Dynamic ubiquitin signaling in cell cycle regulation

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The cell division cycle is driven by a collection of enzymes that coordinate DNA duplication and separation, ensuring that genomic information is faithfully and perpetually maintained. The activity of the effector proteins that perform and coordinate these biological processes oscillates by regulated expression and/or posttranslational modifications. Ubiquitylation is a cardinal cellular modification and is long known for driving cell cycle transitions. In this review, we emphasize emerging concepts of how ubiquitylation brings the necessary dynamicity and plasticity that underlie the processes of DNA replication and mitosis. New studies, often focusing on the regulation of chromosomal proteins like DNA polymerases or kinetochore kinases, are demonstrating that ubiquitylation is a versatile modification that can be used to fine-tune these cell cycle events, frequently through processes that do not involve proteasomal degradation. Understanding how the increasing variety of identified ubiquitin signals are transduced will allow us to develop a deeper mechanistic perception of how the multiple factors come together to faithfully propagate genomic information. Here, we discuss these and additional conceptual challenges that are currently under study toward understanding how ubiquitin governs cell cycle regulation.

Introduction

Cell proliferation is a continuous cycle of DNA synthesis and subsequent chromosome separation. Posttranslational modifications of effector proteins ensure that these major events and their transitions are orchestrated so that genomic information is preserved. The covalent conjugation of the small protein ubiquitin through a process called ubiquitylation plays a critical role in the overall regulation of cell division. It is well established that ubiquitylation is a signal for protein degradation by the proteasome (Fig. 1, A and B), with special importance in assuring ordered and well-timed cell cycle transitions (Teixeira and Reed, 2013; Bassermann et al., 2014). However, ubiquitylation is not necessarily linked to protein degradation, and in recent years, an increasing number of nonproteolytic outcomes of protein ubiquitylation have been reported to play important cellular roles (Komander and Rape, 2012). Proteasome-independent regulation of an ubiquitylation target is achieved by changes in protein–protein interactions, subcellular localization, or enzyme activity (Fig. 1 B). As opposed to the irreversible fate of degradation, nonproteolytic outcomes of ubiquitylation allow for functional fine-tuning, dynamically and reversibly responding to intracellular cues instead of requiring de novo protein synthesis.

Ubiquitin conjugation to its targets requires the concerted action of an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase. The latter binds specifically to the substrate and promotes the transfer of ubiquitin to one of its lysine residues (see text box for an overview of E3 ligases involved in cell cycle regulation). Because of multiple reactive sites on ubiquitin, more moieties may be added, establishing complex oligomers or chains (Fig. 1 A). This enables that multiple ubiquitin topologies generate individual signals, which are collectively referred to as the ubiquitin code (Komander and Rape, 2012). This code is read by downstream factors containing ubiquitin-binding domains, referred to as readers or decoders, which specifically recognize the chain topology and induce the appropriate signal (Husnjak and Dikic, 2012). For example, a polyubiquitin chain in which ubiquitin conjugates via its lysine-48 (K48) and/or K11 residues is read and as a result rapidly degraded by the 26S proteasome, an irreversible process that is often observed in cell cycle transitions (Grice and Nathan, 2016). Conversely, a monoubiquitin moiety or K63-linked chain can recruit factors that allow for a specific localized response, such as the recruitment of a DNA damage–tolerant polymerase to a site of replication stress (García-Rodríguez et al., 2016). In many cases, ubiquitylated proteins first need to be extracted from interacting partners or chromatin, a function typically attributed to the ATPase valosin-containing protein (VCP)/p97 (Cdc48 in yeast; Meyer et al., 2012; Franz et al., 2016). Importantly, specific proteases termed deubiquitylating enzymes (DUBs) can cleave off ubiquitin moieties and reverse the signal (Lim et al., 2016).

In this review, we summarize the main ubiquitin-mediated regulatory mechanisms that are believed to fine-tune DNA replication and segregation. We emphasize how E3 ubiquitin ligases orchestrate these processes in space and time, with

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Abbreviations used: APC/C, anaphase-promoting complex/cyclosome; CRL, cullin-RING E3 ligase; DUB, deubiquitylating enzyme; ESCRT, endosomal sorting complexes required for transport; FACT, facilitates chromatin transcription; MCC, mitotic checkpoint complex; PCNA, proliferating cell nuclear antigen; SAC, spindle assembly checkpoint; SLBP, stem-loop binding protein; UBD, ubiquitin-binding domain; VCP, valosin-containing protein.

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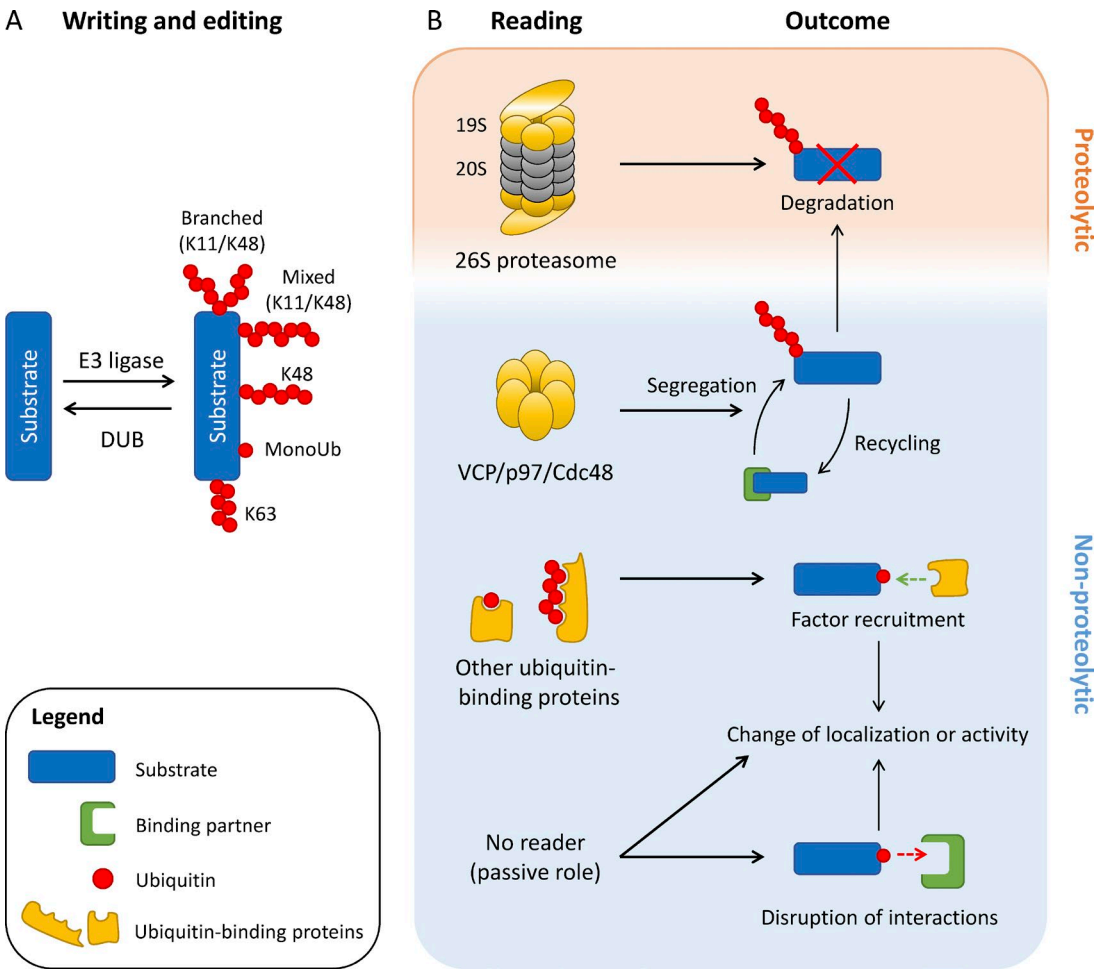


Figure 1. **Writing, reading, and editing ubiquitin.** (A) Ubiquitin is added to a substrate protein (writing ubiquitin) by E3 ligases, and ubiquitin moieties can be removed (editing ubiquitin) by deubiquitylating enzymes (DUBs). Protein degradation is mostly associated with polyubiquitin chains, in which ubiquitin moieties attach to each other via homotypic lysine-48 (K48) linkages or heterotypic K11/K48 linkages (mixed chain or branched; see Ubiquitin signals produced by CRLs and the APC/C text box). The result of K63-linked polyubiquitylation is distinct and, together with monoubiquitylation (monoUb), it is associated with nonproteolytic outcomes. (B) Ubiquitylation produces a signal that is often dependent on effector proteins or complexes (ubiquitin readers). These include the proteasome, which is a proteolytic machine, or the segregase VCP/p97 (Cdc48 in yeast), which extracts proteins from chromatin, cellular compartments, or protein complexes for recycling or degradation. Other ubiquitin-binding proteins can fulfill a specific function with nonproteolytic outcomes when they are recruited to ubiquitylated substrates (e.g., damage tolerance by error-prone polymerases), potentially altering the localization or activity of the ubiquitylated substrate. By affecting protein interactions or conformations, ubiquitylation may directly alter protein localization or activity. A challenge in present research is to distinguish between a passive effect of ubiquitylation and the action of an unidentified ubiquitin reader.

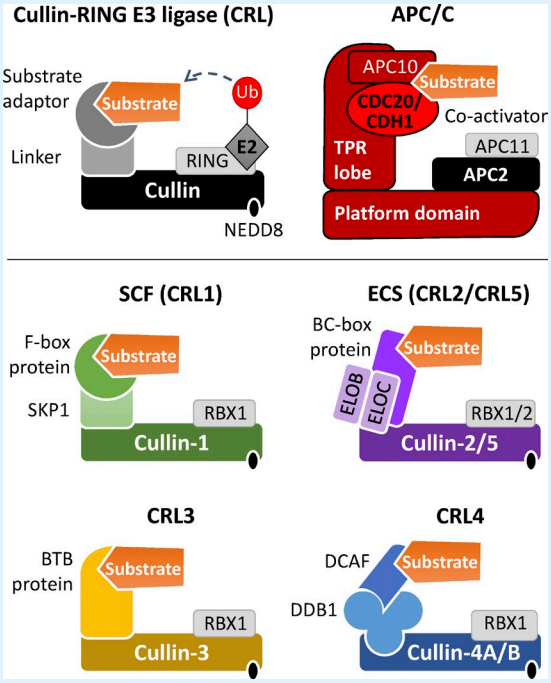
a special focus highlighting nonproteolytic consequences of ubiquitylation. We aim to pinpoint current research challenges and suggest novel research approaches to decipher the complex ubiquitin-dependent network orchestrating cell cycle regulation.

Dynamic control of DNA replication by ubiquitin

A cell duplicates its genomic information during S phase. Synthesis of the complementary DNA strands begins at localized replication origins, which are established during mitosis and G1 during replication licensing (Fragkos et al., 2015). After DNA duplex unwinding by the replicative helicase, the polymerases (Pol) Polε and Polδ elongate the "leading" and "lagging" DNA strands (Fig. 2 A). Once the duplication of a DNA stretch is complete, replication is terminated and components are removed from chromatin. Ubiquitylation impacts all stages of DNA replication (Moreno and Gambus, 2015; García-Rodríguez et al., 2016). Past research has focused on the global degradation of replication effectors when their function is no longer needed. For example, to prevent rereplication by prematurely assembling origins on newly replicated DNA, replication licensing factors are degraded in S phase, and cells degrade DNA replication factors such as the nuclease FEN1 after replication is complete (Guo et al., 2012; Moreno and Gambus, 2015). Altogether, the prevailing paradigm suggests that degradation of replication effectors is required to restrict their function to a narrow temporal window.

Regulation of lagging-strand synthesis. In recent years, localized proteolytic and several nonproteolytic ubiquitin-mediated regulatory processes have been discovered to regulate replication (Table 1 summarizes nonproteolytic cell cycle ubiquitylation events). An example of replication fine-tuning through selective and localized degradation arises during the process of lagging-strand synthesis (Fig. 2 B). The discontinuous synthesis of DNA requires a constant exchange of factors to prime, elongate, process, and ligate the so-called Okazaki fragments. Priming is performed by Polα, which synthesizes a RNA primer that is removed during the maturation step. Polδ

Cullin-RING and APC/C E3 ligases



Of the three described E3 ligase families, HECT, RING, and RING-between-RING E3 ligases (Spratt et al., 2014), the bulk of cell cycle regulation is performed by RING E3 ligases. In particular, the major family Cullin-RING E3 ligases (CRLs) and the anaphase-promoting complex/cyclosome (APC/C) take up most known cell cycle ubiquitylation events. CRLs use one of the six cullin proteins encoded by the human genome as a scaffolding subunit that brings together the ubiquitin-loaded E2 enzyme and the substrate. The E2 enzyme is recruited by the C-terminally bound RING subunit (RBX1 or RBX2). Substrates associate to CRLs via an N-terminal receptor module composed of a variable substrate-specific adaptor and a cullin-bound linker subunit, except in the case of CRL3. CRLs are activated by modification with NEDD8, termed neddylation, and they associate dynamically with regulators that modulate the neddylation state, block substrate access, or promote substrate receptor release and exchange (Lydeard et al., 2013). CRLs are thus regarded as modular, dynamic assemblies with substrate-specific adaptors that associate and dissociate in a regulated manner to ensure timely and specific substrate ubiquitylation (Craney and Rape, 2013). Specific adaptors are linked to individual cullins. CRL1 or SCF (SKP1–CUL1–F-box) E3 ligases contain an F-box protein; CRL3 contains a Broad complex, Tramtrack, and Bric-a-brac (BTB) domain–containing protein; and CRL4 has a DDB1- and CUL4-associated factor (DCAF) protein (Lydeard et al., 2013). Subdivided into CRL2 and CRL5, the Elongin B-C–CUL2/CUL5–SOCS box protein (ECS) E3 ligases recruit BC-box–containing adaptors, in particular VHL-box and SOCS-box proteins (Cai and Yang, 2016). Multiple cell cycle transitions critically depend on SCF E3 ligases, in particular for targeting degradation of cyclin-dependent kinase (CDK) inhibitors such as p27 and WEE1 at G1/S and G2/M, respectively. CRL4 complexes have been described for their functions in preventing DNA re-replication, whereas CRL3 is probably the most emergent CRL in cell cycle control, in particular by regulating mitosis. Several nonproteolytic functions of CRL3 and CRL4 complexes are now attributed (Table 1; Teixeira and Reed, 2013; Bassermann et al., 2014). DNA replication is one of the few cell cycle functions currently attributed to ECS E3 ligases (Table 1 and main text).

Although the APC/C is closely related to CRLs and contains the cullin-homology subunit APC2 (Yu et al., 1998), it is structurally divergent. The APC/C is composed of at least 14 different subunits, including the RING subunit APC11, plus one of two coactivators (CDC20 and CDH1) that also participate in substrate binding (Sivakumar and Gorbsky, 2015). The APC/C operates in mitosis and G1 and is mostly known for its ability to degrade mitotic cyclins and other mitotic factors so that chromosomes are separated and mitotic exit ensues (Zhou et al., 2016).

functions during lagging-strand synthesis for consecutive extension of the primer and also for gap-filling during nick translation, a far less processive event (Zheng and Shen, 2011). It appears that in humans the composition of the four-subunit Polδ enzyme (Polδ4) is altered in order to promote this activity shift. Recent evidence argues that the cullin RING ligase (CRL) $CRL4^{CDT2}$ mediates the destruction of the regulatory p12 subunit of Polδ4 during S phase (Cullin-RING and APC/C E3 ligases text box; Zhang et al., 2013), resulting in the formation of Polδ3, which has specialized properties such as increased proofreading activity. Polδ3 was also associated with gap-filling during DNA repair (Lee et al., 2012). Hence, one model is that the conversion from Polδ4 to Polδ3 generates a polymerase that is more suitable for gap-filling during Okazaki fragment processing (Fig. 2 B), explaining how the processivity of Polδ is locally adjusted (Lin et al., 2013; Lee et al., 2014), with local Polδ4 clearance important for the proper execution of DNA replication. Moreover, there is also a role for nonproteolytic ubiquitylation in lagging-strand synthesis through modulation of protein–protein interactions. MCM10 is a replication fork scaffolding protein involved in the recruitment of the replicative polymerases. Early evidence in yeast suggested that di-monoubiquitylation of MCM10 changes its interactions. Although the affinity of MCM10 for the primase Polα decreases, dimonoubiquitylation likely facilitates the recruitment of the elongating Polε/δ because of the concomitant increased MCM10 affinity to proliferating cell nuclear antigen (PCNA), the sliding clamp that brings these polymerases to DNA (Das-Bradoo et al., 2006; Thu and Bielinsky, 2014). Whether analogous mechanisms also regulate this switch in higher eukaryotes remains to be established.

Control of chromatin assembly during DNA replication. Recent work also uncovered a crucial nonproteolytic role for ubiquitin signaling in regulating the dynamic nucleosomal chromatin structure at advancing replication forks (Fig. 2, C and D). Nucleosome histones must be evicted from DNA and deposited in a semiconservative manner onto new DNA strands and the remaining gaps filled with newly synthesized histones. Thus, nucleosome assembly during S phase necessitates an adequate histone supply (Alabert and Groth, 2012), regulated through transcriptional induction and histone mRNA maturation by the processing factor stem-loop binding protein (SLBP; Fig. 2 D). Interestingly, histone mRNA processing is activated by human $CRL4^{WDR23}$ through multimonoubiquitylation of SLBP (Brodersen et al., 2016). Indeed, cells lacking WDR23 or SLBP exhibit severe DNA replication defects caused by slow replication forks, suggesting that incorporation of newly synthesized histones is tightly coupled to fork progression. How ubiquitylation mechanistically impacts SLBP function remains to be investigated, but it is conceivable that ubiquitylation regulates its binding to interacting partners or directly affects enzymatic activity (Lampert et al., 2017). After S phase, SLBP is rapidly degraded by $SCF^{cyclin\ F}$ complexes (Dankert et al., 2016), and this proteolytic destruction is critical for genome maintenance upon genotoxic stress. Thus, nonproteolytic and proteolytic regulation of SLBP by ubiquitin cooperate in space and time to restrict histone synthesis to S phase and thereby maintain genome stability.

Both histone eviction and deposition require so-called histone chaperones. Available data suggest that nonproteolytic ubiquitin signaling mediated by cullin-4 and its putative yeast homologue, Rtt101 (Zaidi et al., 2008), coordinate histone-

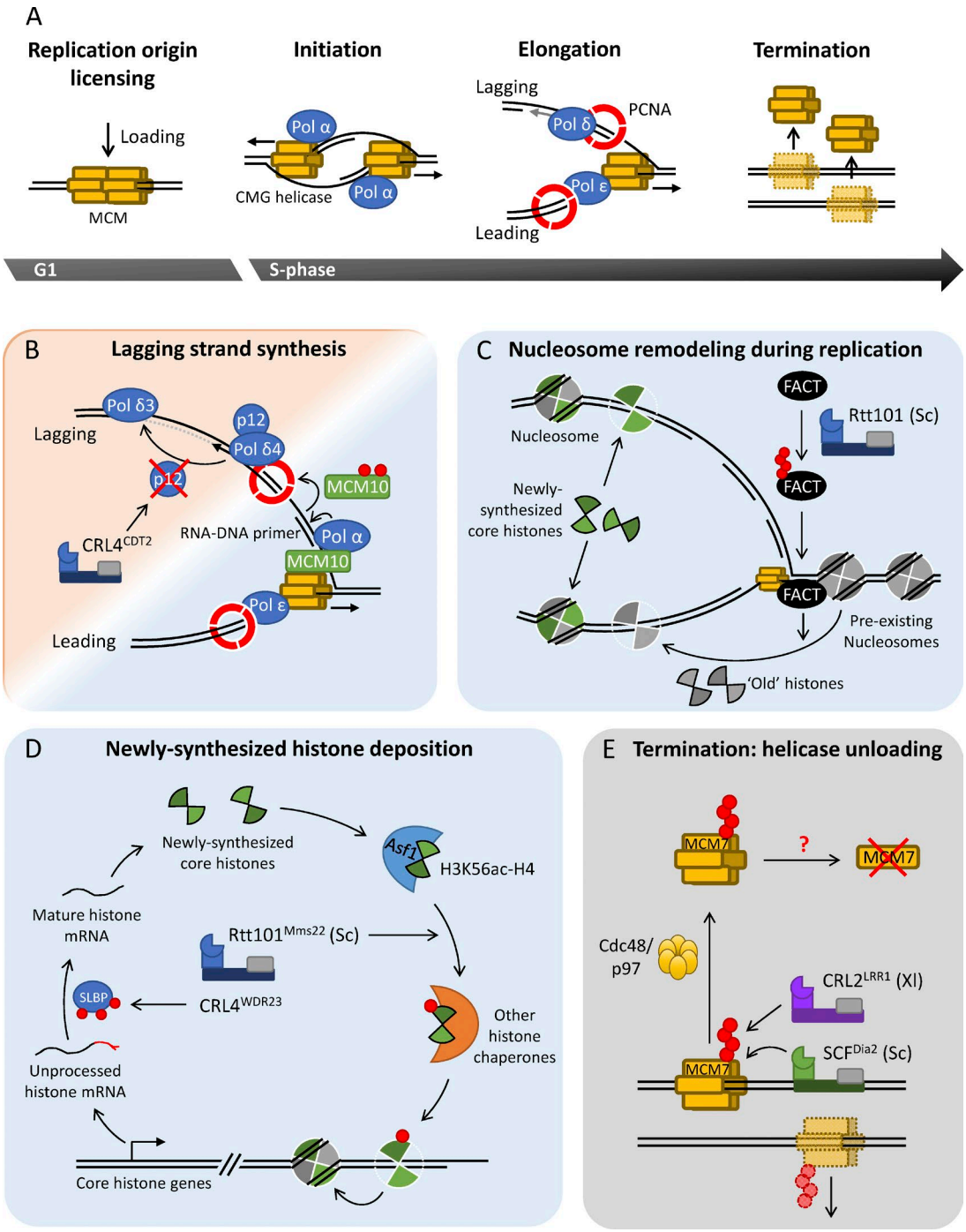


Figure 2. **The dynamic regulation of unperturbed DNA replication by ubiquitin.** Proteolytic and nonproteolytic mechanisms are depicted with light orange and light blue background, respectively, and gray if a determination is incomplete. (A) Overview of the primary events occurring during DNA replication. Activation of the active CMG helicase (CDC45, MCM hexamer, GINS complex) induces the recruitment of the sliding clamp PCNA (depicted in red), which serves as an interaction platform to tether DNA polymerases to chromatin (Moldovan et al., 2007). (B) Polymerase switches occurring in lagging strand synthesis are mediated by ubiquitylation. (C and D) Concomitant with DNA replication, nucleosomes are disassembled and reassembled in a semi-conservative manner, incorporating newly synthesized histones, which requires nondegradative ubiquitylation. (E) Termination of chromosomal replication in yeast and *Xenopus* requires Cdc48/p97 for CMG eviction from the chromatin. Red crosses depict targets of proteasomal degradation, and red circles depict ubiquitin. Ac, acetylation; Sc, *Saccharomyces cerevisiae*; Xl, *Xenopus laevis*.

related processes by acting either on histone chaperones or on histones themselves. Rtt101 is required to target the histone chaperone facilitates chromatin transcription (FACT) complex to the replication fork through nonproteolytic polyubiquitylation of the FACT Spt16 subunit (Fig. 2 C; Han et al., 2010). The same E3 ligase promotes the deposition of newly synthesized histone H3–H4 dimers by ubiquitylating new, acetylated histone H3. The consequence is a switch in interactions between H3–H4 and the respective histone chaperones that allows their loading onto nucleosomes (Fig. 2 D; Han et al., 2013). A recent study clarified that Rtt101 is indeed tethered to replisomes to locally restrict its function to the vicinity of the replication fork

(Buser et al., 2016). In humans, $CRL4^{CDT2}$ is also recruited to active forks (Havens and Walter, 2009; Havens et al., 2012) and may thus perform an equivalent function.

Unloading of the replicative helicase. Rtt101 is not the only resident E3 ligase functioning at yeast replication forks. The replisome also binds the SCF^{Dia2} E3 ligase (Morohashi et al., 2009), further underscoring the importance of local ubiquitylation of factors in the normal progression of replication forks. In the case of SCF^{Dia2}, the best described function is to promote the termination of DNA replication (Fig. 2 E). Hence, although Rtt101 is necessary during fork progression, SCF^{Dia2} rather operates when chromosomal replication is finished. Because binding of SCF^{Dia2} to the fork is important, it appears that SCF^{Dia2} in some way senses when replisome

Table 1. **Nonproteolytic ubiquitylation: Selected substrates of E3 ubiquitin ligases that operate in an unperturbed cell cycle**

Phase and substrate	E3 ligase	Chain topology	Evidence	Role of ubiquitylation (or deubiquitylation, if indicated)	Counteracting DUB	Reference
G1						
PALB2	$CRL3^{KEAP1}$	MultimonoUb?	vv (uPD), int, vt, m	Prevents BRCA1-PALB2-BRCA2 complex assembly, inhibiting homology-directed DNA repair	USP11	Orthwein et al., 2015
S						
Histone H2A	RING1A,B	MonoUb	vv (ChIP, IF)	Pericentromeric DNA replication	Multiple; not tested for this function	Bravo et al., 2015; Lim et al., 2016
Histone H2B	BRE1	MonoUb	vv (ChIP), m	Promotes nucleosome reassembly and/or stability	—	Trujillo and Osley, 2012
Histone H3	$Rtt101^{Mms22}$ (Sc); CRL4?	MultimonoUb	vv (sIP), vv (Wb, ChIP), int, vt, m	Promote H3 deposition in newly synthesized DNA	—	Han et al., 2013
MCM3	$CRL3^{KEAP1}$	MultimonoUb?	vv (sIP), int	Undetermined	—	Mulvaney et al., 2016
MCM7	SCF^{Dia2} (Sc), $CRL2^{LRR1}$ (Xl)	K48-linked (degradation uncertain)	vv (sIP, uPD), vt	Replication termination: Disassembly of the replicative CMG helicase	—	Maric et al., 2014; Moreno et al., 2014; Dewar et al., 2017
MCM10	? (Sc)	DimonoUb	vv (sIP)	Promote PCNA recruitment for elongation during DNA replication	—	Das-Bradoo et al., 2006
SLBP	$CRL4^{WDR23}$	MultimonoUb	vv (K-GG), vt, m, int	Promote histone mRNA expression	—	Brodersen et al., 2016
Spt16	Rtt101 (Sc)	K63-linked chain	vv (sIP, uPD), int, vt	Stabilizes FACT complex at replication origins to promote MCM binding	—	Han et al., 2010
S and G2						
Aurora A	$CRL3^{KLHL18}$	?	vv (sIP), int, vt	Activation of centrosomal Aurora A to promote mitotic entry	—	Moghe et al., 2012
TOP2A	BRCA1	K63-linked chain?	vv (sIP), int	Increase decatenation activity of topoisomerase IIα	—	Lou et al., 2005
TOP2A	RNF168	K63-linked chain	vv (sIP), int, vt, m	Promote DNA decatenation by increasing topoisomerase IIα chromatin association	USP10	Guturi et al., 2016
Mitosis						
Aurora B	$CRL3^{KLHL21}$	MonoUb?	int, vt, m	Promote UBASH3B-dependent Aurora B translocation to the spindle midzone in anaphase	—	Maerki et al., 2009; Krupina et al., 2016
Cyclin B1	?	K63-linked chain	vv (sIP), int	Stabilize cyclin B1	—	Zhang et al., 2015
Dishevelled DVL3	?	K63-linked chain	DUB[a]: vv (sIP, uIP), m	DUB: Promotes spindle orientation, by promoting correct localization of NuMA/dynein at the cell cortex	CYLD	Yang et al., 2014
NuMA	BRCA1?	K63-linked chain	DUB[a]: vv (sIP)	DUB: Promotes spindle assembly by stimulating the incorporation of NuMA into spindle poles	BRISC complex	Yan et al., 2015
PLK1	$CRL3^{KLHL22}$	MonoUb?	int, vt, m	Remove PLK1 from the kinetochore upon chromosome bi-orientation	USP16	Beck et al., 2013; Zhuo et al., 2015
Survivin	?	K63-linked chain	DUB[a]: vv (sIP)	DUB: Dissociates Survivin and the CPC from centromeres	USP9X	Vong et al., 2005
Late M/early G1						
CENP-A (Dm)	$CRL3^{RDX}$	?	vv (uIP), vt	Stabilize CENP-A to promote its incorporation into centromeres	—	Bade et al., 2014
CENP-A	$CRL4^{COPS8}$, $CRL4^{RBBP7}$?	MonoUb	vv (sIP), vt, m	Promote interaction with the HJURP histone chaperone and CENP-A loading at centromeres	—	Mouysset et al., 2015; Niikura et al., 2015

Shown substrates are not thought to be targeted for proteasomal degradation. Depicted E3 ligase/substrate pairs refer to human proteins, unless indicated. If known, the type of ubiquitylation topology is indicated. A question mark denotes unknown information or a speculative hypothesis. ChIP, chromatin immunoprecipitation; Dm, *Drosophila melanogaster*; IF, immunofluorescence; int, E3 ligase interaction with substrate; K-GG, ubiquitin profiling; m, mutagenesis of ubiquitylated sites (lysine to arginine); monoUb, monoubiquitylation; Sc, *Saccharomyces cerevisiae*; sIP, substrate immunoprecipitation and ubiquitin detection; uPD or uIP, ubiquitin pull-down or immunoprecipitation and substrate detection; vt, in vitro ubiquitylation assays; vv, in vivo (method indicated between parentheses); Wb, Western blot; Xl, *Xenopus laevis*; —, not described.
[a]Available evidence designates the function of the DUB, not an E3 ligase.

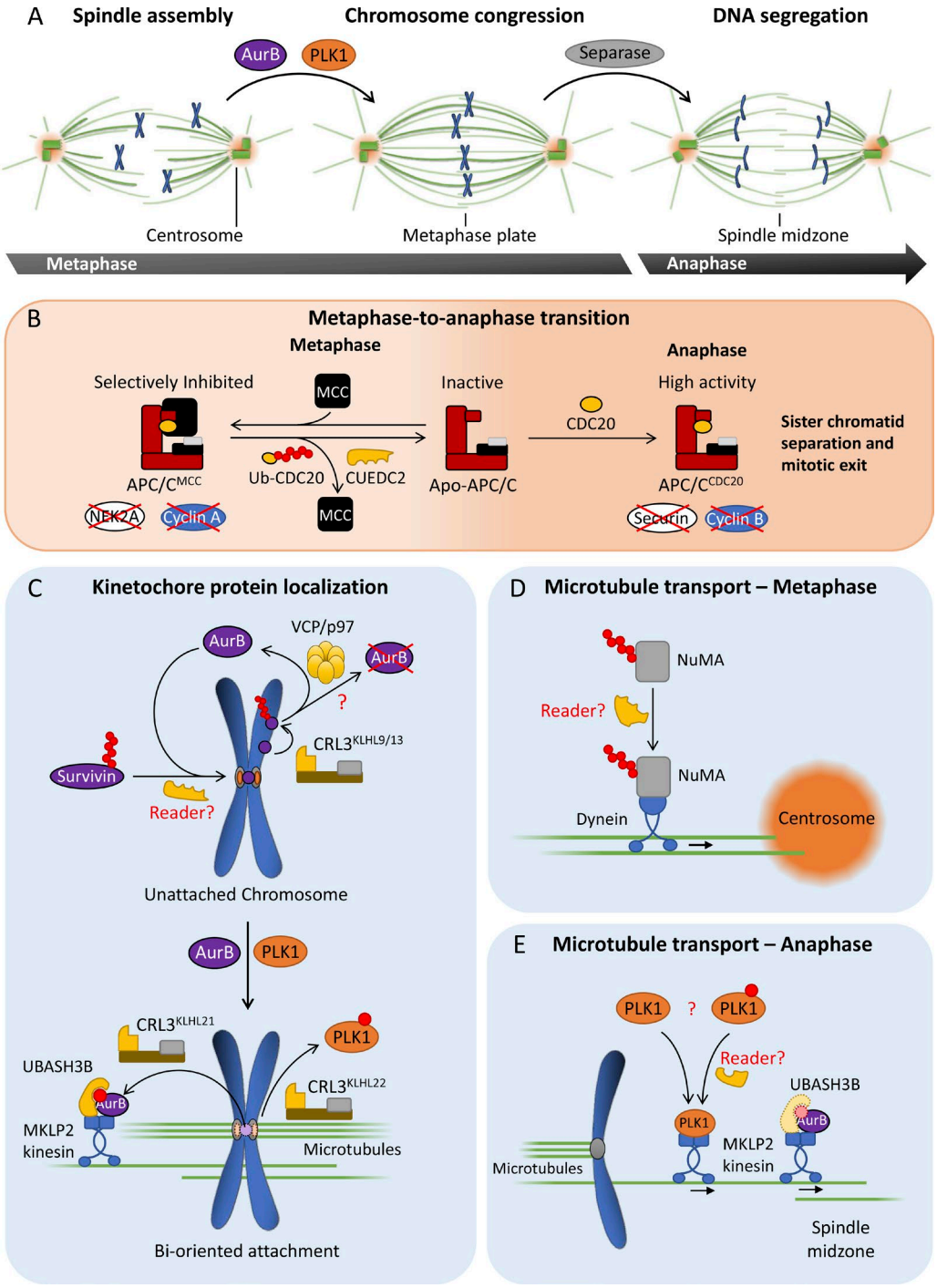


Figure 3. **Ubiquitin in the regulation of protein dynamics and localization in mitosis.** Proteolytic and nonproteolytic mechanisms are depicted with light orange and light blue background, respectively. (A) Upon mitotic entry, chromosomes condense and the cell assembles a bipolar mitotic spindle. The kinases Aurora B (AurB) and PLK1 both contribute to the establishment of correct, bioriented, kinetochore–microtubule attachments by destabilizing incorrect attachments and stabilizing correct ones, respectively. (B) Transit to anaphase occurs when the APC/C is no longer inhibited by the MCC and is activated by CDC20. MCC disassembly is promoted by autoubiquitylation of CDC20 within the APC/C-bound MCC and by the ubiquitin reader CUEDC2. The irreversibility of this transition necessitates cyclin B destruction, as otherwise the SAC is reactivated (Clijsters et al., 2014; Rattani et al., 2014; Vázquez-Novelle et al., 2014). (C) Kinetochore recruitment and exclusion of the chromosomal passenger complex (CPC), which includes Aurora B and Survivin, depend on nonproteolytic ubiquitylation. Exclusion of PLK1 from the kinetochore in case of bioriented microtubule attachments also depends on its ubiquitylation. (D and E) Microtubule transport can be promoted by cargo ubiquitylation, as is the case for the spindle assembly factor NuMA and Aurora B. Whether the ubiquitylation of PLK1 promotes its transport to the spindle midzone has not yet been determined. Red crosses depict targets of proteasomal degradation, red circles depict ubiquitin, and purple circles depict Aurora B kinase.

function is complete, after which it ubiquitylates the Mcm7 subunit of the replicative helicase (Maculins et al., 2015). Mcm7 ubiquitylation promotes the extraction of the replicative helicase from DNA by Cdc48/p97 and hence the disassembly of the entire replisome, thereby terminating replication (Maric et al., 2014; Moreno et al., 2014). A similar mechanism exists in

Ubiquitin signals produced by CRLs and the APC/C

Unlike the other known classes of E3 ligases, RING E3 ligases work by facilitating the direct transfer of ubiquitin from the E2 to the substrate lysine residue. A different E2 enzyme may be used to initiate and elongate a polyubiquitin chain (Deshaies and Joazeiro, 2009; Ye and Rape, 2009). Alternatively, as for a subset of CRLs, an independent E3 ligase may be recruited to catalyze the initiation step (Scott et al., 2016). The E2 enzyme used for chain elongation is the major determinant of ubiquitin chain topology (Deshaies and Joazeiro, 2009; Ye and Rape, 2009). In the case of CRLs, UBCH5 E2 enzymes allow for mono or multimonoubiquitylation, whereas CDC34 drives chain extension, forming canonical K48-linked polyubiquitin chains (Lydeard et al., 2013; Grice and Nathan, 2016).

Surprisingly, the metazoan APC/C appears to be the major cellular source of atypical K11-linked polyubiquitin chains, which is part of a signal for proteasomal degradation. The APC/C makes use of the E2 enzymes UBE2C and UBE2S to initiate and elongate these atypical chains, respectively (Jin et al., 2008; Garnett et al., 2009; Williamson et al., 2009; Matsumoto et al., 2010; Min et al., 2015; Brown et al., 2016). Despite considerable effort, a consensual structure for K11-linked chains is lacking (Bremm et al., 2010; Matsumoto et al., 2010; Castañeda et al., 2013). Nevertheless, recent studies clarified that homotypic K11 chains are not sufficient to signal proteasome-mediated degradation. Rather, heterotypic K11/K48–polyubiquitinated proteins are efficient proteolytic signals (Grice et al., 2015). Moreover, it was also observed that several ubiquitin chains can be extended from preformed ubiquitin oligomers, constituting branched K48/K11–polyubiquitin chains that appear to be better signals for proteasomal recognition (Meyer and Rape, 2014). These branched chains were suggested to facilitate the degradation of prometaphase APC/C substrates, a mitotic stage characterized by low APC/C activity (Meyer and Rape, 2014). The ability of the human APC/C to synthesize heterotypic ubiquitin chains does not appear to be conserved across all eukaryotes, as at least yeast APC/C substrates are modified with canonical K48-linked polyubiquitin (Rodrigo-Brenni and Morgan, 2007). Yeast might instead make use of complementary mechanisms that reassure the similarly ordered degradation pattern (Lu et al., 2014, 2015a).

Despite the importance of K11 chains as a degradation signal, the respective E2 UBE2S is not essential for cyclin B1 degradation (a canonical APC/C substrate; Garnett et al., 2009; Dimova et al., 2012), leading to the conclusion that multimonoubiquitylation can also constitute a signal for proteasomal degradation (Dimova et al., 2012). Indeed, single-molecule kinetic studies support the view that multimonoubiquitylation can efficiently induce substrate binding to the proteasome (Lu et al., 2015b). Hence, it appears that higher local concentration of ubiquitin moieties enhances binding to proteasomal ubiquitin readers, even though binding to the proteasome does not necessarily correlate with an increased rate of degradation (Lu et al., 2015b; Yau and Rape, 2016). Future research will likely reveal the determinants of the commitment of a substrate to degradation once it is bound to the proteasome.

Xenopus laevis, and the E3 ligase was recently identified to be $CRL2^{LRR1}$ (Moreno et al., 2014; Dewar et al., 2017). Of note, $CRL2^{LRR1}$ seems to be specifically recruited to the chromatin at the time of termination instead of being tethered to the replisome like SCF^{Dia2} (Dewar et al., 2017). It is currently unclear whether the K48-polyubiquitylated Mcm7 subunit is targeted to the proteasome or recycled.

Ubiquitin in DNA replication: Open questions. Altogether, ubiquitin can be used to signal specific and consequential modulation of the DNA replication machinery, especially for lagging-strand synthesis factor switching and nucleosome reassembly. Both proteasomal and nonproteolytic pathways can contribute to this behavior. Importantly, the fine-tuned response requires reversible effects, because a modified protein must be rapidly unmodified or replaced to initiate a new synthesis cycle. After local factor degradation, a sufficiently large protein pool must be available to allow dynamic regulation, as in the case of Polδ4 (Lee et al., 2014). Local degradation and replenishment of factors is experimentally challenging to identify, and the process of local cell cycle effector regulation may be more common than current evidence suggests. Likewise, nonproteolytic ubiquitylation is expected to rely on DUBs that remove the modification. However, only a few DUBs have been identified to date that regulate DNA replication, all but ensuring that a new chapter of discovery awaits. For example, is a DUB also tethered to the replication fork to reverse MCM10 dimonoubiquitylation? At which point is newly synthesized histone H3 deubiquitylated? Furthermore, we do not understand how regulatory ubiquitylation signals are translated into their response and which ubiquitin readers are involved. Finally, there are certainly more ubiquitylation substrates and perhaps more E3 ligases with functions in DNA replication awaiting discovery. For example, in *Caenorhabditis elegans*, the $CRL2^{LRR-1}$ complex regulates DNA replication, and in *Xenopus*, the homologous E3 ligase is involved in replication termination (Ossareh-Nazari et al., 2016; Dewar et al., 2017). The direct substrates mediating DNA replication regulation are not known, and whether either function of $CRL2^{LRR1}$ is conserved in humans remains to be tested. Finally, although the replicative helicase subunit MCM3 is ubiquitylated, the biological significance of this regulation is elusive despite considerable study (Mulvaney et al., 2016). Collectively, many questions remain to be answered, especially in identifying the players that erase and read critical ubiquitin signals during S phase.

Ubiquitin regulation of DNA segregation

Sister chromatids are segregated during mitosis in a process that involves chromosome condensation, nuclear envelope breakdown in animal cells, and centrosome separation to opposite poles. The activity of cyclin-dependent kinase 1, with its positive regulator cyclin B (CDK1/cyclin B), is the main trigger of these events (Gavet and Pines, 2010). In addition, the centromere of condensed chromosomes plays an important role in the assembly of kinetochores that mediate chromosome–spindle attachments and allow chromosome congression at the metaphase plate (Fig. 3 A). Finally, the spindle assembly checkpoint (SAC) monitors microtubule–kinetochore attachments to ensure faithful separation of sister chromatids.

Regulating APC/C E3 ligase activity. Well-timed protein degradation is a common event in the cell cycle, known to drive mitotic entry (G2/M) as well as the metaphase-to-anaphase transition (Teixeira and Reed, 2013; Bassermann et al., 2014). A frequent general question in these and other cell cycle processes is what defines the functional time window of an E3 ligase. In principle, either the activity of the E3 ligase may itself be regulated, or the substrate binding to the E3 ligase may depend on third-party factors such as kinases or scaffolding proteins. Mitosis provides a remarkable example of how an E3 ligase can be dynamically regulated, in this case to tightly coordinate the status of kinetochore–microtubule attachments with the onset of chromosome separation. It is long known that the metaphase-to-anaphase transition is driven by the E3 ligase anaphase-promoting complex/cyclosome (APC/C; see Cullin-RING and APC/C E3 ligases text box), activated by its subunit CDC20 (Teixeira and Reed, 2013; Bassermann et al., 2014). High APC/C^{CDC20} activity triggers anaphase and mitotic exit by mediating the degradation of cyclin B and securin, an inhibitor of the protease separase that cleaves the cohesin complex holding sister chromatids together (Hirano, 2015). Before anaphase, APC/C^{CDC20} is kept inhibited by the SAC until appropriate kinetochore–microtubule attachments are established for all

chromosomes. A critical product of the SAC is the mitotic checkpoint complex (MCC), which inhibits APC/C^{CDC20} activity to prevent premature separation of sister chromatids (Lischetti and Nilsson, 2015).

Further studies provided deeper mechanistic insight into the dynamic regulation of the APC/C^{CDC20} E3 ligase (Fig. 3 B). Surprisingly, the APC/C^{CDC20} can itself promote the release of its inhibitor MCC through autoubiquitylation of CDC20, a process antagonized by the DUB USP44 (Reddy et al., 2007; Stegmeier et al., 2007). More recently, it was clarified that CDC20 ubiquitylation is brought about by a peculiar structural rearrangement, triggering CDC20 destruction and MCC disassembly (Mansfeld et al., 2011; Varetti et al., 2011; Foster and Morgan, 2012; Yamaguchi et al., 2016). Rather than occurring only at the point of anaphase onset, a model has been proposed in which constant MCC disassembly during metaphase generates a pool of uninhibited APC/C that can either rebind the MCC when unattached kinetochores are present or bind free CDC20 and thus be activated, triggering anaphase onset (Fig. 3 B). This dynamic view of APC/C release from inhibition is complemented by other specific mechanisms of MCC extraction (Westhorpe et al., 2011; Miniowitz-Shemtov et al., 2015; Kaisari et al., 2017). Interestingly, MCC disassembly is enhanced by the ubiquitin reader CUEDC2 (Fig. 3 B; Gao et al., 2011). Although experimental evidence demonstrated that the ubiquitin-binding domain (UBD) of CUEDC2 is important for its function, the ubiquitylated factor to which CUEDC2 binds remains to be determined. The UBD is not required for constitutive binding to CDC20, but we speculate that it might be the key in detecting CDC20 ubiquitylation to trigger MCC release from the APC/C. As a result, CDC20 would be available to the proteasome, with subsequent MCC disassembly.

Ordered degradation of the targets of a single E3 ligase. Another concept currently in focus is the pattern of ordered degradation of substrates of a single E3 ligase. Such pattern was observed for S phase targets of $CRL4^{CDT2}$ and is established by distinct substrate binding affinities to the E3 ligase (Coleman et al., 2015). APC/C^{CDC20} likewise represents a prime example of coordinated sequential degradation of E3 ligase substrates, though it does not make use of identical mechanisms. Early observations debated that despite the fact that the MCC precludes the degradation of its late metaphase substrates, MCC-bound APC/C^{CDC20} can ubiquitylate other targets in prometaphase, namely cyclin A and the kinase NEK2A (Fig. 3 B; den Elzen and Pines, 2001; Geley et al., 2001; Hames et al., 2001). Thus, the very same E3 ligase mediates the destruction of several substrates at different time points. The mechanistic basis for selective substrate targeting includes increased affinity of the early substrates for APC/C binding, and APC/C^{CDC20} can generate branched ubiquitin chains that are better signals for proteasomal degradation (Meyer and Rape, 2014; Boekhout and Wolthuis, 2015; Di Fiore et al., 2015; Lu et al., 2015a). A summary of the current information on proteolytic ubiquitin signals generated by the APC/C and CRLs can be found in the respective text box.

Fine-tuning kinetochore protein localization. Other E3 ligases operate in mitosis, providing critical regulation often through nonproteolytic ubiquitylation. These signals during mitosis contribute to the remarkable resilience of the system so that cells readily adapt to changing conditions such as

Table 2. **Proposed human ubiquitin readers with cell cycle functions**

UBD	Ubiquitin binding mode	Number of UBD-containing proteins (cell cycle associated/total)	Examples with cell cycle functions	Ubiquitin-binding role in cell cycle function[a]	Reference
UBA	MonoUb, polyUb (predominant for K48)	14/55	UBQLN2,[b] KPC2,[b,c] FAF1,[d] UBASH3B,[c] BRSK1/2, LATS1/2, MARK4	KPC2 (E3 ligase subunit): Promotes the transfer of ubiquitylated p27 to the proteasome; UBASH3B: Targets ubiquitylated Aurora B to microtubules in mitosis	Hara et al., 2005; Krupina et al., 2016
CUE	MonoUb	1/13	CUEDC2[c]	CUEDC2: Promotes MCC release from APC/C^{CDC20}	Gao et al., 2011
UIM	MonoUb, polyUb (K48, K63)	6/28	DDI1,[b] RPN10,[b] Epsin-1, MAT1, alpha4[c]	alpha4: Prevents polyubiquitylation of the PP2A catalytic subunit	Kong et al., 2009; McConnell et al., 2010
UBP	PolyUb	1/3	USP39	None described	—
NZF	MonoUb, polyUb (K63)	2/7	NPL4,[d] HOIL-1	None described	—
UEV	MonoUb	1/2	TSG101	None described	—
UBAN	DimonoUb (M1)	2/7	ALIX, Optineurin	None described	—
WD40 (subset)	MonoUb	1/4	BUB3	None described	—
Unique or uncharacterized domains	Various	10/21	RPN13,[b] VCP,[d] UFD1,[d] NUP62, ERα, SMURF2[c]	SMURF2 (E3 ligase): Stabilize ubiquitylated substrate binding to promote polyUb[e]	Ogunjimi et al., 2010
Domains not found in cell cycle regulators	Various	0/22	—	—	—
Total	—	38/162	—	—	—

We considered UBD-containing proteins annotated in UniProtKB/Swiss-Prot, a curated protein database (UniProt Consortium, 2015), together with a manual literature search. Our criteria include (1) proteins containing UBDs described previously (Husnjak and Dikic, 2012), (2) other proteins annotated as binding ubiquitin, and (3) manual exclusion in case of covalent ubiquitin binding (e.g., E2s or ubiquitin modification) and of active DUBs. Assignment of cell cycle–related functions was determined by gene ontology and complemented with manual literature search. Ubiquitin-binding mode is according to Husnjak and Dikic (2012). MonoUb, monoubiquitylation; polyUb, polyubiquitylation; —, not applicable.

[a]Excluding proteins involved in general proteasome function or that of VCP/p97.

[b]Proteasome subunit, or proteasome associated (mostly according to Grice and Nathan, 2016).

[c]Proteins have a reported cell cycle function for their UBDs that is distinct from general proteasome or VCP/p97 functions.

[d]VCP/p97 component or cofactor (Meyer et al., 2012).

[e]Probably a general mechanism, which includes its cell cycle functions.

the status and quality of kinetochore–microtubule attachments. The importance of these ubiquitylation signals is twofold. Ubiquitylation triggers removal of factors from local chromosomal pools when their function is no longer required, and it can promote microtubule transport of effectors to their new sites of action. During metaphase, correct kinetochore–microtubule attachments must be stabilized, whereas erroneous attachments are destabilized in order to prevent chromosome instability. These processes are coordinated by two kinases, PLK1 and Aurora B (Zitouni et al., 2014; Krenn and Musacchio, 2015). Interestingly, the mitotic localization of Aurora B is regulated by nonproteolytic ubiquitylation at multiple points, including for its microtubule-mediated translocation (Fig. 3, C and E). Aurora B works at the kinetochore to destabilize incorrect microtubule attachments. The VCP–p97 complex ensures exclusive kinetochore localization by removing Aurora B from chromosomal arms, possibly after $CRL3^{KLHL9\text{-}KLHL13}$-mediated polyubiquitylation (Ramadan et al., 2007; Sumara et al., 2007; Dobrynin et al., 2011). In anaphase, Aurora B translocates to the spindle midzone, a process initiated by $CRL3^{KLHL21}$-dependent monoubiquitylation of Aurora B at attached kinetochores. Remarkably, this ubiquitin signal is decoded by the UBA-containing protein UBASH3B, which recruits ubiquitylated Aurora B to microtubules in the vicinity of the attached kinetochore (Maerki et al., 2009; Krupina et al., 2016). The microtubule-dependent translocation of Aurora B to the spindle midzone in anaphase is mediated by the kinesin MKLP2 (Gruneberg et al., 2004). Indeed, UBASH3B tethers MKLP2 and ubiquitylated Aurora B, thereby promoting microtubule-dependent Aurora B translocation (Fig. 3, C and E). Whether ubiquitylated Aurora B first needs to be extracted by VCP/p97 remains to be investigated.

Although PLK1 stabilizes correct kinetochore–microtubule attachments, its removal from kinetochores is required for faithful metaphase progression (Liu et al., 2012). Bipolar attachment creates tension across the kinetochore, and recent data suggest that this may activate $CRL3^{KLHL22}$ to trigger rapid removal of PLK1 (Fig. 3 C; Beck et al., 2013). Ubiquitylation is counteracted by the DUB USP16, and thus a balance between $CRL3^{KLHL22}$ and USP16 ensures the correct localization and function of PLK1 (Zhuo et al., 2015). This balance provides plasticity to this system, as ubiquitylation can be added or removed to fine-tune the localization of a subpopulation of PLK1. Because $CRL3^{KLHL22}$ regulates PLK1 by nonproteolytic ubiquitylation, probably by monoubiquitylation, its displacement from kinetochores likely depends on a dedicated ubiquitin-binding protein such as VCP/p97. Because PLK1 is also translocated by the MKLP2 kinesin to the spindle midzone in anaphase (Neef et al., 2003), it also needs to be clarified whether ubiquitylated PLK1 is similarly recognized and translocated by UBASH3B or whether this process requires a different reader.

DUBs reveal additional roles of ubiquitin in microtubule transport. Kinase translocation in anaphase is not the only example of how protein ubiquitylation determines cargo for mitotic microtubule-based transport. Two studies reported that the DUBs CYLD and the BRISC complex are involved in the assembly and positioning of the mitotic spindle by regulating the function of the spindle assembly factor NuMA (Yang et al., 2014; Yan et al., 2015). NuMA promotes the tethering of microtubules to the spindle poles and also to the cell cortex and is transported to these sites along microtubules by cytoplasmic dynein (Radulescu and Cleveland, 2010). The BRISC complex appears to deliver ubiquitylated NuMA to spindle poles, indicating that NuMA ubiquitylation likely promotes its transport by dynein (Fig. 3 D; Yan et al., 2015). Cytoplasmic dynein was previously implicated in the transport of ubiquitylated protein aggregates, tethered by the ubiquitin-binding protein HDAC6, and perhaps another reader transports NuMA in a similar fashion (Kawaguchi et al., 2003; Ouyang et al., 2012).

Ubiquitin in mitosis: Open questions. Overall, in mitosis, ubiquitin operates to ensure genome integrity and well-timed DNA segregation by essentially two pathways. First, the peculiar regulation of APC/C by autoubiquitylation provides the necessary flexibility for the cell to quickly recognize changing conditions in the kinetochore–microtubule attachment state. Second, the plasticity of PLK1, Aurora B, and NuMA ubiquitylation ensures that the spindle is correctly assembled and that proper kinetochore–microtubule attachments are established. Today, cell cycle research faces the challenge of understanding how the observed dynamicity in ubiquitylation is achieved. The increased knowledge of APC/C^{CDC20} regulation might facilitate understanding of how other E3 ligases are regulated in space and time. For example, it seems that $CRL3^{KLHL22}$ dynamically responds to microtubule–kinetochore tension to ubiquitylate PLK1, but the underlying mechanism remains elusive (Beck et al., 2013). To which extent other cell cycle E3 ligases are regulated in a comparable dynamic fashion will likely demand considerable research efforts. For instance, the APC/C E3 ligase was an early discovery in cell cycle research (Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995), yet APC/C regulation is still an area of active investigation. Dynamic ubiquitylation can also be modulated at the level of the substrate by DUBs, but information regarding how their activity is modulated is mostly lacking. As another pressing and relatively obscure topic, further functional analysis will be required to identify specific readers involved in mitotic processes regulated by nonproteolytic ubiquitin signals. Finally, it will be of interest to determine whether ubiquitin-dependent microtubule motor binding is an ordinary feature in microtubule cargo transport.

Perspective: Reading ubiquitin signals

In this review, we summarized examples in which both proteolytic and nonproteolytic ubiquitin signals regulate cell cycle events. Ubiquitylation of key factors can be reversible, either by a DUB or through the rapid replenishment of a locally degraded factor, such as p12 or CDC20. Despite a growing catalog of nonproteolytic ubiquitin signals, surprisingly little is known about the mechanisms underlying cell cycle regulation that go beyond proteasome targeting. Although monoubiquitylation is widespread (Nakagawa and Nakayama, 2015), assessing nonproteolytic ubiquitin signals and elucidating how ubiquitin mechanistically alters the activity of a given target requires detailed understanding of the underlying process. Therefore, reading the information encoded in ubiquitin chains is now a major challenge in cell cycle research for nondegradative outcomes. The action of CUEDC2 and UBASH3B, in addition to VCP/p97, provides the first clues toward a more comprehensive understanding. We have summarized information regarding cell cycle proteins with UBDs and discovered that ∼25% of the putative human ubiquitin readers are also proteins associated with cell cycle regulation (Table 2). Nevertheless, in the majority of these cases, we do not yet understand the role of the UBD or that of the ubiquitylated binding proteins and subsequent response in the context of the cell cycle. For example, the yeast MCC component BUB3 can bind ubiquitin, but how it contributes

to APC/C regulation remains elusive (Pashkova et al., 2010). Other examples are the endosomal sorting complexes required for transport (ESCRT)–related proteins TSG101 and ALIX, which regulate cytokinesis (Morita et al., 2007). Although their interaction with ubiquitin needs to be investigated (Bishop et al., 2002; Dowlatshahi et al., 2012), ALIX and other ESCRT proteins recruit ESCRT-III to promote cytokinetic abscission (Christ et al., 2016). Interestingly, ESCRT-III is directed to the reforming nuclear envelope by a VCP/p97-dependent mechanism to aid in nuclear envelope reformation after chromosome segregation (Olmos et al., 2015). Although speculative, it is thus possible that binding of ALIX to an ubiquitylated factor may similarly help to recruit ESCRT-III during late mitosis. Our efforts to compile cell cycle–associated readers (Table 2) are likely incomplete, and it is therefore clear that much remains to be discovered before the underlying processes of nonproteolytic ubiquitylation are well understood.

Technically, addressing nondegradative ubiquitylation can be a challenging task. In particular, when the bulk levels of a given target protein remain unchanged, it can be difficult to experimentally distinguish local degradation of a small but specific pool from ubiquitin-dependent changes promoting protein translocations and/or activity changes. Tagging specific proteins with a photoswitchable fluorescent protein (Zhou and Lin, 2013) and/or pulse-chase–type labeling with stable protein markers provide powerful tools to visualize ubiquitin-dependent translocations. The identification of specific ubiquitin readers may require siRNA or CRISPR-based screenings and/or mutagenesis of their UBDs. Because of the lack of tools for their detection, another technically challenging task is addressing the synthesis and functions of heterotypic (including branched) polyubiquitin chains in vivo. Ubiquitin linkage in polyubiquitin chains is often distinguished by linkage-specific polyubiquitin antibodies, but they cannot discern between homotypic and heterotypic chains. To overcome this limitation, bispecific bivalent antibodies that simultaneously and exclusively bind two distinct types of ubiquitin linkages within the same polyubiquitin chain have been developed (Rape, M., personal communication). Perhaps research will also lead to the identification of specific ubiquitin readers for these noncanonical linkages that in addition to their functional characterization could be exploited and employed to discriminate linkage types. We believe that new tools will be required to decipher the ubiquitin code. Despite the numerous challenges, it is clear that studying the roles of proteins that noncovalently bind ubiquitin will continue to shed light into how the complex network of ubiquitin-dependent signals cooperate to perpetually drive cells through ordered cycles of DNA synthesis and separation.

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