Evolution and Function of the Mitotic Checkpoint

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The mitotic checkpoint evolved to prevent cell division when chromosomes have not established connections with the chromosome segregation machinery. Many of the fundamental molecular principles that underlie the checkpoint, its spatiotemporal activation, and its timely inactivation have been uncovered. Most of these are conserved in eukaryotes, but important differences between species exist. Here we review current concepts of mitotic checkpoint activation and silencing. Guided by studies in model organisms and our phylogenomics analysis of checkpoint constituents and their functional domains and motifs, we highlight ancient and taxa-specific aspects of the core checkpoint modules in the context of mitotic checkpoint function.

Mitosis, Kinetochores, and the Mitotic Checkpoint

Accurate distribution of the replicated genome during mitosis is essential for the formation of genetically identical daughter cells. Errors in this process lead to genomic instability by causing aneuploidy and structural chromosome aberrations, both hallmarks of cancer (Gordon et al., 2012). Error-free chromosome segregation relies on dynamic linkages between chromosomes and the plus ends of spindle microtubules in a manner that connects sister chromatids to opposite spindle poles. Such bioriented attachments are provided by large multiprotein complexes called kinetochores that are assembled on centromeric DNA (Cheeseman and Desai, 2008). Kinetochores attach to microtubules predominantly via the KMN network, a complex of eleven proteins that contains at least two microtubule-binding activities, provided by the Ndc80 complex and Knl1 (Cheeseman and Desai, 2008; Lampert and Westermann, 2011). Different evolutionary taxa have distinct additional factors that act in concert with the KMN network (Lampert and Westermann, 2011).

The mitotic checkpoint (MC, also called the spindle assembly checkpoint [SAC]) is a molecular safeguard mechanism that prevents premature chromosome segregation until all kinetochores have obtained connections to spindle microtubules (Musacchio and Salmon, 2007). There is some degree of debate about whether the checkpoint can distinguish unattached kinetochores from non-bioriented chromosomes, and we refer interested readers to some recent reviews on this matter (Khodjakov and Pines, 2010; Nezi and Musacchio, 2009). Kinetochores respond to lack of attachment by catalyzing the production of a molecular inhibitor of the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that drives sister chromatid separation and mitotic exit by directing Securin and Cyclin B, respectively, for proteasomal degradation (Pines, 2011) (Figure 1A). As long as unattached kinetochores persist, the APC/C remains inactive toward these substrates and cells are stuck in a mitotic state with connected sister chromatids. The core machinery of the checkpoint comprises the APC/C inhibitor, also known as the mitotic checkpoint complex (MCC), as well as the proximal proteins that ensure its assembly by unattached kinetochores (Figure 1A). The MCC is a complex of Mad2, BubR1/Mad3, and Bub3 that is directly bound to the essential APC/C cofactor Cdc20. In addition, Bub1, Mps1, and Mad1 promote Cdc20 inhibition either directly through phosphorylation (Bub1) or indirectly through stimulating MCC assembly (Mps1 and Mad1) (Musacchio and Salmon, 2007). Several additional, sometimes taxa-specific, kinetochore proteins have been included in the group of checkpoint proteins and may aid in fine-tuning or amplifying checkpoint signals (see below).

Once the checkpoint is satisfied by attachment of the final kinetochore, the block on APC/C-Cdc20 by the MCC is quickly released, a process known as checkpoint silencing. This involves disassembly of the MCC, an active process that requires ubiquitination by the APC/C and a protein known as p31^{comet} (Hardwick and Shah, 2010). In addition, checkpoint proteins are removed from kinetochores by the dynein motor with the aid of kinetochore dynein recruiters such as Spindly and the Rod-Zwilch-ZW10 (RZZ) complex. Furthermore, phosphorylation events critical for MC function are reversed by kinetochore-localized protein phosphatases such as PP1 (Hardwick and Shah, 2010) (Figure 1B).

With this review, we aim to provide an overview of the molecular workings of the MC and distill its core principles. To this end, we complement insights from experiments in various model organisms with our phylogenomics analysis of the MC machinery. This evolutionary perspective aids in distinguishing ancient from modern mechanisms and helps to uncover previously underappreciated concepts of the MC signaling pathway.

Evolution of the MC and Its Auxiliary Proteins

We used the publicly available genomes of 60 eukaryotes from all supergroups except rhizaria (Supplemental Experimental Procedures), to search for homologs of proteins from the core and auxiliary MC modules, including the MCC (Mad2, BubR1/Mad3, Bub3), kinetochore MC scaffolds (Mad1, Knl1), and kinases (Bub1, Mps1), as well as the contributing protein complex RZZ, the primary MC target Cdc20, and components of MC silencing mechanisms (Spindly, p31^{comet}) (Figure 2;

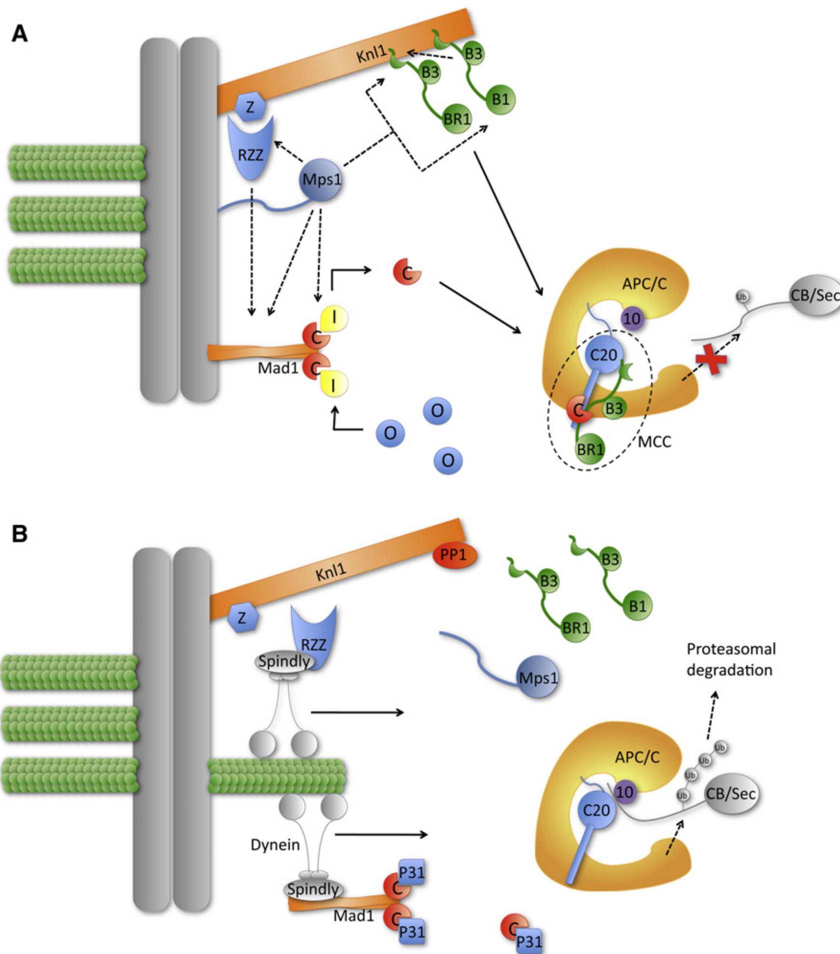

Figure 1. The Mitotic Checkpoint and Checkpoint Silencing

(A) Unattached kinetochores recruit Mad1, Bub1 (B1), BubR1/Mad3 (BR1), Bub3 (B3), and the RZZ complex (RZZ) either directly or indirectly via the MC scaffold Knl1/Zwint-1 (Z). The combined actions of these proteins and protein complexes promotes conversion of O-Mad2 (O) into C-Mad2 (C) through an intermediate state (I) after its dimerization with Mad1-bound C-Mad2. Soluble C-Mad2 and BubR1/Mad3 then bind the APC/C coactivator Cdc20 (C20), blocking its substrate binding sites and repositioning Cdc20 away from the APC/C subunit Apc10 (10). As a result, APC/C-mediated ubiquitinations (Ub) of Cyclin B (CB) and Securin (Sec) are inhibited, maintaining sister chromatid cohesion and a mitotic state. Various steps in these processes are under control of Mps1.

(B) Attachment of vertebrate kinetochores causes dynein-dependent poleward stripping of MC proteins such as Mad1/Mad2, Spindly, and the RZZ complex. Mps1, Bub1, and BubR1/Mad3 are additionally dislodged from attached kinetochores. After satisfaction of the MC, when all kinetochores have achieved stable attachments, the MCC is disassembled by the action of p31^{comet} (P31), resulting in APC/C-Cdc20 activity toward Cyclin B and Securin, followed by their proteasomal degradation. Mitotic exit further requires reversal of MC phosphorylations by PP1-like phosphatases that bind to the N terminus of Knl1.

see also Supplemental Experimental Procedures, Table S1, Figures S1–S3, and Supplemental Sequence File available online). We complemented our data with recent phylogenomic analysis of the APC/C by showing presence or absence of Apc1 (scaffold), Apc2 (cullin-domain), and Apc11 (RING-finger) homologs (Eme et al., 2011). For more in-depth analysis of evolution of functional domains within the identified homologs, we focused on a selection of species from different classes (indicated in bold in Table S1), representing the best-characterized species in the supergroups, as well as most of the common model organisms (Figure 2).

In general, our analyses indicate that most checkpoint components are ancient and were likely present in the last eukaryotic common ancestor (LECA). The exception is Spindly, with recognizable homologs only in most ophistokonta except for dikaryan fungi. Please note that we cannot formally exclude the possibility that poor genome annotation is an occasional reason for our inability to identify homologs in certain species. Although the core MC components can be found in at least one species in every supergroup, some may have been specifically lost in distinct supergroups or in major subbranches: Knl1 in chromalveolata and excavata, p31^{comet} in primitive fungi, and Zwilch in most but not all species that lack Spindly. In addition, some single-celled eukaryotes appear to lack one or more of the essential APC/C subunits, and most or all of the core MC components could not be found in the genomes of such species (e.g., *Encephalitozoon cuniculi*, *Plasmodium falciparum*, and *Cryptosporidium parvum*). Some organisms that contain APC/C subunits and Cdc20 are devoid of core MC components (*Paramecium tetraurelia*, *Tetrahymena thermophila*, *Leishmania major*, and *Trypanosoma brucei*). Certain eukaryotes may therefore do without a surveillance mechanism for chromosome segregation or may have evolved alternative ways of delaying cell division in the presence of unattached kinetochores. We will discuss these and other interesting evolutionary patterns in relation to established protein function in the following sections and expand it with insights obtained from detailed inspection of the evolution of functional protein domains within a subset of proteins.

The Inhibitor and Its Target: MCC and Cdc20

Polyubiquitination of Cyclin B and Securin by the APC/C requires destruction signals including a D(estruction) box (RxxLxxxx[EDNQ]) and/or KEN box (KEN) that are recognized by Cdc20. Recent structural insights have shown that the related cofactor Cdh1 and the APC/C subunit Apc10 form a bipartite D-box receptor that positions the substrate for catalysis by the Apc11/Apc2 catalytic core (Buschhorn et al., 2011; da Fonseca et al., 2011; Schreiber et al., 2011). Recognition of D or KEN boxes is provided by distinct surfaces on the WD40 repeat domain in Cdc20 and Cdh1 (Chao et al., 2012). An additional IR tail and an amino-terminal C box anchor the cofactor to the APC/C (Yu, 2007). Finally, Cdc20 itself has either a D or KEN

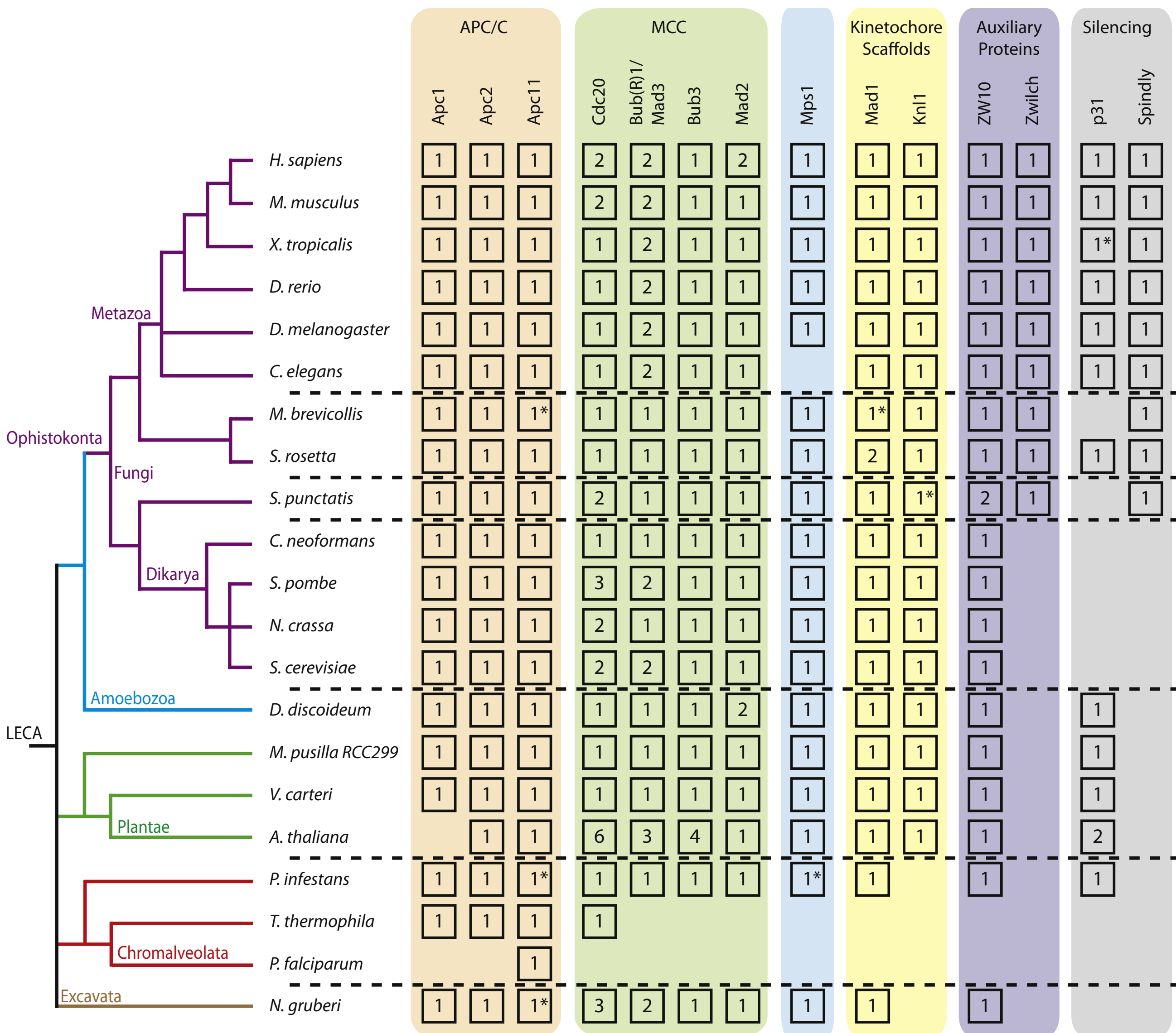

Figure 2. Homologs of the Core and Auxiliary MC Proteins
Schematic representation of eukaryotic tree of life in which a selection of eukaryotic species from the five different supergroups is indicated on the left. Checkpoint proteins are grouped in different functional groups (MCC, Mps1, kinetochore scaffolds, auxiliary proteins, silencing), and, whenever present, the number of homologs is indicated in black boxes (for gene IDs, see Table S1; for protein sequences, see Supplemental Sequence Files). Data on APC/C subunit homologs are adapted from (Eme et al., 2011); asterisks indicate potential homologs of MC subunits in genomic DNA from nonannotated genes (see Supplemental Experimental Procedures and Figure S3).

box sequence in its amino-terminal region that is required for its degradation during later stages of mitosis (Yu, 2007).

The MCC inhibits substrate recognition by the APC/C by repositioning Cdc20 away from the Apc10 subunit, by blocking the KEN-box binding site, and by partially blocking the D-box binding site in Cdc20 (Chao et al., 2012; Herzog et al., 2009). This is achieved by a concerted effort of Mad2 and BubR1/Mad3. Mad2 directly interacts with Cdc20 through a motif preceding the WD40 repeat domain (Chao et al., 2012). Binding of Mad2 to Cdc20 disturbs interactions between Cdc20 and the APC/C (Yu, 2007) but, more importantly, allows BubR1 to bind Cdc20 (Kulukian et al., 2009). BubR1 has an amino-terminal KEN box that engages the KEN-box binding site in Cdc20 in a pseudosubstrate manner (Burton and Solomon, 2007; Chao et al., 2012; Sczaniecka et al., 2008). Additional interactions of the BubR1 tetratricopeptide repeat (TPR) domain with the WD40 repeat domain in Cdc20 sterically hinders access of substrate D-box sequences to the D-box binding site in Cdc20 (Chao et al., 2012). Finally, it has been proposed that a second KEN box in BubR1, carboxy-terminal to the TPR domain, directly engages the APC/C and may thus contribute to the inhibitory activity of MCC (Lara-Gonzalez et al., 2011).

Evolution, Function, and Regulation of Cdc20

Cdc20 is found in one or multiple copies in virtually all genomes that we analyzed (Figure 2; Table S1). Most essential domains in

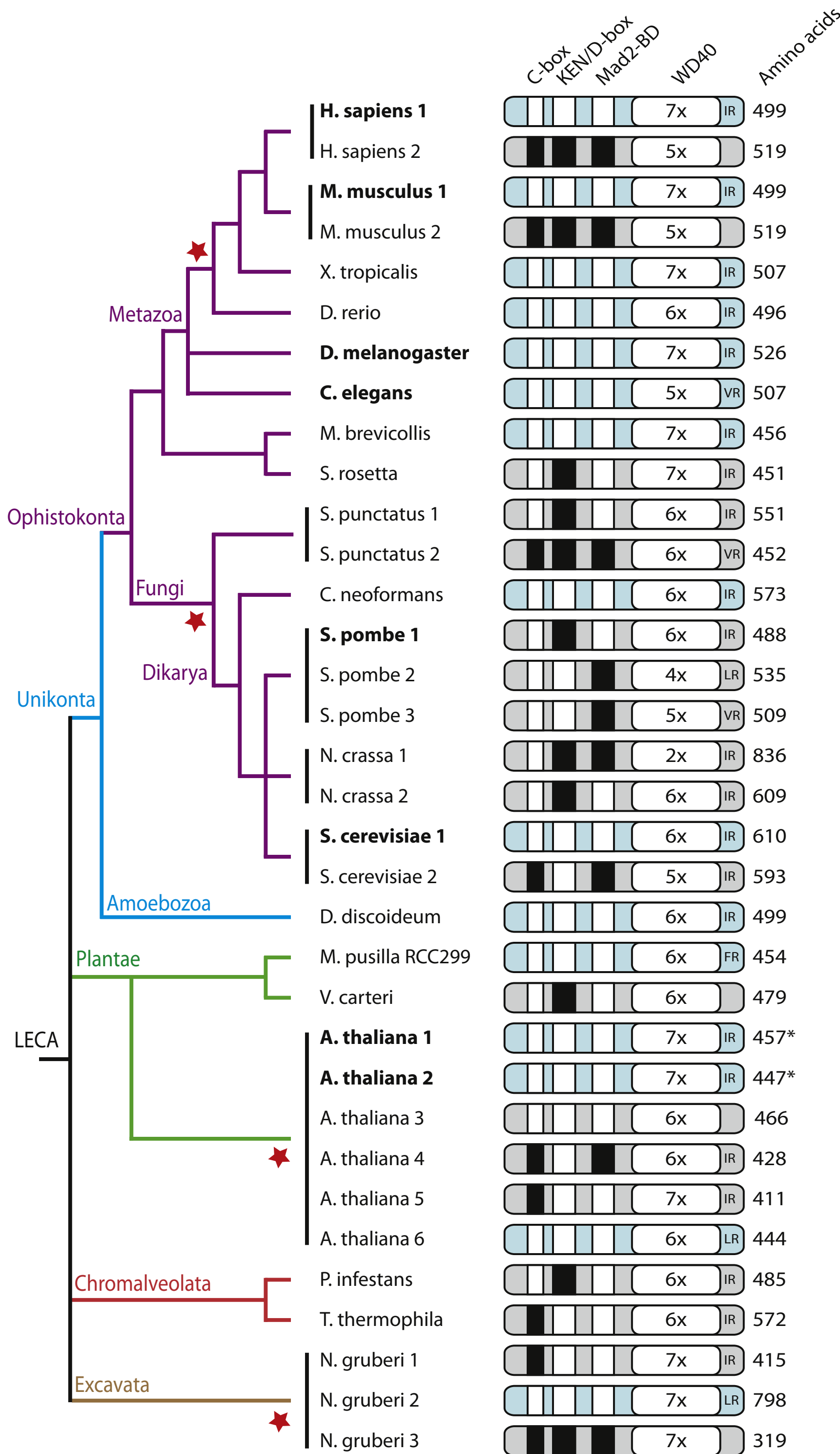

Figure 3. Cdc20 Homologs in the Eukaryotic Tree of Life

Schematic representation of eukaryotic tree of life with Cdc20 homologs from species listed in Figure 2. Indicated for every homolog are the presence (white box) or absence (black box) of the C box (DR[YF]IP), KEN/D box (KEN/RxxLxxxx [EDNQ]), Mad2-binding domain ([KR][IV]LxxxP), the number of predicted WD40 repeats (using SMART-EMBL), the presence of an IR tail ([IVLF]R), and the ORF length in amino acids. Species in bold indicate experimentally confirmed Cdc20 homologs, blue protein bodies indicate homologs containing all essential domains, and the *A. thaliana* Cdc20 genes that are expressed are indicated by an asterisk. Red star shapes indicate probable gene duplication events based on phylogenetic alignments.

Cdc20 have been strongly conserved during evolution, including the Mad2-binding motif, C box, WD40 repeats, IR tail, and, to a lesser extent, the degradation motifs (D and KEN box) (Figure 3; Figure S1). Interestingly, in many species with multiple Cdc20 paralogs, only one contains all the domains that in animals and fungi are required for the function and regulation of Cdc20.

In budding and fission yeast, some of the other Cdc20-like proteins have meiosis-specific functions (Kimata et al., 2011; Tsuchiya et al., 2011). These paralogs have no Mad2-binding motif (Figure 3), raising the question of whether they are regulated by the state of kinetochore attachment. The Cdc20B paralog in humans (*H. sapiens* 2 in Figure 3) is highly degenerated. Besides a recent report that an intronic region in the gene encodes a miRNA that regulates proliferation (Lizé et al., 2010), it is unknown whether human Cdc20B or similarly degenerate Cdc20 proteins in other organisms have a cellular function.

Feedback Control of the MC: Ubiquitination of Cdc20 by the APC/C

Cdc20 expression is restricted to late S phase, G2, and early mitosis. This restriction is imposed by Cdh1, which recognizes Cdc20 as an APC/C substrate in anaphase, leading to persistent low Cdc20 protein levels in G1 and early S phase (Yu, 2007). Besides ensuring the absence of Cdc20 postanaphase, ubiquitination of Cdc20 has also been implicated in regulating MC function. Multiubiquitination (monoubiquitination on multiple residues) of Cdc20 by the APC/C was proposed to cause MCC dissociation and MC silencing (Reddy et al., 2007). A nonubiquitinatable mutant of Cdc20, however, still allows MCC dissociation upon MC satisfaction, challenging this notion of feedback inhibition (Mansfeld et al., 2011). Rather than multiubiquitination, Cdc20 seems to undergo polyubiquitination and subsequent degradation continuously, a process that is balanced by Cdc20 protein synthesis (Nilsson et al., 2008; Varetti et al., 2011; Zeng et al., 2010). This turnover could assist the MC in

maintaining mitotic delays by keeping APC/C activity toward its relevant substrates low (Nilsson et al., 2008; Pan and Chen, 2004), or it could promote a certain rate of formation and disassembly of MCC-APC/C complexes to allow timely mitotic exit as soon as MCC production at kinetochores stops. The latter hypothesis is supported by evidence that Cdc20 turnover is aided by p31^{comet}, a structural Mad2 mimic that opposes MC function (Varetti et al., 2011). These two proposed models are difficult to reconcile, and further detailed studies will be required to clarify the role of Cdc20 degradation in mitosis. Regardless of the exact consequences of Cdc20 ubiquitination, it will be informative to examine whether the destruction motifs in Cdc20 contribute to this: p31^{comet} does not necessarily co-occur in evolution with Cdc20 homologs containing such destruction motifs (e.g., *Neurospora crassa*, *Volvox carteri*, and *Phytophtora infestans*) (Figures 2 and 3). If destruction motifs are critical for Cdc20 turnover, this may suggest that p31^{comet} has other functionalities in addition to promoting Cdc20 turnover. Conversely, in budding yeast, where Cdc20 turnover was described initially (Pan and Chen, 2004), we could not identify a p31^{comet} homolog, indicating that Cdc20 turnover can occur by p31^{comet}-independent mechanisms.

Catalyzing MCC Production

An essential feature of the MC is the ability of Mad2 to bind Cdc20. Mad2 interacts with Cdc20 only when in a "closed" conformation (C-Mad2), production of which is catalyzed by unattached kinetochores through the action of Mad1. Mad1 and Mad2 localize to unattached kinetochores in mitosis (Musacchio and Salmon, 2007), and a significant pool of free Mad2 is present in the cytoplasm of mitotic cells (Chung and Chen, 2002). Cytoplasmic Mad2 is in an "open" conformation (O-Mad2) that has low affinity for Cdc20 but can be converted to C-Mad2 by virtue of dimerizing with Mad1-bound C-Mad2 at unattached kinetochores (De Antoni et al., 2005; Nezi et al., 2006). Structural conversion of O- to C-Mad2 then allows it to bind Cdc20 and ensures efficient MCC formation. Although this conversion and subsequent MCC formation can be strikingly recapitulated in vitro (Kulukian et al., 2009; Vink et al., 2006), efficient MCC formation in cells seems to require additional inputs from kinetochores. Mitotic delays in cells that express an artificial Mad1 protein that is maintained on attached kinetochores depends on kinetochore kinases, and targeting Mad1 to nonkinetochore chromosomal regions is not sufficient to delay mitosis (Maldonado and Kapoor, 2011). One possible explanation for this is that the kinetochore-localized MC kinase Mps1 aids Mad2 conversion by promoting Mad2 dimerization (Hewitt et al., 2010). In normal conditions, Mps1 further impacts Mad1-Mad2 function by promoting Mad1 localization to kinetochores (Lan and Cleveland, 2010). Clarifying the mechanism for this will require identification of the Mad1 receptor at kinetochores. Interestingly, the amino-terminal region of Mad1 that is required for its kinetochore binding was allowed to diverge during evolution (Figure S2). It has been suggested that this region determines checkpoint sensitivity, because the less-robust checkpoint in rodent cells can be made more stringent by ectopic expression of human Mad1 or a hybrid of murine Mad1 with a human amino-terminal domain (Haller et al., 2006).

The conversion of O-Mad2 to C-Mad2 relies on several features within the Mad1-Mad2 complex, including a Cdc20-like Mad2-binding motif in Mad1, Mad1 homodimerization, and a HORMA domain in Mad2 that is required for both Cdc20 and Mad1 binding in a mutually exclusive manner (Musacchio and Salmon, 2007). The Mad2 HORMA domains are highly similar between species in all supergroups analyzed, suggesting strict conservation of the Mad2-Cdc20 interface. Much like the Mad2-binding motif in Cdc20 and the HORMA domain in Mad2, the Mad2-binding motif in Mad1, when present, is highly conserved (Figure S2). Mutation of this motif abrogates MC activity (Maldonado and Kapoor, 2011). Interestingly, the Mad2-binding motif in Mad1 is absent from Mad1 homologs in *Salpingoeca rosetta*, *Micromonas pusilla*, and *Naegleria gruberi* (Figure S2). The related motif can be found in their Cdc20 homologs, suggesting that fundamentals of Mad2 binding have in principle not been altered in these species. If their Mad1 is nevertheless capable of binding Mad2, examining how may provide additional insight into molecular aspects of this interaction. Potentially important in this regard is the recent identification of S187 phosphorylation in fission yeast Mad2 that affects the Mad1-Mad2 interaction (Zich et al., 2012). Given the high conservation of the position of this serine in Mad2 homologs, such a regulatory mechanism for Mad2 function may be ancient.

The APC/C Pseudosubstrate Inhibitor within the MCC

Human BubR1 was identified as a Bub1-like gene mutated in chromosomally unstable colon cancer cell lines but was later recognized as the functional equivalent of the budding yeast spindle checkpoint protein Mad3p (Elowe, 2011). Mad3/BubR1 and Bub1 share extensive sequence homology and domain architecture. Both contain a TPR domain followed by a Gle2-binding sequence (GLEBS) motif, and in vertebrates and *Drosophila* both contain an unusual carboxy-terminal Ser/Thr kinase domain (Bolanos-Garcia and Blundell, 2011). This similarity stems from the fact that LECA contained a single protein, to which we refer as Madbub, that possessed the shared domains as well as the amino-terminal KEN box characteristic of Mad3/BubR1-like proteins (Suijkerbuijk et al., 2012) (Figure 4). Madbub subsequently took distinct paths of evolution: it either remained a Madbub and diverged little or it underwent a gene duplication event on multiple (probably nine) independent occasions. Duplication was followed either by loss of one of the copies, as in the case of some relatives of *Saccharomyces cerevisiae* (Murray, 2012), or by a striking example of parallel subfunctionalization, during which retainment of the KEN box or kinase domain was mutually exclusive in the vast majority (seven of nine) of cases. These parallel subfunctionalization events gave rise to present-day Bub1-BubR1/Mad3 paralogs (Figure 4) (Suijkerbuijk et al., 2012). Insightful exceptions to this rule are insects and vertebrates. The KEN-box-containing protein retained a kinase domain in vertebrates, but this domain was allowed to degenerate to a pseudokinase that is highly sensitive to destabilization by amino acid substitutions in various regions of the domain (Suijkerbuijk et al., 2012). Because destabilization is propagated to the whole protein, this liability may have contributed to selection for truncating mutations in so many nonvertebrate species (Figure 4). In *D. melanogaster*, however, the KEN-box-containing protein

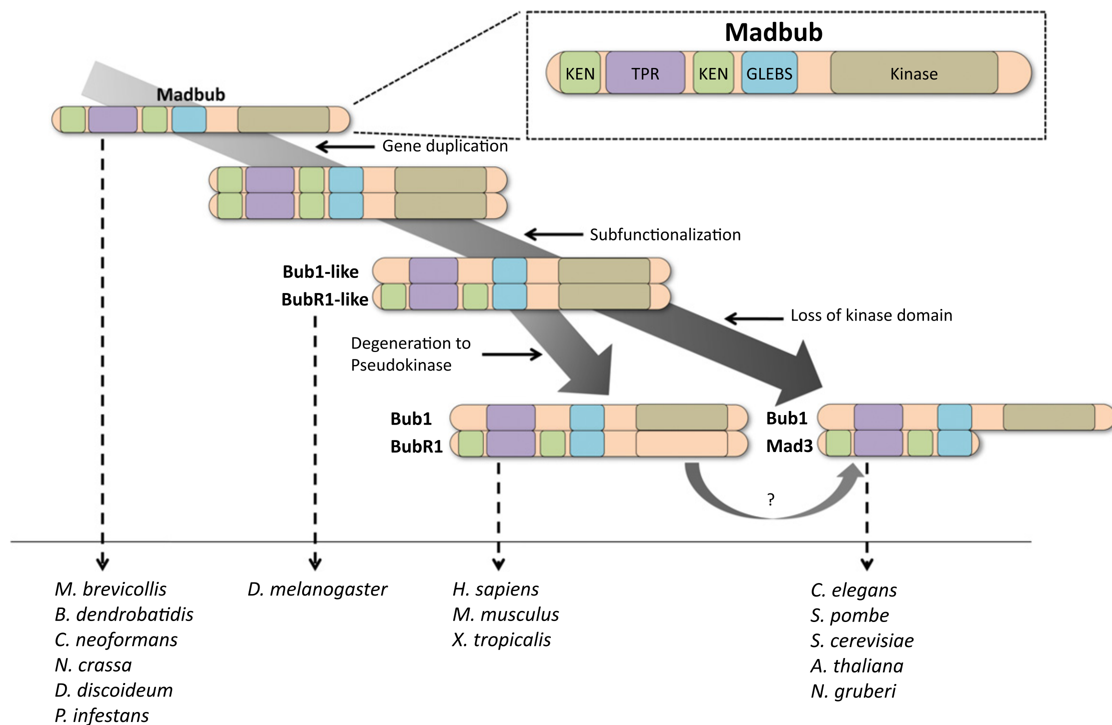

Figure 4. Proposed Model for Evolution of the Madbub Family

LECA possessed a Madbub protein containing the predominant functional domains (see inset: two KEN boxes, TPR domain, GLEBS motif, and a kinase domain). Madbubs are still present in numerous organisms, including those indicated at the bottom. At least nine independent gene duplications led to subfunctionalization of a Bub1-like (kinase) and a BubR1-like (KEN box) gene. After the loss of kinase requirement in the KEN-box protein, the kinase either degenerated into a pseudokinase (vertebrates) or was shed altogether. One notable exception is the Drosophilids, in which the KEN box was lost from one paralog, but the kinase was maintained with almost identical sequence in both.

retained a proper Madbub-like, and therefore Bub1-like, kinase domain. *D. melanogaster* BubR1 may have kinase function, which is unique in eukaryotes, or may alternatively be in a transition state, one that is predicted to have occurred between a gene duplication event and evolution toward either a pseudokinase or shedding of the kinase domain (Figure 4).

The Fate of a Paralog: Evolution and Function of Bub1

Whereas the role of the KEN-box-containing Mad3/BubR1-like proteins in the MC is well defined, it is less so for the paralogs that retained the kinase domain. These Bub1-like kinases can be found in at least one copy in most eukaryotes examined, either as part of Madbub proteins or of the KEN-box-lacking paralog that originated after evolution from Madbub gene duplications (Figure 2) (Suijkerbuijk et al., 2012). Given the evidence from gene disruptions in mice, *Drosophila*, and both model fungi, Bub1 appears to be essential for MC function (Musacchio and Salmon, 2007). Whether this is mediated by kinase activity is unclear. Studies in human cells, *S. pombe*, and *X. laevis* extracts show that Bub1 kinase activity promotes but is not absolutely required for a robust MC response (Chen, 2004; Klebig et al., 2009; Yamaguchi et al., 2003), while the MC in *S. cerevisiae* responds properly when the Bub1 kinase domain is removed altogether (Fernius and Hardwick, 2007; Warren et al., 2002). Human Bub1 was found to modify Cdc20 on multiple residues in vitro, causing reduced APC/C activity (Tang et al., 2004). Some of these are relatively well conserved, but functional analysis of phosphomimetic substitutions in the background of inactive Bub1 in various organisms will be needed to clarify whether Cdc20 phosphorylation by Bub1 is conserved and part of the core MC. Bub1 kinase activity does have a conserved role in non-MC processes, such as centromere localization of Shugoshin via phosphorylation of T121 on the histone H2A (Kawashima et al., 2010). A recent study of Bub1 function in human cells pinpointed a short sequence, dubbed conserved domain I (CDI), as crucial for the MC (Klebig et al., 2009). Although it is unknown how CD1 has impact on MCC formation, it may have been part of LECA Madbub, as we can recognize CD1 in some Madbub homologs (Suijkerbuijk et al., 2012).

Kinetochore Scaffolds for the Mitotic Checkpoint

Both Mad3/BubR1 and Bub1, as well as the Madbub proteins, have a highly similar TPR domain that interacts with the KMN network member Knl1. This interaction was mapped to the convex surface of the TPR domains and to two "KI" motifs in Knl1, which we and others can recognize only in vertebrate Knl1 homologs (Figure 5) (Bolanos-Garcia and Blundell, 2011; Bolanos-Garcia et al., 2011; Kiyomitsu et al., 2011; Krenn et al., 2012). The mode of Knl1-Bub interactions may be quite flexible and may rely on other motifs in nonvertebrates, because *D. melanogaster* Bub1 interacts with Knl1/Spc105, which is devoid of a clear KI1 motif (Schittenhelm et al., 2009). Knl1 depletion in human and fungal cells prevents Bub1 and BubR1/Mad3 kinetochore binding and checkpoint activation (Kiyomitsu et al.,

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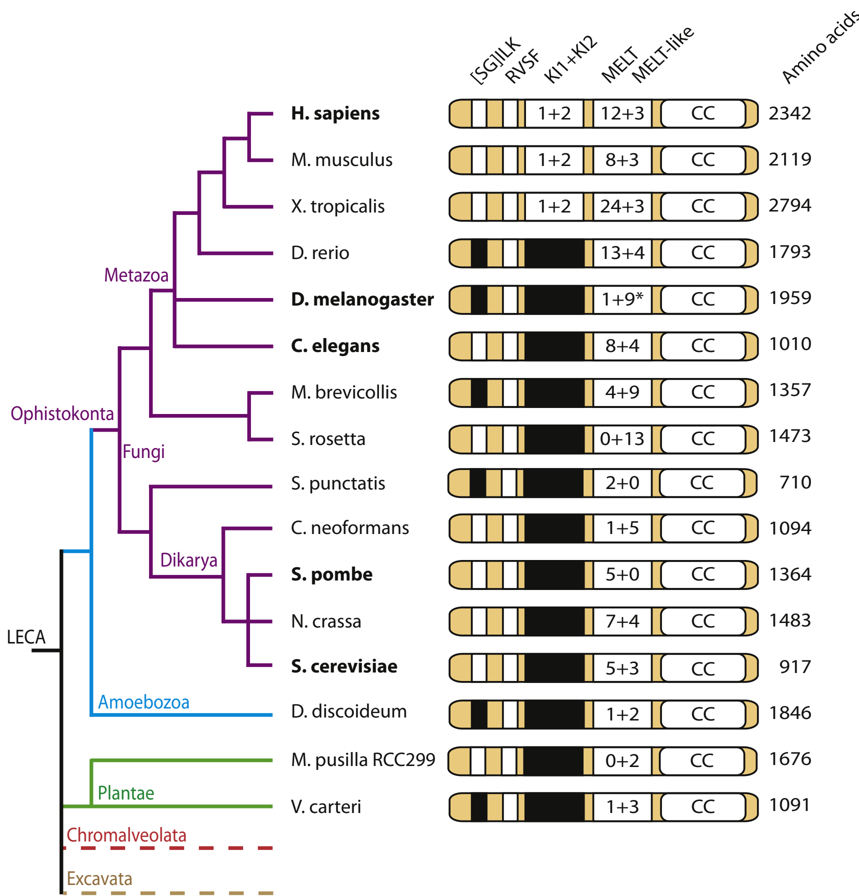

Figure 5. Knl1 Homologs in the Eukaryotic Tree of Life

Schematic representation of eukaryotic tree of life with Knl1 homologs from species listed in Figure 2. Indicated for every homolog are the presence (white box) or absence (black box) of the [SG]ILK and RVSF motifs, the KI motifs, the number of MELT (M[ED][ILVM][ST]) and MELT-like Mxxx (x = 2 out of 3 amino acids are D or E) motifs, the presence of a coiled coil (using the EMBnet coils server), and the ORF length in amino acids. Species in bold indicate experimentally confirmed homologs, and the asterisk indicates the presence of *Drosophila*-specific motifs.

2007; London et al., 2012; Shepperd et al., 2012). This is, however, independent of the interaction between the TPR domains and Knl1. Mutating this interface does not prevent localization of the Bubs and has only minor effects on the MC response (Bolanos-Garcia et al., 2011; Krenn et al., 2012). The functional relevance of the Bub-Knl1 interaction is unclear but may involve, at least for Bub1, an allosteric mechanism for kinase activation (Krenn et al., 2012). The essential role of Knl1 in localizing the Bubs to kinetochores is likely mediated by Bub3, a small globular protein that interacts with the Bub1/BubR1 GLEBS motifs. Like Knl1 depletion, mutating the GLEBS motif in either Bub1 or BubR1 prevents Bub3 binding, abrogates kinetochore localization of both Bubs, and disrupts their various functions in mitosis (Bolanos-Garcia and Blundell, 2011). Oddly, however, Bub3 depletion in human cells inhibits kinetochore localization of BubR1 but not Bub1 (Logarinho et al., 2008). Whether this reflects greater sensitivity of BubR1 to reductions in Bub3, possibly because of differences in kinetochore residence time (Howell et al., 2004), or whether this reflects a possible Bub3-independent role of the Bub1 GLEBS motif is presently unknown. Because the interaction of the Bub1 GLEBS motif to Bub3 leaves limited space for other interaction partners (Larsen et al., 2007), we favor the former possibility. To make matters more complicated, Bub1 is required for BubR1 localization, but not vice versa (Johnson et al., 2004; Klebig et al., 2009). Unraveling the intricate relationship between BubR1, Bub1, Knl1, and Bub3 will be of great interest. One recent insight may be a significant step forward in this regard: in fungi, Bub3 and Bub1 kinetochore localization depends on intact MELT motifs of Knl1 (London et al., 2012; Shepperd et al., 2012).

Knl1 may act as a molecular MC scaffold on more levels than localizing the three Bubs. Through its C-terminal region, Knl1 binds the kinetochore protein Zwint-1 that, in turn, localizes the RZZ complex to kinetochores (Kiyomitsu et al., 2011). Because RZZ promotes Mad1 kinetochore binding (Karess, 2005), Knl1 likely affects the ability of kinetochores to efficiently catalyze MCC formation. Additionally, Knl1 binds PP1 phosphatase through a SILK-RVSF motif near its N terminus. While this interaction is required to stabilize kinetochore-microtubule interactions in human cells (Liu et al., 2010), it silences MC signaling from attached kinetochores in fungi and *C. elegans* (Espeut et al., 2012; Meadows et al., 2011; Rosenberg et al., 2011).

Knl1 displays poor overall sequence conservation, which may explain its propensity, more than other MC components, to escape identification in our homolog searches (Table S1). Despite this, the SILK and RVSF motifs, as well as MELT motifs (defined as M[ED][ILVM][ST]), are well conserved in most identifiable Knl1 homologs, as is a defining C-terminal coiled coil (Figure 5). A striking observation is that the MELT motifs diverge highly in numbers, ranging from 0 (*S. rosetta* and *M. pusilla*) to 24 (*Xenopus tropicalis*). Their functional relevance likely goes beyond Bub recruitment, because a MELT-mutated Spc7/Knl1 in *Schizosaccharomyces pombe* results in profoundly more chromosome segregation problems than deletion of Bub3 (Shepperd et al., 2012). Interestingly, Spc105/Knl1 in Drosophilids have repeats of a slightly distinct motif, which seem largely dispensable for Spc105/Knl1 function in *D. melanogaster* (Schittenhelm et al., 2009). The MELT motifs are thus quite enigmatic, and uncovering their role in mitosis and the reasons for their highly variable numbers in different species will be a great value in our understanding of the connections between the KMN network, microtubule attachments, and the MC.

Regulating the MCC: Essential and Conserved Contributions of Mitotic Kinases

Efficient formation of MCC in cells depends both directly and indirectly on kinase activities. Mps1 and Bub1 were among the original genes found to control the MC in *S. cerevisiae* (Musacchio and Salmon, 2007). In contrast with Bub1, inhibition of Mps1 ablates MC activity in all organisms tested (Lan and Cleveland, 2010; Musacchio and Salmon, 2007) and is therefore the only undisputed MC kinase. Nevertheless, activity of several other kinases, such as Aurora B, Cdk1, and PRP4, also affects MC function, but current evidence supports the notion that they do so predominantly by regulating Mps1 (Montembault et al., 2007; Morin et al., 2012; Saurin et al., 2011).

Mps1 orchestrates many events that contribute to APC/C inhibition, including localization of Bub1, BubR1, RZZ, and Mad1 to unattached kinetochores (in various organisms) (Lan and Cleveland, 2010), Mad2 phosphorylation (in fission yeast) (Zich et al., 2012), and Mad2 dimerization and MCC stabilization (in human cells) (Hewitt et al., 2010; Maciejowski et al., 2010). Recent studies have shown that Mps1 promotes Bub1 recruitment and subsequent MC activity in human cells and in budding and fission yeast by phosphorylating Knl1 on multiple of its MELT motifs (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). The Bubs, however, interact with KI rather than MELT motifs. Because MELT phosphorylations also recruit Bub3, a likely scenario is that Mps1 controls Bub1 (and BubR1/Mad3) localization by promoting the interaction of Bub3 with Knl1, possibly by ensuring Bub3-pMELT binding. This could also contribute to the role of Mps1 in localization of Mad1 to unattached kinetochores, because Bub1 depletion prevents Mad1 kinetochore binding (Musacchio and Salmon, 2007). A MELT-phosphomimetic Knl1 retains Bub1 on kinetochores in the absence of Mps1. In contrast, this mutant Knl1 cannot force kinetochore recruitment of Mad1 under those conditions, suggesting that Mps1 has Bub1-independent mechanisms for recruiting Mad1 (Shepperd et al., 2012). This is supported by *D. melanogaster*, in which Spc105/Knl1 possesses species-specific MELT-like motifs that lack the phosphorylatable threonine (Schittenhelm et al., 2009), while Mad1 localization remains Mps1 dependent (Althoff et al., 2012) (Figure 5). Interestingly, these motifs contain an excess of negative charges (D or E), possibly bypassing phosphodependency of Bub recruitment, but excluding MELT-dependent control of this by Mps1. Our analysis of Knl1 homologs has revealed additional MELT-like methionine-based motifs (methionine followed by two or three acidic residues) in other organisms as well (Figure 5). Given the conservation of Mps1 function and MELT motifs in Knl1, we hypothesize that MELT phosphorylation by Mps1 is a fundamental MC regulatory principle. Whether the widely differing number of MELT and MELT-like motifs in species has any relation to this (for instance by adding levels of control or variable distance between PP1 and Bub [N-terminal] and the RZZ [C-terminal] binding sites) and, if so, how, are intriguing questions. Answers will require detailed insight into which MELT motifs are truly essential for mediating the impact of Mps1 on Bub localization and MC function. Based on species like *Spizellomyces punctatus* and *Dictyostelium discoideum*, we predict one or two will suffice.

Strikingly, although Mps1 is well conserved and essential for error-free chromosome segregation in all organisms tested, no sequence homolog in *C. elegans* can be detected (Figure 2). It is possible that the Mps1 homolog exists but diverged so much as to escape our detection. Alternatively, perhaps the fast-evolving nematode has bypassed a requirement for Mps1 in regulating MCC formation/function, for instance by modifying the mechanism of (Knl1-dependent) Bub3 localization. Finally, a distinct kinase may have replaced Mps1 in nematodes. In this respect it is of interest to note that Mps1 shares significant overlap in consensus phosphorylation sequence with the kinetochore-localized kinase Plk1 (Dou et al., 2011), which is expressed in *C. elegans* (Chase et al., 2000).

Auxiliary MC Proteins: The RZZ Complex

The heterotrimeric RZZ complex plays an essential part in recruitment of Mad1/Mad2 to unattached kinetochores in human and *Drosophila* cells (Karess, 2005). In contrast to Mad1 and Mad2, however, the RZZ subunit Zwilch does not seem to have been retained in many species besides ophistokonta, indicating that RZZ function is a fairly recent add-on to the core MC (Figure 2). This may point to evolution in more complex eukaryotes toward a multiprotein kinetochore interface for Mad1 binding that includes RZZ (Kim et al., 2012). Whereas Zwilch is never found without co-occurrence of an identifiable ortholog of ZW10, the opposite is frequently observed, suggesting a non-RZZ function of ZW10. In support of this, ZW10 is involved in vesicle trafficking in interphase, during which it is part of the conserved NRZ complex that contains Nag and Rint1 in addition to ZW10 (Civril et al., 2010). Homology between Rod and Nag and lack thereof between Zwilch and Rint1 (Civril et al., 2010) indicates that the RZZ may have arisen from NRZ by initially replacing Rint1 with Zwilch, causing it to be retained in those organisms that utilized RZZ for distinct functions. RZZ is coupled to kinetochores via an interaction between ZW10 and Zwint-1 that in turn binds the C terminus of Knl1 (Karess, 2005; Kiyomitsu et al., 2011). In contrast to Zwilch participation, the ZW10-Zwint-1 interaction is likely ancient, as Zwint-1, like NRZ, is suggested to regulate interphasic vesicular trafficking (van Vlijmen et al., 2008). Functions of ZW10-containing complexes furthermore involve the minus-end-directed microtubule motor dynein. ZW10 directly binds the dynactin subunit p50/dynamitin, and, as a result, RZZ ensures kinetochore localization of dynein, required for both chromosome movements and checkpoint silencing (Karess, 2005). RZZ therefore promotes checkpoint activation while simultaneously setting the stage for checkpoint silencing. Because both ancient interphasic and more recent mitotic functions of ZW10 depend on dynein, recruitment of dynein to kinetochores may have provided an important selective force driving ZW10 toward RZZ evolution. Perhaps more complex kinetochore-microtubule interactions benefit from more ways to ensure inhibition of MCC production.

Releasing the Brake: MC Silencing

APC/C activation upon attachment of the final kinetochore is very rapid, suggesting a switch-like release from the MC-inhibited state (Clute and Pines, 1999). MC silencing occurs on two levels: local shutdown of MCC production upon kinetochore attachment and global reversion of APC/C inhibition upon stable

attachment of the final kinetochore (Figure 1B). Below, we will briefly outline different checkpoint silencing mechanisms, their mode of action, and to what extent they have been conserved throughout evolution.

Inhibiting MCC Production upon Kinetochore-Microtubule Interaction

Microtubule attachment depletes essential checkpoint components, including Mad1/Mad2, from kinetochores in a dynein-dependent manner. This is a critical step in MC silencing, because kinetochore-tethered Mad1 is sufficient to delay mitotic exit after full chromosome biorientation is achieved (Maldonado and Kapoor, 2011). Essential to this is the Spindly protein, which depends on RZZ for kinetochore localization and which localizes dynein to kinetochores via its so-called Spindly-box motif [GNSxFxEVxD] (Barisic et al., 2010; Gassmann et al., 2010). Besides a receptor for dynein, Spindly, like RZZ, is also cargo, and it was recently suggested that in fact removal of Spindly from kinetochores is a primary function of dynein in MC silencing (Gassmann et al., 2010). It was proposed that Spindly prevents an undefined dynein-independent pathway for Mad1/Mad2 removal from attached kinetochores and that dynein-dependent removal of Spindly allowed this unknown pathway to operate. Spindly appears to be an ophistokont invention and shows a strong correlation with the presence of Zwilch homologs being absent from, for example, dikaryan fungi (Figure 2; Table S1). This observation is likely related to a role for dynein at mitotic kinetochores, which is nonexistent in either *S. cerevisiae* or *S. pombe*. Because such organisms nevertheless presumably also deplete Mad1/Mad2 from attached kinetochores, it has been speculated that the unknown dynein-independent Mad1/Mad2 removal pathway that Spindly normally prevents is an ancient one (Gassmann et al., 2010). A major challenge for the future will be to examine whether such a dynein-independent pathway for clearing MC proteins from kinetochores exits, what its molecular identity is, and how RZZ and Spindly affect its function. Given the conserved nature of Knl1, it may involve a recently defined MC silencing mechanism that relies on direct interaction of Knl1 with microtubules (Espeut et al., 2012).

Undoing the Actions of MC Kinases

Besides physically removing MC proteins from kinetochores as soon as they engage a microtubule, MC silencing requires dephosphorylation of essential MC targets. A PP1-like phosphatase is needed for the ability of yeast cells to exit from an MC-induced cell-cycle delay (Hardwick and Shah, 2010). Specific PP1 isoforms localize to bioriented kinetochores via the N-terminal SILK and RVSF motifs in Knl1 (Espeut et al., 2012; Liu et al., 2010; Meadows et al., 2011; Rosenberg et al., 2011). This specific interaction contributes to MC silencing in budding and fission yeast, as well as in *C. elegans* (Espeut et al., 2012; Meadows et al., 2011; Rosenberg et al., 2011), but it is unknown whether this is also true for vertebrates, in which a dynein-dependent MC silencing mechanism has evolved. Phosphatases are nevertheless likely required for exit from an MC arrest in human cells, as persistent kinetochore Mps1 maintains MC signaling from attached and bioriented kinetochores in metaphase (Jelluma et al., 2010). Similar reversal of Mps1-mediated phosphorylations also contributes to PP1's role in MC silencing in budding yeast (Pinsky et al., 2009), which could involve dephosphorylation of the Knl1/Spc105 MELT motifs (London et al., 2012).

Freeing the APC/C: Disassembly of MCC-APC/C Complexes

Once all kinetochores have achieved stable attachments to spindle microtubules, what remains for cells to initiate anaphase is releasing APC/C inhibition by MCC. As outlined in our discussions on Cdc20, this process requires APC/C-dependent ubiquitination and the actions of the Mad2-mimetic p31^{comet}. The same surface on Mad2 interacts with both p31^{comet} and Mad3/BubR1, suggesting that p31^{comet} actively disrupts MCC stability by competing out Mad2 (Chao et al., 2012; Westhorpe et al., 2011). This may simply be achieved by the observed high affinity of p31^{comet} for C-Mad2 (Vink et al., 2006), but it somehow also involves Cdk1-dependent phosphorylation of Cdc20 (Miniowitz-Shemtov et al., 2012). How Cdk1, the APC/C, and p31^{comet} collaborate to ensure efficient MCC disassembly is presently unclear but may involve, for example, Cdk1- and APC/C-mediated relaxation of structural constraints to allow more efficient p31^{comet}-dependent exclusion of Mad2 from MCC. It will be of additional interest to examine how rapid disassembly by this pathway is regulated by kinetochore attachment. p31^{comet} is located exclusively on unattached kinetochores with a residence time identical to Mad2, leading to a proposed model in which p31^{comet} is modified by unattached kinetochores in order to prevent its premature action on MCC disassembly (Hagan et al., 2011). As postulated before (Yang et al., 2007), our analysis shows that Mad2 and p31^{comet} are probably paralogs that have arisen by a pre-LECA gene duplication (Figure 2; Supplemental Experimental Procedures). In contrast to the widespread maintenance of MCC throughout evolution, p31^{comet} was apparently lost in many species, which is particularly apparent in fungi (Figure 2; Table S1). Unlike most other fungi, the higher basidiomycete fungi *Ustilago maydis* contains p31^{comet} and has a metazoa-like open mitosis and anaphase B-like spindle elongation (Steinberg and Perez-Martin, 2008). Examining the p31^{comet} homolog in *U. maydis* cell division may provide intriguing insights into its mitotic functions and may help to reveal why p31^{comet} was allowed to disappear from the genomes of some organisms while it was retained by others.

Concluding Thoughts and Future Directions

In this review, we have attempted to integrate current knowledge on the molecular workings of the MC with our evolutionary analysis of key MC (silencing) proteins and their functional domains and motifs. Inspired by this, we propose an outline of the ancient MC and its functional modules (Figure 6), a core that is conserved in species that utilize the MC and that was likely present in LECA. The various species-specific additions, deletions, and/or modifications to this core may be related to fundamental differences between mitoses in these organisms. Such differences include but are by no means limited to: open versus closed mitosis, holocentric versus point centromeres, the size of kinetochores and the amount of microtubules connecting these to the mitotic spindle, the number of chromosomes to be segregated, the size of the cells, and the amount of cells that make up the organism. Future studies on the relation between such differences and MC function will be of interest not only from an

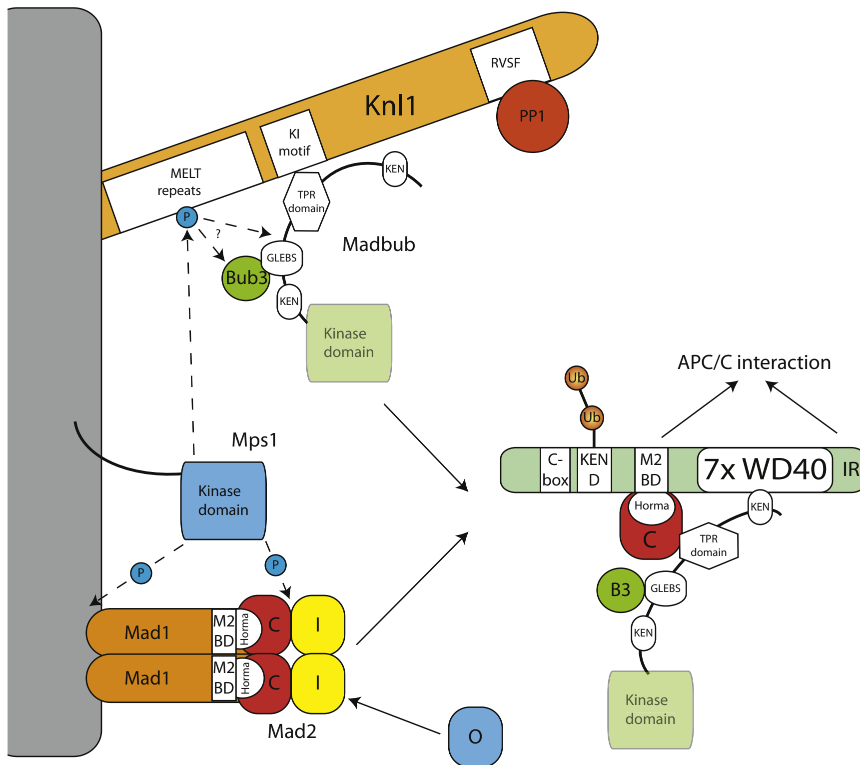

Figure 6. The Ancient and Conserved Core MC Proteins and Their Domains/Motifs
Phosphorylation of one or more MELT motifs on Knl1 by Mps1 recruits Bub3 and the Madbub protein. Mps1, together with the Madbub protein, further ensures kinetochore binding of Mad1 that interacts with the Horma domain of Mad2, allowing subsequent structural conversion of Mad2 into a closed form. C-Mad2 and Madbub/Bub3 assemble onto Cdc20 via various indicated domain/motif interactions to inhibit Cdc20 activity. PP1-mediated checkpoint silencing occurs through its interaction with the RVSF motif in Knl1. See text for further details.

evolutionary perspective but also from the perspective of understanding the MC and its adaptability. Many additional outstanding questions remain in relation to the conserved MC activation and silencing mechanisms. How and where is the MCC formed? How is the signal amplified from individual kinetochores, and, possibly in relation to this, how are MC kinases activated and what are their critical substrates? How is MCC action reverted upon MC satisfaction, especially considering the poor conservation of p31^{comet}? How does the state of attachment of kinetochores translate to recruitment or removal of MC proteins? The lack of kinetochore dynein and Spindly/RZZ in most species points to another, more ancient, mechanism that might or might not be retained in all eukaryotes. Binding of MC proteins like Bub1, Mad3/BubR1, and Mps1 with KMN network components are intriguing interactions on which silencing mechanisms could act to affect MC activity, but it is unknown whether such interactions are directly sensitive to microtubules. Uncovering which principles are ancient will require significant efforts in establishing sensitive real-time and biochemical assays for measuring MC activity, kinetochore changes upon microtubule binding, and MCC assembly and disassembly in a variety of model organisms. Because evolution has done most of the experiments for us, it may further be worthwhile to adopt less widely used and possibly even novel model organisms into this exciting field of research.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, one sequence file, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2012.06.013.

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