Title

Guiding mitotic progression by crosstalk between post-translational modifications

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Abstract

Cell division is tightly regulated to disentangle copied chromosomes in an orderly manner and prevent loss of genome integrity. During mitosis, transcriptional activity is limited and post-translational modifications (PTMs) are responsible for functional protein regulation. Essential mitotic regulators, including polo-like kinase 1 (PLK1) and cyclin-dependent kinases (CDK) as well as the anaphase promoting complex/cyclosome (APC/C), are members of the enzymatic machinery responsible for protein modification. Interestingly, communication between PTMs ensures the essential tight and timely control during all consecutive phases of mitosis. Here, we present an overview of current concepts and understanding of crosstalk between PTMs regulating mitotic progression.

Keywords

Mitosis, post-translational modifications, crosstalk, phosphorylation, ubiquitin, SUMO

Regulation of mitosis by crosstalk between PTMs

Ever since Rudolf Virchow claimed that 'all cells come from cells' (*omnis cellula e cellula*) in 1855 [1], it has been a challenge to understand the process of cell division. Since cells are the building blocks of all organisms, their capability to divide is essential for organisms to reproduce themselves. For all multicellular organisms, cell division is also crucial for growth and tissue renewal. Although ample elements remain to be discovered, decades of research have significantly increased our knowledge about the cell cycle and enabled categorization of the complex processes during interphase and mitosis. During interphase the cell grows and prepares for replication (G1 phase), chromosomes are replicated (S phase) and the cell expands further and prepares for mitosis (G2 phase). Throughout these phases chromosomes are decondensed and the microtubules are organized in a radial array from the centrosome. During mitosis the sister chromatids are segregated into two nascent cells, ensuring that each daughter cell inherits one complete set of chromosomes. Finally, cell division is completed when the intercellular bridge between the daughter cells is disconnected via abscission during cytokinesis.

As uncontrolled cell proliferation can result in genetic instability and thereby have detrimental effects, the complex process of cell division is tightly controlled. Regulatory proteins strictly monitor progression through the consecutive cell cycle phases and are able to stop cell cycle progression at specific checkpoints when a defect is encountered. To enable timely repair of the identified problem and prevent mitotic catastrophe, a rapid response is essential. Therefore, the cell utilizes the fascinating mechanism of post-translational modifications (PTMs). Modification by a PTM can directly change the function of a target protein, for example through

influencing protein activity, changing its localization, inducing a conformational change or affecting its interaction partners. Currently, over 600 different PTM types are categorized in Uniprot and other databases, including non-reversible modifications such as protein cleavage as well as reversible modifications [2-4]. Here, we will focus on modification of amino acids by the latter group of PTMs. Amino acids can be modified by chemical groups as well as small proteins, including ubiquitin and the ubiquitin-like family. While chemical modifications (such as phosphorylation) are regulated via single steps (Figure 1A), conjugation of small proteins (including ubiquitin and small ubiquitin-like modifier (SUMO)) to a target occurs through an enzymatic cascade involving an activating E1, conjugating E2 and E3 ligase (Figure 1B). Interestingly, ubiquitin and ubiquitin-like PTMs can modify themselves, resulting in polyPTM chains on the target protein. Two ubiquitin E2s were recently shown to work together and utilize their individual catalytic architecture to enable the formation of polyubiquitin chains [5]. Furthermore, cooperation between different ubiquitin E3 ligases has been reported. The first E3 ligase was found to be responsible for (multi-) monoubiquitylation, while the second E3 ligase modified the conjugated ubiquitin to form polyubiquitin chains on the target protein (E3-E3 tagging cascade) [6].

Advances in the field of proteomics have enabled the identification of a plethora of cell cycle regulators as targets for PTMs, including phosphorylation, ubiquitylation and SUMOylation [7-12]. Functional analysis has revealed that these PTMs are able to influence each other through crosstalk (Box 1 and Figure I), which increases the complexity of the system and expands the ability of PTMs to tightly and timely control the cell cycle.

During mitotic progression each sequential phase and transition requires high level dynamic protein regulation. Since most transcriptional activity is inhibited during mitosis [13], post-translational modifications are essential for the required regulation of proteins. Complex crosstalk networks interconnect different PTM pathways and provide flexibility in protein state, which is essential all throughout mitosis. Here, we will focus on mitotic regulation by PTMs and illustrate the extent of their crosstalk throughout the sequential mitotic phases.

Prophase: crosstalk regulating chromosome condensation

Accurate chromosome segregation during cell division is facilitated by condensing the chromosomes into compact structures in prophase and maintaining these structures throughout mitosis, which prevents chromosomes from becoming entangled during cytokinesis potentially leading to DNA damage. The fundamental role in chromosome condensation is carried out by two multiprotein complexes, condensin I and condensin II. Both complexes contain the same pair of structural maintenance of chromosome (SMC) subunits SMC2 and SMC4, which are combined with a distinct set of non-SMC subunits. These complexes are able to positively supercoil DNA and thereby condense chromosomes [14]. Large-scale phosphoproteomic screens identified numerous cell cycle-regulated phosphorylation events with essential roles during chromosome condensation [15,16].

Functional analysis has revealed how these modifications communicate with other PTMs, for example resulting in crosstalk on condensin II regulating inhibition, establishment and maintenance of chromosome condensation throughout cell cycle progression (Figure 2) [17]. In *Drosophila* cells, phosphorylation of the non-SMC condensin II subunit CAP-H2 by casein kinase I α (CK1α) during interphase provides

a phosphodegron motif, which is recognized by the ubiquitin E3 ligase skp1-cullin-F box (SCF)^{slimb} (homolog of the human SCF$^{\beta\text{-TrCP}}$) [18]. Subsequent ubiquitylation results in degradation of the subunit and thereby inhibits condensin II activity in interphase [19]. This phosphorylation-dependent ubiquitylation event on condensin II is a textbook example of positive crosstalk (type 8) between two different PTMs by an enzymatic reader containing a specific PTM binding domain. During mitosis, condensin II is activated via phosphorylation of the non-SMC subunit CAP-D3 by cyclin-dependent kinase 1 (CDK1) to establish chromosome condensation [20]. However, initial activation is insufficient to maintain condensation when CDK1 levels decrease during metaphase-to-anaphase transition, while phosphatases remain active [17]. Interestingly, phosphorylation by CDK1 provides its targets with a recognition site (S[pS/T]P) for the polo-box domain (PBD) in polo-like kinase 1 (PLK1) [21,22]. Initial priming of CAP-D3 by CDK1 therefore results in subsequent phosphorylation of all non-SMC subunits of condensin II by PLK1 at [D/E]x[S/T] motifs. This positive crosstalk (type 10) between PTMs on different subunits of the complex results in hyperactivation of condensin II and thereby ensures continuation of chromosome condensation throughout mitosis [23].

Interestingly, the kinase activity of PLK1 itself is controlled via a complex crosstalk network involving the aurora A-bora complex [24]. Similar to CAP-D3, phosphorylation of bora by CDK1 primes the protein for recognition and subsequently phosphorylation by PLK1 (positive crosstalk type 8). The resulting phosphodegron motif is recognized by the ubiquitin E3 ligase SCF$^{\beta\text{-TrCP}}$, which subsequently ubiquitylates bora (positive crosstalk type 8) [25,26]. As this modification by ubiquitin results in proteasomal degradation and thereby decreases the activity of the aurora A-bora complex, its ability to phosphorylate and thereby activate PLK1 is reduced

(negative crosstalk type 6). This complex crosstalk network involving enzymatic readers as well as the modification of enzymatic machinery might provide a negative feedback mechanism to reduce PLK1 activity when required, for example during chromosome decondensation at the end of mitosis.

Recently, histone modifications were identified as additional driving forces in yeast for chromosome hypercondensation in a condensin-independent manner [27]. Phosphorylation of histone H3 S10 by aurora B recruits the histone deacetylase Hst2p to remove the acetyl group from histone H4 K16, which subsequently enables the histone H4 tail to interact with close nucleosomes and promote chromosome condensation. This phosphorylation dependent-deacetylation event illustrates that chromosome condensation is also regulated by negative crosstalk (type 10) via an enzymatic reader involved in yet another modification, namely acetylation.

Although research efforts have mostly focussed on the functional relevance of phosphorylation events during chromosome condensation, the field has started to unravel the importance of other modifications and their crosstalk during this process of which several examples are described above. A recent analysis combining all available human SUMO proteomics data revealed that all subunits of both condensin complexes have been identified as SUMO substrates by mass spectrometry [28], indicating direct regulation of the condensin complexes by SUMOylation. Although the role for SUMO on most condensin subunits has not been functionally studied, the non-SMC subunit of condensin I Ycs4 (yeast homologue of CAP-D2) was found to be dynamically modified by SUMO during mitosis [29]. Interestingly, condensin I SUMOylation is dependent on a loss of phosphorylation via the phosphatase Cdc14 (yeast homologue of Cdc14A and Cdc14B), while inactivation of the yeast SUMO

protease Smt4 (the functional equivalent of mammalian SENP6 and SENP7) results in condensin localization defects [30]. Although the exact crosstalk mechanism remains to be discovered, these results suggest a role for negative crosstalk between phosphorylation and SUMOylation in chromosome condensation. As many phosphorylation sites for CAP-D2 were identified surrounding the SUMO acceptor lysines on the C-terminal site of the protein, we speculate that phosphorylation could influence a recognition motif for the SUMO conjugation machinery (crosstalk type 8).

In addition to their regulation by phosphorylation and SUMOylation, recent proteomic studies revealed that both SMC subunits of the condensin complexes are also modified by acetylation, monomethylation and ubiquitylation [31-37]. Interestingly, acetylation as well as ubiquitylation of SMC2 K114, K222, K677, K1160 and SMC4 K268 and K607 was identified [31,35-38], suggesting potential negative crosstalk (type 5) between these two PTMs on the condensin subunits. These data indicate important roles for these PTMs in condensin complex regulation. Nevertheless, the functional relevance of these modifications as well as their potential crosstalk currently remains unexplored.

Prometaphase: crosstalk regulating kinetochore complex formation

During prometaphase the nuclear envelop breaks down and microtubules connect to the chromosomal centromeres via kinetochore complexes. Accurate formation of the centromere-kinetochore-microtubule interaction is essential for mitotic progression, as the complex is responsible for chromosome alignment and segregation in subsequent phases. In prometaphase the kinetochore ensures exclusive connection of sister chromatids to opposite spindle poles and monitors inaccurate attachments [39-41]. As the kinetochore has over 100 potential subunits, various modifying enzymes

as well as crosstalk mechanisms are involved in recruitment, assembly and maintenance of this complex structure.

Before the kinetochore can be assembled, the centromeric chromatin has been primed for recruitment of the histone 3 variant CENP-A during late mitosis of the previous division and early G1. Proteomic analysis revealed that centromeric chromatin is modified by distinct PTMs, which have been shown via functional analyses to communicate through crosstalk [42]. Ubiquitylation of histone H2B K120 recruits the Set1 methyltransferase complex, which is then able to methylate histone H3 K4 (positive crosstalk type 10) [43,44]. Additional priming by histone H3 K9 acetylation acts as an AND logic gate, resulting in recruitment of the Mis18 complex via a potential tandem modular PTM recognition domain (positive crosstalk type 9). Subsequent phosphorylation of the Mis18 complex by PLK1 results in CENP-A deposition at the centromeric region [45-48]. Premature loading of CENP-A is prevented by phosphorylation of CENP-A itself and the Mis18 complex via CDK1 [49,50]. In contrast to the positive communication between these kinases on condensin II, these data indicate the presence of negative crosstalk (type 5) between CDK1 and PLK1 at the centromeric chromatin. This area between the sister chromatids containing CENP-A and histone H3 as well as the chromosomal passenger complex (CPC), including the kinase aurora B, is referred to as the inner centromere (Figure 3A). CENP-A deposition primes the inner centromere for recruitment of the inner kinetochore, consisting of the constitutive centromere-associated network (CCAN).

Function and characteristics of the CCAN subunits enable their division into subcomplexes, namely CENP-H-I-K-L-M-N, CENP-O-P-Q-R-U, CENP-T-W-S-X and CENP-C, which are dynamically assembled together during S phase to form the inner kinetochore (Figure 3B) [39]. Interestingly, CENP-I is polySUMOylated upon

incorrect kinetochore assembly, which has been described to prime the protein for recognition by the SUMO-targeted ubiquitin ligase (STUbL) RNF4 via its SUMO-interaction motifs (SIMs) [51]. Subsequent ubiquitylation by this reader protein (positive crosstalk type 8) and proteasomal degradation of CENP-I would explain the observed displacement of CENP-H-I-K as well as the CENP-O complex from kinetochores, which inhibits kinetochore assembly and kinetochore-microtubule interaction [52]. Interestingly, kinetochore destabilization upon SUMO-ubiquitin crosstalk is antagonized by the SUMO protease SENP6 (negative crosstalk type 5), which only targets SUMOylated CENP-I in correctly assembled kinetochores and leaves the inaccurately formed complexes for destruction to ensure proper kinetochore formation [51,52].

Subsequently, CENP-T-W-S-X and CENP-C recruit the outer kinetochore, which directly binds the spindle microtubules during mitosis (Figure 3C). The basis of the outer kinetochore consists of the KMN network, which includes the Knl1 complex (Knl1-Zwint), the Mis12 complex (Mis12-Dsn1-Nnf1-Nsl1) and the Ndc80 complex (Ndc80-Nuf2-Spc24-Spc25). After phosphorylation of the Mis12 complex subunit Dsn1 by aurora B, its ubiquitylation by the CENP-C binding ubiquitin E3 ligase Mub1/Ubr2 and subsequent degradation by the proteasome is prevented (negative crosstalk type 5) [53]. Upon this phosphorylation-dependent stabilization of Dsn1, the complete Mis12 complex interacts with CENP-C and recruits both the Knl1 and Ndc80 complexes [54,55]. Subsequent microtubule binding is regulated by the balance between phosphorylation by aurora B and dephosphorylation by phosphatase such as PP2A of various kinetochore subunits, which will be discussed later [56]. Interestingly, dephosphorylation and thereby inactivation of the kinase itself by PP2A is prevented via aurora B acetylation (positive crosstalk type 5) [57].

Interestingly, almost all members of the CCAN subcomplexes have been identified as phosphorylation as well as ubiquitylation targets by mass spectrometry, which could indicate regulation by group modification [7,35-37,58-62]. Several subunits are also modified by other PTMs, including methylation of CENP-C and CENP-H as well as SUMOylation of CENP-C, CENP-I and CENP-R [34,51,63,64]. These specific modifications on certain CCAN members suggest explicit individual regulation of a particular protein function. Interestingly, several identified SUMO acceptor sites in CENP-C and CENP-R are located in a phosphorylation-dependent SUMOylation motif (KxExpSP) [65,66], indicating potential positive crosstalk (type 8) between these PTMs at the inner kinetochore. Although the field has started to unravel the functional relevance of these modifications and their crosstalk, as described above for CENP-I, functional studies are essential to improve our understanding of CCAN regulation by crosstalk between PTMs.

Metaphase: crosstalk regulating chromosome alignment

Upon accurate formation of the centromere-kinetochore-microtubule structure, the chromosomes align at the spindle equator during metaphase. However, to accomplish chromosome movement, each chromosome has to be correctly captured by two microtubules from opposing poles. This process of kinetochore-microtubule attachment is controlled by two feedback mechanisms, namely error correction and the spindle assembly checkpoint (SAC), which both involve the chromosomal passenger complex (CPC) located at the inner centromere [67]. The CPC consists of the regulatory subunit aurora B and three non-enzymatic subunits survivin, INCENP and borealin, whose localization is dependent on positive crosstalk [68]. Along the

length of the chromosomes where the paired sister chromatids are connected, SUMOylated TOP2A has been reported to recruit the kinase haspin, which results in phosphorylation of histone H3 T3 via positive crosstalk (type 10) on distinct targets via an enzymatic reader (Figure 4A) [69,70]. At the outer kinetochore, phosphorylation of the Knl1 complex by the kinase Mps1 recruits the Bub1 complex (Bub1-Bub3-BubR1), which then phosphorylates histone H2A T120 (positive crosstalk type 10) [70,71]. While the CPC subunit survivin is recruited to phosphorylated histone H3 T3, borealin interacts via its phosphorylation by CDK1 with the Sgo1 and Sgo2 proteins recruited to phosphorylated H2A T120 [72-74]. At the inner centromere, where both histone modifications overlap and therefor both CPC subunits are actively recruited, kinase activity of aurora B is initiated [68,70]. PolySUMOylation of aurora B promotes its autophosphorylation (positive crosstalk type 6), which then enhances its kinase activity towards its substrates (positive crosstalk type 6) [75]. Phosphorylation of INCENP causes a conformational change in aurora B and thereby fully activates the kinase (positive crosstalk type 10), which is essential for aurora B functionality during metaphase [76].

Aurora B is subsequently involved in the process of error correction upon the formation of incorrect kinetochore-microtubule attachments. Its phosphorylation of various key outer kinetochore complexes, including Knl1, Ndc80 and Ska subunits, interferes with their binding to each other as well as microtubules. When both sister kinetochores are connected to the same spindle pole, inter-kinetochore stretching is minimal and local aurora B activity hereby disrupts centromere-kinetochore interactions. However, when via trial-and-error correct bi-orientation of sister chromosomes is accomplished, increased inter-kinetochore stretching reduces local aurora B activity at the kinetochore region. The balance shift towards

dephosphorylation by phosphatases such as PP1 and PP2A promotes the stabilization of correctly bi-orientated kinetochore-microtubule attachments (Figure 4B) [77]. Interestingly, in yeast reducing the phosphorylated levels of the Ska complex homologue was found to be additionally regulated via a complex crosstalk mechanism (types 10 and 5). Upon recruitment of the methyltransferase Set1 to the kinetochore by ubiquitylation of histone H2B, methylation of the Ska complex inhibits its phosphorylation by aurora B [78,79].

Aurora B is also involved in monitoring kinetochore-microtubule interactions via recruitment of spindle assembly checkpoint (SAC) components. During early mitosis, the SAC prevents segregation of duplicated chromosomes until each sister chromatid is properly attached to an appropriate microtubule and aligned at the spindle equator [56]. Unlike the individual and local regulation of kinetochore-microtubule attachments by the process of error correction, the SAC signal is able to extend beyond the kinetochore and prevent mitotic exit upon the recognition of a single incorrect attachment. Until all kinetochore-microtubule interactions are accomplished in a bi-orientated manner, the mitotic checkpoint complex (MCC) stalls mitotic progression. Although the MCC itself is composed of Mad2, Bub3, BubR1 and Cdc20 other proteins are involved in SAC activation, including Mad1, Mps1, Bub1 and the rod-zwilch-zw10 (RZZ) complex. Unattached kinetochores recruit Mad1, which is phosphorylated by the kinase Mps1 enabling binding to the MCC subunit Mad2 [80,81]. A conformational change enables MCC formation upon interaction with Cdc20, which dominantly inhibits the function of the ubiquitin E3 ligase anaphase-promoting complex/cyclosome (APC/C) and thereby the ubiquitylation of its substrates (negative crosstalk type 10) [80,82]. Cdc20 was observed to interact with the recently identified ABBA motif, which is present in multiple subunits of the MCC as

well as various APC/C substrates [83]. When all kinetochores are attached properly to opposite spindle poles and aligned at the spindle equator, the MCC is relocated away from the kinetochores and is therefore no longer able to interfere with the interaction between APC/C and its cofactor Cdc20. Interestingly, phosphorylation of Mad2 inhibits its binding to Mad1 [84], which might be involved in inactivating the SAC upon proper microtubule attachment and thereby promote ubiquitylation by the APC/C (positive crosstalk type 10). Additionally, the SAC promoting activity of the kinases aurora B and Mps1 is counteracted by the phosphatases PP1 and PP2A. Dephosphorylation of the Knl1 complex by PP2A, for example, promotes silencing of the SAC via reducing Bub1 complex recruitment and thereby increases APC/C activity (positive crosstalk type 10) [85].

Throughout metaphase many processes controlling kinetochore-microtubule attachments and thereby chromosome alignment are known to be tightly regulated by phosphorylation. Analysing the available mass spectrometry data revealed that, besides their phosphorylation, each member of the CPC has been identified as a target for ubiquitylation as well as SUMOylation [35-37,58,60,64,65,75,86-89]. In addition to the crosstalk between SUMOylation and phosphorylation on aurora B described above, ubiquitin potentially competes with SUMO for all CPC subunits (negative crosstalk type 5), as both PTMs have been identified on the same residues in aurora B (K31, K56, K85, K202, K211, K215 and K306), INCENP (K51 and K327), borealin (K183 and K225) and survivin (K78, K90 and K91) [35-37,64,65,75,87-89]. Since the stoichiometry of ubiquitylation is generally considerably higher compared to SUMOylation, ubiquitin is expected to outcompete SUMO. Ubiquitin is after all a ubiquitous protein. Other PTMs might also be involved in the communication between modifications on CPC

subunits, as INCENP and aurora B can be monomethylated and acetylation was observed on survivin, INCENP and aurora B [32-34,57,90].

Anaphase: crosstalk regulating chromosome segregation

When all correctly bi-orientated chromosomes are located at the spindle equator and the SAC is inactivated, anaphase is initiated (Figure 5). Throughout this process composition of the centromere and kinetochore changes, regulated by crosstalk between PTMs. Dephosphorylation of histone H3 T3 results in dissociation of survivin from centromeres [68,72,91,92]. Upon ubiquitylation of aurora B by the ubiquitin E3 ligase cul3-KLHL21, the kinase is removed from chromosomes by Cdc48 and its adaptor proteins Ufd1-Npl4 [93,94]. Consequently, phosphorylation levels of aurora B substrates in the centromere decrease (negative crosstalk type 6). Subsequent dephosphorylation of INCENP, by Cdc14 in yeast, results in translocation of the CPC from centromeres to the central spindle at metaphase-to-anaphase transition [95]. SUMOylation of Mis18bp1, a subunit of the Mis18 complex at the inner centromere, peaks during mitosis [96]. SUMOylation primes Mis18bp1 for recognition by the STUbL RNF4, resulting in Mis18bp1 ubiquitylation and subsequent degradation by the proteasome (positive crosstalk type 8) [96]. Interestingly, Mis18bp1 is part of a group of mitotic regulators that are co-modified by SUMO and ubiquitin upon inhibition of the proteasome, indicating that RNF4 might regulate the stability of this complete protein cluster by group modification involving crosstalk. Since knockdown of the SUMO E3 ligase PIAS4 as well as the STUbL RNF4 causes chromosome segregation defects [97,98], these enzymes potentially collaborate to SUMOylate and ubiquitylate certain centromere-kinetochore subunits during mitosis to ensure their timely degradation

and prevent chromosome segregation errors due to prolonged centromere-kinetochore persistence.

Segregation of chromosomes and thereby anaphase onset is tightly regulated by the anaphase promoting complex/cyclosome (APC/C), a 1.5-MDa multi-subunit ubiquitin E3 ligase [99]. By assembling branched ubiquitin conjugates containing multiple blocks of K11-linked chains, the APC/C significantly enhances substrate recognition by the proteasome [100]. A diverse array of mechanisms is employed to ensure degradation of these key mitotic regulators in a robust and timely order. Upon inactivation of the spindle assembly checkpoint, ubiquitylation and dephosphorylation of the released cofactor Cdc20 promotes its interaction with the APC/C [101-104]. Interestingly, phosphorylation of various core subunits (including APC1, APC3, APC6 and APC8) by mitotic kinases CDK1 and PLK1 promotes the formation of active APC/C^{cdc20} and thereby ubiquitylation of its substrates (positive crosstalk type 6) [82,105-107]. For anaphase onset, APC/C^{cdc20} ubiquitylates securin and thereby marks the protein for proteasomal degradation [82,108]. During early mitosis, securin inactivates the protease separase by forming a complex. Upon securin degradation, separase is liberated from the complex, but still inactivated by phosphorylation [108]. APC/C^{cdc20} also ubiquitylates cyclin B, which results in its proteasomal degradation and subsequently inactivation of CDK1 (negative crosstalk type 6). Whereas CDK1 inactivates separase by phosphorylation during early mitosis, inactivation of CDK1 as a result of cyclin B degradation now enables protein phosphatases to remove the phosphate groups and activate separase [82]. Consequently, separase cleaves the cohesin complexes between centromeres and thereby facilitates chromosome segregation towards spindle poles during anaphase. The simultaneous degradation of securin

and cyclin B by the APC/C^{cdc20} thereby couples chromosome segregation with the dissolution of complexes that monitor microtubule attachment during mitotic exit [109]. Interestingly, SUMOylation of the platform subunit APC4 increases APC/C activity towards a subset of targets, including kif18B, which promotes timely metaphase-to-anaphase transition via positive crosstalk (type 6) (our unpublished data).

While the cohesin complex is responsible for chromosome cohesion establishment as well as maintenance, in yeast cohesion is additionally conserved by the regulator Pds5 [110]. During mitosis, phosphorylation of the yeast SENP2 homologue Ulp2 by its PLK1 homologue Cdc5 inhibits its deSUMOylation activity (positive crosstalk type 6) [111]. Subsequently, SUMOylation of Pds5 peaks during mitosis and promotes sister chromatin separation [112].

Although modification of some APC/C subunits by ubiquitylation or phosphorylation has already been shown to affect complex formation, almost all subunits of the APC/C have been identified as targets for ubiquitylation as well as phosphorylation by now [15,35-37,58-60,113-116]. Due to this extent, the question arises whether all APC/C subunits are regulated in a similar manner, potentially influencing complex stability by complex group modification. Besides being regulated by the balance between (de)phosphorylation and (de)ubiquitylation, the APC/C is modified by other PTMs. Although SUMOylation of various subunits has been identified upon different treatments [64,65], APC4 is the only subunit modified by SUMO under control conditions (our unpublished data). Furthermore, the subunits APC1, APC4, APC8 and Cdc20 have been found to be monomethylated [32-34,117] and acetylation was observed of APC7, APC8, APC10 and APC12 [31,35,118]. However, whether these modifications are part of a crosstalk mechanism by influencing the activity of the APC/C and thereby

affecting ubiquitylation of its substrates or have other functional relevance, remains to be determined.

Telophase/cytokinesis: crosstalk to complete cell division

After promoting chromosome segregation, a shift in the balance between phosphorylation and dephosphorylation facilitates exit from mitosis (Figure 6A). Various crosstalk mechanisms together regulate inactivation of the kinases CDK1 and Mastl (Greatwall), while activating phosphatases PP1 and PP2A. During late mitosis, the yeast homologue of PLK1 Cdc5 phosphorylates the phosphatase Cdc14, which releases it from its binding partner Cfi/Net1 [119]. Subsequently, Cdc14 is able to dephosphorylate various targets, including Sic1, Swi5 and Cdh1 (negative crosstalk type 6) [120]. As phosphorylation of Sic1 primes the protein for recognition by ubiquitin E3 ligases, ubiquitylation and consequently proteosomal degradation (positive crosstalk type 8), dephosphorylation by Cdc14 stabilizes Sic1 protein levels [120]. Dephosphorylation of Swi5 promotes its transport into the nucleus, where it stimulates Sic1 transcription [82]. Subsequently, the accumulated protein levels of dephosphorylated Sic1 help to inactivate CDK1. Inactivation of CDK1, via dephosphorylated Sic1 and ubiquitin-dependent cyclin B degradation, prevents Cdh1 phosphorylation (negative crosstalk type 10 and 6 respectively) [121]. As Cdh1 phosphorylation on distinct sites is responsible for cell cycle-regulated localization as well as preventing its binding to APC/C, dephosphorylation by Cdc14 promotes the replacement of Cdc20 and the APC/C^{cdh1} complex is formed [108,120,122]. Subsequently, this complex ubiquitylates specific substrates, including Cdc20, and thereby promotes their proteosomal degradation (positive crosstalk type 6) [123]. Decreased CDK1 levels additionally promote PP1 activity, which counteracts phosphorylation of

Mastl by CDK1 [124,125]. The resulting inactivation of Mastl opposes PP2A inactivation by phosphorylation of Arpp-19 (negative crosstalk type 6), which is essential for mitotic exit. Degradation of particular microtubule-, kinetochore- and centromere-related proteins helps mitotic kinases and phosphatases to relocalize the CPC to the spindle midzone and regulates the mitotic spindle process during telophase. The central spindle contains bundled plus-ends of antiparallel microtubules that form an organized structure [68]. Although the nuclear envelope already begins to reform and the DNA decondenses during telophase, cell division is not completed until cytokinesis.

Cytokinesis requires the selection of an appropriate abscission site following the assembly and constriction of an equatorial contractile ring consisting of myosin, actin and other cytoskeletal filaments [68]. Through interaction with the CPC containing the active kinase aurora B, various accessory proteins locate to the division site during cytokinesis. Although the mechanistic details of contractile ring constriction and abscission in animal cells are not completely understood [68,126,127], the process is regulated by crosstalk between PTMs at the abscission site [128,129]. Ubiquitylation of PLK1 and aurora A by the APC/C^{cdh1} complex targets the kinases for proteasomal degradation in anaphase, which results in decreased substrate phosphorylation (negative crosstalk type 6) and allows formation of a mature spindle midzone [130,131]. As discussed above, ubiquitylated aurora B results in removal of the kinase from centromeres. Upon degradation of aurora A, ubiquitylated aurora B relocates to the spindle midzone and guides cytokinesis by phosphorylation of various targets, including centralspindlin (positive crosstalk type 6) [94]. Phosphorylation of this latter protein complex promotes its clustering and accumulation on the plus ends of antiparallel microtubules [132,133]. The spindle midzone matures into the midbody and

various proteins involved in microtubule disassembly and abscission are recruited. Interestingly, a rivalry between O-GlcNAcylation and phosphorylation has been observed to regulate the CPC and CDK1 activity to promote accurate cytokinesis (negative crosstalk type 5) [134]. Methylation of dynamic microtubules by the histone methyltransferase Setd2 was reported to regulate cytokinesis [135]. Interestingly, methylation of alpha-tubulin K40 competes with acetylation of the same lysine residue (negative crosstalk type 5). However, the functional relevance of this crosstalk remains to be determined. Finally, various cytokinetic proteins, including anillin (the scaffold protein between actin and myosin), are degraded upon ubiquitylation by the phospho-activated APC/C^{cdh1} complex (positive crosstalk type 6) [136,137]. When the cytoplasm is divided by severing the intercellular bridge during abscission, the daughter cells are completely separated and cell division has finished.

Concluding remarks

Mitosis is an essential cellular process that is highly regulated by a variety of post-translational modifications. Due to the abundance of protein modification by mitotic kinases [7] and the frequently clear effects of phosphate conjugation as a protein activity on-off switch, the role of phosphorylation during mitosis has been well studied. Subsequently, specific ubiquitin E3 ligase complexes were found to be timely regulated to ensure proper mitotic progression by targeting proteins to the proteasome. Recent studies have shown the involvement of other PTMs, including SUMO [9], in cell cycle progression. However, as the outcome of these modifications on target protein functionality is often less predictable, determining the functional relevance of each modified target protein within the process of mitosis is challenging.

Developments in the field of mass spectrometry have enabled to make significant progress in identifying target proteins in an unbiased and proteome-wide manner [138,139]. However, functional analysis will have to reveal the physiological relevance of each modification on each target protein. Within this process, we will have to keep the high levels of crosstalk between PTMs, specifically during mitosis, in mind (see Outstanding Questions).

Throughout this review we have discussed the different types of crosstalk and found examples of the diverse ways in which PTMs communicate during mitotic progression. The cell does not simply use a single general crosstalk principle during this essential process, but rather combines them into a complex crosstalk network. Many key mitotic regulators are modifying enzymes themselves, which contain reader domains for specific modifications (types 8 and 10) or whose activity is regulated by other PTMs (type 6). As the role of these important proteins has generally been studied intensively, some of the resulting crosstalk has been identified in the process. On the contrary, our understanding of the role of modifications on PTMs themselves (type 7) or even the presence of mixed chains during mitosis is very limited. Although the existence and function of single modifications on individual proteins has been and still is investigated extensively, studying multiple modifications and their crosstalk is extremely challenging.

The stoichiometry of certain single modifications, such as SUMOylation, is generally low. Depending on the type of crosstalk involved, the abundance of the co-modified substrate might be even lower. As a result, demonstrating the co-occurrence of PTMs on a protein can be technically difficult. This contrasts with the

high stoichiometry of phosphorylation observed during mitosis for CDK1 and CDK2 substrates [7].

Currently, many purification methods rely on protein digestion before purification of modified peptides and identification by mass spectrometry. Although some co-modified peptides can be identified, most crosstalk information will be lost as a result of the low abundance of co-modified peptides amongst the single modified peptides and the fact that most modifications will be present on different peptides due to the distance between communicating PTMs. Top-down mass spectrometry using intact proteins would be needed to address this. Unfortunately, the sensitivity of this technique is limited. The development of novel strategies to purify and identify proteins co-modified by two distinct PTMs will provide new insights into potential substrates of positive crosstalk.

Recently, we developed a methodology to purify co-modified proteins before their digestion for mass spectrometry analysis enabling the identification of many proteins simultaneously modified by ubiquitin and SUMO [96]. As this strategy can be applied to various combinations of small protein PTMs, it could provide more insight into the co-occurrence of certain modifications on proteins. However, the identification of direct competition or crosstalk between PTMs on different proteins is still very challenging as both modifications are not present at the same time or on a single substrate, eliminating the use of these co-modification purification strategies. To get a better overview of these crosstalk mechanisms, new methods are required that are specifically developed to detect these types of communication between PTMs.

Upon the identification of a list of proteins with co-modification by different PTMs, further analyses for each individual substrate will have to determine whether

direct crosstalk is involved. These functional experiments are generally challenging due to the complex nature of crosstalk. Ideally, genome editing by CRISPR-Cas9 enables us to mutate the acceptor site of the suspected first modification, while the presence or absence of the second modification could be used as a readout. However, this strategy requires knowledge about the involved proteins, acceptor sites and a potential crosstalk mechanism. Additionally, it is less useful to study direct competition between PTMs on the same acceptor site, promiscuous modifications that will 'jump' to an adjacent acceptor site or redundant PTMs involved in group modification of a certain complex. To study crosstalk involving these modifications, the catalytic domain of the suspected modifying or demodifying enzyme could be fused to the substrate to affect the modification of all surrounding proteins and thereby potentially the whole complex. Although this could be an interesting method to study group modifications and their involvement in crosstalk, we have to keep in mind that the results are less specific and could be indirect. Finally, it would be useful to be able to distinguish between redundant and non-redundant PTM events (see Outstanding Questions). Also, some modifications might act individually, not affect protein functionality at all or only under certain conditions. Although the plethora of hypotheses to test and complex crosstalk networks result in a challenging research area, it also provides lots of opportunities for novel discoveries and the development of new techniques.

The significant role of PTMs and their machinery in cell division has made them interesting novel targets for potential anti-cancer therapies [140-144]. Various small molecule inhibitors targeting kinases, as well as proteasome inhibitors targeting the ubiquitin system, are currently already used in the clinic [145-147]. Small compounds

targeting specific components of the ubiquitin or ubiquitin-like PTM machinery are being developed or already available, including the ubiquitin E1 inhibitor MLN7243, the SUMO E1 inhibitor ML-792 and the NEDD8 E1 inhibitor MLN4924 [148-150]. Future research will have to reveal how these inhibitors affect the high level of communication between PTMs (see Outstanding Questions). Importantly, the potential indirect effects of an inhibitor specifically targeting a cancer-promoting modification via crosstalk on other PTMs needs to be investigated. Ongoing detailed functional investigation of PTM crosstalk in mitosis might form a sound basis for translational research, aiming to exploit tumor cell dependency on a functioning mitotic machinery. Ultimately, detailed understanding of cell type and tumor type specific dependency on PTM machinery could be exploited in combination therapies to eradicate tumor cells with limited toxicity due to reduced side effects.

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Figure Legends

Figure 1. Conjugation and deconjugation of target proteins by post-translational modifications. (A) Chemical post-translational modification (PTM) of a target protein is regulated via a single step responsible for protein modification and a single step resulting in protein demodification. (B) Modification by small proteins, including ubiquitin-like proteins (Ubl) such as SUMO, occurs via an enzymatic cascade involving specific activating E1, conjugating E2 and ligating E3 enzymes. This process is reversible via PTM specific proteases.

Figure 2. Regulation of chromosome condensation by crosstalk between PTMs. (A) The supercoiling activity of condensin II is inhibited via interplay between phosphorylation and ubiquitylation on the subunit CAP-H2 to prevent chromosome condensation during interphase (positive crosstalk). (B) To establish chromosome condensation, the CAP-D3 subunit of condensin II is phosphorylated by CDK1. (C) The modification by CDK1 primes the complex for recognition and phosphorylation by PLK1, resulting in hyperactivation to maintain chromosome condensation throughout mitosis (positive crosstalk) . P: phosphorylation, Ub: ubiquitylation.

Figure 3. Regulation of kinetochore complex formation by crosstalk between PTMs. (A) Priming of the centromeric chromatin via positive crosstalk between four different PTMs recruits CENP-A (C-A) to form the inner centromere. (B) Modification of CENP-I by the SUMO-targeted ubiquitin ligase RNF4 influences recruitment of various CENP complexes and thereby the constitutive centromere-associated network (CCAN) at the inner kinetochore. (C) Negative as well as positive crosstalk

between multiple PTMs collectively regulates formation of the outer kinetochore, which is responsible for microtubule binding. Ub: ubiquitylation, M: methylation, A: acetylation, P: phosphorylation, S: SUMOylation, CPC: chromosomal passenger complex, aur B: aurora B, MTs: microtubules.

Figure 4. Regulation of chromosome alignment by crosstalk between PTMs. (A) Positive crosstalk between SUMOylation and phosphorylation promotes the binding of survivin along the length of the chromosomes (1), while phosphorylation-dependent phosphorylation via an enzymatic reader recruits borealin via Sgo1/2 (2). At the inner centromere, where both histone modifications overlap, the chromosomal passenger complex (CPC) is active (3). (B) Incorrect microtubule-kinetochore attachments activate error correction (4) and the spindle assembly checkpoint (5) via aurora B and Mps1 (dashed lines). Upon correct bi-orientation, recruitment of Set1 and phosphatases PP2A and PP1 in combination with the increased kinetochore tension counteracts the activity of these kinases (uninterrupted lines). When Mad2 phosphorylation results in a release of Cdc20 from the mitotic checkpoint complex (MCC), the spindle assembly checkpoint is inactivated and anaphase is initiated via ubiquitylation of APC/C substrates (positive crosstalk). P: phosphorylation, S: SUMOylation, Ub: ubiquitylation, M: methylation.

Figure 5. Regulation of chromosome segregation by crosstalk between PTMs. While ubiquitylation of aurora B results in reduced phosphorylation at the centromere (negative crosstalk) and thereby changes complex composition, SUMO-targeted ubiquitin ligation by RNF4 regulates chromosome segregation via proteasomal

degradation of mitotic regulators such as Mis18bp1 (positive crosstalk) (1). Formation and activation of APC/C^{cdc20} via positive crosstalk results in ubiquitylation and degradation of securin and cyclin B, which activates separase to cleave the cohesin complexes between centromeres and thereby facilitate chromosome segregation (2). S: SUMOylation, Ub: ubiquitylation, P: phosphorylation, CPC: chromosomal passenger complex.

Figure 6. Regulation of the final stages of cell division by crosstalk between PTMs. (A) Ubiquitylation of cyclin B in combination with phosphorylation of Cdc14 results in inactivation of CDK1 and dephosphorylation of Cdh1 via interconnected crosstalk mechanisms. Subsequently, Cdh1 is able to replace Cdc20 as the APC/C cofactor and phosphatases PP1 and PP2A become activated, promoting progression through the final stages of mitosis. (B) Cytokinesis and abscission is regulated via negative crosstalk by ubiquitin-dependent degradation of mitotic kinases, positive crosstalk via aurora B on centralspindlin as well as direct competition between methylation and acetylation on alpha-tubulin. S: SUMOylation, Ub: ubiquitylation, P: phosphorylation, M: methylation, A: acetylation, aur A: aurora A, aur B: aurora B.

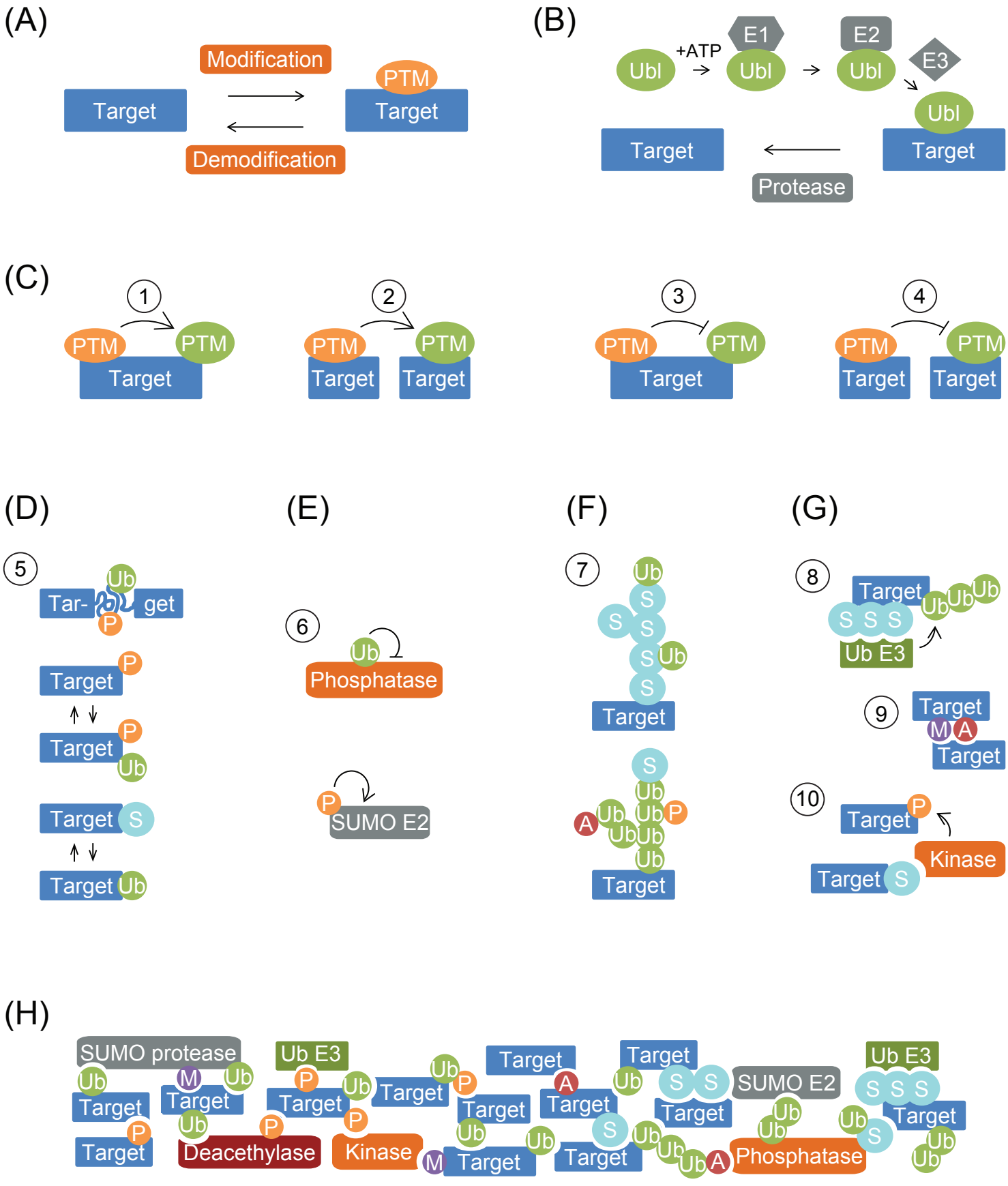
(A)
Modification
Target
PTM
Target
Demodification
(B)
Ubl
+ATP
E1
Ubl
Ubl
E2
Ubl
E3
Ubl
Target
Target
Protease
(C)
1
PTM
PTM
Target
2
PTM
PTM
Target
Target
3
PTM
PTM
Target
4
PTM
PTM
Target
Target
(D)
5
Ub
Tar-
get
P
Target
P
Target
P
Ub
Target
S
Target
Ub
(E)
6
Ub
Phosphatase
P
SUMO E2
(F)
7
Ub
S
S
S
S
Ub
S
Target
S
Ub
A
Ub
Ub
P
Ub
Ub
Ub
Target
(G)
8
Target
Ub
Ub
Ub
S
S
S
Ub E3
9
Target
M
A
Target
10
Target
P
Kinase
Target
S
(H)
SUMO protease
Ub
Target
M
Target
Ub
P
Target
Deacethylase
Ub E3
P
Ub
Target
P
Target
Kinase
Ub
P
Target
Target
A
Target
Ub
Ub
Target
M
Target
Target
Ub
S
S
Target
SUMO E2
Ub
Ub
Phosphatase
Ub E3
S
S
S
Target
Ub
S
Ub
Ub
Ub
A

Figure 1

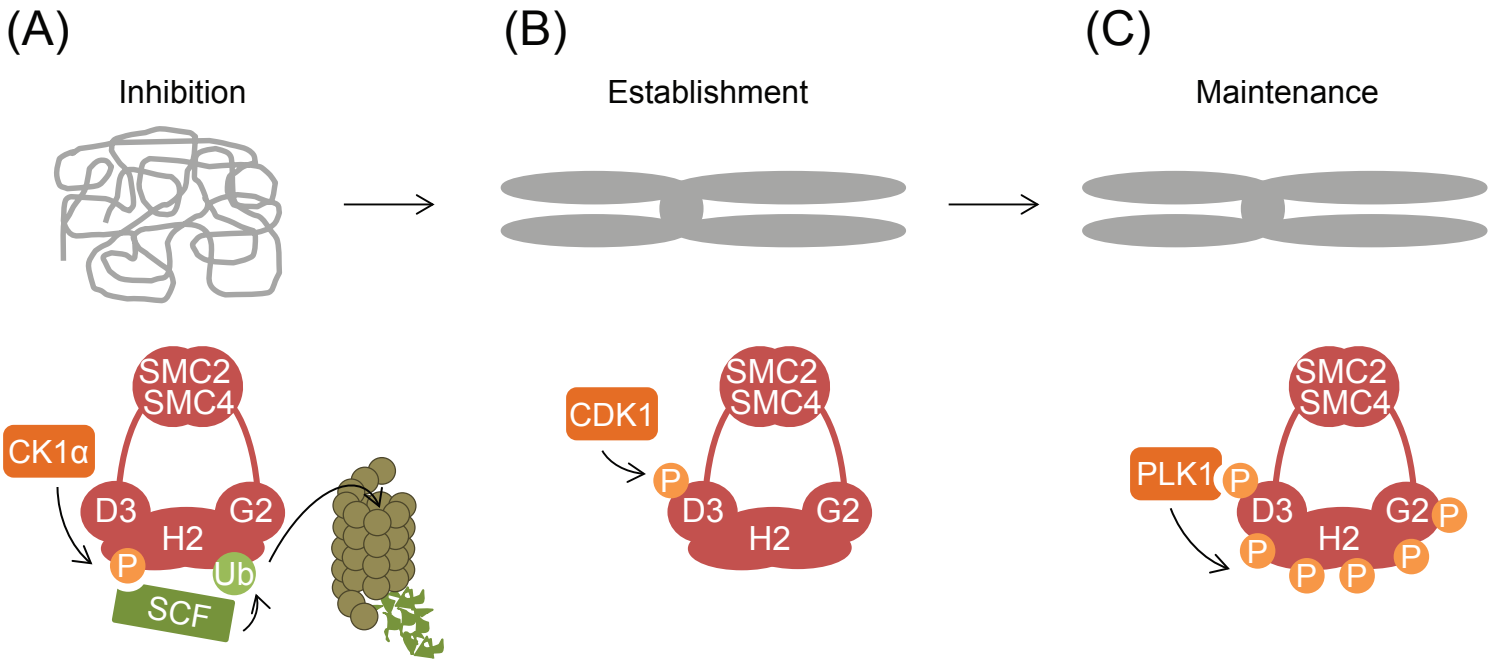
(A)
Inhibition
(B)
Establishment
(C)
Maintenance
SMC2
SMC4
CK1α
D3
H2
G2
P
Ub
SCF
CDK1
PLK1

Figure 2

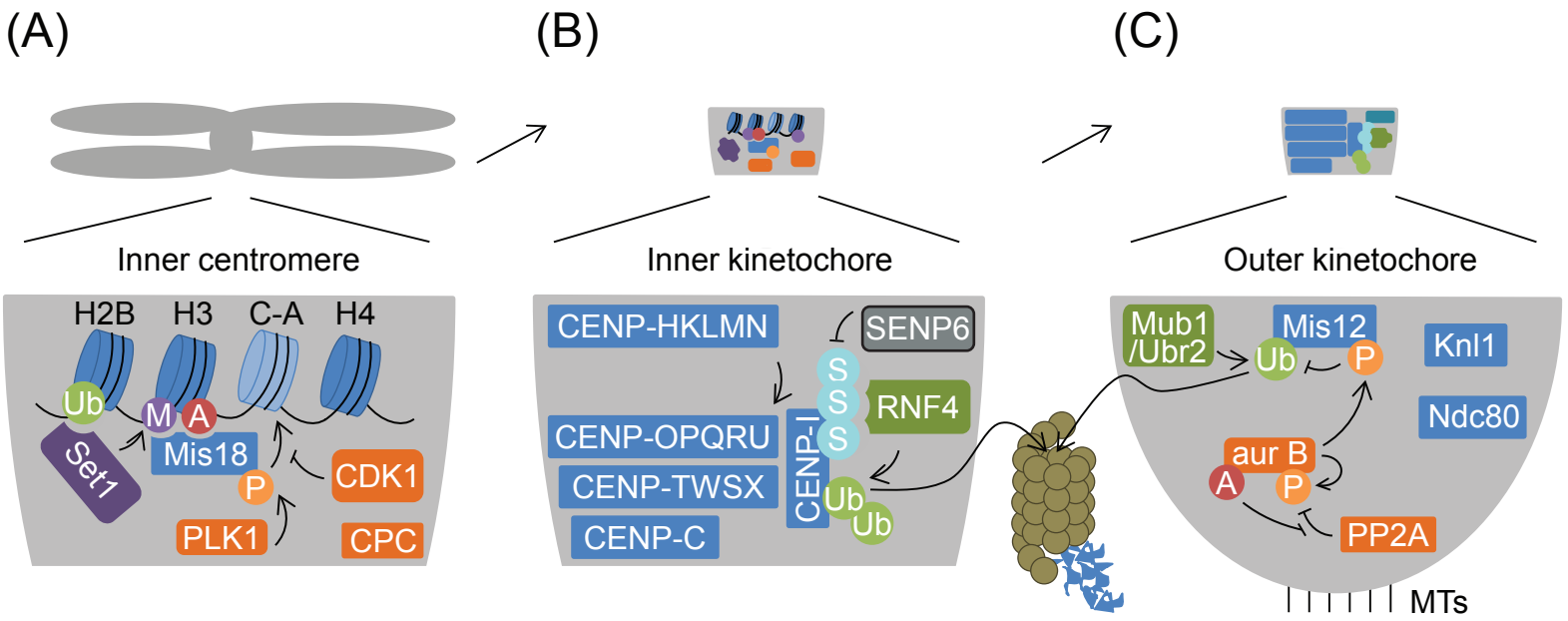
(A)
(B)
(C)
Inner centromere
Inner kinetochore
Outer kinetochore
H2B
H3
C-A
H4
Ub
M
A
Set1
Mis18
P
CDK1
PLK1
CPC
CENP-HKLMN
SENP6
S
S
S
RNF4
CENP-OPQRU
CENP-I
CENP-TWSX
Ub
Ub
CENP-C
Mub1
/Ubr2
Mis12
Ub
P
Knl1
Ndc80
aur B
A
P
PP2A
MTs

Figure 3

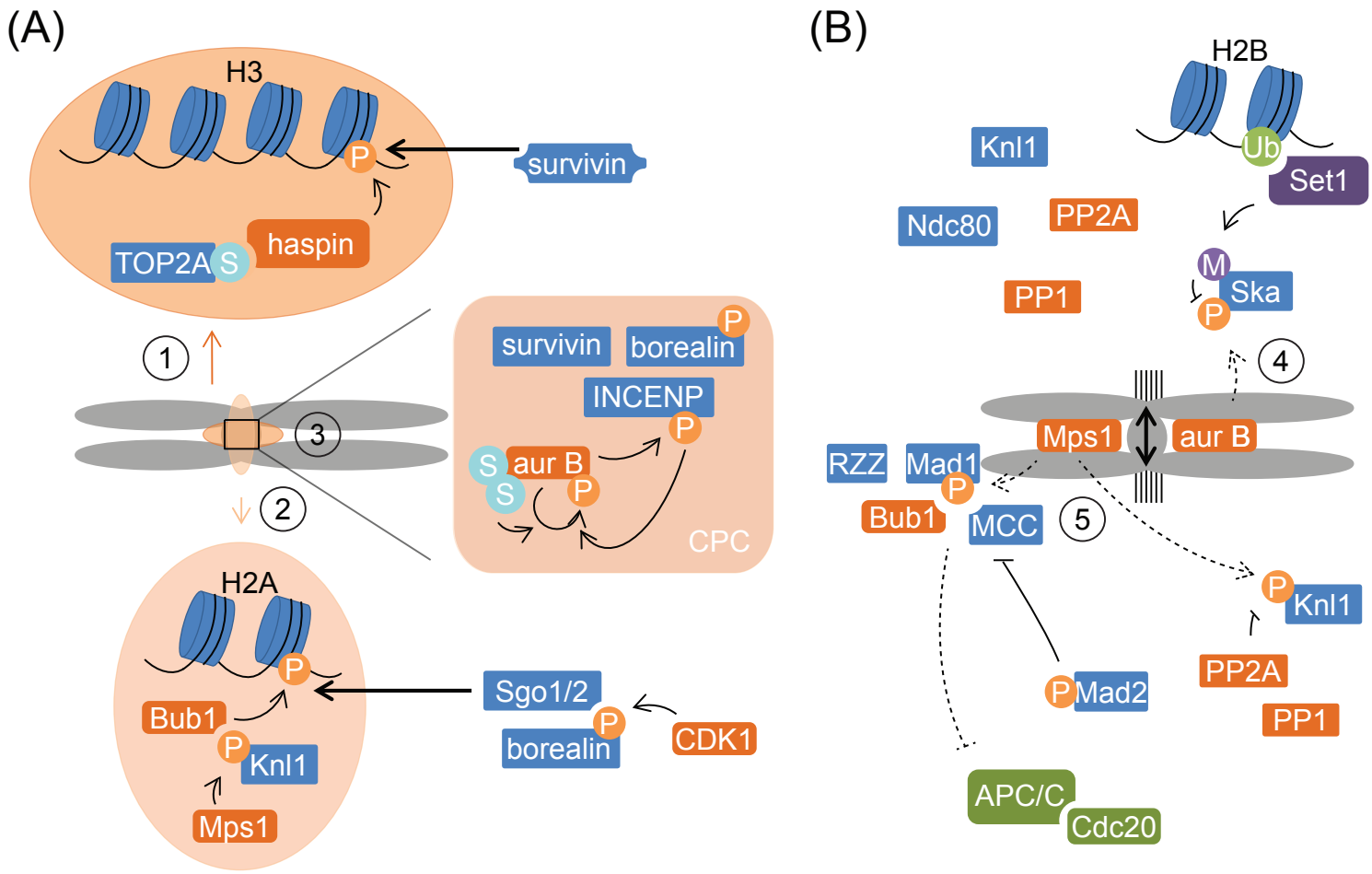
(A)
H3
P
survivin
TOP2A
S
haspin
1
2
3
survivin
borealin
P
INCENP
P
S
aur B
S
P
CPC
H2A
P
Bub1
P
Knl1
Mps1
Sgo1/2
P
borealin
CDK1
(B)
H2B
Ub
Set1
Knl1
Ndc80
PP2A
M
Ska
P
PP1
4
Mps1
aur B
RZZ
Mad1
P
Bub1
MCC
5
P
Knl1
PP2A
PP1
P
Mad2
APC/C
Cdc20

Figure 4

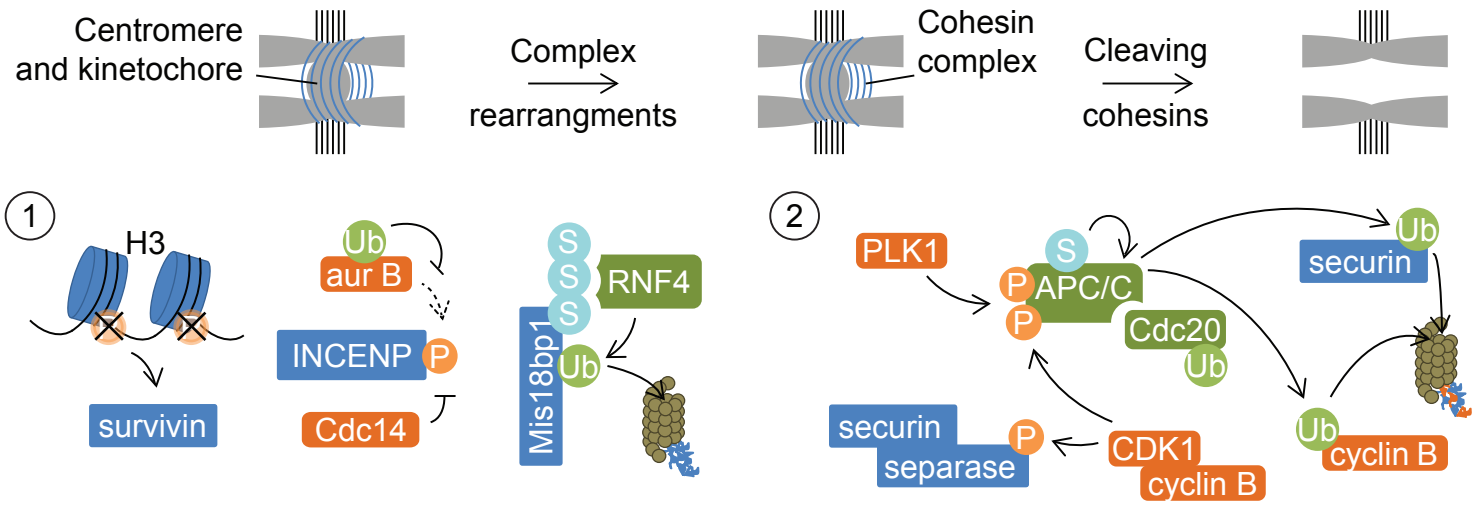
Centromere
and kinetochore
Complex
rearrangments
Cohesin
complex
Cleaving
cohesins
1
H3
survivin
Ub
aur B
INCENP
P
Cdc14
S
S
S
RNF4
Mis18bp1
Ub
2
PLK1
S
P
P
APC/C
Cdc20
Ub
Ub
securin
securin
P
separase
CDK1
cyclin B
Ub
cyclin B

Figure 5

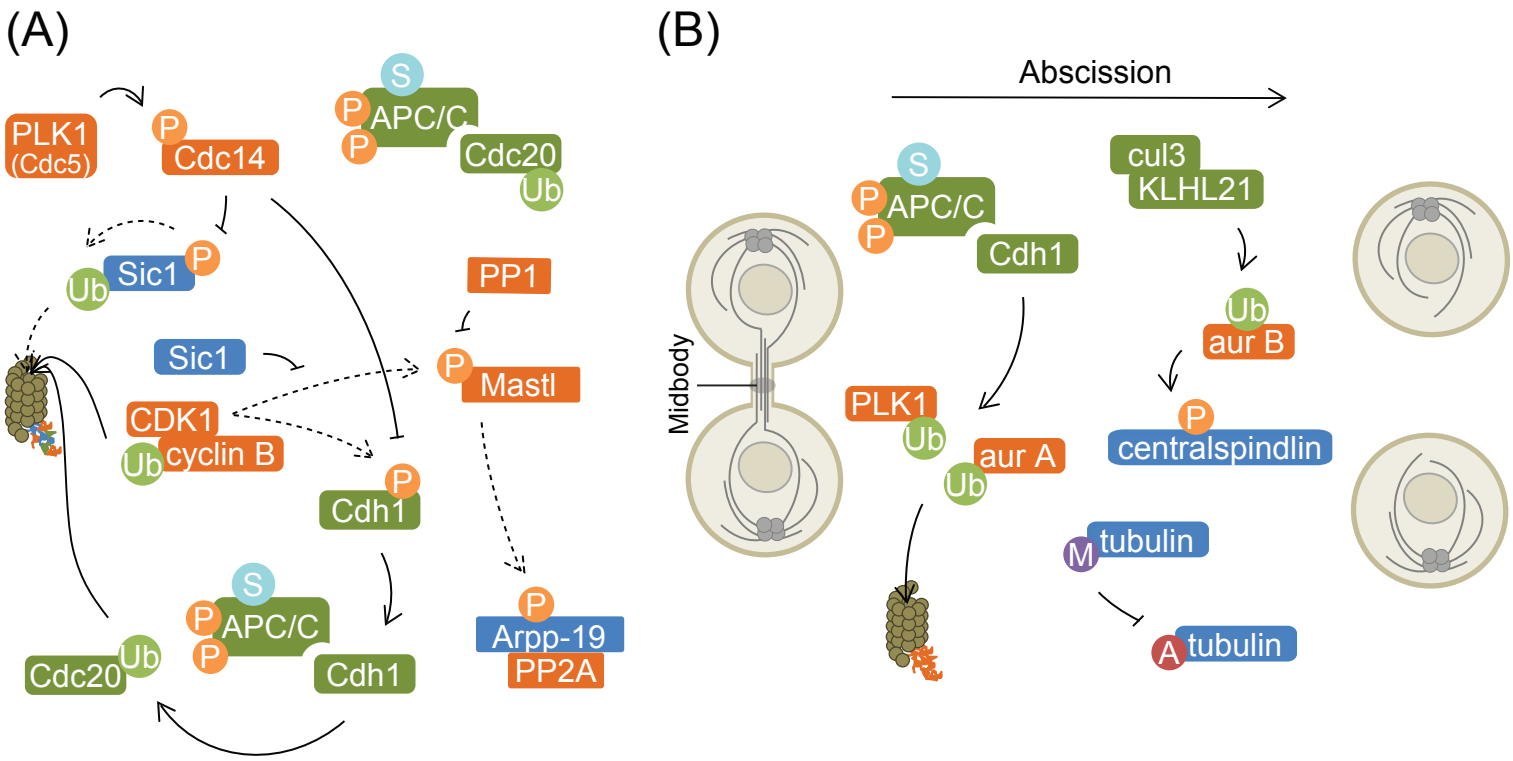
(A)
PLK1
(Cdc5)
P
Cdc14
S
P
APC/C
P
Cdc20
Ub
Ub
Sic1
P
PP1
Sic1
P
Mastl
CDK1
Ub
cyclin B
P
Cdh1
S
P
APC/C
P
Cdc20
Ub
Cdh1
P
Arpp-19
PP2A
(B)
Abscission
Midbody
S
P
APC/C
P
Cdh1
cul3
KLHL21
Ub
aur B
P
centralspindlin
PLK1
Ub
aur A
Ub
M
tubulin
A
tubulin

Figure 6