Published in final edited form as:
Cell Cycle. 2009 February 1; 8(3): 338–344.

The Spindle Assembly Checkpoint in *Caenorhabditis elegans* — One Who Lacks Mad1 Becomes Mad One.

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Abstract

The spindle assembly checkpoint (SAC) monitors the microtubule attachment status of the kinetochore and arrests cells before anaphase until all pairs of sister kinetochores achieve bipolar attachment of microtubules, thereby ensuring faithful chromosome transmission. The evolutionarily conserved coiled-coil protein MAD1 has been implicated in the SAC signaling pathway. MAD1 forms a complex with another SAC component MAD2 and specifically localizes to unattached kinetochores to facilitate efficient binding of MAD2 to its target, CDC20, the mitotic substrate-specific activator of the anaphase promoting complex or cyclosome (APC/C). Thus, MAD1 connects 2 sequential events in the SAC signaling pathway – recognition of unattached kinetochores and inhibition of APC/C activity. However, the molecular mechanisms by which it specifically localizes to unattached kinetochores are largely unknown. Studies in multicellular organisms have revealed the role of MAD1 in development and tumor suppression, but the precise time at which MAD1 activity is required is unknown. Investigation of cellular and organismic functions of MAD1 in the simple multicellular organism *C. elegans* identified functional interactors of MAD1 in both kinetochore-oriented SAC signaling and kinetochore-independent cell cycle regulation. Studying the function of SAC components in *C. elegans* provides a new molecular insight into the SAC-regulated cell cycle progression in a context of a multicellular organism.

Keywords

Spindle assembly checkpoint (SAC); cell cycle; kinetochore; MAD1; *C. elegans*

Core components of the SAC machinery

To maintain the fidelity of chromosome transmission during the cell division cycle, eukaryotic cells have evolved the spindle assembly checkpoint (SAC) pathway, which monitors kinetochore–microtubule attachment and arrests cells before anaphase by inhibiting the anaphase promoting complex or cyclosome (APC/C) until all chromosomes achieve amphitelic attachment of microtubules. Thus, all eukaryotic cells rely on the SAC to coordinate the cell division cycle and timing of sister chromatid separation [reviewed in Musacchio and Salmon (2007), May and Hardwick (2006) and Varetti and Musacchio (2008)].[1-3]

The SAC signaling pathway is mediated by evolutionarily conserved proteins, including MAD1, MAD2, MAD3 (called BUBR1 in mammals: BUBR1 is structurally related to MAD3 but possesses the BUB1-like kinase domain), BUB1, and BUB3. These proteins were originally identified in budding yeast by genetic screens for mutants that exhibit increased sensitivity to microtubule-depolymerizing drugs.[4, 5] Although lack of these proteins reduces the fidelity of chromosome transmission,[6] none of them is essential for normal cell cycle progression in yeast.[4, 5] These proteins become essential only when the microtubule–kinetochore attachment is compromised.[7, 8] On the other hand, their homologs are indispensable in mammals [reviewed

in Foijer et al (2008)].[9] Studies on genetically modified mice revealed that the lack of any SAC component causes the early-stage embryonic lethal phenotype because of more p53-dependent apoptotic cells.[10] At the cellular level, mammalian cells lacking MAD2 or BUBR1 have a shorter mitosis due to precocious sister chromatid separation,[11] suggesting that mammalian MAD2 and BUBR1 pace the progression of mitosis in addition to safeguarding against chromosome missegregation when a spindle or kinetochore defect is present. In addition to MAD and BUB proteins, MPS1 kinase also plays a central role in the SAC pathway.[12-14] Metazoan cells also require the ROD-ZW10-ZWILCH (RZZ) complex, which recruits dynein to the kinetochore, for SAC activation [reviewed in Karess (2005)].[15] The RZZ complex is not conserved in budding yeast, reflecting the more complex feature of the kinetochore-SAC signaling in higher eukaryotes. Recent studies have shown that the chromosomal passenger complex, comprising Aurora B kinase, inner centromere protein (INCENP), survivin, and borealin (or Dasra-B), is a key player in the SAC pathway [reviewed in Vader et al (2007)].[16] In addition, NEK2A, CDK1, PLK, PRP4, and TAO1 kinases regulate SAC function.[17-21]

Kinetochore localization of the MAD1–MAD2 complex

Most SAC proteins associate with unattached kinetochores or those incorrectly bound to microtubules (monotelic, syntelic, or merotelic attachment).[4, 5, 22-25] In mammalian cells, all SAC proteins appear to localize to the kinetochores during prometaphase, but MAD1 and MAD2 dissociate from the kinetochore on microtubule attachment.[24, 26] In budding yeast, kinetochore localization of MAD1 and MAD2 can hardly be detected during the normal cell cycle; it becomes detectable only when kinetochore–microtubule attachment is compromised by mutational or chemical disruption of the spindle.[25] The 2 main hypotheses that explain which features of the kinetochore status are monitored by the SAC are “tension generated between sister kinetochores” and “microtubule attachment”, but it is not known whether these features are recognized by the same pathway or by 2 distinct pathways. However, it is clear that MAD1 and MAD2 specifically recognize the microtubule attachment status of the kinetochore. Extensive analysis of the molecular dependency of kinetochore assembly by fluorescence microscopy–based cytologic analysis in combination with RNAi-mediated depletion of proteins has revealed the kinetochore-associated proteins required for the kinetochore localization of MAD1–MAD2: BUB1 and MPS1 kinases have been implicated to function upstream of MAD1–MAD2,[12, 27] and the RZZ complex and outer kinetochore structural proteins CENP-I and CENP-F are also required for the kinetochore localization of MAD1–MAD2.[28-31] Mislocalization of MAD1–MAD2 caused by depletion of these proteins coincides with SAC deficiency,[11, 24] strongly suggesting that the kinetochore localization of MAD1–MAD2 is a crucial step for SAC activation. However, which protein functions as the kinetochore receptor of MAD1–MAD2 or whether MAD proteins need to be posttranslationally modified for specific localization to unattached kinetochores are still not known.

Protein complexes that compose the SAC components

The downstream target of the SAC is the APC/C [reviewed in Sczaniecka and Hardwick (2008)].[32] Inhibition of APC/C activity by the SAC machinery causes mitotic substrates of the APC/C (such as securin and cyclin B) to accumulate, thereby preventing separation of sister chromatids and mitotic exit. MAD2, BUBR1/MAD3, and BUB3 have been biochemically copurified with CDC20 as constituents of the protein complex termed the mitotic checkpoint complex (MCC), which inhibits the ubiquitin ligase activity of the APC/C.[33-35] MAD1 is not a constituent of the MCC, but forms a complex with MAD2. The MAD1–MAD2 complex localizes to the unattached kinetochore during prometaphase or when microtubule–kinetochore attachment is disrupted, whereby it functions as a receptor for cytosolic MAD2 to facilitate the efficient binding of MAD2 to CDC20.[36, 37] During interphase, the MAD1–MAD2 complex

localizes to the nuclear periphery, presumably by interacting with the nuclear pore complex.[26, 38] However, whether this localization is required for SAC function or for any other function during interphase is unclear. Mad1 has also been identified in a protein complex containing Bub1 and Bub3 in budding yeast.[39] Unlike MCC, which constitutively exists through the cell cycle, formation of the Mad1–Bub1–Bub3 complex increases under conditions that activate the SAC, suggesting that association of Mad1 with Bub1 is crucial to initiate the kinetochore-oriented SAC signaling pathway. Consistent with the physical interaction of Mad1 and Bub1 in budding yeast, human MAD1 has been shown to be phosphorylated by human BUB1 kinase *in vitro*.[40] MAD1 is extensively phosphorylated in an MPS1 kinase–dependent manner when the SAC is active.[41] MAD1 also binds the NEK2A kinase that is required for SAC activation.[17] However, the precise sites for or the timing of phosphorylation on MAD1 and the functional significance of these phosphorylation events in SAC activation remain to be elucidated.

Conservation of the SAC pathway in *C. elegans*

In *C. elegans*, most key players of the SAC signaling pathway are functionally and structurally conserved [reviewed in Kitagawa (2009)].[42] The only major player that has not been identified in the *C. elegans* genome is Mps1 kinase (Table 1). Nocodazole-induced cell cycle delay in mitosis has been detected in fixed premeiotic germ cells as an increase in the number of nuclei that react with the phosphorylated histone H3 antibody, demonstrating the presence of the SAC in proliferating germ cells.[43] Although it has been difficult to prove the presence of SAC function in *C. elegans* embryos because of lack of an apparent mitotic-arrest phenotype,[44, 45] recent time-lapse experiments that use live imaging of embryos expressing a transgene fused with fluorescence markers have enabled the precise measurement of the intervals between nuclear envelope breakdown and anaphase onset, nuclear envelope reformation, or chromosome decondensation.[46, 47] These studies have revealed that chemical or mutational disruption of microtubules induces SAC-dependent extension of mitotic duration by as much as two- or three-fold. The spindle damage–induced SAC activation coincides with the association of MAD proteins with the unattached kinetochores on holocentric chromosomes, where the kinetochore is assembled along the entire length of the mitotic chromosomes in a sequence-independent manner.[42, 48, 49]

Thus, although *C. elegans* is a holocentric organism, the role of the kinetochore as a center of signal generation for SAC activation is conserved, making *C. elegans* embryos a valuable model for studying the molecular mechanism of activation of the SAC signaling pathway.

Characterization of the loss-of-function phenotype of SAC components

In *C. elegans*, depletion of any of the SAC proteins overrides the spindle defect induced–extension of the mitotic duration but does not affect mitotic duration during normal cell cycle progression of blastomeres in early-stage embryos.[46] This suggests that SAC in the *C. elegans* does not have a role in regulating the timing of anaphase onset during the normal cell cycle, at least in early-stage embryos. However, RNAi depletion of BUB-1 or the RZZ components (ROD-1, ZW10^{CZW-1}, and ZWILCH^{ZWL-1}) causes embryonic lethality, presumably because of severe defects in chromosome segregation.[49-51] Consistent with the RNAi phenotype, worms homozygous for the *bub-1* deletion allele (*bub-1Δ*) result in embryonic lethality.[52] Indispensability of these genes may reflect the essential role of BUB-1 and the RZZ complex in kinetochore function, which is distinct from SAC function.

Although all MAD proteins (MAD1^{MDF-1}, MAD2^{MDF-2}, and MAD3^{SAN-1}) are dispensable for normal cell cycle progression in early-stage embryos (i.e., in embryos depleted of MAD proteins, intervals of each cell cycle stages and the time course profile of the pole–pole distance, which reflects the status of the kinetochore–microtubule attachment, are indistinguishable from those in untreated embryos; chromosome condensation, chromosome congression at the

metaphase plate and synchronous sister chromatids separation normally occur)[46] (Yamamoto and Kitagawa unpublished data) , lack of each MAD protein results in distinct phenotypes with differing magnitudes of severity. The *mdf-1* deletion (*mdf-1Δ*) homozygotes produced from the *mdf-1Δ* heterozygotes grow to adults; however, despite the dispensability of MAD1^{MDF-1} in early-stage embryos, the *mdf-1Δ* homozygotes cannot be maintained beyond the third generation because defects in larval development and fertility increase in severity with every generation,[43] suggesting that MAD1^{MDF-1} has some essential roles in cell cycle regulation during postembryonic development. Homozygotes for the *mdf-2* deletion allele (*mdf-2Δ*) are maintainable, but have severely reduced brood size, reduced viability of progeny, and genome instability, reflected by the high incidence of the male (Him) phenotype.[52, 53] The strain homozygous for the deletion allele of *san-1* (*san-1Δ*) is also viable and does not exhibit any severe observable phenotype except for slow growth and moderate reduction of brood size at a low penetrance.[52-54] Thus, MAD3^{SAN-1} is dispensable under normal conditions despite its crucial role in spindle damage–induced or anoxia-induced mitotic delay.

Identification of a kinetochore receptor of MAD1^{MDF-1} by an RNAi-based synthetic genetic screen

In budding yeast, lack of an SAC component becomes fatal only in the presence of the kinetochore–microtubule defect. Therefore systematic comprehensive screens for genes whose depletion result in synthetic lethality in combination with deletion mutants of SAC components by using a collection of deletion alleles of nonessential genes – synthetic genetic array (SGA) or synthetic lethal analysis by microarray (SLAM) – have identified many genes that play a role in chromosome transmission or mitotic spindle function.[7, 8] Studying synthetic genetic interaction with SAC components is also an effective approach in *C. elegans*. Particularly, *san-1* is an ideal query gene for screening synthetic genetic interactors because it is dispensable for viability but necessary for proper response to certain stresses such as spindle damage or anoxia. As seen in synthetic genetic interaction in yeast, RNAi-mediated depletion of known components of spindles, kinetochore, and cohesion result in phenotypes that are embryonic lethal or have reduced number of progeny in combination with *san-1Δ*.[48, 52, 54] Interestingly, unlike the yeast SGA analysis that did not identify synthetic genetic interactions between any 2 SAC components assayed, depletion of SAC genes *mdf-1* or *bub-3* are synthetically lethal with *san-1Δ*,[48, 52, 54] indicating some redundancy in the SAC function of MAD3^{SAN-1}, MAD1^{MDF-1}, and BUB-3. *spdl-1*, a *C. elegans* homolog of the kinetochore-specific dynein recruiter Spindly, was identified as a gene whose depletion is synthetically lethal with *san-1Δ* in a screen of an RNA feeding library comprising bacterial clones expressing dsRNAs of 86% of genes encoded by the *C. elegans* genome.[48] Although many *san-1* synthetic genetic interactors have also shown genetic interaction with *mdf-1* or *mdf-2*, depletion of *spdl-1* is synthetically lethal with *san-1Δ* but not with *mdf-1Δ* or *mdf-2Δ*.[48] This property suggests that SPDL-1 functions in the MAD1^{MDF-1}-mediated pathway.

The role of SPDL-1 in the SAC pathway has been further characterized by using a simple assay system in which a mono-polar spindle is set up in the second mitotic division, thereby inducing an SAC-dependent mitotic delay in embryonic cells. In this system, the mono-polar spindle is formed by RNAi-mediated depletion of ZYG-1 kinase, which is required for centrosome duplication. In ZYG-1–depleted embryos, microtubules emanating from 1 pole are attached to the kinetochore assembled on the poleward side of the sister chromatids (i.e., monotelic attachment). Therefore, each pair of sister chromatids has both attached and unattached kinetochores and contrasts the molecular configurations of attached and unattached kinetochores. Under such a condition, the mitotic duration is extended in a SAC dependent manner up to 2 to 3 fold of that in normal cells with chromosomes which achieved bipolar spindle attachment.[48, 49] SPDL-1 functions downstream of the RZZ complex and upstream of MAD1^{MDF-1}–MAD2^{MDF-2} in a linear hierarchic molecular dependency for kinetochore

localization.[48, 49] In ZYG-1–depleted embryos that have mono-polar spindles, SPDL-1 localizes only to the unattached kinetochore, regardless of the presence of the RZZ complex on both attached and unattached kinetochores.[48] Furthermore, SPDL-1 and MAD1^{MDF-1}–MAD2^{MDF-2} coimmunoprecipitate *in vivo*.[48] These observations suggest that SPDL-1 senses the microtubule attachment status of the kinetochore and functions upstream of MAD1^{MDF-1} as a part of the kinetochore receptor of the MAD1^{MDF-1}–MAD2^{MDF-2} complex. Depletion of BUB-1 eliminates the accumulation of MAD1^{MDF-1} at the unattached kinetochore without interfering with the kinetochore localization of SPDL-1,[48] suggesting that MAD1^{MDF-1} requires BUB-1 to interact with SPDL-1. Therefore, SPDL-1 functions particularly in the initial step of the SAC pathway – recognition of the kinetochore–microtubule attachment and recruitment of MAD1^{MDF-1} to the unattached kinetochore.

Thus, an RNAi-based screen for synthetic genetic interactors of SAC components in *C. elegans* followed by cytological analysis of the protein dynamics by using early-stage embryonic cells with monopolar spindles is an effective approach to identify factors required for SAC activation.

Tight genetic interaction between MAD1^{MDF-1} and APC components

Although SAC function is not necessary for cell cycle regulation during early-stage embryogenesis, *mdf-1Δ* homozygotes exhibit severe developmental defects and infertility, which result in loss of descendants after a few generations. This genetic lethality of *mdf-1Δ* homozygotes is suppressed by the reduction in APC/C activity, which is caused by hypomorphic mutations in the APC/C component *emb-30* (*C. elegans* homolog of APC4) or in the APC activator *fzy-1* (*C. elegans* homolog of CDC20).[55, 56] Additional *mdf-1Δ* suppressors have been isolated and characterized, and 1 of the mutants has been mapped in a newly identified APC5-like protein.[57] Most of the other suppressor mutants themselves exhibit extended mitotic duration in early-stage embryos with increased level of IFY-1 (*C. elegans* ortholog of Securin),[57] suggesting that APC/C activity is downregulated in them. These results suggest that the essential function of MAD1^{MDF-1} is to regulate APC/C activity to a level appropriate for proper development. Notably, 2 of the suppressor mutants do not alter mitotic duration, and their molecular characterization remains to be done. Reciprocally, Stein *et al.* (2007) demonstrated that the meiosis I metaphase arrest phenotype of *mat-3*(*or180*), a temperature-sensitive allele of the APC/C component *mat-3* (a *C. elegans* homolog of APC8) was suppressed by a dominant mutant allele *mdf-1*(*av19*) or *mdf-1Δ*,[53] suggesting that MAD1^{MDF-1} has a critical role in maintaining APC/C activity at the appropriate level during meiosis I division. Complete loss of MAD1^{MDF-1} not only suppresses the meiotic defect of *mat-3*(*or180*) embryos but also causes embryonic lethality in a *mat-3*(*or180*) background.[53] On the other hand, embryogenesis of the *mat-3*(*or180*); *mdf-1*(*av19*) double mutant is normal.[53] Therefore, the *mdf-1*(*av19*) allele is considered a separation-function mutant, that is, deficient in SAC function but functional in other roles that become essential in a *mat-3* (*or180*) background.

Cross-talk between the SAC pathway and PI3K-AKT signaling pathways

Extensive analysis of the *mdf-1Δ* phenotype has revealed that MAD1^{MDF-1} is required for the starvation-induced mitotic arrest of primordial germ cells.[58] In *C. elegans*, during postembryonic development, unfavorable environmental conditions cause global interruption of the reproducible pattern of cell division. For example, hatchlings exposed to the starved condition enter the diapause stage, termed L1 diapause.[59-63] The cell cycle of many somatic precursors is suspended during L1 dipause. Although most cells are arrested in the G_0/G_1 stage in a cyclin-dependent kinase inhibitor, CKI-1(*C. elegans* KIP)–dependent manner,[61, 64-66] the primordial germ cells (PGCs), in contrast, are arrested in prophase in a PTEN^{DAF-18}, *C.*

elegans PTEN(Phosphatase and tensin homolog)–dependent manner.[59] Fine genetic analysis revealed that proliferation of germ cells is positively regulated by the PI3K^{AGE-1}-AKT kinase signaling pathway and negatively regulated by the phosphatase activity of PTEN^{DAF-18}, which antagonizes PI3K^{AGE-1} activity.[59] Watanabe et al. found that hemizygosity of *mdf-1* caused inappropriate germ cell proliferation in larvae exposed to the starved condition[58] and demonstrated that MAD1^{MDF-1} functions as one of the downstream targets of the PI3K^{AGE-1}-AKT kinase signaling pathway in response to nutritional status,[58] exemplifying that the SAC functions as a cell cycle regulator during postembryonic development by cross-talking with other signaling pathways. Since the inappropriate germ cell proliferation observed in *mdf-1Δ* hemizygotes is suppressed by hypomorphic mutants of APC/C components, it is likely that MAD1^{MDF-1} causes the mitotic arrest in response to starvation by inhibiting APC/C activity. This finding has brought a new perspective to the field of the SAC-mediated cell cycle regulation in several ways. First, this is the first example of kinetochore-independent cell cycle regulation by an SAC component; that is, MAD1^{MDF-1} senses not only the kinetochore–microtubule attachment status but also the environmental cue. In germ cells arrested in prophase, MAD1^{MDF-1} does not associate with chromosomes but does localize to the nuclear periphery, where it senses the AKT-1 mediated nutrient signal and triggers APC/C inhibition.[58] Second, MAD1^{MDF-1} functions in a specific cell lineage (germ cells) at a specific developmental stage (L1 diapause) rather than affecting every cell. This observation has been made possible only because of using *C. elegans*, in which, from the first mitotic cell division, each round of the cell cycle of every cell is temporary and spatially controlled to follow genetically programmed cell divisions. Also, an individual animal undergoes a nearly invariant developmental process so that alteration of any cell cycle progression during development by deregulation of the specific pathway can be readily investigated. Third, MAD1^{MDF-1} causes the "prophase" arrest of PGCs despite the fact that the SAC is known as the checkpoint that normally acts in prometaphase. Our finding of the role of MAD1^{MDF-1} in starvation-induced prophase arrest of PGCs suggests that MAD1^{MDF-1} links the signaling pathways that mediate various developmental or environmental cues and the SAC pathway to achieve coordinated cell division.

Future directions

Since SAC components were first identified in budding yeast in 1991, great strides have been made in understanding the molecular mechanism of the SAC signaling pathway. However, several issues still need to be resolved, some which can be addressed specifically by taking advantage of the power of *C. elegans*, as described below.

Kinetochore-oriented SAC signaling

A major goal of SAC-related research will be to elucidate how the kinetochore–microtubule attachment is sensed by the SAC. Toward this end, it is important to determine the molecular configuration of the kinetochore protein complex that physically interacts with SAC proteins and elucidate the molecular events that occur in the initial step of the SAC signaling pathway. An RNAi-mediated synthetic genetic screen for genes required for SAC activation identified SPDL-1, which specifically localizes to unattached kinetochores and recruits MAD1^{MDF-1}, presumably via physical interaction. The presence of the RZZ complex at the kinetochore is required but not sufficient for kinetochore localization of SPDL-1, suggesting that there are factors that regulate the interaction of SPDL-1 and the RZZ complex in response to the microtubule attachment status of the kinetochore. Taking into account the observation that SPDL-1 localizes to the kinetochore from prometaphase until anaphase during normal cell cycle, it is more likely that SPDL-1 is specifically excluded from attached kinetochores rather than being actively recruited to unattached kinetochores by interacting with other proteins. Future work should focus on identifying the factors that inhibit the interaction between SPDL-1

and the RZZ complex when microtubules attach to the kinetochore. The factors that regulate the interaction between SPDL-1 and the MAD1^{MDF-1}–MAD2^{MDF-2} complex also need to be identified. The hypothesis that phosphorylation of MAD1^{MDF-1} or SPDL-1 by BUB-1 kinase regulates the interaction between SPDL-1 and the MAD1^{MDF-1}–MAD2^{MDF-2} complex needs to be tested. Immunoprecipitation-mass spectrometry using the tandem-affinity purification (TAP) [also known as localization and tandem affinity purification (LAP) system][67] will identify additional physical interactors of SPDL-1, the RZZ complex, and MDF-1. Because SPDL-1 was identified as a *san-1* synthetic genetic interactor, it is expected that the remaining uncharacterized *san-1* synthetic genetic interactors isolated by the RNAi screen will include factors that function specifically in the MAD1^{MDF-1}-mediated pathway. These physical or genetic interactors of SAC components will be good candidates for the regulation of protein–protein interactions between SPDL-1 and the RZZ complex or SPDL-1 and MAD1^{MDF-1}.

SPDL-1 shares a short conserved amino acid sequence and has similar molecular functions, such as dynein recruitment to the kinetochore, with Spindly family proteins.[48, 49, 68, 69] However, the role of SPDL-1 in SAC activation in *C. elegans* is different from that of Spindly in *Drosophila melanogaster*: in contrast to Spindly whose depletion activates the SAC and causes accumulation of MAD2 proteins at the kinetochore in neuronal cells of *Drosophila* 69, SPDL-1 needs to exist for SAC activation in *C. elegans* embryos.[48, 49] Therefore, in other organisms, other proteins besides Spindly may play the role of SPDL-1 in recruiting MAD1^{MDF-1} to unattached kinetochores. Determination of how the association of SPDL-1 with the RZZ complex, or with the MAD1^{MDF-1}–MAD2^{MDF-2} complex, is regulated in response to the microtubule attachment status of the kinetochore will provide a molecular insight into the evolutionarily conserved event of MAD1 recruitment to the kinetochore, which connects 2 sequential events – recognition of unattached kinetochores and inhibition of APC/C activity - in the SAC signaling pathway.

Kinetochore-independent function of MAD1^{MDF-1}

MAD1^{MDF-1} functions as a downstream target of the PI3K^{AGE-1}-AKT kinase signaling pathway in regulation of germ cell proliferation and is required to sustain the prophase arrest of PGCs in response to starvation. This finding leads us to hypothesize that MAD1^{MDF-1} links the signaling pathways that mediate various environmental signals and the SAC pathway to inhibit APC/C activity to cause cell cycle arrest. At the same time, the following questions have arisen from this finding:

- What is the mechanism of kinetochore-independent activation of MAD1^{MDF-1}? Starvation induced-prophase arrest of PGCs requires MAD1^{MDF-1}, which localizes to the nuclear periphery in PGCs. Comparison of global and germ-cell specific RNAi experiments has revealed that MAD1^{MDF-1} functions autonomously of cell in PGCs.[58] Therefore future work should focus on how MAD1^{MDF-1} at the nuclear periphery transmits the signal from the PI3K^{AGE-1}-AKT kinase pathway to the APC/C. Some components of the nuclear pore complex interact with MAD1^{MDF-1} at the nuclear pore,[38] suggesting that they may have a role in MAD1^{MDF-1}-mediated signaling.

- What is the downstream target of the APC/C to arrest primordial germ cells in prophase? Inappropriate germ cell proliferation observed in *mdf-1Δ* hemizygotes can be suppressed by mutational reduction of the APC/C activity.[58] It needs to be determined whether accumulation of IFY-1, the *C. elegans* securin, is responsible for prophase arrest of PGCs or whether there are other prophase-specific APC/C targets. A genetic screen for mutants that suppress the inappropriate PGC proliferation phenotype of *mdf-1>Δ* hemizygotes may be a reasonable approach to take, because mutations that stabilize APC/C substrates should suppress the phenotype caused by hemizygosity of *mdf-1Δ*.

- Whether phosphorylation of MAD1^{MDF-1} by AKT kinase has any physiological functions in other cell types? Two AKT phosphorylation sites, T523 and T672, have been identified on MAD1^{MDF-1}.[58] The phosphomimietic mutant MAD1^{MDF-1}T523D is defective in binding to MAD2^{MDF-2} in the yeast two-hybrid assay,[58] suggesting that phosphorylation of T523 affects the SAC activity of MAD1^{MDF-1}. However, the functional significance of phosphorylation at T672 is not yet known. Furthermore, although the PI3K^{AGE-1}-AKT kinase signaling pathway regulates many biological processes,[70-77] it remains to be determined whether phosphorylation of MAD1^{MDF-1} is involved in regulating these processes. The transgenic strain expressing MAD1^{MDF-1}T523AT672A in an *mdf-1Δ* background is a powerful system to address this question.

Summary

The conserved SAC component MAD1 plays a crucial role in the initial step of the kinetochore-oriented SAC signaling pathway. Therefore, identifying and characterizing the factors that regulate MAD1 activity will help elucidate the molecular mechanisms of the recognition of kinetochore–microtubule attachment by the SAC. Furthermore, in *C. elegans*, Mad1 has a role in kinetochore-independent cell cycle regulation, which suggests that MAD1 has a role in cell cycle regulation during postembryonic development. Thus, the study of MAD1 in the context of multicellular organisms will provide new insights into the mechanism by which SAC contributes to the spatiotemporal regulation of cell division during development. Furthermore, genetic evidence suggests a causal involvement among deregulated or compromised SAC, chromosome instability (CIN), and cancer. This has led investigators to focus on the critical role of SAC genes as tumor suppressors. Therefore, for cancer research, studies of genes that regulate MAD1 activity in multicellular organisms are important to understand the mechanisms of development of cancer and many genetic diseases.

Acknowledgements

This work was supported by the Cancer Center Support Grant CA021765 from the National Cancer Institute and by the American Lebanese Syrian Associated Charities (ALSAC).

Abbreviations

SAC, Spindle assembly checkpoint; APC/C, Anaphase promoting Complex/Cyclosome; RZZ, ROD-ZW10-ZWILCH; MCC, Mitotic Checkpoint Complex; PGCs, primordial germ cells.

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Table 1

Nomenclature for the human and *C. elegans* genes discussed in this article

	Human	***C. elegans***	***C. elegans* protein**
SAC components	MAD1	*mdf-1*	$MAD1^{MDF-1}$
	MAD2	*mdf-2*	$MAD2^{MDF-2}$
	MAD3	*san-1*	$MAD3^{SAN-1}$
	BUB1	*bub-1*	BUB-1
	BUB3	*bub-3*	BUB-3
	MPS1	Not identified	-
	ZW10	*czw-1*	$ZW10^{CZW-1}$
	ROD	*rod-1*	ROD-1
	ZWILCH	*zwl-1*	$ZWILCH^{ZWL-1}$
APC/C components	APC4	*emb-30*	$APC4^{EMB-30}$
	APC8	*mat-3*	$APC8^{MAT-3}$
	CDC20	*fzy-1*	$CDC20^{FZY-1}$
PI3K-AKT pathway	PI3K	*age-1*	$PI3K^{AGE-1}$
	AKT kinase	*akt-1, akt-2*	AKT-1, AKT-2
	PTEN	*daf-18*	$PTEN^{DAF-18}$
CDK inhibitor	KIP	*cki-1*	CKI-1