# Regulation of the metaphase-to-anaphase Transition

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# Introduction

The cell division cycle consists of a series of temporally and spacially regulated events. In many eukaryotes, cell proliferation depends on the ubiquitin ligase (E3) activity of the Anaphase Promoting Complex / Cyclosome (APC/C). APC/C is essential for the separation of the sister chromatids in anaphase, for the exiting of mitosis and the resulting division into two cells. During S-phase, APC/C is essential in the initiation of the steps leading to DNA replication.

As all E3 ligases, APC/C performs its functions by assembling polyubiquitin chains on its substrate proteins, which targets them for destruction by the 26S proteasome. Destruction is mostly associated to inactivation of a protein or complex, but can also lead to the activation of a protein or complex. The most important examples of the former are the proteolysis of mitosis-specific Aurora kinases, Polo-like kinase-s (Plk1), mitotic A- and B-type cyclins, and interestingly the critical co-activator of APC/C in mitosis Cdc20. The prominent examples of the latter are the destruction of securin, the inhibitor of separase, a protein that cleaves cohesion, and geminin, a protein that inactivates the replication factor CTD1. In this paper I will focus on the essential mitotic functioning of APC/C, including a detailed description of the current understanding of the factors regulating the functioning.

The goal of mitosis is to take the duplicated DNA, condensed in chromosomes, and distribute them evenly, such that each daughter cell has a complete copy of the genome. Video microscopy has shown that the time taken for all chromosomes to bi-orient at the so called metaphase plate can vary considerably between cells, however, the interval between bi-orientation of the last chromosome (metaphase) and sister chromatid separation (anaphase) is relatively invariant. Crucially, cells rarely, less than 1 in 100.000divisions, start sister chromatid separation while there still exist at least one chromosome that has not bi-oriented. Furthermore, disassembly of microtubules by the addition of poisons such as nocodazole delays sister chromatid separation, as well as exit from the mitotic state, for many hours. This suggests that cells are capable of monitoring the attachment of kinetochores to microtubules and prevent activation of the APC/C and hence separase when kinetochores are unoccupied by microtubules. This surveillance mechanism is known as the spindle assembly checkpoint or SAC. Failure of the SAC results in aneuploidy, daughter cells having an incorrect number of chromosomes, resulting in malignancy and birth defects. Given the importance of the SAC process, it is understandable, as well as astonishing, that even one uncaptured chromosome pair can delay anaphase.

Unattached kinetochores in prometaphase activate the SAC by triggering a pathway that ultimately inhibits APC/C. The effector of the SAC is the mitotic checkpoint complex (MCC), which binds directly to APC/C. The MCC, containing Cdc20 and three other SAC proteins, Mad2, BubR1 (Mad3 in yeast), and Bub3, binds to APC/C as a pseudosubstrate inhibitor.

Other SAC components include Mad1, and the kinases Bub1, Aurora B, and Mps1. These proteins are required to amplify the SAC signal or the rate of SAC formation. Furthermore, Rod, ZW10, Zwilch (together in RZZ complex) p31comet, MAPK, CDK1-cyclin-B, PLK, CENP-E, and dynein, together with dynein associated proteins as spindly, dynactin CLIP and LIS1 are identified to regulate the SAC in higher eukaryotes.

FRAP studies show that during prometaphase, Cdc20 and all SAC proteins localize at kinetochores, many of them in a dynamic manner, suggesting that the kinetochores are a catalytic platform for MCC production. Remarkably, APC/C and cyclin-B are also shown to locate at kinetochores, and spindle apparatus, during prometaphase.

When all kinetochores become attached to microtubules and are under tension, the transition from metaphase to anaphase happens very quickly: 5-6 minutes in mammalian tissue culture cells. The time scale of this progression is much shorter than the predicted timescale of spontaneous Cdc20:Mad2 dissociation from the MCC, suggesting that the dissociation of parts of the MCC from APC/C is an active process. There are several mechanisms proposed to silence the inhibition of APC/C.

A detailed description of both the inhibition of APC/C and the silencing of the inhibition can be found below. Firstly, however, I will describe the APC/C complex and its cofactors, including their roles in APC/C-mediated ubiquitylation.

# APC/C complex: Structure and function

***Structure.*** APC/C is a huge E3 enzyme, depending on the organism, composed of twelve to eighteen subunits, together building a molecular machine of about 1 MDa in size. APC/C can be divided in four functional blocks: a structural block consisting of proteins rich in tetrico-peptide repeats (TPR), a scaffolding block, a catalytic block, and activator block. The TPR proteins are thought to modulate activator regulation[[1-3](#_ENREF_1)]. Scaffolding proteins link the TPR block to the catalytic block, perhaps optimizing spacing and orientation, in order to have the most efficient catalysis. The catalytic block includes RING finger APC11, and the cullin-like APC2, and functions together with the processivity factor APC10. Finally, the activators are members of the Cdc20 (cell division cycle) family of tryptophan-aspartate (WD) repeat proteins [[4](#_ENREF_4)]. A 2D representation of the APC/C can be found in figure 1. APC/C can only (poly)ubiquitylate substrates with the help of ubiquitin-activating enzyme (E1), two ubiquitin-transferring enzymes (E2), and a co-activator protein.

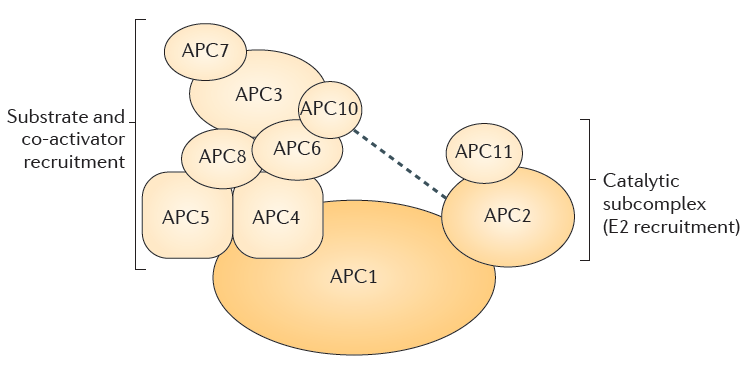


Figure 1: Schematic representation of APC/C subunits. The catalytic subcomplex is linked by a scaffolding subcomplex (APC1) to a structural subcomplex. The structural subcomplex of APC/C is responsibly for substrate and co-activator recruitment. Figure 1 is taken from [[5](#_ENREF_5)].

***APC/C enzymology***. APC/C uses two E2’s in tandem: a member from the E2C family or UBCH5 to initiate the ubiquitin chain on the substrate, and an ubiquitin-conjugating enzyme (Ubc1 in yeast; UBE2S in humans) to extend the chain [[4](#_ENREF_4), [6](#_ENREF_6)]. E2C null mutation studies have shown that E2C is vital in fission yeast and *Drosophila melanogaster [*[*7*](#_ENREF_7)*,* [*8*](#_ENREF_8)*]*. However, depletion studies in human cells show conflicting results [[9](#_ENREF_9)]. Recent studies have shown that the depletion of UBE2S has a much more significant effect than the depletion of UBCH10 (E2C member) [[6](#_ENREF_6)].

The use of two E2s by the APC/C is conserved between species. However it is unknown why there is conservation, considering the difference in the linkage specificity of polyubiquitin between species. In budding yeast, APC/C creates Lys48-linked polyubiquitin chains [[10](#_ENREF_10)], whereas in Xenopus leavis and human APC/C generates predominantly Lys11-linked ubiquitin chains, or mixed Lys11 and Lys48-linked chains [[6](#_ENREF_6)]. A possible explanation for the difference would be that Lys11-linked chains distinguish APC/C substrates from subtrates from other ligases, and therefore allows regulation by specific de-ubiquitylation enzymes (DUBs) after release from APC/C. The X-ray crystal structure of K11-linked chains shows a compact structure like K48-linked ubiquitin chains [[11](#_ENREF_11)]. However, the hydrophobic patches which mediate the inter-ubiquitin interactions in K48-linked chains are surface exposed in K11-linked chains. One question that comes to mind is whether all APC/C are in complex with the E2’s.

***APC/C co-activators.*** In addition to the E2 enzymes, APC/C activity is highly dependent on the association of one of several co-activators, during specific periods in the cell cycle. The ones best studied, and active in mitosis, are Cdh1 and Cdc20. Both proteins are characterized by sequence elements; a C-box and IR-tail mediating the binding to APC/C; and a C-terminal WD40 domain, mediating the binding of the substrates of APC/C [[5](#_ENREF_5)].

***3D structure***. Structural data from EM single particle 3D reconstruction studies show that APC/C has a roughly asymmetric triangular structure with a central cavity[[12-14](#_ENREF_12)]. Although there is conflicting information, nowadays this cavity is thought to be the catalytic site[[12](#_ENREF_12), [14](#_ENREF_14)]. Many crystallographic studies have focused on isolated subunits or mall sub-complexes of APC/C. A recent study has assigned the EM envelope to specific APC/C subunits[[15](#_ENREF_15)], as shown in figure 2. This study confirms the notion that the cavity is the location of interaction between substrate and D-Box receptor (Doc1/APC10) subunit of the APC/C and ensures positioning of the substrate for catalysis by the APC2/Apc11 catalytic core.



*Figure 2: Subunit organization and pseudo-atomic model of APC/C from different angles. Atomic coordinates of Cdc16-Cdc26, Cdc23, Cdc27, Apc2, Apc11, Apc10 and Cdh1 are docked in the 11 Å cryo-EM map of [14]. Image taken from [*[*15*](#_ENREF_15)*].*

***APC/C Substrate Specificity*** Short conserved sequence motifs, commonly referred to as degrons, are used by subunits of APC/C to recognize destruction candidates. At least eight different APC/C targeting degrons in substrates have so far been identified. Mutations in degrons often stabilize substrates, and addition of degrons to proteins promotes ubiquitination[[16](#_ENREF_16)]. Many of the degrons are quite common motifs in the proteome. Therefore the mere presence of a degron, does not make a protein a candidate for APC/C destruction. Interestingly, bona fide degrons are often found in disordered regions, as is the case for securin and cyclin B[[17](#_ENREF_17)].

The most common degrons are the D-box and the KEN-box. The D-box was firstly identified in cyclin-B [[16](#_ENREF_16)], and is characterized by RxxLxxxN. A D-box is found in substrates for APC/CCdc20 and for APC/CCdh. The KEN box is recognized by APC/CCdh1, characterized by KENxxxN [[18](#_ENREF_18)].

Other identified degrons are the TEK box [[19](#_ENREF_19)], the A box[[20](#_ENREF_20), [21](#_ENREF_21)], the CRY box[[22](#_ENREF_22)], the GxEN[[20](#_ENREF_20)], the O-box[[23](#_ENREF_23)], and a Spo13 specific sequence[[24](#_ENREF_24)]. These degrons might not be conserved between species, and are less common in APC/C substrates than D-box or KEN-box. Furthermore, these degrons are often found in substrates that also have other degrons, making their contribution to APC/C substrate recognition unclear.

An interesting observation is that although the D-box in the N-terminus of cyclin-B is nine aminoacids long, a much longer stretch around the D-box is required for APC/C to recognize it when attached to another protein [[25](#_ENREF_25), [26](#_ENREF_26)].

Most degrons do not seem to be post-translationally modified, which suggest that it is APC/C itself which is activated to recognize them at different times in the cell cycle. Exeptions are: the D-boxes of Cdc6 and Pds1, both phosphorylated to prevent recognition [[27](#_ENREF_27), [28](#_ENREF_28)]; and interestingly, the acetylation of Bubr1 at a Lysine close to its KEN-box protects it from degradation, possibly by prevention of ubiquitylation [[29](#_ENREF_29)].

Substrate specificity has been proposed to be conferred by the N-terminal of the E2C family [[30](#_ENREF_30)].

An EM single particle 3D reconstruction study identifies Cdh1 and Apc10 as the D-box co-receptor[[14](#_ENREF_14)]. The substrate binds between the WD40 B-propeller domain of Cdh1 and the b-sandwich of APC10 (Doc1). The formation of a D-box co-receptor is consistent with earlier findings [[31-34](#_ENREF_31)]. Cdh1 shifts towards APC10 upon binding of the substrate. Biochemical data suggest that Cdc20 binds to APC/C in the same way as Cdh1 to form a similar D-box co-receptor with APC10 [[35](#_ENREF_35)]. However, this has not been confirmed structurally.

***Activation of APC/C.*** The activity of APC/C varies throughout the cell cycle. It is low in S and G2 phase, and high in mitosis and G1. Nuclear envelope breakdown (NEBD) and mitotic entry are triggered by activation of CyclinB1-Cdk1. Cdc20-dependent activity emerges immediately after NEBD, at the same time as APC/C get phosphorylated [[36](#_ENREF_36)]. This suggests an activating role for phosphorylation. Both APC/C and cyclinB1-Cdk1 have been shown to recruit to the kinetochores during prometaphase, suggesting APC/C could get activated at kinetochores [[37](#_ENREF_37), [38](#_ENREF_38)]. B-type cyclins phosphorylate several APC/C subunits, enhancing its affinity for Cdc20[[39](#_ENREF_39)].

In budding yeast, cyclin-CDK-mediated phosphorylation determines which co-activator binds to APC/C. By changing the cyclin-CDK phosphorylation sites allows Cdh1 to bind to APC/C during mitosis, making Cdc20 dispensable [[40](#_ENREF_40)].

Whether cyclin-CDK-mediated phosphorylation is the only activation mechanism of APC/C is unclear. Conflicting studies exists about the activating role of PLK1, or the Plk1-dependent degradation of early mitotic inhibitor 1 (Emi1) [[5](#_ENREF_5)].

Binding of co-activators might also have a function in activating APC/C. Fusing the N-terminal domain of cdc20 to NEK2, leads to NEK2 degradation in absence of Cdc20 [[41](#_ENREF_41)].

Finally the binding of co-activators might induce a conformational change in APC/C, resulting in better arrangement of substrate and E2. This is seen in vertebrates [[12](#_ENREF_12)], but has not been detected for yeast APC/C [[14](#_ENREF_14)].

# Inhibition of APC/CCdc20 by Spindle Assembly Checkpoint.

When the APC/C is activated, it is necessary that the degradation of securin and cyclin-B is delayed until all chromosomes have correctly aligned. This is achieved by the SAC, through multiple strategies which inhibit APC/CCdc20; including the stoichiometric binding to and sequestration of Cdc20 by Mad2, BubR1, and Bub3, as well as phosphorylation of Cdc20 by Bub1. The main effector of SAC is MCC. In this chapter I will firstly describe the structure of MCC, after which different generation pathways of anaphase inhibitors (i.e. Cdc20:Mad2, Cdc20:BubR1:Bub3, or MCC) will be described. Finally, the transduction and amplification of the anaphase inhibitors will be discussed, including the role of several kinases.

***Structure of MCC*** A recent structural study on the MCC (crystal structure of Cdc20, mad2 and mad3) shows that Mad2 and mad3 bind to Cdc20 directly [[42](#_ENREF_42)], see figure 3 and 4. The Mad3 KEN-box binds to the KEN-box recognition site on Cdc20, blocking its function in cdc20-mediated substrate recognition by APC/C. The MCC crystal structure corresponds closely to the MCC density found in an earlier electron-microscope derived density map of APC/CMCC [[13](#_ENREF_13)] The D-box site of Cdc20 is still directed towards APC10(Doc1). However, compared to APC/CCdh1-D-box, the D-box of cdc20 in APC/CMCC is shifted downwards towards APC5. Possibly facilitating the ubiquitylation of Cdc20, and preventing the formation of a bipartite substrate recognition site with APC10. The ubiquitylation hypothesis is further strengthened by the fact that the KEN-box of Mad3 involved in binding Cdc20 is known to promote Mad3-dependent APC/C-mediated degradation of Cdc20. Suggesting that the binding of Mad3 has a role in positioning Cdc20 for autoubiquitylation. Mad2 possibly has a role in the repositioning of Cdc20 as well. Binding of Mad2 to the n-terminal of Cdc20 possibly prevents the neighboring C-box to find its binding site at the APC/C, which is necessary for Cdc20-dependent stimulation of APC/C.



*Figure 3: Structure of S. pombe MCC trimer. Mad3 is shown in teal, Mad2 in green and Cdc20 in yellow. The KEN box of Mad3 is shown in red. The picture is taken from [[42](#_ENREF_42" \o "Chao, 2012 #40)].*



*Figure 3: Pseudo-atomic structure of human APC/CMCC.The crystal structure of MCC (see figure 3), as well as other known crystal structures of APC/C subunits, are docked into the density map of [*[*13*](#_ENREF_13)*] Image taken from [*[*42*](#_ENREF_42)*].*

***Kinetochore independent MCC formation.***

Even though all SAC proteins can be recruited to kinetochores of S. cerevisiae [[43](#_ENREF_43)], MCC is detectable in checkpoint defective cells [[44](#_ENREF_44), [45](#_ENREF_45)]. A detailed analysis of mitotic timing in mammalian cells, depleted of different SAC components, shows that Mad2 and BubR1 depletions affect the overall timing of mitosis in a kinetochore-independent manner [[46](#_ENREF_46)]. On the other hand, the depletion of Mad1, Bub3, and certain other kinetochore proteins, result in SAC deficiency, without affecting the duration of mitosis. This suggests that there might be two phases in the SAC control of mitosis. Phase 1: Mad2 and BubR1, proteins that bind Cdc20 directly – suggesting sequestration of Cdc20 as inhibiting mechanism, control inhibition of APC/CCdc20. Phase 2: the kinetochore-dependent SAC regulates mitotic timing. Alternatively, the difference in timing may simply reflect differences in the penetrance of inhibition by the used methods. In support of this, Mad1 antibody injection caused similar acceleration of mitosis as Mad2 antibody injection did. Possibly therefore, there is no such thing as two phases.

Furthermore, Mad3 is recruited to kinetochores in most organisms. However, in *C. elegans*, this is not the case. It has to be considered that in the same species, the effect of depletion of Mad3 can be suppressed by overexpression of Mad2 (makes me question whether depletion was 100%) [[47](#_ENREF_47)]. However, overexpression of Mad2 does not compensate for depletion of Mad3 in mammalian cells . In mammalian cells, it has been shown that the N-terminus of BubR1 is enough to act as an APC/C inhibitor[[48](#_ENREF_48)].

Finally, it could be the case that APC/C is sensitized by unattached prometaphase kinetochores. There is evidence that APC/C gets recruited to unattached kinetochores [[38](#_ENREF_38)]. Furthermore, one study shows that MCC is present in interphase, but does not inhibit APC/C [[49](#_ENREF_49)]. These results have not been confirmed, and in vitro addition of Mad2 and BubR1 to interphase APC/C does show functional inhibition [[50](#_ENREF_50)].

***Kinetochore dependent MCC formation.***

Localization of all SAC proteins to unattached kinetochores strongly suggests that kinetochores contribute to MCC formation. A detailed description of the mechanisms leading to the generation, and amplification of the MCC signal by unattached kinetochores will follow.

***Mad2 kinetics at kinetochore***. All MCC constituents cycle on and off kinetochores with high turnover rates (t½ < 25 s) [[51](#_ENREF_51)]. Kinetochore Mad2 exists in two independent pools; one stably bound the kinetochore, the other more mobile [[52](#_ENREF_52)]. Mad2 binds tightly to Mad1. Mad1 is required for Mad2 localization at the kinetochore, is a stable resident of the kinetochore during prometaphase, where it is known to form dimers [[51](#_ENREF_51), [53](#_ENREF_53)]. Upon binding to Mad1, Mad2 switches from an open Mad2 conformation (O-Mad2, from seatbelt analogy) to a closed C-Mad2 conformation. The closed form Mad2 is able to recruit an extra O-Mad2 molecule, and induce the transition from O-Mad2 to an intermediate state I-mad2, which is destined to turn into C-Mad2 upon binding of Cdc20 (or Mad1). Evidence for this model shows that when dimerization of Mad2 is inhibited by point mutations, the catalytic effect of kinetochores on APC/C inhibition is lost [[54](#_ENREF_54)]. Interestingly, FRAP studies show that the turnover rate of Cdc20, is close to the turnover rate of Mad2, suggesting that the slow pool of Cdc20 can leave the kinetochore bound to Mad2. The conversion of Mad2 from open to closed conformation bound to Cdc20 is rate limiting in the formation of MCC [[54-56](#_ENREF_54)]. The exact cellular stoichiometry of the MCC complex is under debate. Herzog et al 2009 show that 50% of APC/C is bound to complete MCC. Other studies however, show that a complex of Cdc20, Bub3 and Mad3 is bound to the majority of APC/C [[54](#_ENREF_54), [57](#_ENREF_57)]. The binding of Mad2-less MCC is capable of inhibiting APC/C, but less strongly than the complete MCC [[54](#_ENREF_54)]. Mad2 possibly detaches from the MCC complex during purification, although that should have been the case for Herzog et al as well.

***Mad2 template model*** One of the most remarkable feature of mitoses is that just one unattached kinetochore can delay metaphase-anaphase transition. Mechanistically a system able to do has to have the following properties: the rate at which the inhibition signal arrives at and associates to the target complex, should be higher than its removal (destruction, competition, or spatial removal). A study of Rieder et al showed that the inhibition of APC/C by MCC is diffusion limited [[58](#_ENREF_58)]. This could mean that the active, regarding metaphase-anaphase transition, APC/C is relatively close to the kinetochores.

A postulated system that potentially could achieve strong enhancement of the MCC signal is the Mad2 template model. Previously it has been shown how the formation of C-Mad2 is achieved at the kinetochore. Cdc20 can trigger the same conformational change in Mad2, as Mad1 can. The Kd of the interaction of O-Mad2 with Cdc20 is about 100 nM, implying that binding of these two proteins occurs spontaneously at cellular concentration[[59](#_ENREF_59)]. The Kon, however, is three to four orders of magnitude slower than normal protein association rates [[59](#_ENREF_59)]. The generation of C-Mad2 (or I-Mad2) by Mad1:C-Mad2 complexes might lower this energetic barrier. Because Cdc20:C-Mad2 bind and activate free O-Mad2, just as Mad1:C-Mad2 can, and is able to diffuse freely through the cell, Cdc20:C-Mad2 might have an autocatalytic, prion like-reaction within the cell, triggering the formation of Cdc20-C-Mad2 copies. Models describing such autocatalytic systems predicted that it is indeed the case that such a system would be able to inhibit APC/C, even when only one kinetochore is unattached. However, the quick response rate to the binding of the last kinetochore would not be possible, due to too high concentration of the inhibitor [[60](#_ENREF_60)]. A one-step amplification system would however be able to silence and respond to the final binding [[61](#_ENREF_61), [62](#_ENREF_62)].

***BubR1/Mad3.*** BubR1 inhibits the activity of APC/CCdc20 more efficiently than Mad2 does, *in vitro* [[50](#_ENREF_50), [54](#_ENREF_54), [63](#_ENREF_63)]. BubR1 uses a TPR-domain in the N-terminal to bind to the kinetochore, a conserved GLEBS to mediate the binding of Bub3, and has a C-terminal kinase domain [[64](#_ENREF_64), [65](#_ENREF_65)]. The latter is not conserved between species, and not required for APC/C inhibition. BubR1 kinase activity might be required for other aspects of kinetochore signaling or chromosome alignment [[66](#_ENREF_66), [67](#_ENREF_67)]. As mentioned above, BubR1 forms a complex with Cdc20 through it KEN-box. This binding possibly shifts the location of Cdc20, blocking the formation of a bipartite D-box substrate-binding site with APC10. Interestingly, BubR1 itself does not undergo APC/CCdc20 dependent degradation, suggesting that BubR1 is a pseudo-substrate inhibitor of APC/CCdc20 [[68](#_ENREF_68), [69](#_ENREF_69)]. Acetylation of K250 has been suggested to allow BubR1 to escape the APC/CCdc20 dependent degradation [[29](#_ENREF_29)].

***Inhibition of Cdc20 by Mad2 and BubR1.*** The binding of Mad2 and BubR1 to Cdc20 simultaneously inhibits APC/CCdc20 more efficient than either alone [[50](#_ENREF_50), [54](#_ENREF_54)]. Depletion of both affects the duration of mitosis more, than either alone. This suggests that both have a residual inhibitory function in absence of each other. An interesting theory is that firstly Mad2 and BubR1 bind to Cdc20 simultaneously, to achieve potent APC/CCdc20 inhibition. In later stages, Mad2 dissociates from APC/C, leaving bound BubR1 to inhibit securin polyubiquitylation. This would explain the observation that APC/C is often not bound to the complete MCC, but to Cdc20:BubR1:Bub3 [[57](#_ENREF_57)]

***BubR1 and CENP-E.*** BubR1 interact with, and phosphorylates CENP-E (a microtubule-plus-end-directed motor, that contributes to chromosome alignment) [[70](#_ENREF_70), [71](#_ENREF_71)]. Studies show that in absence of CENP-E, few unattached chromosomes are unable to sustain SAC activity [[72](#_ENREF_72)]. However, if all chromosomes are unattached, absence of CENP-E does not impair a valid SAC response [[72](#_ENREF_72)]. This suggests that CENP-E is involved in SAC amplification. CENP-E binds and activates BubR1 kinase activity in vitro [[73](#_ENREF_73)]. This activity is repressed upon microtubule binding [[73](#_ENREF_73)].

***Bub1.*** Bub1 could have an equally important role as the Mad1 in the formation of APC/C inhibitor complexes. Like Mad1, Bub1 binds stably to kinetochores in mitosis, and targets other mitotic regulators to the kinetochores, including Bub3, Mad3 and Plk1 [[52](#_ENREF_52), [74](#_ENREF_74), [75](#_ENREF_75)]. Ectopic targeting of Bub1 to telomeres specifically recruits Mad3 and Bub3 to telomeres, suggesting a scaffolding role for Bub1 [[75](#_ENREF_75)]. The N-terminal TPR domain of human Bub1 and BubR1 interacts with KNL1/Blinkin, through adjacent but conserved motif in the N-terminal domain of Blinkin [[64](#_ENREF_64), [76](#_ENREF_76)]. Remarkable is that this kinetochore targeting of Bub1 and BubR1 is dependent on Bub3, which is mechanistically not understood [[77](#_ENREF_77)]. Whether Mad1:Mad2 scaffold and Bub1 scaffold interact directly at kinetochores is unknown, although some studies have identified a Mad1:Bub1 complex [21,22]. However, the catalytic effect of kinetochore-bound Mad1-Mad2 has been shown, whereas there was no observed kinetochore-induced catalytic effect of BubR1 and Bub3 [[54](#_ENREF_54)]. This discrepancy is observed in vitro, which does not imply that the effect does not occur in vivo.

Bub1 might also be involved in the phosphorylation of Cdc20, although this remains a contentious issue. Some reports have suggested that Bub1’s catalytic activity is very important: the Bub1-mediated phosphorylation of Cdc20 at S153 inhibits the functioning of APC/CCdc20 in cells [[78](#_ENREF_78)]. Like BubR1, Bub1 has a KEN-box in the N-terminal domain, enabling the recruitment of Cdc20 to Bub1, resulting in efficient phosphorylation [[79](#_ENREF_79)]. Depletion of Bub1 or expression of the kinase-dead form of Bub1 abolishes Cdc20 phosphorylation and suppresses the SAC [[78](#_ENREF_78)]. Another independent study showed that the kinase-dead Bub1 mutant is less effective in rescuing a cell from Bub1 depletion than normal Bub1 is [[80](#_ENREF_80)]. Opposing evidence suggests that Bub1 has little effect on SAC response [[65](#_ENREF_65)]. Other proteins that can phosphorylate Cdc20 are Mapk and Cdk1 [[81](#_ENREF_81), [82](#_ENREF_82)]. It is currently unknown whether the phosphorylation of Cdc20 plays a role in the formation of MCC, or how the phosphorylation plays a role in the deactivation of APC/CCdc20.

**MPS1.** Mps1 is an evolutionarily conserved protein kinase required for the SAC and chromosome bi-orientation [[83](#_ENREF_83" \o "Yamagishi, 2012 #227)]. Mps1 has a function in the localization of Mad1 and Mad2 to kinetochores [[84](#_ENREF_84" \o "Abrieu, 2001 #32)]. Mps1 has two complementary roles in SAC regulation. Firstly, upon mitotic entry *cytoplasmic* Mps1 is activated through phosphorylation by Cdk1-cyclin, where it promotes the association of Mad2 and BubR1 to Cdc20 [[85](#_ENREF_85" \o "Maciejowski, 2010 #34), [86](#_ENREF_86" \o "Morin, 2012 #51)]. Secondly, upon the localization of Mps1 to kinetochores, Msp1 promotes several key events: it recruits Mad1:C-Mad2 to kinetochores [[87](#_ENREF_87" \o "Hewitt, 2010 #24), [88](#_ENREF_88" \o "Jelluma, 2010 #28)]; it recruits cytosolic O-Mad2, where it gets converted to C-Mad through binding to Mad1:C-Mad2; and phosphorylates the conserved MELT motifs of the kinetochore component KNL1/Blinkin [[89](#_ENREF_89" \o "London, 2012 #52)], which in its turn recruits Bub1 to kinetochores. The localization of Mps1 itself to kinetochores requires the kinetochore protein complex Ndc80 [[90](#_ENREF_90" \o "Martin-Lluesma, 2002 #230)], and Msp1’s N-terminal domain, including phosphorylation of T12 and S15 [[86](#_ENREF_86" \o "Morin, 2012 #51)].

Mps1 activation at kinetochores is dependent on the trans-autophosphorylation events; chemically induced Mps1-dimers are sufficient to activate Mps1 [[91](#_ENREF_91" \o "Kang, 2007 #231)], which is in agreement with the observation that Mps1 forms oligomers in mitotic human cells [[87](#_ENREF_87" \o "Hewitt, 2010 #24)]. Inhibition of the kinase activity of Mps1 promotes the accumulation of Mps1 to kinetochores, suggesting an negative feedback meachanim regulating the recruitment of Mps1 to kinetochores [[88](#_ENREF_88" \o "Jelluma, 2010 #28)].

# Spindle Assembly Checkpoint Silencing

The silencing of the SAC is achieved by different mechanisms, varying between organisms. The four mechanisms I will describe are: the functioning of dynein, the functioning of p31comet, the role of ubiquitylation, and finally the role of dephosphorylation. Every process has an effect, which can be amplified, however it is very important to get an understanding of when and where different components meet.

**Dynein.** One of the key processes of silencing the SAC, is the removal, or stripping, of Mad1, Mad2, Mad3, Bub1, RZZ, Mps1 and other checkpoint proteins away from the kinetochore, towards the poles [[92](#_ENREF_92), [93](#_ENREF_93)]. In metazoans, this process requires the dynein/dynactin complex, a minus-end-directed microtubule motor [[93](#_ENREF_93), [94](#_ENREF_94)]. Retention of Mad1:Mad2 at fully attached kinetochores is sufficient to provoke a metaphase arrest [[95](#_ENREF_95)]. Inhibiting dynein at metaphase kinetochores results in the return of Mad2 to approximately 25% of the level of unattached kinetochores without a loss in the number of attached kinetochores [[93](#_ENREF_93), [94](#_ENREF_94)]. Which raises the question, whether this would be enough for an effective SAC response. The stripping of Bub1 and Mps1 antagonizes the recruitment of their associated checkpoint proteins to the kinetochores.

Recently, growing evidence suggests that spindly and the RZZ complex are regulating the stripping process [[96](#_ENREF_96), [97](#_ENREF_97)]. RZZ is transported from kinetochores to the spindle poles during checkpoint silencing. Spindly is the protein that regulates the dynein-mediated transport of Rod and Mad2 at metaphase [[96](#_ENREF_96)]. Spindly itself is also transported to the spindle poles following microtubule attachment. Moreover, a conserved region in spindly is critical in the targeting of dynein to kinetochores [[96](#_ENREF_96), [97](#_ENREF_97)]. Depletion of spindly reduces localization of dynein at kinetochores, but more importantly it prevents the removal of the checkpoint proteins away from kinetochore, resulting in a prolonged metaphase arrest [[96](#_ENREF_96), [97](#_ENREF_97)].

**P31comet.** P31comet has a tertiary fold very similar to C-Mad2 [[98](#_ENREF_98)]. It is able to form a stable complex with C-Mad2 specifically, where it binds at C-Mad2’s dimerization interface [[99](#_ENREF_99)]. P31comet can block the functioning of C-Mad2 in two ways. Firstly, p31comet bound to Mad1:Mad2 prevents further activation of Mad2. This model is in agreement with the observation that p31comet moves towards spindle poles, in a way similar to Mad2 [[100](#_ENREF_100)]. On the contrary, a fluorescence recovery experiment shows that the kinetics of the cycling of p31comet is very similar to the mobile pool of Mad2, suggesting p31comet is not bound Mad1:Mad2, but most likely to the second C-Mad2 molecule [[101](#_ENREF_101)]. This would suggest that the Mad2 in Mad1:Mad2 is protected from p31comet capping, until late metaphase.

Secondly, p31comet can bind to Cdc20:C-Mad2, inhibiting recruitment and activation of Mad2 (as suggested above). Furthermore, this binding is also neutralizing the Mad2-inhibitory effect on APC/CCdc20, through destabilization of MCC. MCC is present in free form, and in APC/C bound form. Both pools are regulated very differently during mitotic exit [[101](#_ENREF_101)]. This study shows that free MCC is disassembled ahead of APC/CMCC, independently of both APC/C and proteasome activities. APC/CMCC disassembly requires APC/C and proteasome activity, and is inextricably linked to Cdc20 degradation. P31comet aids in the ubiquitylation of Cdc20 [[102](#_ENREF_102)], suggesting a possible mechanism explaining the difference. The crystal structure of Chao et al shows that p31comet has to compete for Mad3 interactions with Mad2, which in buried in MCC . Hershko and coworkers showed that an ATP dependent process, possibly the CDK-driven phosphorylation of Cdc20, aids in the release of Cdc20:Mad2:p31comet from the MCC [[103](#_ENREF_103), [104](#_ENREF_104)]. I can imagine that the phosphorylation of Cdc20 induces a conformational change in MCC, enabling p31comet to find its binding site at Mad2, which completes the dissociation of Cdc20:Mad2:p31comet from MCC. Which raises the question whether the turnover rate of Cdc20 within “one” MCC molecule is high?

Independently of which function p31comet has, in vivo, depletion of p31comet only delays the start of anaphase, suggesting that it has a stabilizing role, instead of an essential silencing role [[105](#_ENREF_105)]. It could be the case that p31comet counteracts the MCC formation, such that the cell has a shorter response time to the bi-orientation of the last kinetochore (more important as cells increase in size). Overexpression of p31comet results in adaptation; exit from mitosis while SAC is active [[105](#_ENREF_105)].

**Ubiquitylation.** APC/C is the target of the SAC, however it also has a function in the degradation of Mps1 and Bub1 [[106-108](#_ENREF_106)]. A recent study has shown that a small molecule named TAME, prevents the activation of APC/C by Cdc20 and Cdh1, resulting in checkpoint arrest [[109](#_ENREF_109)]. Interestingly, this inactivation is dependent on an active SAC. Depletion of Mad2 alleviates the TAME induced arrest. This could mean that TAME prevents checkpoint silencing, but allows the degradation of cyclin B in absence of an active SAC.

In yeast, Cdc20 autoubiquitylation and degradation requires Mad2 and Mad3 [[110](#_ENREF_110)]. It is possible that the repositioning of Cdc20 (see above) by Mad2 and Mad3 binding, promotes autoubiquitylation. Autoubiquitylation is a conserved process in mammalian cells, and stimulated by p31comet and UbcH10. Two recent studies show that Cdc20 ubiquitylation causes the dissociation of Mad2 from Cdc20 [[102](#_ENREF_102), [111](#_ENREF_111)]. Moreover, Usp44 (a DUB), prevents the autoubiquitylation, stabilizing the Cdc20:Mad2 interaction [[111](#_ENREF_111)]. However, both studies did not use an elongation factor in their *in vitro* studies.

A later study of showed that a lysine-less Cdc20 mutant still shows dissociation of Mad2 from Cdc20 [[57](#_ENREF_57)], which would be suggest the contrary to the findings [100, 101]. First of all, it has to be noted that one of the lysines is involved in the binding to Mad3, which means that the mutation possibly influenced the formation of MCC [[105](#_ENREF_105)]. Furthermore, it could be possible that the observation in [57] is due to the phosphorylation process described in the p31comet silencing section.

**Dephosphorylation.** The spontaneous turnover of phosphoproteins and mitotic subcomplexes is too slow to account for the abrupt activation of APC/C [[112](#_ENREF_112)]. This hints at the existence of a switch-like mechanism, triggering checkpoint silencing. Expression of phospho-mimetic mutants of kinetochore and checkpoint and checkpoint proteins, constitutively activates the SAC, suggesting that dephosphorylation is an essential of checkpoint silencing [[113-115](#_ENREF_113)]. Moreover, it is the only silencing mechanism that is phylogenetically conserved. The disruption of the kinetochore-associated protein phosphatase PP1 results in a metaphase arrest [[116](#_ENREF_116)]. In yeast, PP1 is required for checkpoint silencing, independently of its role in kinetochore-microtubule attachment [[117](#_ENREF_117)]. Yeast becomes hypersensitive to checkpoint activation after expressing an inactive PP1, and overexpression of PP1 prevents checkpoint activation [[118](#_ENREF_118)]. PP1 is regulated by the activity of the kinase Aurora B, they might form an antagonistic pair. Aurora B prevents the localization of PP1 catalytic subunits by phosphorylating motifs, conforming to the sequence RVXF (X is any amino acid that is not negatively charged or phosphorylated), that mediate PP1 localization [[119](#_ENREF_119)]. Examples of such motifs are found in the kinetochore proteins KNL1 and Cenp-E [[115](#_ENREF_115), [116](#_ENREF_116)]. This might affect the kinases involved in the SAC. CENP-E is involved in the silencing of BubR1, possibly through PP1. Bub1 binds directly to phosphorylated KNL1 [[89](#_ENREF_89)]. Dephosphorylation of KNL1 by PP1 would release Bub1 from the kinetochores. Dephosphorylation of the N-terminal of Msp1 releases Msp1 from the kinetochore, a process essential for checkpoint silencing [[88](#_ENREF_88)].

# Concluding remarks.

Much has been achieved over the last decades towards an understanding of the molecular mechanisms regulating the metaphase-anaphase transition. The cell uses intricate signal transduction pathways to sense both unattached and untense kinetochores, and to temporally block the functioning of APC/CCdc20. The checkpoint proteins are targeted to the kinetochore, where they get conformationally and enzymatically activated. Upon activation these proteins inhibit APC/CCdc20 through various mechanisms. Once all kinetochores are properly attached and bi-oriented, the checkpoint is inactivated, also through various mechanisms. In the last year, many structural questions are answered, which gives the power to develop more precise tools to influence the metaphase-anaphase transition.

Many question regarding the checkpoint remain unanswered. In the next chapter I will discuss a few of those questions. Future studies might be able to answer them, aiding to the understanding of this fascinating and intriguing process of cell division.

# Open questions.

In order to get a deeper understanding of the relative importance of the various mechanisms contributing to the SAC and the silencing of the SAC, quantification of various processes would be necessary. For example, measuring the exact dissociation rate of MCC from APC/C, or the change of this rate upon addition of p31comet. All complexes are relatively stable, and their binding could be assessed by surface plasmon resonance. Quantification of the dissociation process will shed light on both the mechanism for inhibition of APC/CCdc20 and for the silencing of the inhibition. The dissociation rate limits the distance between the last unattached kinetochore and any APC/C molecule (see equation 1).

The mean displacement of a particle in a cell <r>2 is proportional to time *t*:

<r>2 = 6 D t (1)

D is the diffusion constant of the particle [[120](#_ENREF_120)].

A large question I would like to answer is: would it be easier for SAC to reach proper APC/C inhibition and perhaps faster, if APC is localized? Limiting the distance between a potentially active APC/C and kinetochores would limit the diffusion time, which is becomes more important as the size of a cell increases. Furthermore, the required rate of MCC formation necessary to inhibit APC/C can be lower, since APC/C has to be inhibited in a smaller volume. The disadvantage is that you need securin and cyclin-B close to spindle poles as well.

APC/C molecules could be localized individually; through active transport, through binding, or by limiting the accessible space for APC/C. Evidence suggests that first method of localization could be achieved though an active localization of APC/C at for instance the spindle poles [[51](#_ENREF_51)]. The latter could possibly be achieved through the formation of a fusiform spindle structure by Megator (human homologue Tpr), together with Skeletor and Chromator, independently of the microtubules [[121](#_ENREF_121)]. Interestingly, the second observation would explain why inhibitory signal does not propagate to the cytoplasm as observed by Rieder et al. more than a decade ago [[58](#_ENREF_58)].

**Localization at the spindle poles.** The advantage of having APC/C localizing at spindle poles is that there is a possibility to quickly transport any signal via the tubules. It does have to be noted that Mad1:Mad2 is transported to the spindle poles upon binding of the microtubules. If this complex is not inhibited somehow, it could function as a template just as Cdc20:Mad2 does. No research has into the disabling of Mad1:Mad2 after its transportation from the kinetochores has been done so far.

**Localization through compartmentalization.** The net of Megator forms at approximately a third of the radius of the *Drosophila* cells, decreasing the maximal diffusion time by a factor ten, reducing the necessary concentration of MCC by a factor of ~ 30. As noted above, this net will inhibit free diffusion of MCC or MCC templates, which is in accordance to Rieder et al. [[58](#_ENREF_58)]. However, Rieder et al also observed that once one spindle is going into anaphase, the other does as well, independently of the attachment status of its kinetochores. This means that there is a signal triggering the onset of anaphase. This means that the net must open at anaphase, such that cohesin can diffuse (or any other anaphase triggering signal). It seems like this is could be the case [[121](#_ENREF_121)], but it is not very clear. Another observation that could potentially be explained by the net is the 50% MCC-free APC/C [[13](#_ENREF_13)], this fraction is the APC/C outside of the net.

Another observation that puzzles me is the structural mimicry between p31 and mad2. A random evolution leading to similar structures would be astonishing. Research in lab of G.J.P.L Kops has shown that the conserved region between the p31comet from different species has the highest sequence homology with Mad2. This would suggest that somewhere in the past the p31comet gene got duplicated, and mutated towards p31comet afterwards. Some organisms use p31comet for checkpoint silencing where others do not. A possible reason would be either the size of the cell or perhaps the type of organism. One can understand that the improper chromosome segregation has less of an impact on yeast as a species, than it would on multicellular species such as humans: one improperly dividing cell has less of an influence on the others.

During mitosis, even one unattached kinetochore can delay anaphase. In order to study the implications of one kinetochore being unattached for a longer period of time, it would be necessary to gain selective control over the attachment of microtubules to the kinetochores. One way to achieve selective non-binding is through photo-crosslinking a depolymerase to one or a few kinetochores. It is possible to selectively ablate the spindles bound to one kinetochore [[122](#_ENREF_122)], therefore using a lower intensity laser it might be possible to link the photo-crosslinker to one kinetochore. To increase the chance of having a depolymerase in the vicinity of the radiated kinetochore, the depolymerase should be linked to a Mad1 antibody [[54](#_ENREF_54)] (the actual photo-crosslinker is located at the antibody). The antibody should be adjusted to have a lower affinity for Mad1, in order to avoid binding of depolymerase to all kinetochores.

The presence of a microtubule depolymerase will upset the functioning of a cell it is necessary to remove it from the cell. A possible way is to achieve this is by pre-targeting the depolymerase for destruction, but inhibiting its destruction initially by inhibiting the proteasome by MG132. The targeting for destruction will be achieved through *in vitro* or perhaps *in vivo* polyubiquitylation of the deploymerase by adding a D-box to one of the termini as in [[25](#_ENREF_25), [26](#_ENREF_26)]. Critical to this setup is that the photo-crosslinking does not happen to all kinetochores at the same time. Therefore I would be necessary to have a focal area of the laser to be smaller than 10 μm2, as well as the affinity of the antibody for the kinetochore. If the latter is too high, all kinetochores will remain unattached.

The final issue I would like to point out regards the step from SAC inhibition to cohesin cleft. In the literature the step from polyubiquitylation of securin to the cleaving of cohesin is often described in one or few sentences. Furthermore, the separation of all chromosome pairs is often assumed to occur simultaneously. Is this the case? Is there a critical threshold for the separase concentration that triggers chromosome separation? Securin has to travel from APC/C to the proteasome, and than to cohesin, including the disassembly of MCC all within 6 min. Assuming a diffusion constant of 20 μm2/s for securing as well as separase [[62](#_ENREF_62)], both can maximally travel 190 μm in 5 min, which would most likely be more than enough. The signal from Cdc20:Mad2 most likely also travels with a diffusion constant of 20 μm2/s, so in a 50 μm cell in diameter the maximum delay, the time difference between the bi-orientation of last kinetochore and the time the signal takes to reach an APC/C at the other side of the cell, would be approximately 20 seconds. This might be possible to measure. I suggest a closer examination of the onset of anaphase, especially in the case that one kinetochore does not attach.

# References

1. Kraft, C., et al., *The WD40 propeller domain of Cdh1 functions as a destruction box receptor for APC/C substrates.* Molecular Cell, 2005. **18**(5): p. 543-553.

2. Thornton, B.R., et al., *An architectural map of the anaphase-promoting complex.* Genes & Development, 2006. **20**(4): p. 449-460.

3. Matyskiela, M.E. and D.O. Morgan, *Analysis of Activator-Binding Sites on the APC/C Supports a Cooperative Substrate-Binding Mechanism.* Molecular Cell, 2009. **34**(1): p. 68-80.

4. McLean, J.R., et al., *State of the APC/C: Organization, function, and structure.* Critical Reviews in Biochemistry and Molecular Biology, 2011. **46**(2): p. 118-136.

5. Pines, J., *Cubism and the cell cycle: the many faces of the APC/C (vol 12, pg 427, 2011).* Nature Reviews Molecular Cell Biology, 2011. **12**(8).

6. Williamson, A., et al., *Identification of a physiological E2 module for the human anaphase-promoting complex.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(43): p. 18213-18218.

7. Seino, H., et al., *Two ubiquitin-conjugating enzymes, UbcP1/Ubc4 and UbcP4/Ubc11, have distinct functions for ubiquitination of mitotic cyclin.* Molecular and Cellular Biology, 2003. **23**(10): p. 3497-3505.

8. Mathe, E., et al., *The E2-C vihar is required for the correct spatiotemporal proteolysis of cyclin B and itself undergoes cyclical degradation.* Current Biology, 2004. **14**(19): p. 1723-1733.

9. Rape, M. and M.W. Kirschner, *Autonomous regulation of the anaphase-promoting complex couples mitosis to S-phase entry.* Nature, 2004. **432**(7017): p. 588-595.

10. Rodrigo-Brenni, M.C., S.A. Foster, and D.O. Morgan, *Catalysis of Lysine 48-Specific Ubiquitin Chain Assembly by Residues in E2 and Ubiquitin.* Molecular Cell, 2010. **39**(4): p. 548-559.

11. Bremm, A., S.M.V. Freund, and D. Komander, *Lys11-linked ubiquitin chains adopt compact conformations and are preferentially hydrolyzed by the deubiquitinase Cezanne.* Nature Structural & Molecular Biology, 2010. **17**(8): p. 939-U47.

12. Buschhorn, B.A., et al., *Substrate binding on the APC/C occurs between the coactivator Cdh1 and the processivity factor Doc1.* Nature Structural & Molecular Biology, 2011. **18**(1): p. 6-+.

13. Herzog, F., et al., *Structure of the Anaphase-Promoting Complex/Cyclosome Interacting with a Mitotic Checkpoint Complex.* Science, 2009. **323**(5920): p. 1477-1481.

14. da Fonseca, P.C.A., et al., *Structures of APC/C-Cdh1 with substrates identify Cdh1 and Apc10 as the D-box co-receptor.* Nature, 2011. **470**(7333): p. 274-+.

15. Schreiber, A., et al., *Structural basis for the subunit assembly of the anaphase-promoting complex.* Nature, 2011. **470**(7333): p. 227-+.

16. Glotzer, M., A.W. Murray, and M.W. Kirschner, *Cyclin Is Degraded by the Ubiquitin Pathway.* Nature, 1991. **349**(6305): p. 132-138.

17. Cox, C.J., et al., *The regions of securin and cyclin B proteins recognized by the ubiquitination machinery are natively unfolded.* Febs Letters, 2002. **527**(1-3): p. 303-308.

18. Pfleger, C.M. and M.W. Kirschner, *The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1.* Genes & Development, 2000. **14**(6): p. 655-665.

19. Jin, L.Y., et al., *Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex.* Cell, 2008. **133**(4): p. 653-665.

20. Castro, A., et al., *APC/Fizzy-Related targets Aurora-A kinase for proteolysis.* Embo Reports, 2002. **3**(5): p. 457-462.

21. Littlepage, L.E. and J.V. Ruderman, *Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit.* Molecular Biology of the Cell, 2002. **13**: p. 171A-172A.

22. Reis, A., et al., *The CRY box: a second APC(cdh1)-dependent degron in mammalian cdc20.* Embo Reports, 2006. **7**(10): p. 1040-1045.

23. Araki, M., H.T. Yu, and M. Asano, *A novel motif governs APC-dependent degradation of Drosophila ORC1 in vivo.* Genes & Development, 2005. **19**(20): p. 2458-2465.

24. Sullivan, M. and D.O. Morgan, *A novel destruction sequence targets the meiotic regulator Spo13 for anaphase-promoting complex-dependent degradation in anaphase I.* Journal of Biological Chemistry, 2007. **282**(27): p. 19710-19715.

25. King, R.W., M. Glotzer, and M.W. Kirschner, *Mutagenic analysis of he destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates.* Molecular Biology of the Cell, 1996. **7**(9): p. 1343-1357.

26. Yamano, H., et al., *The role of the destruction box and its neighbouring lysine residues in cyclin B for anaphase ubiquitin-dependent proteolysis in fission yeast: defining the D-box receptor.* Embo Journal, 1998. **17**(19): p. 5670-5678.

27. Mailand, N. and J.F.X. Diffley, *CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis.* Cell, 2005. **122**(6): p. 915-926.

28. Holt, L.J., A.N. Krutchinsky, and D.O. Morgan, *Positive feedback sharpens the anaphase switch.* Nature, 2008. **454**(7202): p. 353-357.

29. Choi, E., et al., *BubR1 acetylation at prometaphase is required for modulating APC/C activity and timing of mitosis.* Embo Journal, 2009. **28**(14): p. 2077-2089.

30. Summers, M.K., et al., *The unique N terminus of the UbcH10 E2 enzyme controls the threshold for APC activation and enhances checkpoint regulation of the APC.* Molecular Cell, 2008. **31**(4): p. 544-556.

31. Passmore, L.A., et al., *Doc1 mediates the activity of the anaphase-promoting complex by contributing to substrate recognition.* Embo Journal, 2003. **22**(4): p. 786-796.

32. Passmore, L.A. and D. Barford, *Coactivator functions in a stoichiometric complex with anaphase-promoting complex/cyclosome to mediate substrate recognition.* Embo Reports, 2005. **6**(9): p. 873-878.

33. Carroll, C.W., M. Enquist-Newman, and D.O. Morgan, *The APC subunit Doc1 promotes recognition of the substrate destruction box.* Current Biology, 2005. **15**(1): p. 11-18.

34. Carroll, C.W. and D.O. Morgan, *The Doc1 subunit is a processivity factor for the anaphase-promoting complex.* Nature Cell Biology, 2002. **4**(11): p. 880-887.

35. Izawa, D. and J. Pines, *How APC/C-Cdc20 changes its substrate specificity in mitosis (vol 13, pg 233, 2011).* Nature Cell Biology, 2011. **13**(5): p. 633-633.

36. van Leuken, R., L. Clijsters, and R. Wolthuis, *To cell cycle, swing the APC/C.* Biochimica Et Biophysica Acta-Reviews on Cancer, 2008. **1786**(1): p. 49-59.

37. Chen, Q., et al., *Cyclin B1 is localized to unattached kinetochores and contributes to efficient microtubule attachment and proper chromosome alignment during mitosis.* Cell Research, 2008. **18**(2): p. 268-280.

38. Acquaviva, C., et al., *The anaphase promoting complex/cyclosome is recruited to centromeres by the spindle assembly checkpoint.* Nature Cell Biology, 2004. **6**(9): p. 892-U82.

39. Kraft, C., et al., *Mitotic regulation of the human anaphase-promoting complex by phosphorylation.* Embo Journal, 2003. **22**(24): p. 6598-6609.

40. Goss, V.L., et al., *SAPK/JNK regulates cdc2/cyclin B kinase through phosphorylation and inhibition of cdc25c.* Cellular Signalling, 2003. **15**(7): p. 709-718.

41. Kimata, Y., et al., *A Role for the Fizzy/Cdc20 Family of Proteins in Activation of the APC/C Distinct from Substrate Recruitment.* Molecular Cell, 2008. **32**(4): p. 576-583.

42. Chao, W.C.H., et al., *Structure of the mitotic checkpoint complex.* Nature, 2012. **484**(7393): p. 208-U89.

43. Gillett, E.S., C.W. Espelin, and P.K. Sorger, *Spindle checkpoint proteins and chromosome-microtubule attachment in budding yeast.* Journal of Cell Biology, 2004. **164**(4): p. 535-546.

44. Fraschini, R., et al., *Bub3 interaction with Mad2, Mad3 and Cdc20 is mediated by WD40 repeats and does not require intact kinetochores.* Embo Journal, 2001. **20**(23): p. 6648-6659.

45. Poddar, A., P.T. Stukenberg, and D.J. Burke, *Two complexes of spindle checkpoint proteins containing Cdc20 and Mad2 assemble during mitosis independently of the kinetochore in Saccharomyces cerevisiae.* Eukaryotic Cell, 2005. **4**(5): p. 867-878.

46. Meraldi, P., V.M. Draviam, and P.K. Sorger, *Timing and checkpoints in the regulation of mitotic progression.* Developmental Cell, 2004. **7**(1): p. 45-60.

47. Essex, A., et al., *Systematic Analysis in Caenorhabditis elegans Reveals that the Spindle Checkpoint Is Composed of Two Largely Independent Branches.* Molecular Biology of the Cell, 2009. **20**(4): p. 1252-1267.

48. Malureanu, L.A., et al., *BubR1 N Terminus Acts as a Soluble Inhibitor of Cyclin B Degradation by APC/C-Cdc20 in Interphase.* Developmental Cell, 2009. **16**(1): p. 118-131.

49. Sudakin, V., G.K.T. Chan, and T.J. Yen, *Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2.* Journal of Cell Biology, 2001. **154**(5): p. 925-936.

50. Fang, G.W., *Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex.* Molecular Biology of the Cell, 2002. **13**(3): p. 755-766.

51. Howell, B.J., et al., *Spindle checkpoint protein dynamics at kinetochores in living cells.* Current Biology, 2004. **14**(11): p. 953-964.

52. Shah, J.V., et al., *Dynamics of centromere and kinetochore proteins: Implications for checkpoint signaling and silencing.* Current Biology, 2004. **14**(11): p. 942-952.

53. Sironi, L., et al., *Crystal structure of the tetrameric Mad1-Mad2 core complex: implications of 'safety belt' binding mechanism for the spindle checkpoint.* Embo Journal, 2002. **21**(10): p. 2496-2506.

54. Kulukian, A., J.S. Han, and D.W. Cleveland, *Unattached Kinetochores Catalyze Production of an Anaphase Inhibitor that Requires a Mad2 Template to Prime Cdc20 for BubR1 Binding.* Developmental Cell, 2009. **16**(1): p. 105-117.

55. De Antoni, A., et al., *The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint.* Current Biology, 2005. **15**(3): p. 214-225.

56. Simonetta, M., et al., *The Influence of Catalysis on Mad2 Activation Dynamics.* Plos Biology, 2009. **7**(1): p. 175-188.

57. Nilsson, J., et al., *The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction.* Nature Cell Biology, 2008. **10**(12): p. 1411-U74.

58. Rieder, C.L., et al., *Mitosis in vertebrate somatic cells with two spindles: Implications for the metaphase/anaphase transition checkpoint and cleavage.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(10): p. 5107-5112.

59. Musacchio, A. and E.D. Salmon, *The spindle-assembly checkpoint in space and time.* Nature Reviews Molecular Cell Biology, 2007. **8**(5): p. 379-393.

60. Doncic, A., E. Ben-Jacob, and N. Barkai, *Evaluating putative mechanisms of the mitotic spindle checkpoint.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(18): p. 6332-6337.

61. Struder, L., et al., *Large-format, high-speed, X-ray pnCCDs combined with electron and ion imaging spectrometers in a multipurpose chamber for experiments at 4th generation light sources.* Nuclear Instruments & Methods in Physics Research Section a-Accelerators Spectrometers Detectors and Associated Equipment, 2010. **614**(3): p. 483-496.

62. Sear, R.P. and M. Howard, *Modeling dual pathways for the metazoan spindle assembly checkpoint.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(45): p. 16758-16763.

63. Tang, Z., et al., *Mad2-Independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1.* Developmental Cell, 2001. **1**(2): p. 227-37.

64. Bolanos-Garcia, V.M., et al., *Structure of a Blinkin-BUBR1 Complex Reveals an Interaction Crucial for Kinetochore-Mitotic Checkpoint Regulation via an Unanticipated Binding Site (vol 19, pg 1691, 2011).* Structure, 2011. **19**(12): p. 1895-1895.

65. Bolanos-Garcia, V.M. and T.L. Blundell, *BUB1 and BUBR1: multifaceted kinases of the cell cycle.* Trends in Biochemical Sciences, 2011. **36**(3): p. 141-150.

66. Lampson, M.A. and T.M. Kapoor, *The human mitotic checkpoint protein BubR1 regulates chromosome-spindle attachments.* Nature Cell Biology, 2005. **7**(1): p. 93-8.

67. Ditchfield, C., et al., *Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores.* Journal of Cell Biology, 2003. **161**(2): p. 267-80.

68. Burton, J.L. and M.J. Solomon, *Mad3p, a pseudosubstrate inhibitor of APC(Cdc20) in the spindle assembly checkpoint.* Genes & Development, 2007. **21**(6): p. 655-667.

69. King, E.M., S.J. van der Sar, and K.G. Hardwick, *Mad3 KEN boxes mediate both Cdc20 and Mad3 turnover, and are critical for the spindle checkpoint.* Plos One, 2007. **2**(4): p. e342.

70. Chan, G.K., et al., *Human BUBR1 is a mitotic checkpoint kinase that monitors CENP-E functions at kinetochores and binds the cyclosome/APC.* Journal of Cell Biology, 1999. **146**(5): p. 941-54.

71. Chan, G.K., B.T. Schaar, and T.J. Yen, *Characterization of the kinetochore binding domain of CENP-E reveals interactions with the kinetochore proteins CENP-F and hBUBR1.* Journal of Cell Biology, 1998. **143**(1): p. 49-63.

72. Weaver, B.A.A., et al., *Centromere-associated protein-E is essential for the mammalian mitotic checkpoint to prevent aneuploidy due to single chromosome loss.* Journal of Cell Biology, 2003. **162**(4): p. 551-563.

73. Mao, Y., A. Desai, and D.W. Cleveland, *Microtubule capture by CENP-E silences BubR1-dependent mitotic checkpoint signaling.* Journal of Cell Biology, 2005. **170**(6): p. 873-80.

74. Qi, W., Z.Y. Tang, and H.T. Yu, *Phosphorylation- and polo-box-dependent binding of Plk1 to Bub1 is required for the kinetochore localization of Plk1.* Molecular Biology of the Cell, 2006. **17**(8): p. 3705-3716.

75. Rischitor, P.E., K.M. May, and K.G. Hardwick, *Bub1 is a fission yeast kinetochore scaffold protein, and is sufficient to recruit other spindle checkpoint proteins to ectopic sites on chromosomes.* Plos One, 2007. **2**(12): p. e1342.

76. Kiyomitsu, T., C. Obuse, and M. Yanagida, *Human Blinkin/AF15q14 is required for chromosome alignment and the mitotic checkpoint through direct interaction with Bub1 and BubR1.* Developmental Cell, 2007. **13**(5): p. 663-76.

77. Elowe, S., et al., *Uncoupling of the spindle-checkpoint and chromosome-congression functions of BubR1.* Journal of Cell Science, 2010. **123**(Pt 1): p. 84-94.

78. Tang, Z.Y., et al., *Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint.* Molecular Cell, 2004. **16**(3): p. 387-397.

79. Kang, J., et al., *Structure and substrate recruitment of the human spindle checkpoint kinase Bub1.* Molecular Cell, 2008. **32**(3): p. 394-405.

80. Rahmani, Z., et al., *Separating the spindle, checkpoint, and timer functions of BubR1.* Journal of Cell Biology, 2009. **187**(5): p. 597-605.

81. D'Angiolella, V., et al., *The spindle checkpoint requires cyclin-dependent kinase activity.* Genes Dev, 2003. **17**(20): p. 2520-5.

82. Chung, E.N. and R.H. Chen, *Phosphorylation of Cdc20 is required for its inhibition by the spindle checkpoint.* Nature Cell Biology, 2003. **5**(8): p. 748-753.

83. Yamagishi, Y., et al., *MPS1/Mph1 phosphorylates the kinetochore protein KNL1/Spc7 to recruit SAC components.* Nature Cell Biology, 2012. **14**(7): p. 746-52.

84. Abrieu, A., et al., *Mps1 is a kinetochore associated kinase essential for the vertebrate mitotic checkpoint.* Molecular Biology of the Cell, 2001. **12**: p. 409A-409A.

85. Maciejowski, J., et al., *Mps1 directs the assembly of Cdc20 inhibitory complexes during interphase and mitosis to control M phase timing and spindle checkpoint signaling.* Journal of Cell Biology, 2010. **190**(1): p. 89-100.

86. Morin, V., et al., *CDK-Dependent Potentiation of MPS1 Kinase Activity Is Essential to the Mitotic Checkpoint.* Current Biology, 2012. **22**(4): p. 289-295.

87. Hewitt, L., et al., *Sustained Mps1 activity is required in mitosis to recruit O-Mad2 to the Mad1-C-Mad2 core complex.* Journal of Cell Biology, 2010. **190**(1): p. 25-34.

88. Jelluma, N., et al., *Release of Mps1 from kinetochores is crucial for timely anaphase onset.* Journal of Cell Biology, 2010. **191**(2): p. 281-290.

89. London, N., et al., *Phosphoregulation of Spc105 by Mps1 and PP1 Regulates Bub1 Localization to Kinetochores.* Current Biology, 2012. **22**(10): p. 900-906.

90. Martin-Lluesma, S., V.M. Stucke, and E.A. Nigg, *Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2.* Science, 2002. **297**(5590): p. 2267-70.

91. Kang, J., et al., *Autophosphorylation-dependent activation of human Mps1 is required for the spindle checkpoint.* Proc Natl Acad Sci U S A, 2007. **104**(51): p. 20232-7.

92. Maiato, H., et al., *The dynamic kinetochore-microtubule interface.* Journal of Cell Science, 2004. **117**(Pt 23): p. 5461-77.

93. Howell, B.J., et al., *Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation.* Journal of Cell Biology, 2001. **155**(7): p. 1159-72.

94. Wojcik, E., et al., *Kinetochore dynein: its dynamics and role in the transport of the Rough deal checkpoint protein.* Nature Cell Biology, 2001. **3**(11): p. 1001-7.

95. Maldonado, M. and T.M. Kapoor, *Constitutive Mad1 targeting to kinetochores uncouples checkpoint signalling from chromosome biorientation.* Nature Cell Biology, 2011. **13**(4): p. 475-U291.

96. Gassmann, R., et al., *Removal of Spindly from microtubule-attached kinetochores controls spindle checkpoint silencing in human cells.* Genes & Development, 2010. **24**(9): p. 957-971.

97. Barisic, M., et al., *Spindly/CCDC99 is required for efficient chromosome congression and mitotic checkpoint regulation.* Molecular Biology of the Cell, 2010. **21**(12): p. 1968-81.

98. Yang, M.J., et al., *p31(comet) blocks Mad2 activation through structural mimicry.* Cell, 2007. **131**(4): p. 744-755.

99. Xia, G.H., et al., *Conformation-specific binding of p31(comet) antagonizes the function of Mad2 in the spindle checkpoint.* Embo Journal, 2004. **23**(15): p. 3133-3143.

100. Habu, T., et al., *Identification of a MAD2-binding protein, CMT2, and its role in mitosis.* Embo Journal, 2002. **21**(23): p. 6419-6428.

101. Hagan, R.S., et al., *p31(comet) acts to ensure timely spindle checkpoint silencing subsequent to kinetochore attachment.* Molecular Biology of the Cell, 2011. **22**(22): p. 4236-4246.

102. Reddy, S.K., et al., *Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation.* Nature, 2007. **446**(7138): p. 921-925.

103. Teichner, A., et al., *p31(comet) promotes disassembly of the mitotic checkpoint complex in an ATP-dependent process.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(8): p. 3187-3192.

104. Miniowitz-Shemtov, S., et al., *Role of phosphorylation of Cdc20 in p31(comet)-stimulated disassembly of the mitotic checkpoint complex.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(21): p. 8056-8060.

105. Varetti, G., et al., *Homeostatic Control of Mitotic Arrest.* Molecular Cell, 2011. **44**(5): p. 710-720.

106. Palframan, W.J., et al., *Anaphase inactivation of the spindle checkpoint.* Science, 2006. **313**(5787): p. 680-4.

107. Qi, W. and H. Yu, *KEN-box-dependent degradation of the Bub1 spindle checkpoint kinase by the anaphase-promoting complex/cyclosome.* Journal of Biological Chemistry, 2007. **282**(6): p. 3672-9.

108. Cui, Y., et al., *Degradation of the human mitotic checkpoint kinase Mps1 is cell cycle-regulated by APC-cCdc20 and APC-cCdh1 ubiquitin ligases.* Journal of Biological Chemistry, 2010. **285**(43): p. 32988-98.

109. Zeng, X. and R.W. King, *An APC/C inhibitor stabilizes cyclin B1 by prematurely terminating ubiquitination.* Nature Chemical Biology, 2012. **8**(4): p. 383-392.

110. Pan, J. and R.H. Chen, *Spindle checkpoint regulates Cdc20p stability in Saccharomyces cerevisiae.* Genes & Development, 2004. **18**(12): p. 1439-1451.

111. Stegmeier, F., et al., *Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities.* Nature, 2007. **446**(7138): p. 876-881.

112. Vanoosthuyse, V. and K.G. Hardwick, *Overcoming inhibition in the spindle checkpoint.* Genes Dev, 2009. **23**(24): p. 2799-805.

113. Huang, H., et al., *Phosphorylation sites in BubR1 that regulate kinetochore attachment, tension, and mitotic exit.* Journal of Cell Biology, 2008. **183**(4): p. 667-80.

114. Kemmler, S., et al., *Mimicking Ndc80 phosphorylation triggers spindle assembly checkpoint signalling.* Embo Journal, 2009. **28**(8): p. 1099-110.

115. Kim, Y., et al., *Aurora kinases and protein phosphatase 1 mediate chromosome congression through regulation of CENP-E.* Cell, 2010. **142**(3): p. 444-55.

116. Liu, D., et al., *Regulated targeting of protein phosphatase 1 to the outer kinetochore by KNL1 opposes Aurora B kinase.* Journal of Cell Biology, 2010. **188**(6): p. 809-20.

117. Vanoosthuyse, V. and K.G. Hardwick, *A Novel Protein Phosphatase 1-Dependent Spindle Checkpoint Silencing Mechanism.* Current Biology, 2009. **19**(14): p. 1176-1181.

118. Pinsky, B.A., C.R. Nelson, and S. Biggins, *Protein Phosphatase 1 Regulates Exit from the Spindle Checkpoint in Budding Yeast.* Current Biology, 2009. **19**(14): p. 1182-1187.

119. Hendrickx, A., et al., *Docking motif-guided mapping of the interactome of protein phosphatase-1.* Chem Biol, 2009. **16**(4): p. 365-71.

120. Leondes, C.T., *Computational methods in biophysics, biomaterials, biotechnology and medical systems : algorithm development, mathematical analysis, and diagnostics*2003, Boston: Kluwer Academic Publishers.

121. Qi, H.Y., et al., *Megator, an essential coiled-coil protein that localizes to the putative spindle matrix during mitosis in Drosophila.* Molecular Biology of the Cell, 2004. **15**(11): p. 4854-4865.

122. LaFountain, J.R., Jr., C.S. Cohan, and R. Oldenbourg, *Functional states of kinetochores revealed by laser microsurgery and fluorescent speckle microscopy.* Molecular Biology of the Cell, 2011. **22**(24): p. 4801-8.