbody by a sequential recruitment of its targeting factors. In particular, during late cytokinesis, PLK1 degradation leads to dephosphorylation of centrosomal protein CEP55 and its consequent relocalization from the spindle midzone to the dark zone at the midbody through interaction with MKLP1. CEP55 recruits the ESCRT-I subunit tumor susceptibility gene 101 (TSG101) and the ESCRT-associated protein apoptosis-linked gene-2-interacting protein X (ALIX) that also localize at the dark zone to the midbody (Morita et al., 2007). TSG101 and ALIX then recruit ESCRT-III subunit, also called CHMPs, to cortical ring at both sites of the midbody (Elia et al., 2011). Later in cytokinesis, a second and smaller pool of ESCRT-III proteins accumulates at the abscission site, approximately 1 μm away from the center of the midbody, where acute membrane constriction and abscission occur. CHMPs recruitments at the secondary ingression is essential for cytokinetic specific localization of spastin, a microtubule severing AAA-ATPase. Indeed, spastin localizes at the midbody by binding the ESCRT subunits CHMP1B and IST1, and mediates microtubule scission at the end of the abscission process (**Fig. 7**) (Connell et al., 2009). ESCRT-III also interacts with membrane via PtdIns3P-binding protein FYVE-CENT and TTC19. During the final stages of abscission, the ESCRT-III depolymerization factor vacuolar protein sorting-associated protein 4 (VPS4) accumulates at the midbody where mediates the disassembly of the complex following the membrane scission (Agromayor et al., 2009).

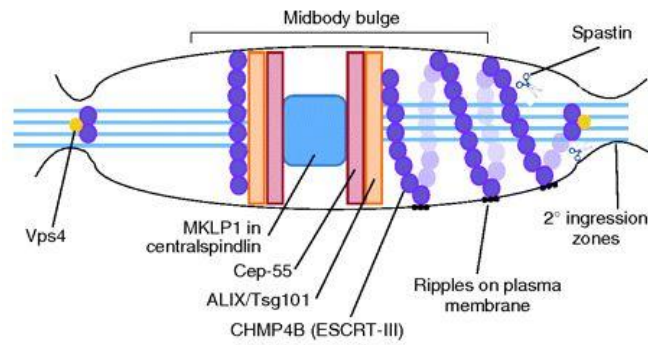


Figure 7. The ESCRT complex in abscission.

The centralspindlin component recruits Cep-55 to the midbody. Cep-55 in turn recruits ESCRT-I protein Tsg101 or ALIX. Tsg101 and the ESCRT-III subunit CHMP4B (purple spheres) are sequentially recruited into the center of the intercellular bridge. Later in cytokinesis, CHMP4B concentrates at the narrow secondary abscission zones, closely followed by Vps4 (yellow circles). ESCRT-III subunits extend towards these sites of cortical constriction forming filaments, which extend towards the site of secondary ingression. ESCRT-III can then recruit the microtubule-severing enzyme spastin (scissors) (Modified by Neto and Gould, 2011).

About 10-20 minutes before abscission, the cortex adjacent to the midbody constricts. This secondary ingression of the cell cortex is essential for abscission. Guizetti and colleagues, by electron microscopy analysis of late stage of cytokinesis, revealed that cortical constriction zone contains membrane associated 17 nm diameter filaments forming large intertwined helices surrounding the intercellular bridge (Guizetti et al., 2011). The ESCRT-III machinery is a candidate of the 17 nm diameter filaments because co-localizes with the constriction zones and is required for the formation of these filaments. Thus far, three models are proposed to clarify how ESCRT-III polymers may contribute to abscission: ESCRT-III may grow filament spirals that decrease their diameter when they extend away from the midbody to constrict the intercellular bridge (Guizetti et al., 2011). Alternatively, part of an ESCRT-III filament helix may be released, possibly by VPS4, to slide away from the midbody while constricting the cortex (Elia et al., 2012). Otherwise, an ESCRT-III-independent process leads to membrane constriction, which is then captured and stabilized by ESCRT-III (Schiel et al., 2012).

In summary abscission proceeds by a secondary ingression of the cell cortex, involving of 17 nm diameter filaments spanning the intercellular bridge. The prevailing model suggest the involvement of the ESCRT-III machinery in abscission. Moreover, the membrane scission model was enriched by the finding that spastin, a microtubule severing enzyme, directly

binds to ESCRT-III subunit at the secondary ingression. Thus, it was proposed that the ESCRT machinery couples microtubule severing to the physical cleavage of the membrane. However, how ESCRT-III adapts to the large membrane tube of the intercellular bridge and how these large structures constrict remain important open questions. Moreover, understanding the mechanism by which the plasma membrane ultimately splits and how vesicle or wound healing contributes to abscission will require further investigation.

1.4 Cytokinesis failure

Cytokinesis, the last step of cell division, proceeds throughout subsequent stages each owing to the previous one, that requires a complex interplay among a still enlarging number of regulatory and effector components related to cytoskeleton, chromosome, cell cycle, lipid raft, vesicle, and membrane trafficking factors. Cytokinesis failure can arise through defects in any of the four stages in cytokinesis. Inhibition or excessive activation of different cytokinesis components can give rise to distinct phenotypes, including precocious ingression before the chromosomes have been separated, regression of the furrow giving rise to binucleated cells, or stabilization of the cytoplasmic bridge where daughter cells remain connected. High fidelity cytokinesis is crucial to ensure that both daughter cells inherit a diploid set of chromosomes. Cytokinesis failure and the resulting tetra- and poly-ploidization promote chromosomal and genomic instability, both hallmarks of cancer (Ganem et al., 2007).

Inhibition or regression of the cleavage furrow induces both formation of binucleated and persistence of connections between daughter cells with formation of long intercellular bridges (LIBs) and syncytial-like structures (Normand and King, 2010). Faithful cell division requires tight coordination with chromosome segregation. Before cytokinesis starts, chromosomes need to be cleared from the cleavage plane to avoid being damaged during furrow ingression (Eggert et al., 2006; Glotzer, 2005). The initiation of chromosome segregation and cytokinesis both depend on the activation of E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C). The APC/C promotes mitotic exit by targeting securin, an inhibitor of the protease separase that destroys the cohesive link between sister chromatids, for proteasome-mediated degradation. APC/C also acts by targeting the CDK1 coactivator cyclin B for degradation leading to CDK1 inactivation. Thus, dephosphorylation of many CDK1 substrates promotes cytokinetic furrow ingression. While chromosome

segregation normally completes early after anaphase onset, it can be severely delayed by lagging or bridged chromosomes. Segregation defects have been estimated to occur in about 1% of dividing somatic cells, and at higher incidence in transformed cells (Cimini et al., 2003). Similarly, abscission must occur only after the complete separation of sister chromatids. Chromosome bridges can result from dysfunctional telomeres (Maser and DePinho, 2002; Stewenius et al., 2005), DNA double-strand breaks (Acilan et al., 2007), or from misregulated chromosome cohesion (Cimini et al., 2003) or decatenation (Chan et al., 2007). Circumstances that cause chromosome segregation errors have shown to delay abscission. In animal cells, Aurora B kinase controls the abscission delay induced by chromosome bridges by a regulatory pathway termed “NoCut”. In the presence of chromosome bridges, at the midbody, Aurora B remains active for a much longer duration after cleavage furrow ingression, which may contribute to abscission delay (Mathieu et al., 2013; Bembenek et al., 2013; Steigemann et al., 2009). Aurora B mediates the phosphorylation of MKLP1 and this phosphorylation is maintained for a longer duration in the presence of chromosome bridges, thus stabilizing the interaction between the ingression furrow and the midbody and prevents the furrow regression in cells with chromosome bridge (Steigemann et al., 2009). Aurora B also regulates the abscission delay through the ESCRT-III subunit CHMP4C. It has been hypothesized that phosphorylated CHMP4C is recruited early at the midbody and may out-compete other ESCRT-III subunit in binding to their targeting factors such as ALIX and CHMP4B, thus inhibiting the assembly of the ESCRT-III filaments required for abscission (Agromayor et al., 2013; Capalbo et al., 2012; Carlton et al., 2012).

2 Histone H2B

Histones are constitutive component of nucleosomes. The nucleosome is the basic unit of chromatin packing in eukaryotes, consisting of a segment of DNA wound around histone proteins. In particular, the nucleosome core consists of about 147 base pairs (bps) of DNA wrapped around a histone octamer consisting of two copies of each core histones H2A, H2B, H3 and H4 (**Fig. 8A**). The nucleosomal structure repeats with a regular periodicity every 160-220 bps. This conformation is termed the 10 nm fiber or “beads on a string”, referring to the way chromatin appeared on electron micrographs when it was first visualized in 1973 (Olins and Olins, 1974). To compact DNA, multiple nucleosomes further assemble into higher-order

structures, which are stabilized by histone H1 (linker histone) that binds and rearranges the DNA between nucleosome units (Luger et al., 1997; Li and Reinberg, 2011).

Histones have long amino-terminal (N-term) tails protruding from the nucleosome, that are post-translationally modified at several places. Modifications of the tails include acetylation, phosphorylation, methylation, ubiquitination, sumoylation, and ADP-ribosylation. These modifications can usually be read by other proteins (*e.g.*, chromatin-associated proteins, transcription factors, regulatory proteins) and act in diverse biological processes such as gene regulation, DNA replication, DNA damage repair and chromosome condensation (Strahl et al., 2000; Jenuwein et al., 2001; Bannister et al., 2011).

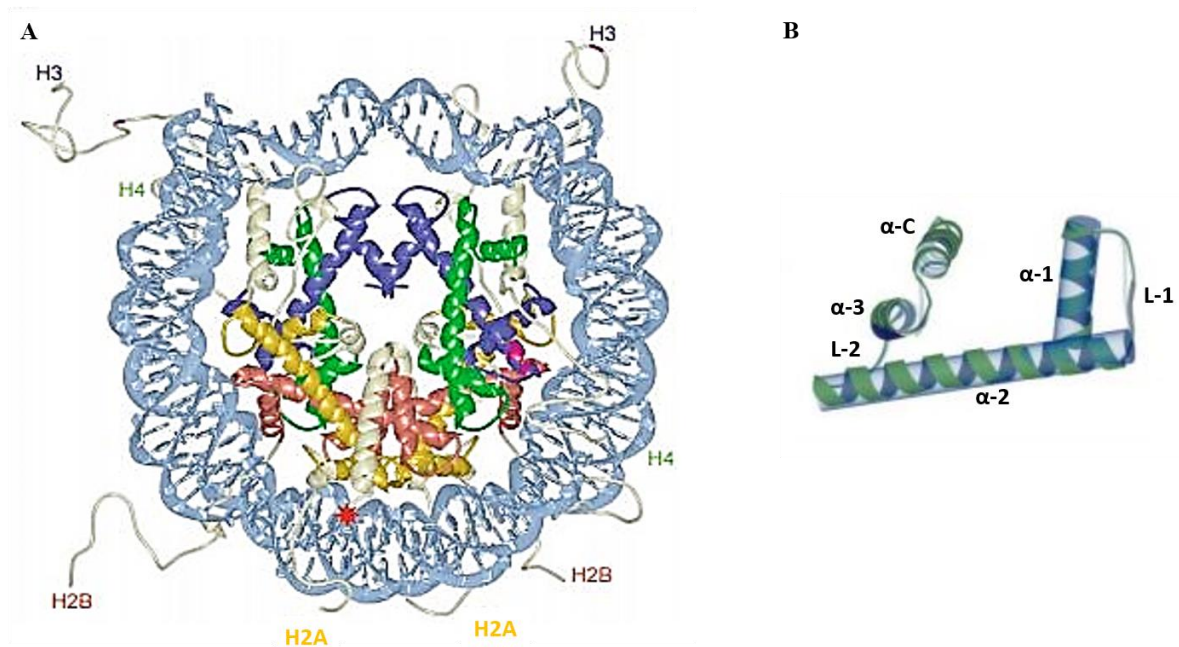


Figure 8. Nucleosome and histone H2B structure.

(A) The histone fold domains of H2A, H2B, H3, and H4 are colored in yellow, red, blue, and green respectively; histone tails and extensions are shown in white; DNA is shown in light blue (modified by Luger, 2003). (B) Histone H2B domain ($\alpha 1$, $\alpha 2$, $\alpha 3$ and αC connected by two loop L-1 and L-2) are shown.

Histone H2B is a small, highly basic protein component of the nucleosome core. The structure of histone H2B (126 aa) is characterized by a structured conserved domain, histone fold (HF), and a flexible non-structured tail at the N-term protruding outward from the nucleosome.

The HF is directly responsible for the formation of the octamer and DNA contacts and is formed by three α -helices ($\alpha 1$, $\alpha 2$, and $\alpha 3$) connected by two short loops (L1 and L2). In addition to the helices of the HF domain, histone H2B is characterized by a C-term helix (αC) extremely well ordered (**Fig. 8B**). This helix is exposed on the surface of the histone octamer and plays an important role in defining the surface on the nucleosome core particle (White et al., 2001). The flexible N-term tail is exposed also on the nucleosome surface, protrudes away from the DNA, and is an acceptor for a variety of posttranslational modifications (Luger et al., 1997; Harp et al., 2000; Luger, 2001).

Among different modifications, it has been shown that phosphorylation of the N-term tail of histone H2B at serine 14 (H2B-S14^P) by caspase-3-activated mammalian sterile twenty kinase 1 (Mst1) specifically correlates with the onset of apoptosis (Cheung et al., 2003). Ahn and colleagues extended these results to yeast by demonstrating that Sterile twenty kinase, a yeast orthologue of Mst1, directly phosphorylates H2B at serine 10 (H2B-S10^P) in a hydrogen peroxide-induced cell death pathway (Ahn et al., 2005a). Phosphorylation of histone H2B at S14 in human and S10 in yeast has been also linked to chromatin condensation during meiosis (Ahn et al., 2005b). Furthermore, H2B phosphorylation on the H2B N-term tail, but not other histone tails, were shown to be essential for chromatin condensation in a *Xenopus* cell-free system (de la Barre et al., 2001).

In mammals, histone genes represent an unusual family of genes. Indeed, there are different functional copies of the genes for each of the four core histone proteins. The metazoan core histone genes are clustered together in the genome. There are two major histone gene clusters on chromosome 6p21–p22 (*HIST1*) and 1q21 (*HIST2*) and one minor cluster on 1q42 (*HIST3*). Each of these genes has a different nucleic acid sequence and produces different histone variants, which share aa sequence homology and core structural similarity to a specific class of major histones so-called canonical. Indeed, histone variants shown only a few aa substitutions (Marzluff et al., 2002). There are 21 histone *H2B* genes reported for human that produce 16 histone variants (Mariño-Ramírez et al., 2011; Khare et al., 2011). Among different H2B variants, it was found that both variants H2B.A (also called TH2B) (Zalensky et al., 2002) and H2B.F-WT (Churikov et al., 2004) are involved in a process that mediates chromatin compaction during spermatogenesis (*e.g.*, when spermatogonia are initially converted into spermatocytes and subsequently into spermatids through meiosis). To date, it is unclear the role of the other histone variants and whether their expression varies between tissues, cell types, or different physiological conditions. Recently, it has been shown that particular H2B variants are expressed at different levels induced under

specific cellular circumstances, but further studies are necessary to elucidate their functions (Kari et al., 2013).

During S-phase, a large amount of histones must be rapidly synthesized, about 10^8 molecules of each core histone protein. Histone mRNA levels increase 35-fold as cells enter S-phase and decrease again at the end of the S-phase (Olsey, 1991). Studies on histone-exchange and -deposition kinetic revealed that H2B is the most rapidly and abundantly exchanged among the core histones (Kimura and Cook, 2001). Histone H2B transits in and out of the nucleosome more rapidly than other core histones, such as H3, H4, and H2A. Thus, about 3% of the total amount of H2B is exchanged within 6 min, $\approx$ 40% within 130 min, and $\approx$ 50% by 8.5 hours (Kimura, 2005).

In addition to chromatin-related functions, a few other extrachromosomal localization and activities of specific histones have been described. Extrachromosomal H2B was shown to be involved in the antiviral innate immune responses (Kobiyama et al., 2010). In apoptotic conditions, histone H1.2 is discharged from the nucleus into the cytoplasm and induces cytochrome C release from mitochondria (Konishi et al., 2003). Phosphorylated histone H1 has also been found in the cytoplasm of M phase HeLa cells (Bleher and Martin, 1999), while H3-S10^P has been shown to co-stain with Aurora B in mitosis and cytokinesis (Song et al., 2007).

3 H2B and HIPK2 localize at the midbody: a new extrachromosomal activity of histone H2B

Recently in our laboratory, Rinaldo and colleagues have discovered a new extrachromosomal localization and activity of histone H2B. They found that both H2B and Homeodomain interacting protein kinase 2 (HIPK2) localize at the midbody, the intercellular bridge connecting two daughter cells during cytokinesis (**Fig. 9**). At the midbody, phosphorylation of histone H2B at serine 14, mediated by HIPK2 is essential for faithful cytokinesis (Rinaldo et al., 2012).

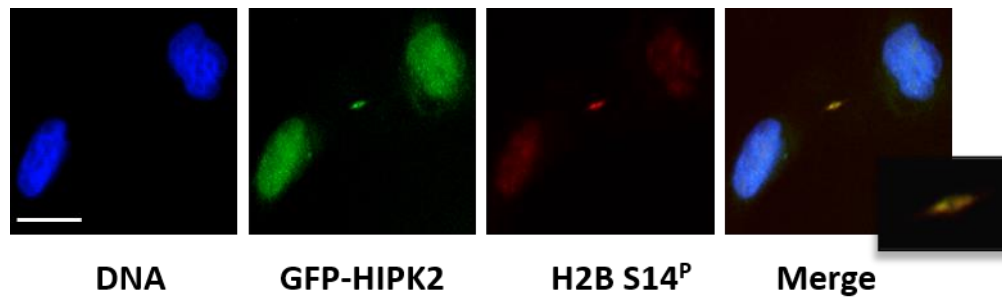


Figure 9. Localization of EGFP-HIPK2 and H2B-S14^P in HeLa Cells.

EGFP-HIPK2 (green) and histone H2B-S14^P (red) localize at the intercellular bridge. DNA is marked with DAPI (blue) (modified by Rinaldo et al., 2012).

Serine/threonine kinase HIPK2 plays important roles in the regulation of proliferation and apoptosis during development and in cell response to DNA damage (reviewed in Rinaldo et al., 2007; D’Orazi et al., 2012). This kinase can either interact with homeobox proteins by acting as transcriptional corepressors or with other types of transcription factors by acting as coactivators or corepressors, depending on the promoters or the cellular context. Indeed, HIPK2 was shown to phosphorylate itself *in vitro* and binds and phosphorylates a still enlarging body of targets, including transcriptional regulators (D’Orazi et al., 2002; Hofmann et al., 2002; Zhang et al., 2003; Wiggins et al., 2004; Wee et al., 2008; Hikasa et al., 2010), chromatin modifiers (Zhang and Wang 2007; Bracaglia et al., 2009), signal transducers (Kanei-Ishii et al., 2004; Ritterhoff et al., 2010), and E3 components of SUMO ligases (Roscic et al., 2006; Swarup and Verheyen, 2011). In DDR, HIPK2 exerts its effects by binding and phosphorylating an increasing array of transcription factors and coregulators. The p53 tumor suppressor is among the first non-homeotic transcription factors identified as HIPK2 target. HIPK2 activity enhances the p53-mediated transcriptional activation of proapoptotic factor such as PIG3, BAX, and NOXA, as well as the repression of the anti-apoptotic factor Galectin-3 (D’Orazi et al., 2002; Hofmann et al., 2002; Di Stefano et al., 2004b; Cecchinelli et al., 2006a). In the presence of DNA damage induced by UV irradiation or antineoplastic treatments (*e.g.*, doxorubicin or cisplatin), HIPK2 specifically phosphorylates human p53 at Ser46 and mouse p53 at Ser58 and this kinase activity is required for the induction of apoptosis (D’Orazi et al., 2002; Hofmann et al., 2002; Moller et al., 2003; Di Stefano et al., 2004b; Cecchinelli et al., 2006b). In addition, HIPK2 can promote apoptosis by targeting factors, such as the CtBP transcriptional co-repressor (Zhang et al., 2003), and by modulating the activity of other proteins directly or indirectly related to

apoptosis, such as the p53 family members p73 and p63 (Kim et al., 2002; Lazzari et al., 2011) and the p53 inhibitor MDM2 (Wang et al., 2001; Di Stefano et al., 2004 a).

In contrast to their association with DNA as kinase involved in DDR and as chromatin structural component, respectively, both HIPK2 and H2B localize at the midbody independently from the presence of chromosome bridges, DDR and/or apoptosis. The completion of cytokinesis by abscission needs to await complete clearance of chromatin from the cleavage plane. Several defects such as dysfunctional telomeres, DNA double strand breaks (DSBs), misregulated chromosome segregation, cohesion, or decatenation can result in the persistence of chromosomal bridges that are visualized by the presence of nucleosomal histones (Steigemann et al., 2009 and references therein). Interestingly, histone H2B localizes at the spontaneous chromosome bridges of HeLa cells, but it was not phosphorylated at S14 and was not immuno-stained for HIPK2. In contrast, all the apparently normal telophases have both H2B-S14^P and HIPK2 colocalized with midbody markers, indicating an extrachromosomal function for the modified histone (Rinaldo et al., 2012).

Furthermore, cytokinetic-specific localization of H2B is independent of HIPK2 but the absence of the kinase results in loss of H2B-S14 phosphorylation, prevention of cell cleavage and accumulation of tetra- and poly-ploid cells that contribute to tumorigenicity (Rinaldo et al., 2012; Valente et al., 2015). Of relevance, the sole expression of a phosphomimetic H2B-S14D mutant in HIPK2-null cells is sufficient to overcome cytokinesis defects and restores cell division and proliferation without any induction of apoptosis (Rinaldo et al., 2012).

These data show that HIPK2 controls cytokinesis through extrachromosomal H2B-S14 phosphorylation, but the mechanisms through which H2B contributes to cell division is still unknown.

AIM

Cytokinesis is a complex process that requires the interplay of many components and regulatory factors. Its failure is known to lead to genetically unstable states, such as tetra- and poly-ploidization, considered a critical step in tumourigenesis. Histones are assembled into nucleosomes, the basic units of DNA packaging in eukaryotes, as core (H3, H4, H2A, and H2B) and linker (H1) histones. Histones have N-terminal tails that protrude from DNA and their post-translational modifications regulate chromatin structure. In addition to chromatin-related functions, a few extrachromosomal activities of specific histones have been described. Recently, our laboratory demonstrated that histone H2B localizes at the midbody, the intercellular bridge connecting two daughter cells during cytokinesis, in a DNA-independent manner. At the midbody, phosphorylation of H2B at serine 14 is mediated by HIPK2, a kinase involved in DNA damage response and development, whose activity is required for cytokinesis and prevention of tetraploidization. In HIPK2-defective cells, expression of a phosphomimetic H2B-S14D mutant is able to rescue cytokinesis defects, cell division, and proliferation, suggesting that H2B phosphorylation at serine 14 is required for cell division.

This unexpected finding uncovers a new role of extrachromosomal histone H2B in cell division and prevention of tetraploidization. However, the mechanism through which H2B contributes to cytokinesis is still unknown.

The aim of this work is to dissect the extrachromosomal functions and activity(ies) of histone H2B in cytokinesis. This will have clear implications for the understanding of cell division and of its failure as promoter of tetra- and poly-ploidization, conditions that can generate chromosomal instability.

RESULTS

H2B LOCALIZATION DURING MITOSIS

Histone H2B and its phosphorylated form at serine 14 (H2B-S14^P) were previously reported to localize at the midbody, the intercellular bridge that connects two daughter cells in the last step of cell division. In addition, both H2B and H2B-S14^P co-localize with well-characterized midbody markers, such as α - and β -tubulin and Aurora B kinase. The midbody localization of both H2B and H2B-S14^P has been confirmed by Western blot analysis of midbodies isolated and extracted from HeLa cells enriched in telophase (Rinaldo et al., 2012).

At the onset of this study, in search of the role of extrachromosomal H2B, we asked if localization of H2B is only restricted to the midbody. The subcellular distribution of histone was re-examined using a new rabbit monoclonal antibody that detects the H2B phosphorylated at S14. Phosphorylated histone H2B-S14 has been linked to DNA damage response (DDR) and chromatin condensation in apoptosis (Cheung et al., 2003; Ahn et al., 2005). Thus, we first evaluated the specificity of the new anti-H2B-S14^P antibody (Ab) by testing its capability to detect apoptotic chromatin in ultraviolet (UV)-irradiated HeLa cells. Immunofluorescence (IF) analysis revealed that anti-H2B-S14^P Ab recognize the apoptotic chromatin, as expected (**Fig. 10**).

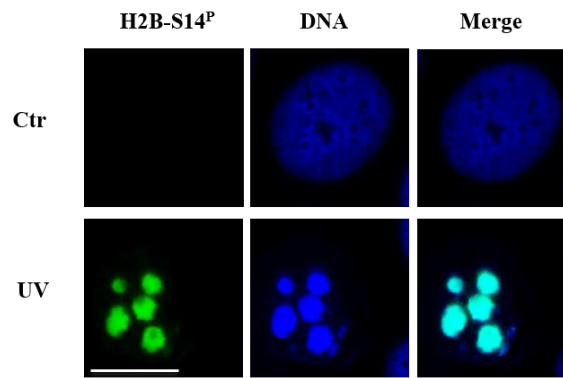


Figure 10. H2B Antibody Specificity.

The anti-H2B Ab specificity was demonstrated in UV-irradiated HeLa cells. Immunostaining of HeLa cells was performed in the untreated (upper panels) or the irradiated (lower panels) with the anti-H2B-S14^P Ab (green). DNA was stained with Hoechst (blue). Scale bar is 10 μ m.

Next, we analyzed H2B-S14^P localization during mitosis. IF analysis showed uncharacterized areas of localization of H2B-S14^P. During **metaphase**, H2B-S14^P forms unclear aggregates that do not co-localize with kinetochores, as assessed by antibody anti-CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia), a kinetochore marker (Williams et al., 1984) (data not show). In **anaphase**, H2B-S14^P accumulates at the central spindle, remains associated with the spindle midzone during cleavage furrow contraction and thereafter accumulates at the midbody, in the flanking zone (**Fig. 11A**). Interestingly, in **late telophase**, H2B-S14^P shows an asymmetric distribution, with a partially re-localization at the secondary ingression site, where acute membrane constriction and abscission occur (**Fig. 11B**).

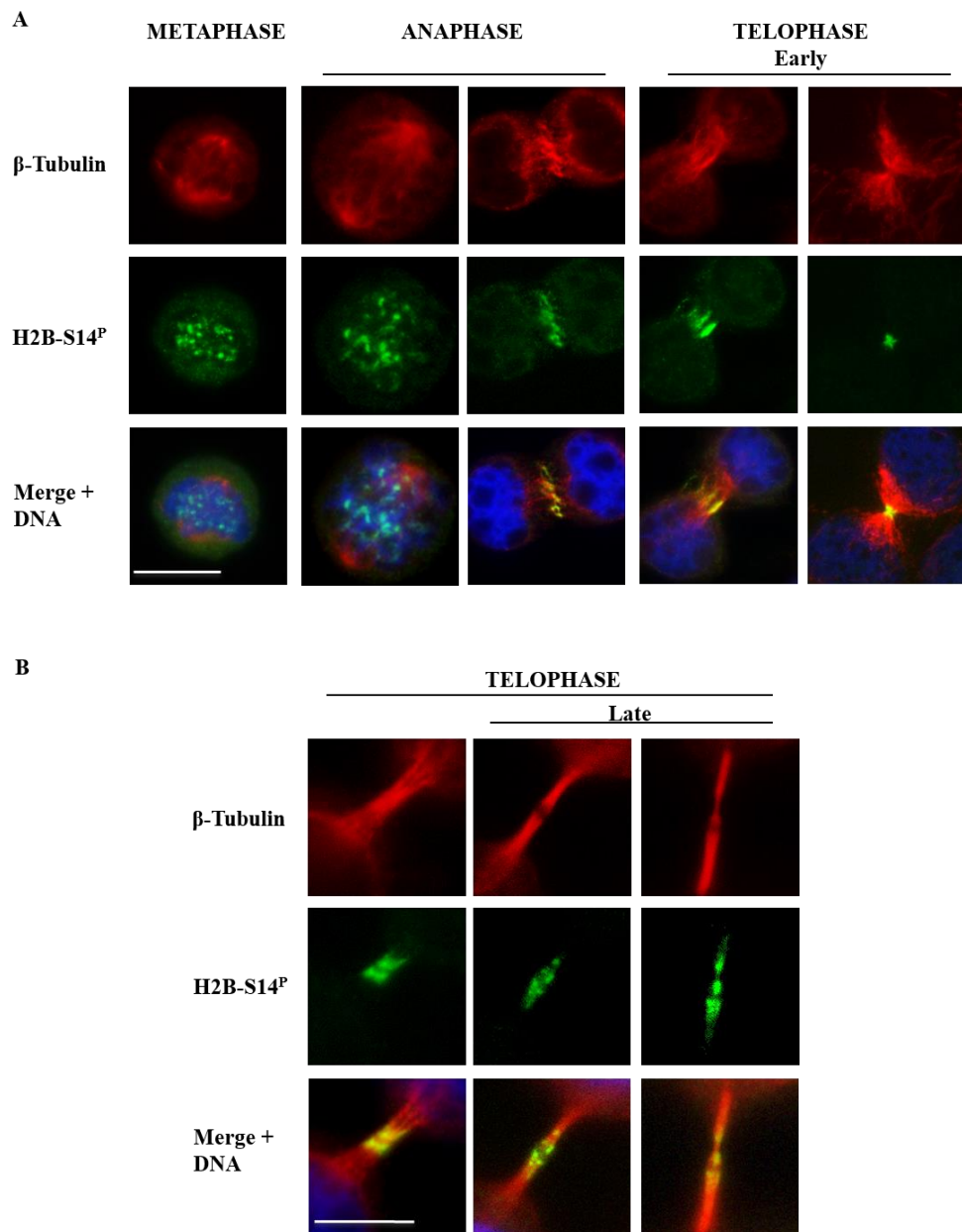


Figure 11. Localization of endogenous histone H2B-S14^P.

(A) Localization of H2B-S14^P nuclear aggregates in metaphase, central-spindle in anaphase, flanking zone at the midbody in telophase. Scale bar is 10 μ m. (B) asymmetric localization of histone H2B-S14^P in late telophase. Scale bar is 5 μ m. Microtubules were marked with anti- β -tubulin Ab (red); H2B was marked with anti-H2B-S14^P Ab (green), DNA was marked with Hoechst (Blue).

The H2B-S14^P localization at the midbody was confirmed by transmission electron microscopy (TEM) analysis of telophase-enriched HeLa cells. Positive controls were obtained analyzing damaged HeLa cells characterized of apoptotic chromatin. Immunogold labeled anti-H2B-S14^P Ab revealed that phosphorylated form of histone H2B at S14 localizes at the

midbody associated with vesicle-like structures at one arm of the midbody, the so-called flanking zone, and with microtubule (**Fig. 12**) at the opposite arm. This latter aspect may explain to the asymmetric distribution of H2B-S14^P showed by IF.

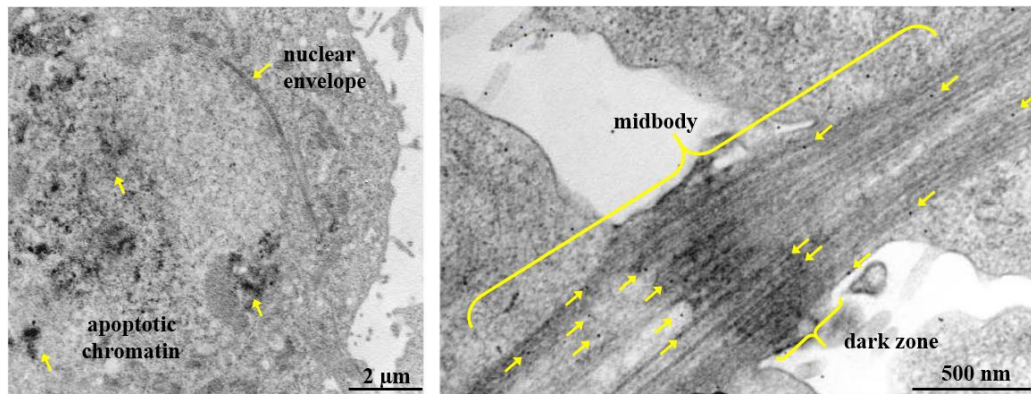


Figure 12. TEM, localization of endogenous histone H2B-S14P in telophase.

(Left panel) positive Ctr: anti-H2B-S14^P Ab gold-conjugated marks apoptotic chromatin. Scale bar is 2 μm; (right panel) asymmetric localization of histone H2B-S14^P in telophase, associated with different sub-structures at the opposite site of the midbody. Scale bar is 500 nm.

Taken together, these results show that histone H2B-S14^P localizes in specific areas in different steps of cell division, from metaphase to telophase. Furthermore, in late telophase, at the abscission onset, H2B-S14^P show an asymmetric distribution probably due to a specific sub-structural localization.

CYTOKINESIS-SPECIFIC LOCALIZATION OF HISTONE H2B IS INDEPENDENT OF RNA

Different types of DNA and chromosomal dysfunctions can result in chromatin localization at the cleavage plane, the so-called chromosome bridges (Steigemann et al., 2009). Because of the role of histone H2B as structural component of chromatin, Rinaldo and colleagues have first evaluated the relationship between localization of histone H2B at the midbody and the presence of DNA. They found that the cytokinetic specific localization of histone H2B is independent of chromosome bridges (Rinaldo et al., 2012). Since DNA is not involved in

H2B midbody localization, we asked whether RNA might be responsible from the H2B cytokinetic localization. This hypothesis was also stimulated by recent observation that DDR focus formation requires structural support of site-specific DICER and DROSHA RNA products (Francia et al., 2012). Thus, to assess whether RNA contributes to H2B midbody localization and/or activity, mitosis-enriched HeLa cells were permeabilized with different percentages of TWEEN 20 and treated with ribonuclease A (RNase A) that catalyzes the degradation of RNA; PBS was used as negative control (**Fig 13A**). Positive control was obtained by assessing loss of DDR-induced 53BP1 foci in RNase A treated HeLa cells after γ -irradiation (**Fig. 14**), as previously reported (Francia et al., 2012). After treatments, we analyzed H2B localization by IF (**Fig. 13B**) and both treated and control cells showed H2B at the midbody indicating that H2B midbody localization is independent of single-strand RNA (ssRNA).

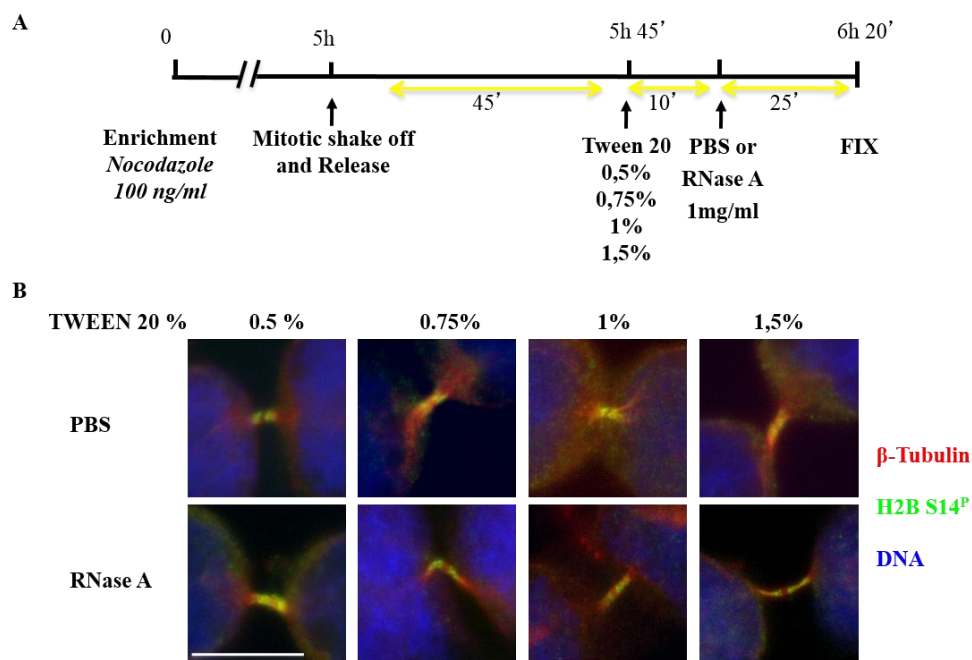


Figure 13. H2B-S14^P localization in RNase A-treated HeLa Cells.

(A) Schematic representation of both mitosis-enrichment and RNase A treatment protocols. (B) Localization of H2B-S14^P in telophase was assessed in HeLa cells treated with PBS or RNase A 1mg/ml. Microtubules were marked with anti- β -tubulin Ab (red); H2B was marked with anti-H2B-S14^PAb (green), DNA was marked with Hoechst (Blue). Scale bar is 10 μ m.

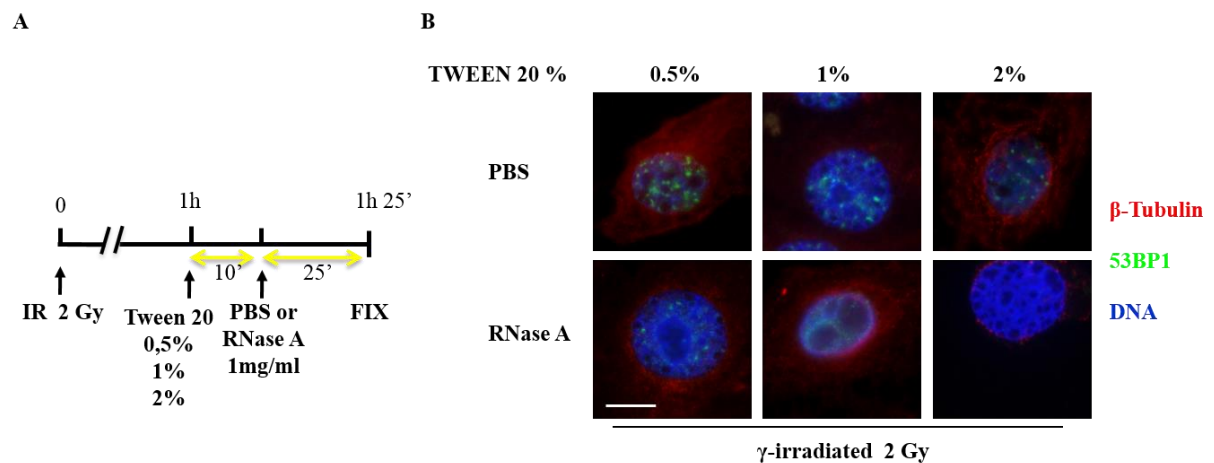


Figure 14. 53BP1 localization in RNase A-treated HeLa Cells.

(A) Schematic representation of irradiation and RNase A treatment protocols; (B) localization of 53BP1 (green) at DDR foci was assessed in irradiated HeLa cells treated with PBS or RNase A 1mg/ml. Microtubules were marked with anti- β -tubulin Ab (red) and DNA was marked with Hoechst (Blue). Scale bar is 10 μ m.

Because of the disappearance of histone H2B localization in early stages of cytokinesis already in control cells, it was not possible to analyze the relationship between RNA and H2B localization in metaphase or at the contractile ring in anaphase (**Fig. 15**).

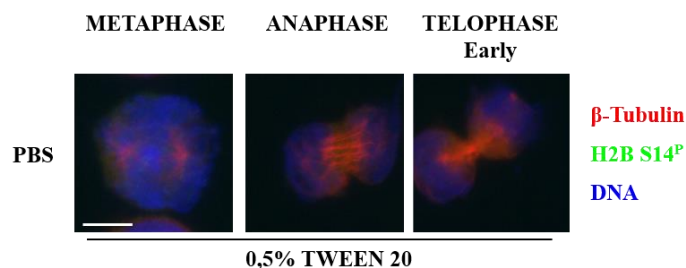


Figure 15. H2B-S14P localization in RNase A-treated HeLa Cells.

H2B-S14P localization disappears in early stages of cytokinesis. Microtubules were marked with anti- β -tubulin Ab (red); H2B was marked with anti-H2B-S14P Ab (green), DNA was marked with Hoechst (Blue). Scale bar is 10 μ m.

To further analyze the relationship between nucleic acids and midbody localization of H2B, we evaluated the presence of histone H2B at the midbody in mitotic enriched HeLa cells. First, we confirmed that localization of H2B at the midbody is independent of DNA (Rinaldo et al., 2012) by using DNase I. As expected, midbody localization of H2B was

revealed both in control and treated cells. Next, we treated the mitotic-enriched HeLa cells with RNase H and RNase III, which respectively bind and cleave hybrid DNA/RNA and double-strand RNA (dsRNA). The cytokinetic specific localization of histone H2B was not affected after degradation of both hybrid DNA/RNA and dsRNA (**Fig. 16**).

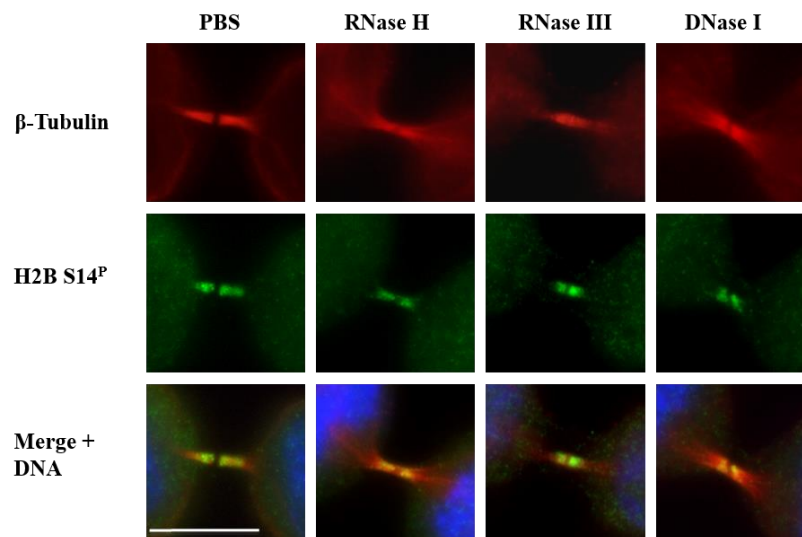


Figure 16. H2B-S14^P localization in RNase H/III and DNase I treated HeLa Cells.

Localization of H2B-S14^P in telophase was assessed in HeLa cells treated with PBS or RNase H/ RNase III/ DNase I. Microtubules were marked with anti- β -tubulin Ab (red); H2B was marked with anti-H2B-S14^PAb (green), DNA was marked with Hoechst (Blue). Scale bar is 10 μ m.

Eventually, we studied the role of non-coding RNA by using HCT116 cells wild-type or knockout for DICER, an enzyme that catalyzes the cleavage of non-coding RNA involved in gene silencing (Manni et al., 2009; Cummins et al., 2006). No defect in H2B localization was observed in DICER-knockout HCT116 cells respect to the wild-type counterparts (**Fig 17**).

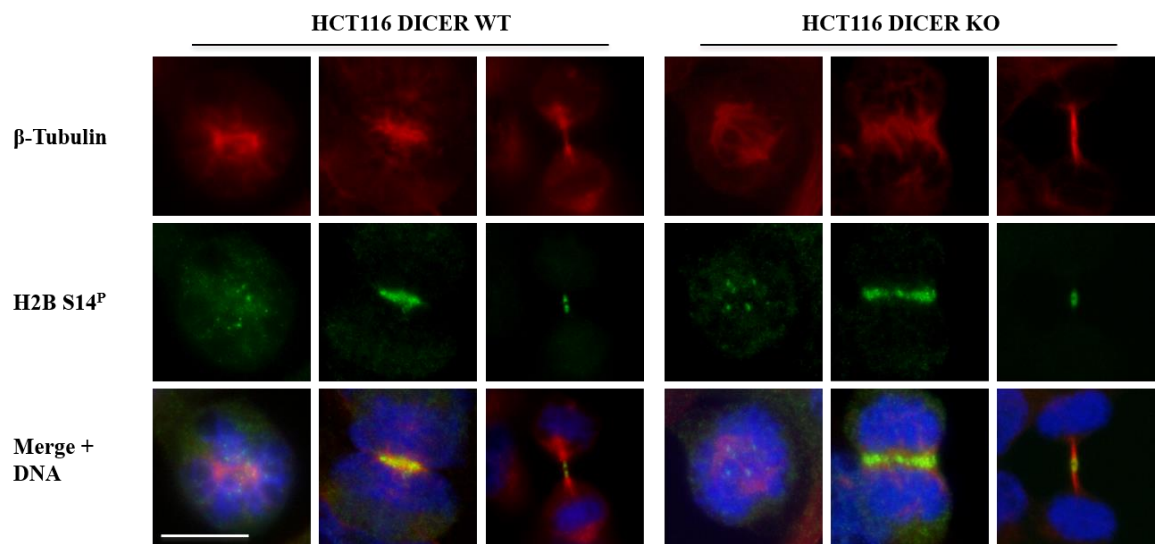


Figure 17. Localization of endogenous H2B-S14^P in DICER WT and DICER KO HCT116 cells.

Localization of H2B-S14^P in HCT116 DICER WT and KO: nuclear aggregates in metaphase, central-spindle in anaphase, flanking zone at the midbody in telophase. Microtubules were marked with anti- β -tubulin Ab (red); H2B was marked with anti-H2B-S14^PAb (green), DNA was marked with Hoechst (Blue). Scale bar is 10 μ m.

H2B DEPLETION CAUSES ABSCISSION DEFECTS AND DELAY

To study the activity of extrachromosomal histone H2B in cytokinesis, HeLa cells were depleted for histone H2B. As constitutive component of chromatin, during S-phase it must rapidly synthesize large amounts of histones, approximately 10^8 molecules of each core histone protein. Histone mRNA levels increased 35-fold as cells enter S-phase and decreasing again at the end of S-phase (Olsey, 1991). Thus, first of all, we have optimized a protocol that ensures cell viability and, at the same time, an efficient depletion of extrachromosomal histone H2B. We have interfered histone H2B expression in HeLa cells by transfecting commercially available H2B short interfering RNAs (siRNA). There are 21 histone H2B genes reported for human that produced 16 histone variants (Mariño-Ramírez et al., 2011; Khare et al., 2011). We have tested combination of 9 H2B variant-specific siRNAs (*i.e.*, H2B.B; H2B.D; H2B.E; H2B.G; H2B.K; H2B.H; H2B.I; H2B.J; H2B.2E) at different concentrations and times and in HeLa cells seeded at different confluence. Control depletion was obtained by universal negative control (Ctri) sequences. Among a variety of protocol (data not shown), because of the high transcription rate of histones and the requirement to maintain nucleosome integrity to allow cell division, two pulses of siRNA transfection, the

second 24 hours from the first one, was needed to obtain a successful depletion of extranucleosomal H2B (**Fig. 17A**) without affecting cell viability. HeLa cells were depleted with a double-pulse of a combination of H2B variant-specific siRNAs (H2Bi) and their related Ctri and analyzed by IF to assess cytokinesis failure. Compared to Ctri, depletion of H2B results in the accumulation of cytokinetic defects, such as prevention of cell cleavage, abscission defects, and accumulation of LIBs. These latter structures are long and often convoluted and resemble elongated midbodies. We also observe tubulin disorganization presumably linked to LIBs formation and the stretching conditions triggered by abscission defects (**Fig. 17B**).

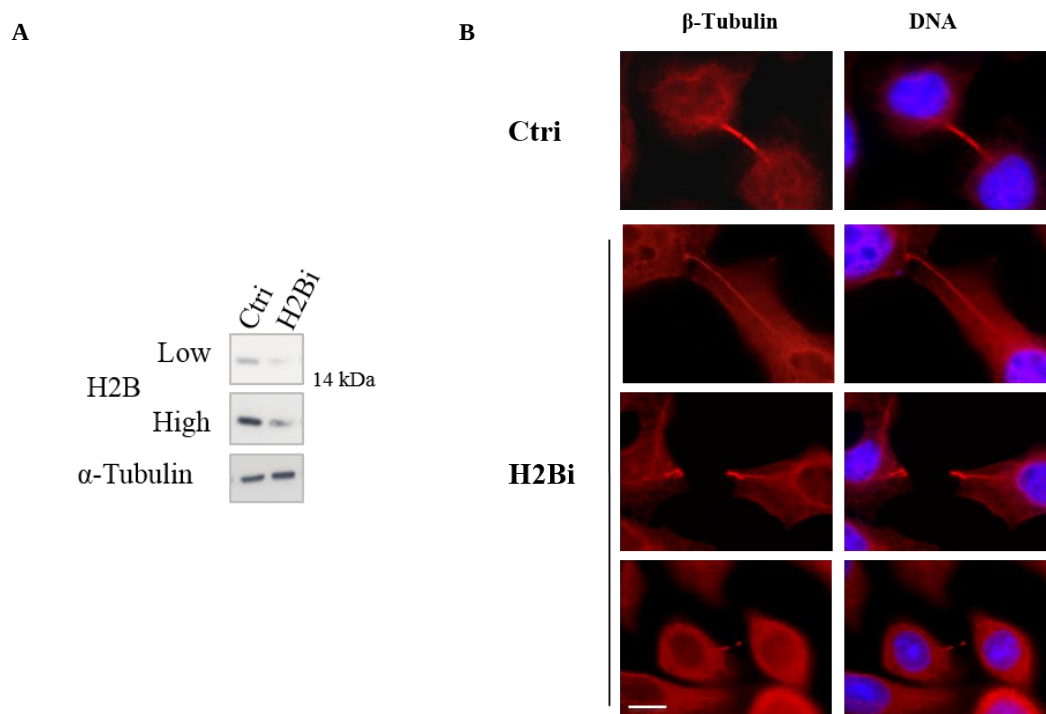


Figure 17. H2B depletion in HeLa cells result in tubulin disorganization.

HeLa cells were depleted for histone H2B with a combination of 9 variant-specific siRNAs (H2Bi) or with universal negative control (Ctri). (A) A representative WB for the indicated proteins is shown. (B) Representative immunofluorescence images of Ctri and H2Bi HeLa cells stained with anti- β -Tubulin Ab (red) and Hoechst (blue). The presence of LIBs (H2Bi top panels), abscission defects (H2Bi middle panels) and tubulin disorganization (H2Bi bottom panels) is detectable in the H2Bi cells compared to the Ctri. Scale bar is 10 μ m.

Microscopy analysis of fixed asynchronous HeLa cells (n=1,000) after H2B depletion reveal that about $34 \pm 3.5\%$ of telophases are aberrant in H2Bi cells compared to

Ctri (11 ± 3.5 %). Depletion of H2B also results in accumulation of cells that are in the midbody stage. Indeed, in asynchronous HeLa cells H2Bi the percentage of cells at the end of cytokinesis is about $15 \pm 2.2\%$ dissimilarity to the $9 \pm 1.6\%$ of telophases detectable in relative Ctri. In contrast, no defects are observed in accumulation of bi- and multi-nucleated cells nor increase in the number of chromosomal bridges (*i.e.*, below 10% in both H2Bi and Ctri HeLa cells). We detected only a slight increase in multinucleated cells in HeLa H2Bi (3.4 ± 0.7 %) in respect to their Ctri (2.8 ± 1.2 %) (**Fig. 18A, B and C**). Furthermore, the aberrant midbodies (n=100) are negative for staining with both anti-H2B Ab (**Fig. 18D**) and anti-H2B S14^P Ab (**Fig. 18E**).

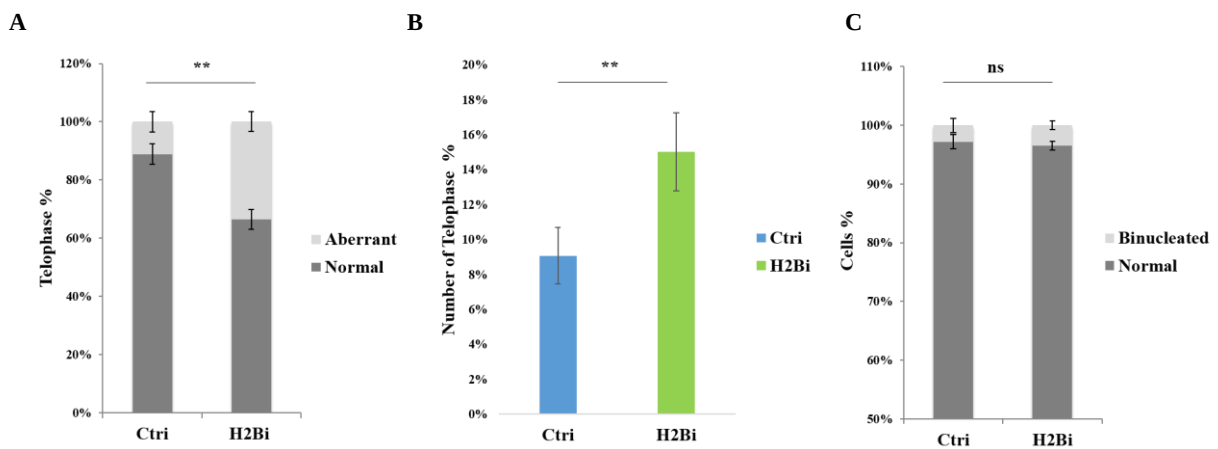


Figure 18A-C. H2B depletion in HeLa cells results in abscission defects.

HeLa cells were depleted for histone H2B with a combination of 9 variant-specific siRNAs (H2Bi) or with universal negative control (Ctri). Cells were stained with anti-H2B Ab or anti-H2B-S14^P (green), Hoechst (blue), and anti- β -tubulin Ab (red) to identify the cytoplasm in interphase and the midbodies or the LIBs in telophase. About 1,000 cells per sample were scored for (A) the presence aberrant telophases, (B) the number of cells in telophase, and (C) the presence of one or two nuclei/cell; data are represented as mean $\pm$ SD (** P-value < 0.01; *** P-value < 0.001; ns P-value > 0.05 by Student *t* test).

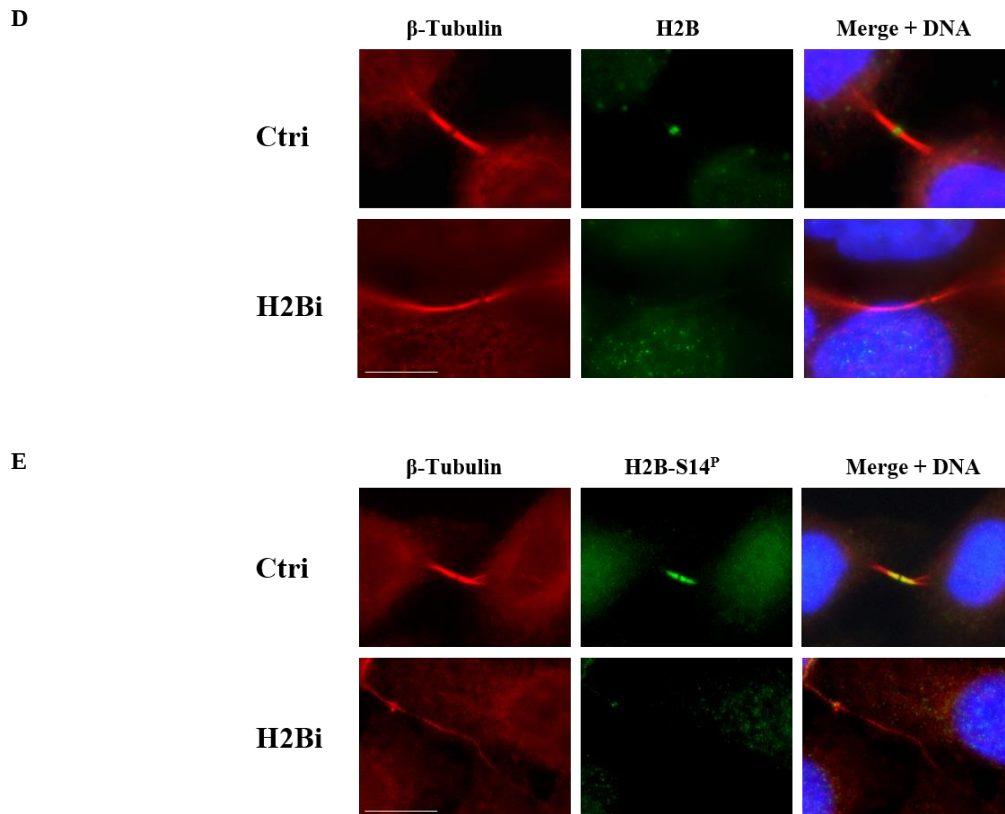


Figure 18D, E. H2B depletion in HeLa cells results in abscission defects.

HeLa cells treated and stained as above were analyzed for cytokinesis defects. Representative images of H2B (D) or H2B-S14^P (E) at the midbody in Ctri and H2Bi HeLa cells. Scale bar is 10 μ m.

Next, to elucidate the cytokinetic function of histone H2B, we analyzed asynchronous HeLa cells H2Bi and Ctri by time-lapse live-cell imaging for 24 hours, starting after the second pulse of siRNA (**Fig. 19A**). We evaluated the cytokinesis time by analyzing the timing from the round up, when cells became rounded after nuclear envelope break down, to cleavage ingression, the easiest recognizable phase of cytokinesis. We also analyzed the timing from cleavage furrow ingression to abscission, when the two daughter cells are physically separated, the so-called abscission time. The timing from the round up to cleavage furrow ingression is about 40 minutes for both H2Bi (n=100) and Ctri (n=150) HeLa cells (**Fig. 19B**). Furthermore, in Ctri cells (n=80), the midbody extend from each daughter cell into the intercellular bridge and abruptly break down approximately 2 hours after cleavage furrow ingression. In contrast, in H2B-depleted cells, this abscission event is severely delayed with the abscission time of H2Bi HeLa cells (n=100) being at least 1 hour longer than their related controls (**Fig. 19C**). Among the persistent intercellular bridges, some become progressively thinner and persist for many hours, often becoming overextended and resembling the LIBs we

see by IF. Although abscission clearly failed in these cells, we did not see re-fusion of daughter cells, has previously observed in HIPK2 depleted HeLa cells (Rinaldo et al., 2012), confirming the observation made by IF and thus indicating that H2B depletion results only in abscission delay.

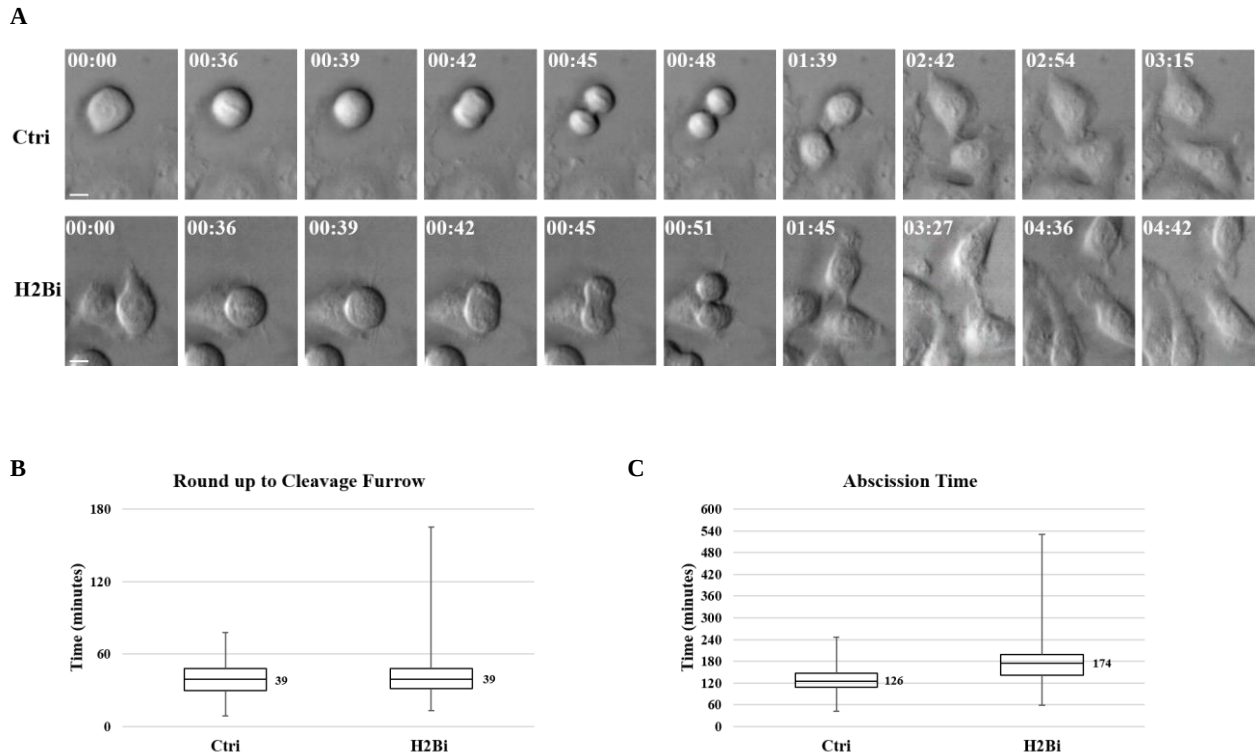


Figure 19. H2B depletion is associated with Abscission failure.

Ctrl and H2Bi HeLa cells were employed as total populations. (A) Stills from time-lapse recording of HeLa Ctrl and H2Bi cells after the second pulse of siRNAs are shown. Ctrl cells divide and return mono-nucleated in about 3 hours while H2Bi cells remain connected by intercellular bridge for several hours before abscission occurs. Timing from round up to cleavage furrow ingression (B) and from cleavage furrow ingression to abscission (abscission time) (C) are reported in box-plot graph. (B) The median of the timing from the round-up to cleavage furrow is 39 minutes both in Ctrl and H2Bi. (C) The median of the abscission time is 126 minutes and 174 minutes for Ctrl and H2Bi cells, respectively. Scale bar is 10 μ m.

Altogether, these data show that H2B depletion by siRNA induces accumulation of cytokinesis-dependent aberrations and impairs abscission that results in abscission delay. These results strongly suggest that histone H2B is required for completion of the late stage of cytokinesis.

H2B DEPLETION ALTERS THE MIDBODY LOCALIZATION OF A FEW ABSCISSION FACTORS

To begin investigating the mechanisms underlying cytokinesis activity of histone H2B, we compared the spatio-temporal localization of a series of structural and functional proteins sequentially recruited during cytokinesis to assure proper cell division. We analyzed the interplay between these proteins and H2B by analyzing their epistatic relationships in H2B-proficient (n=500) and -depleted HeLa cells (n=500). Using specific antibodies previously verified and characterized, we examined:

- 1) proteins associated with **midzone formation**, such as the CPC proteins (Aurora B and INCENP) PLK1, and PRC1;
- 2) proteins associated with **midbody formation** such as PLK1, CPCs, HIPK2, and Citron Kinase;
- 3) proteins associated with **abscission events**, such as CEP55, ALIX, CHMP4B subunit of ESCRT-III machinery, and Spastin.

In all cells analyzed at the early stages of cytokinesis, we observed that the localization patterns for all proteins examined are not affected during midzone formation and cleavage furrow ingression in the H2B depleted cells (**Fig. 20**), indicating that histone H2B is not mainly involved in the early events of cytokinesis.

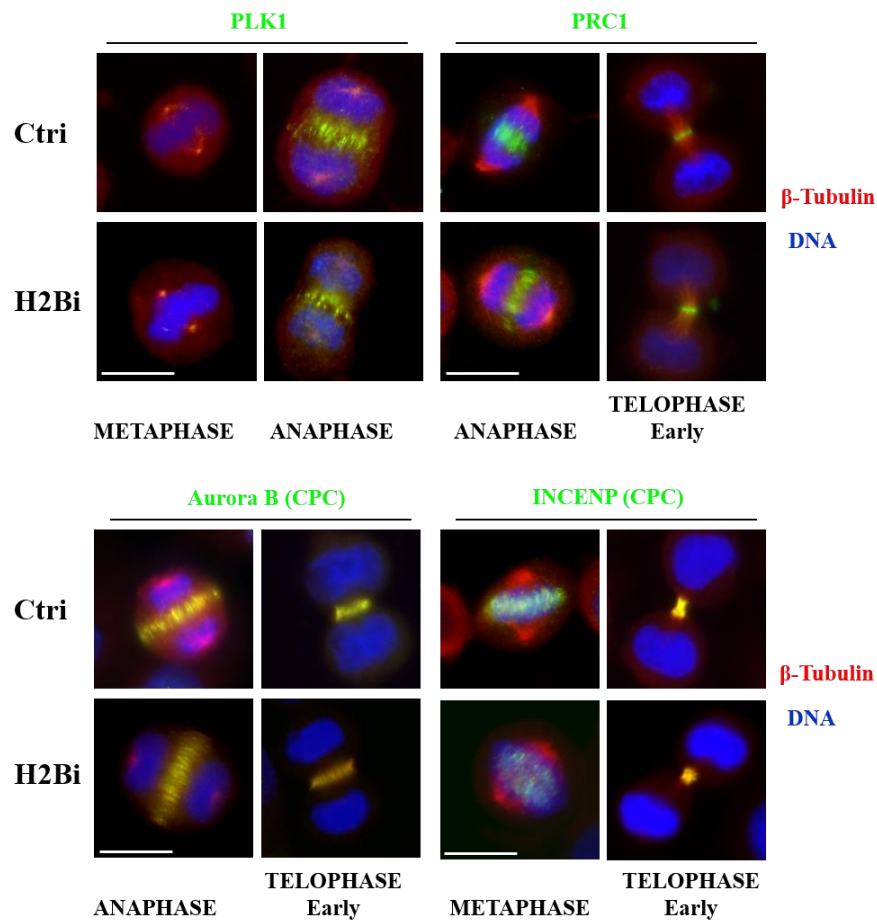


Figure 20. H2B depletion effects on the localization of proteins associated at the early stage of cytokinesis.

Localization of different proteins involved in early stages of cytokinesis was evaluated in H2Bi and Ctrl HeLa cells. Representative images of PLK1, PRC1, Aurora B, and INCENP are shown. The different proteins were marked in green; microtubules were marked with anti-β-tubulin Ab (red); DNA was marked with Hoechst (Blue). Scale bar is 10 μm.

In contrast, in the subsequent stage of midbody formation, PLK1, MKPL1, PRC1, ECT2, CPCs, HIPK2 and Citrone Kinase can be detected at the midbody but their distribution pattern is altered. In particular, MKLP1 and ECT2 appear less sharp than control cells while PLK1, PRC1, CPCs, and HIPK2 becoming dispersed along the midbody microtubules and following the aberrant tubulin structures. These distributions are in sharp contrast to their localizations at the flanking zone in the control cells. Furthermore, the midbody localization at the bulge zone of Citron kinase, a regulator for midbody formation, is not affected by the depletion of histone H2B (**Fig. 21**).

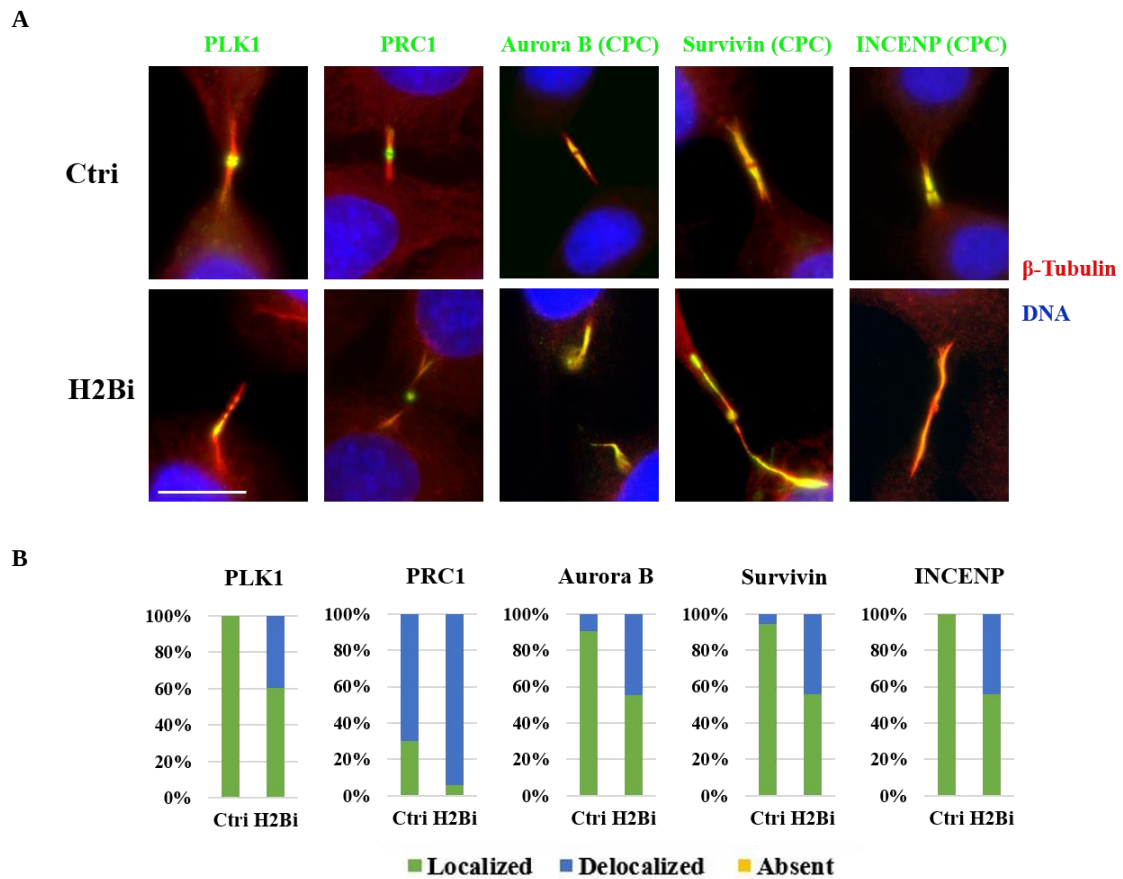
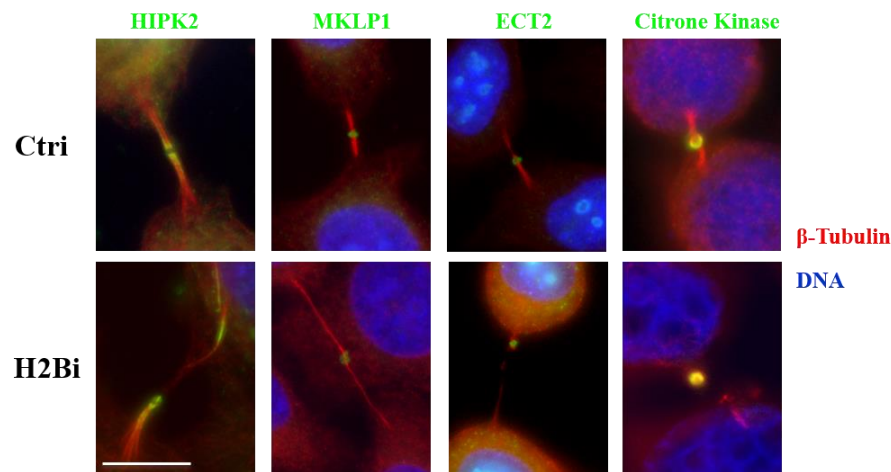


Figure 21A and B. H2B depletion effects on the localization of proteins at the midbody.

Localization of different proteins associated to the midbody was evaluated in H2Bi and Ctrl HeLa. (A) Representative images of PLK1, PRC1, Aurora B, Survivin, and INCENP are shown. (B) The percentage of protein localized, delocalized or absent are indicated in the related graphic. The different proteins were marked in green; microtubules were marked with anti- β -tubulin Ab (red); DNA was marked with Hoechst (Blue). Scale bar is 10 μ m.

C



D

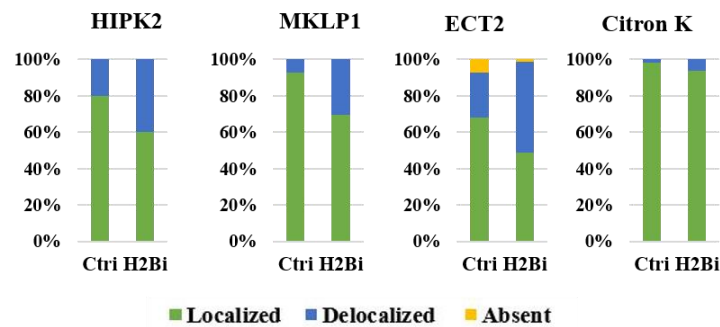


Figure 21. C and D. H2B depletion effects on the localization of proteins at the midbody.

Localization of different proteins associated to the midbody was evaluated in H2Bi and Ctri HeLa cells. (A) Representative images of HIPK2, MKLP1, ECT2, and Citron Kinase are shown. (B) The percentage of protein localized, delocalized or absent are indicated in the related graphic. The different proteins were marked in green; microtubules were marked with anti- β -tubulin Ab (red); DNA was marked with Hoechst (Blue). Scale bar is 10 μ m.

Even more interestingly, by analyzing the proteins involved in abscission, several defects of localization and organization were revealed in H2B depleted cells for the critical abscission factors ALIX, the ESCRT-III subunit CHMP4B, and Spastin, while the localization of CEP55 appeared less sharp than controls cells. Later in cytokinesis, a second and smaller pool of both CHMP4B proteins and Spastin accumulate at the abscission site, approximately 1 μ m away from the center of the midbody, where acute membrane constriction and abscission occur. This latter localization is completely missing in the H2B depleted cells (Fig. 22).

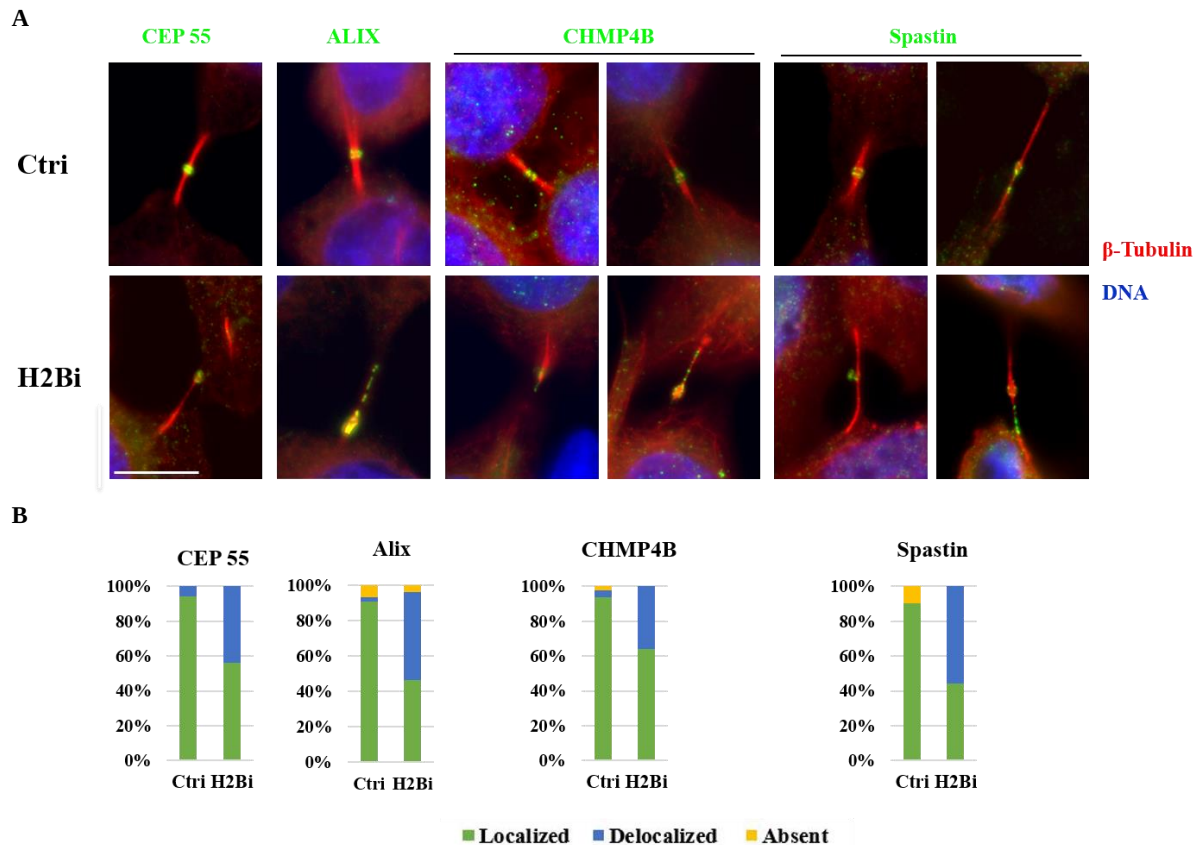


Figure 22. H2B depletion effects on the localization of proteins involved in abscission.

Localization of different proteins associated to the midbody was evaluated in H2Bi and Ctrl HeLa cells. (A) Representative images of CEP55, ALIX, CHMP4B, and Spastin are shown. (B) The percentage of protein localized, delocalized or absent are indicated in the related graphic. The different proteins were marked in green; microtubules were marked with anti- β -tubulin Ab (red); DNA was marked with Hoechst (Blue). Scale bar is 10 μ m.

Overall, these results indicate that histone H2B plays a role at the terminal stage of cytokinesis by controlling the morphology of the midbody and by organizing the localization of a few critical regulators of the abscission

H2B INTERACTS WITH THE ESCRT-III SUBUNIT CHMP4B

To get hints on the mechanisms through which H2B controls abscission, we start investigating the relationships between the ESCRT-III subunit CHMP4B and H2B. This finding was stimulated because of HIPK2 has been previously identified as a possible interacting partner of CHMP4B in a yeast two-hybrid screen (Tsang et al., 2006). This

interaction might be of interest because the ESCRT-III machinery has been proposed to mediate midbody abscission. In particular, it has been shown that, late in cytokinesis, the cortex adjacent to the midbody constricts in a secondary ingression. This secondary ingression is essential for abscission and contains membrane-associated filaments of 17 nm in diameter, forming large intertwined helices surrounding the intercellular bridge. The ESCRT-III machinery is a candidate component of the 17 nm filaments because it co-localizes with the constriction zones and is required for the formation of these filaments (Guizetti et al., 2011).

We performed *in vitro* binding experiments by testing CHMP4B-HIPK2 and CHMP4B-H2B interactions. Surprisingly, we could not confirm the interaction between HIPK2 and CHMP4B by the GST pull-down assay (data not shown), suggesting that the original binding observed by a yeast two-hybrid screening was a false-positive or might requires post-translational modifications that are not present in the GST recombinant proteins. In contrast, we found a strong H2B-CHMP4B binding that was present with different affinity both at low and high salt concentration (*i.e.*, 150mM and 450mM NaCl, respectively). Persistence of binding in high salt conditions suggests that the interaction is not only based on charged interactions between the highly basic histone H2B and the acid C-term end of CHMP4B (**Fig. 23**).

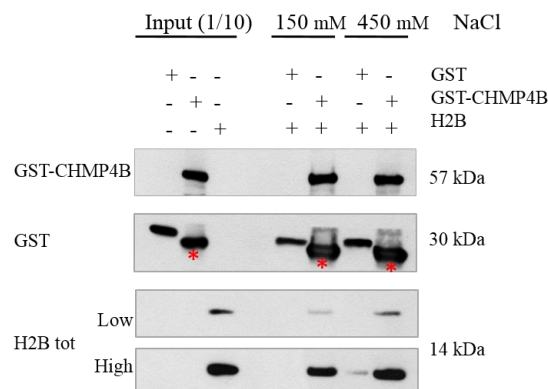


Figure 23. H2B binds CHMP4B.

GST-pull down was performed to analyze the binding between recombinant, commercially available GST-CHMP4B and H2B. GST alone was used as negative control. WBs were performed to detect the indicated proteins. Asterisks (*) indicate GST sub-products of GST-CHMP4B.

In addition, we analyzed the interaction between phosphorylated form of histone H2B and CHMP4B. To evaluate the capability of a kinase active domain of HIPK2 to phosphorylate recombinant His-tagged histone H2B, we performed kinase assay as described by Rinaldo and colleagues (Rinaldo et al., 2012) (**Fig. 24A**). Next, we performed cold kinase assay and assessed the different affinity of H2B and H2B-S14^P for binding to CHMP4B at low and high salt concentrations (**Fig. 24B**). Of relevance, we found that, in low salt concentration, interaction between H2B and CHMP4B is significantly increased after phosphorylation of histone H2B at serine 14.

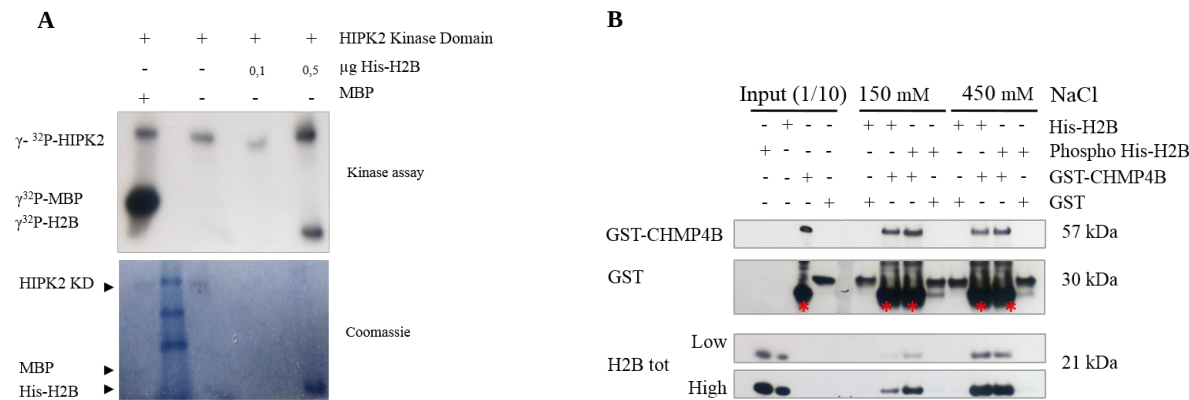


Figure 24. Phosphorylated form of H2B binds CHMP4B.

(A) HIPK2 Kinase Domain (HIPK2 KD) was incubated with recombinant His-H2B protein in the presence of [$\gamma^{32}\text{P}$]-ATP. Kinase reaction products were resolved by SDS-PAGE and analyzed by autoradiography (upper panel) and Coomassie-blue staining (lower panel). (B) Cold kinase assay was performed by using HIPK2 KD on recombinant H2B as in (A), then GST-pull down was performed to analyze the binding between recombinant, commercially available GST-CHMP4B and His-H2B or His-H2B-S14^P. GST alone was used as negative control. WBs were performed to detect the indicated proteins. Asterisks (*) indicate GST sub-products of GST-CHMP4B

Overall, these data support a model for histone H2B function during abscission, possibly through a direct interaction with CHMP4B.

DISCUSSION

A NEW SUBCELLULAR AND SUB-STRUCTURAL LOCALIZATION OF HISTONE H2B-S14^P IN CYTOKINESIS

Our study reveals an unexpected subcellular localization and biological function of histone H2B in cytokinesis. We show that histone H2B localizes in specific areas and in different steps of cell division, from metaphase to telophase. In a previous work, we have demonstrated that histone H2B and its phosphorylated form at serine 14 localizes at the midbody, the intercellular bridge connecting two daughter cells during the last step of cell division. Here, we show that in metaphase, endogenous histone H2B-S14^P forms aggregates that move to the spindle midzone in anaphase. In late anaphase, when cleavage furrow ingresses, histone H2B-S14^P remains associated at the spindle midzone, co-localizes with cleavage furrow and thereafter accumulates at the midbody, in the flanking zone. In late telophase, H2B-S14^P partially re-localizes at the secondary ingression site where acute membrane constriction and abscission occur.

Ultrastructural analysis reveals an unpredicted association between endogenous H2B-S14^P and different sub-structures at the opposite regions adjacent to the midbody. In particular, we show that H2B-S14^P localizes at the midbody in association with vesicle-like structures on one site of the flanking zone and with microtubules at the opposite site.

The midbody serves as a platform to orchestrate the cytoskeleton rearrangements, plasma membrane remodeling, and recruitment of the functional complexes needed for abscission. Recent work from several laboratories has demonstrated that dramatic changes occur in cytoskeleton and endosome dynamics, and are a prerequisite for abscission (Mierzwa and Gerlich, 2014). The intact central spindle is required at the early steps of abscission, while microtubules need to be removed from the intracellular bridge just before abscission. It has been shown that central spindle microtubules play an important role in the delivery and accumulation of secretory organelles and recycling endosomes from anaphase to the intracellular bridge (Goss et al., 2008). Vesicles from Golgi-secretory pathway and from recycling endosome accumulate at the midbody together with several vesicles targeting and

tethering factors required for abscission (Fielding et al., 2005; Gromley et al., 2005; Kouranti et al., 2006). Furthermore, electron microscopy studies showed that vesicles disappear from the intercellular bridge as cytokinesis progresses (Guizetti et al., 2011). Thus, we can speculate that in early telophase, histone H2B localizes at the midbody by a vesicle-like structures that are symmetrically distributed at both flanking zones adjacent to the midbody. At the abscission site, H2B-S14^P remains at the midbody associated with microtubule even when the vesicles disappear, indicating that H2B-S14^P is involved in the last step of cytokinesis.

HISTONE H2B LOCALIZES AT THE MIDBODY INDEPENDENTLY OF NUCLEIC ACIDS

Histones are assembled in the nucleosome as core (H2A, H2B, H3 and H4) and linker (H1) histones. In mitosis, a few site-specific phosphorylations of both linker and core histones are evolutionarily conserved and contribute to chromatin condensation and sister chromatid segregation (Harp et al., 2000). Phosphorylation of histone H2B at S14 in human and S10 in yeast has been linked to DDR and chromatin condensation in apoptosis and meiosis (Cheung et al., 2003; Ahn et al., 2005a and 2005b). All these conditions are associated with chromatin remodeling. In contrast, we have recently found that cytokinesis specific localization of H2B and its phosphorylation at S14 are independent of the presence of chromosomal DNA or apoptosis (Rinaldo et al., 2012).

Increasing number of ncRNA transcripts have been shown to contribute to relevant cellular functions by providing structural support and regulating the activity of different proteins (Clemson et al., 2009; Wutz, 2003; Orom et al., 2010). In DDR, at the sites of DNA damage, Francia and colleagues describe a direct structural role of site-specific, DICER and DROSHA ncRNAs in the assembly of the DNA break-associated foci (Francia et al., 2012). Based on these observations, we asked whether the mitotic, extrachromosomal localization of histone H2B might depend on nucleic acids other than chromatin. We employed several enzymes that catalysis the degradation of nucleic acids *i.e.* RNase A, RNase-III, RNase H and DNase I, which respectively bind and cleave ssRNA, sdRNA, hybrid DNA/RNA, and DNA. As constitutive component of chromatin essential to chromosomal organization/replication during S-phase, in order to study the relationship of histone H2B and nucleic acids only during cytokinesis, we process mitotic-enriched HeLa cells. We could confirm that midbody

localization and cytokinesis function of histone H2B is independent of its interaction with DNA. Indeed, degradation of DNA mediated by DNase I does not impair H2B-S14^P localization at the midbody. Furthermore, here we show that deprivation of both ss- and ds-RNAs and DNA/RNA hybrids do not affect midbody localization of histone H2B-S14^P. Analyses of HCT116 DICER KO cells further confirm that ncRNAs are not involved in extrachromosomal localization and function of H2B both in early stage of cytokinesis and at the midbody. For technical reasons, we could not have information on the relationships between the other nucleic acids and H2B localization in the early stage of cytokinesis. Indeed, we were not able to identify an efficient cell permeabilization protocol able to maintain the presence of H2B in the aggregates we observed from metaphase to anaphase. Thus, it is possible to hypothesis that histone H2B-S14^P is more strictly associated at the midbody, when the microtubules is densely packed, with the progression of cytokinesis.

HISTONE H2B IN THE CONTROL OF ABSCISSION

Cytokinesis is a highly ordered process, requiring a complicated interplay between cytoskeletal, chromosomal, and cell cycle regulatory pathways. Cytokinesis failure is known to lead to genetically unstable states, such as tetra- and poly-ploidization, considered a critical step in tumourigenesis (Ganem et al., 2007; Normand and King, 2010). Here, we show that depletion of histone H2B in HeLa cells results in appearance of different signs of cytokinesis failure such as accumulation of LIBs and syncytium-like cells, tubulin disorganization, increase in the number of cells at the midbody stage, and abscission defects and delay. These signs are usually observed in cells with impaired abscission (Normand and King, 2010; Steigenmann and Gerlich, 2009), strongly supporting a role for histone H2B in this last stage of cell division.

Depletion of critical abscission factors, such as ALIX (Morita et al., 2007) or HIPK2 (Rinaldo et al., 2012), result in the failure of abscission that is frequently associated with accumulation of binucleated cells. Although final cleavage of the midbody clearly failed in H2B-depleted cells, the effect of H2B depletion was different to that seen with manipulations of the other abscission factors. Indeed both IF analysis of fixed cells and time-lapse live cell imaging show only a slight increase of binucleation in our H2Bi HeLa cells. We can speculate to try to explain this behavior. First, it is possible that the point at which H2B is required in abscission is so late and beyond the time at which daughter cells are capable of re-fusing. A

second possibility may be inherent in the H2B chromatin function. Indeed, as constitutive component of chromatin, histone mRNA levels increase 35-fold as cells enter S-phase (Olsey, 1991). In addition, studies on histone exchange and deposition kinetics revealed that H2B is the most rapidly and abundantly exchanged core histone (Kimura and Cook, 2001). Histone H2B transits in and out of the nucleosome more rapidly than other core histones (H3, H4, and H2A), about 3% of total H2B is exchanged within 6 minutes (Kimura, 2005). Despite we have set up a protocol that induce depletion of extrachromosomal histone H2B without affecting cell viability, it is possible that the still large amount of residual exchanging H2B would be enough for a partial recovery of the cytokinetic events. At present, it is not easy to distinguish directly between these two possibilities. A clue might be yield whether a cytokinesis-specific H2B variant can be defined. In fact, 21 human histone *H2B* genes are known to produce 16 histone variants. Thus far, only two of these 16 variants have been ascribed a specific function, *i.e.*, chromatin compaction during spermatogenesis (Zalensky et al., 2002; Churikov et al., 2004). We performed preliminary experiments by using single variant-specific siRNAs and observed the appearance of defects comparable to those we obtained by employing the combination of 9 different siRNAs (*i.e.*, H2B.B, H2B.M, H2B.K, or H2B.E). These results suggest that some H2B variants might be preferentially involved in this function. However, we still have to confirm the latter aspect by performing similar experiments in different cell lines, such as H1299 and U2OS cells that have been show to express different levels of specific H2B variants in different biological conditions (Kari et al., 2014).

Nevertheless, we can conclude that histone H2B is required for resolution of the midbody ad faithful abscission.

INTERPLAY BETWEEN HISTONE H2B AND PROTEIN INVOLVED IN CYTOKINESIS: A DIRECT INTERACTION WITH CHMP4B

Cytokinesis proceeds throughout subsequent stages each owing the previous one, that require a complex interplay among a still enlarging number of regulatory and effectors components. Similarly, in abscission a wide variety of proteins involved in vesicles and proteins trafficking, membrane scission, and cytoskeleton rearrangement are required for physical separation of the two daughter cell, suggesting that the final stage of cytokinesis is just as complex as earlier stages. During the last several years, multiple studies have considerably

increased our understanding about cytokinesis and abscission (Mierzwa and Gerlich, 2014). Although a large list of proteins participating in cytokinesis has been generated, it is still unclear how all of these proteins interact to drive cytokinesis and abscission.

In H2B depleted cells, cleavage furrow specification and ingression proceed normally, but the cell abscission fails to occur. We show that localization of some proteins involved in specification/formation of the midzone, *e.g.* CPCs, PRC1 and PLK1, are unaffected by H2B depletion. At the midbody level, tubulin is disorganized presumably owing to LIBs formation and the stretching conditions triggered by abscission defects. Despite structural components of the midbody, such as CPCs, PLK1, PRC1, ECT2, MKLP1, and HIPK2 still localize at the intercellular bridge, their distribution pattern become dispersed following the aberrant tubulin structures. This is not a general behavior of midbody components since other factors such as Citron Kinase maintains its proper localization. This latter result is of relevance because it indicates that the aberrant localization of the previous factors is not merely due to generic intercellular bridge destruction. In contrast, severe defects of localization and organization are observed for the abscission factors ALIX, ESCRT-III subunit CHMP4B, and Spastin, while the localization of CEP55 appears less sharp than controls cells. An initial, biochemical characterization of the physical interactions among these factors shows that H2B interacts directly with CHMP4B a subunit of ESCRT-III-dependent filaments, recently identified as cortical constriction factors that mediate the secondary ingression at the onset of abscission. In addition, we show that H2B/CHMP4B interaction is significantly increased after phosphorylation of histone H2B at serine 14.

Overall, our results indicate that histone H2B and its phosphorylated form are essential for appropriate localization of critical abscission factors and faithful abscission. Whether the observed H2B/CHMP4B interaction might link the abscission defects and delay with a slower and defective formation of secondary ingressions needs to be verified.

MATERIALS AND METHODS

The experiments have been repeated from three to five times and the results obtained are presented as means $\pm$ SD.

Cells, Culture Conditions

HeLa, HCT116 WT, HCT116-Dicer-KO (cells in which exon 5 of the Dicer gene has been disrupted kindly provided by Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD, USA), were cultured in DMEM with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) and maintained in a humid incubator at 37°C in a 5% CO₂ environment. For live cell imaging, HeLa cells were cultured in DMEM medium without phenol red, supplemented with 10% heat-inactivated FBS (Invitrogen). For DNA damage, subconfluent cells were γ -irradiated (2Gy/dish) by an IBL 437C Irradiator (CIS bio international) or were irradiated with UVB 0.075 J/cm² ray by BIO-Sun Vilbert Lourmat

Western Blot, Kinase Assay

For Western Blotting, TCEs were prepared in RIPA buffer [50mM Tris-HCl (pH 8), 600mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40 and 1mM EDTA] supplemented with protease-inhibitor mix (Roche) and Hal Phosphatase Inhibitor Cocktail (Thermo scientific). Proteins were resolved by SDS-PAGE using NuPAGE® Novex Bis-Tris Gels (Invitrogen), transferred onto nitrocellulose membranes (Bio-Rad), and analyzed with the indicated antibodies (Abs). The following Abs were employed: anti-alpha-tubulin moAb (Immunological Sciences), anti-GST moAb (kindly provided by M. Fanciulli), anti-H2B moAb (ABCAM), HRP-conjugated goat anti-mouse and anti-rabbit (Cappel). Immunoreactivity was determined using the ECL-chemiluminescence reaction (Amersham

Corp) following the manufacturer's instructions. KISase assay was performed as reported in Lazzari et al 2011.

Expression vectors and protein purification

pGEX-GST-HIPK2 (158–557) was obtained by amplifying the 158–557 fragments from the corresponding pEGFP vectors by PCR and cloned into EcoRI/XhoI digested pGEX6p-2rbs (kindly provided by A. Musacchio) (Siepi et al., 2013). Recombinant proteins were produced in *E.coli* BL21 (DE3) (Invitrogen). At the O.D. 0.6–0.8 we added IPTG at final concentration of 1 mM and then re-incubated for 16 hours. GST-protein was purified by using GST-Trap Chelating HP column (GE Healthcare). GST tag was cleaved by using PreScission (GE Healthcare) and purified in a HPLC gel exclusion column (BioFox- 17/1200 Sec, Knauer) and performed size exclusion chromatography. The amount of purified protein can be assessed by UV-visible measurement using $\epsilon_{280\text{nm}} = 48820 \text{ M}^{-1} \text{ cm}^{-1}$.

Enrichment in telophase and RNase A/H/III or DNase I treatment

HeLa cells were enriched in telophase by treatment with nocodazole (100ng/ml for 4 hours) (Santa Cruz) followed by mitotic shake off and nocodazole wash-out. The collected cells were plated on poly-L-lysine coated coverslips and incubated for about 45 min. to reach different cytokinesis stages. Thereafter HeLa cells were permeabilized with (0.5; 0.75; 0.1; 1.5; 2%) Tween 20 in PBS for 10 min at room temperature. RNase A treatment was carried out in 1 ml of 1 mg/ml ribonuclease A from bovine pancreas (Sigma- Aldrich) in PBS for 25 min at room temperature. After RNaseA digestion, To evaluate 53BP1 foci formation cells were plated on poly-L-lysine coated coverslips and irradiated with 2 Gy of ionizing radiation. One hour later HeLa cells were processed as described above. To cleave other nucleic acid we performed the same experiment described above by using recombinant RNase H 25U (Ambion) RNase III 25U(Ambion) or rDNase I 25U (Ambion). Cells were then fixed with 2% paraformaldehyde for 10 min or cold methanol 100% for 5 min at -20°C.

RNA Interference

RNA interference was obtained by using commercially available H2B-specific (H2Bi) stealth RNAi sequences (Invitrogen) (a mix of nine sequences specific for different histone H2B variants) and by universal negative control stealth RNAi Negative Medium GC Duplexes (Invitrogen). Cells were transduced with 90 nM of siRNA using RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. Red fluorescent oligonucleotides (Block-it, Invitrogen) were used to evaluate transfection efficiency.

Immunofluorescence Microscopy

For immunofluorescence experiments, cells were seeded onto poly-L-lysine coated coverslips. Cells were fixed in ice-cold methanol or 2% formaldehyde, washed three times in phosphate buffered saline (PBS), permeabilized in 0.25% Triton X-100 in PBS for 10 min., and then blocked in 0.2% Triton X-100, 5% bovine serum albumin (BSA) in PBS for 60 min. before the required primary Abs were applied. The following Abs were employed: anti-Aurora B Ab (BD-Bioscience) anti-Alix Ab (Santa Cruz), anti-Cep55 Ab (Santa Cruz), anti-Survivin Ab (Epitomix), anti-ECT2 Ab (Santa Cruz), anti-PRC1 Ab (Santa Cruz), anti-MKLP1 Ab (Santa Cruz), anti-HIPK2 Ab (Pieratoni et al., 2007), anti-H2B-S14^P (Cell Signaling), anti-spastin Ab (Santa Cruz), anti-CHMP4B (Proteintech), anti Citron Kinase Ab kindly provided by A. Di Cunto, anti-TSG101Ab (Abcam), anti PLK1 Ab (Santa Cruz), Anti 53BP1 (Abcam), anti INCENP Ab (Epitomix). β -Tubulin-Cy3 (Sigma). Appropriate secondary FITC- or TRITC-conjugated Abs (Alexa-fluor, Invitrogen) were used. DNA was marked with HOECHST 33342 (Sigma) Cells were examined under an Olympus BX53 microscope equipped with epifluorescence and photographs were taken ($\times 100$ objective) using a cooled camera device (ProgRes MF).

Transmission Electron Microscopy

HeLa cells were enriched in telophase by treatment with nocodazole (100ng/ml for 4 hours) followed by mitotic shake off, nocodazole wash-out, and incubation of the collected cells for

about 80 min. to reach telophase stage. Cells were washed three times in PBS and fixed with 2% glutaraldehyde in PBS for 2 h at 4 °C and then resuspended in 2% paraformaldehyde in PBS. Droplets of this sample were directly spotted onto Formvar/carbon-coated copper grids (100 meshes) and incubated with anti-H2B-S14^P monoclonal antibody. After several rinses in PBS-0.1% bovine serum albumin, the grids were incubated with 10-nm diameter colloidal gold particles (prepared by citrate method) conjugated with protein A (Pharmacia, Uppsala, Sweden) and diluted in 1% bovine serum albumin in PBS. Control experiments were performed by omission of the primary antibodies from the labeling procedure. Finally, grids were stained with a solution of 2% methylcellulose and 0.4% uranyl acetate before examination under a Morgagni 268D transmission electron microscope (FEI, Hillsboro, OR, USA) equipped with a Mega View II charge-coupled device camera (SIS, Soft Imaging System GmbH, Munster, Germany) and analyzed with AnalySIS software (SIS)

GST Pull-Down and in Vitro Binding Assay

For in vitro binding experiments recombinant histone H2B (Millipore) or HIS tagged H2B (Proeintech) and recombinat GST-CHMP4B (Proteintech) were incubated over night at room temperature in buffer phosphate pH 7.5 with 150 mM NaCl or 450 mM NaCl. Thereafter, were incubated for 2 hours with Glutathione-Sepharose 4 Fast Flow beads (GE Healthcare). Pull-down GST-tagged proteins were washed three times with buffer phosphate. Bound was analyzed by WB.

Live Cell Imaging

Cells seeded in 35 mm dishes (81156, ibiTreat, Ibidi) and transfected as described above were observed under an Eclipse Ti inverted microscope (Nikon) using a Plan Apo 40x objective (Nikon). During the whole observation, cells were kept in a microscope stage incubator (Basic WJ, Okolab), at 37°C and 5% CO₂. DIC images were acquired every 3 minutes over a 24hrs period by using a DS-Qi1Mc camera and the NIS-Elements AR 3.22 software (Nikon). Image and video processing were performed with NIS-Elements AR 3.22.

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