[Cell Cycle 7:1, 52-56; 1 January 2008]; ©2008 Landes Bioscience

Perspective

Ulp2 and the DNA damage response

Desumoylation enables safe passage through mitosis

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Abbreviations: Ubl, ubiquitin-like protein; SUMO, small ubiquitin-related modifier; ULP, Ubl-specific protease; UD, ULP domain; DSB, double-strand break; APC, anaphase promoting complex

Key words: Ulp2, SUMO, desumoylation, DNA damage checkpoint, adaptation, recovery

The DNA damage checkpoint is a crucial defense mechanism used by cells to withstand DNA damage. Activation of the checkpoint halts the cell cycle at metaphase and allows time for DNA repair prior to cell division. Much effort has been placed on identifying the proteins involved in checkpoint activation and how they elicit the damage response, whereas much less is known about how the checkpoint is silenced and cell division resumes. We recently reported that Ulp2, a SUMO protease, is required for cell division following termination of the DNA damage checkpoint in budding yeast. Here we discuss potential mechanisms by which Ulp2 enables the successful completion of mitosis following DNA damage. We also suggest candidate Ulp2 substrates whose desumoylation may be necessary for cell cycle restart. Finally, given the requirement of Ulp2 for survival in the presence of various metaphase-arresting agents, we suggest that the necessity for Ulp2 following checkpoint termination may not be specific to the DNA-damage response, but rather may indicate a broader role for desumoylation following prolonged metaphase arrest.

Ubiquitin and its cousins, the ubiquitin-like proteins (Ubls), have proven to be among the most ubiquitous and widely deployed post-translational modifiers in eukaryotic cells. Among the Ubls, SUMO (small ubiquitin-related modifier) appears to have the greatest diversity of functions, including roles in transcriptional control, DNA damage regulation, cell cycle progression, nucleocytoplasmic trafficking and senescence, and SUMO is found in all eukaryotes examined to date.[1-4] In humans, there are at least three active SUMO paralogs, while in the yeast *Saccharomyces cerevisiae* there is only one SUMO variant, encoded by the *SMT3* gene, which is essential for viability.[5]

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Submitted: 10/28/07; Accepted: 10/28/07

Previously published online as a *Cell Cycle* E-publication:
www.landesbioscience.com/journals/cc/article/5218

All the SUMO proteins are synthesized in precursor form and can only be attached to substrate proteins after a short C-terminal propeptide has been removed by a SUMO-specific protease. The C-terminus of mature SUMO is activated and then attached to a substrate protein by an enzymatic cascade (Fig. 1). First, SUMO is adenylated by an E1 activating enzyme; the activated C-terminus is subsequently attacked by the active site cysteine of the E1, forming a thioester bond between the two proteins.[6] SUMO is then transferred to the E2 conjugating enzyme, from which it is transferred to a substrate protein, typically by forming an amide bond with a substrate lysine side chain.[7] The E2 generally functions with one of several E3 ligases to identify substrates and catalyze SUMO conjugation.[8]

The regulation of SUMO removal from protein substrates is as important as the regulation of its attachment. SUMO deconjugation is accomplished by SUMO-specific proteases or desumoylating enzymes. The known desumoylating enzymes are conserved from yeast to humans and comprise a specialized family of cysteine proteases called ULPs (SENPs). The yeast *Saccharomyces cerevisiae* has two desumoylating enzymes, Ulp1 and Ulp2, which cleave SUMO from distinct sets of substrates.[9,10] Among the preferred substrates of Ulp2, as well as its mammalian ortholog SUSP1/SENP6, are polySUMO chains.[11,12] In contrast, Ulp1, but not Ulp2, is capable of efficiently cleaving off the C-terminal peptide from the SUMO precursor.[9] A component of ULP substrate specificity is derived from differences in cellular localization. Ulp1 is generally confined to the nuclear side of nuclear pore complexes, whereas Ulp2 is found throughout the nucleus and bound to chromatin.[10,13,14] Specificity differences intrinsic to the proteases themselves are due to differences both within the conserved catalytic ULP domain (UD) and to regions outside the UD (M. Kroetz and M.H., unpublished data).[15]

Humans have six desumoylating enzymes in the ULP/SENP family, with SENP1 and especially SENP2 being the closest orthologs to Ulp1 and SENP6 most closely resembling Ulp2.[16] Metastatic cancer cells have been shown to have decreased levels of SENP1, and the disruption of SENP1 in mice causes embryonic lethality.[17,18] Similarly, the deletion of the *ULP1* gene in yeast is lethal, and disruption of Ulp2 causes a range of phenotypic defects, including

temperature sensitivity, chromosome instability, and hypersensitivity to DNA and spindle damage.[9,10]

A role for Ulp2 in the DNA damage checkpoint

The pleiotropic phenotype of yeast cells lacking Ulp2 implies that this protease is responsible for desumoylating substrates involved in diverse biological pathways. The identification and analysis of specific Ulp2 substrates has proven this to be true, and has indicated roles for Ulp2 in the regulation of chromosome cohesion, spindle elongation, and SUMO chain formation.[11,19,20] Recently, we found that Ulp2 is also required for cell division following termination of the DNA damage checkpoint.[21] The DNA damage checkpoint is a conserved signaling cascade that is activated when cells experience DNA damage and halts the cell cycle at metaphase.[22] This cell cycle block is crucial for allowing cells time to assess and repair DNA damage before the chromosomes segregate in anaphase. While much of the checkpoint activation cascade is known, very little is understood about how cells resume the cell cycle following checkpoint termination. The requirement for Ulp2 implies that desumoylation of one or more substrates is involved.

Previously, it was shown that Ulp2-deleted cells died in the presence of DNA damage, but why this was so remained unclear.[10] Ulp2 could be required for DNA repair or could play a role in the DNA damage checkpoint. Closer examination revealed that in the presence of a DNA double-strand break (DSB), *ulp2*Δ cells permanently arrested as large-budded cells regardless of whether the DNA DSB was repairable or not.[21] In the case where the DSB was repairable, *ulp2*Δ cells could repair the damage by single-strand annealing, implying that a defect in DNA repair was not the cause of the permanent arrest.

When proteins involved in DNA damage checkpoint activation are mutated, cells fail to arrest cell division in the presence of DNA damage. In contrast, when proteins required for checkpoint deactivation and cell cycle resumption are mutated, cells permanently arrest in the presence of DNA damage, similar to the *ulp2*Δ phenotype. Yeast have two mechanisms for resuming the cell cycle following DNA damage checkpoint activation, called recovery and adaptation.[22] Upon successful DNA repair, the "damage signal" is eliminated. Consequently, checkpoint signaling ceases and the block to the cell cycle is removed. This process of checkpoint deactivation and cell cycle resumption is called recovery. Adaptation, on the other hand, describes the process of cell cycle resumption that occurs in the presence of sustained, unrepairable DNA damage.[23] In these instances, a "damage signal" persists, and the cell must bypass the signal in order to silence the checkpoint and restart cell division. Cells that have adapted will eventually die if the DNA damage is severe unless the damage is subsequently repaired, although they typically can undergo several rounds of cell division prior to this occurring. Given the permanent arrest of *ulp2*Δ cells in the presence of either repairable or unrepairable DNA damage, the *ulp2*Δ mutant appears defective for both recovery and adaptation.

If Ulp2 is required for recovery from or adaptation to checkpoint activation, failure to turn on the checkpoint in response to DNA damage should suppress the permanent arrest of the *ulp2*Δ mutant. Indeed, when the checkpoint-activating proteins Mec1,

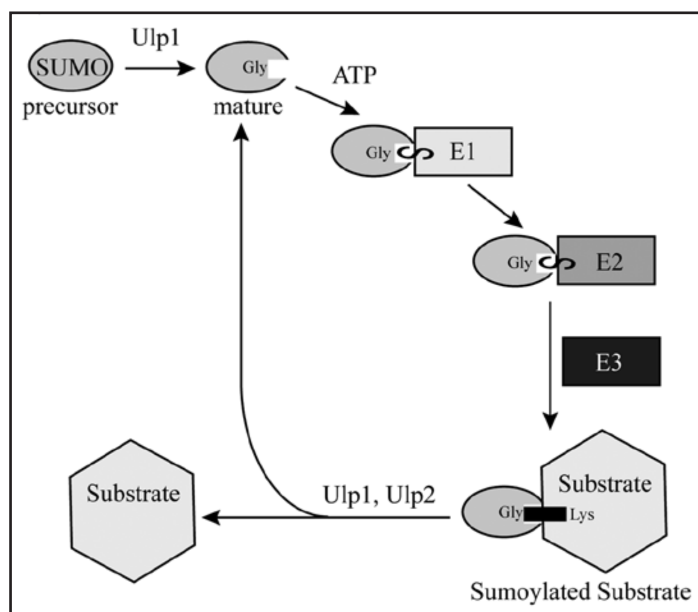

Figure 1. SUMO conjugation and removal. SUMO is synthesized as a precursor protein. A SUMO protease (Ulp1 in *S. cerevisiae*) cleaves SUMO to reveal a C-terminal glycine residue required for substrate conjugation. The E1 activating enzyme adenylates SUMO and creates a high-energy thioester bond (indicated by "~"). SUMO is then passed to the E2 conjugating enzyme. Finally, with the help of an E3 ligase, SUMO is covalently bonded to a lysine residue on the substrate (indicated by shaded rectangle). SUMO can be removed from substrates by SUMO proteases (Ulp1 and Ulp2 in *S. cerevisiae*).

Rad53, Dun1, or Pds1 (Fig. 2A) were deleted, cells failed to arrest in the presence of a DNA DSB, even when Ulp2 was also deleted.[21] Interestingly, this was not the case when the checkpoint-activating protein Chk1 was deleted. Rather, like the *ulp2*Δ single mutant, *chk1*Δ *ulp2*Δ cells permanently arrested following a DNA DSB. The DNA damage checkpoint pathway is a bifurcated signaling cascade (Fig. 2A), with Mec1 kinase phosphorylating and activating both Rad53 and Chk1 kinases.[22] Rad53 then phosphorylates and activates the Dun1 kinase, and Chk1 does the same to the Pds1 (securin) protein. Downstream activities of Pds1 and Dun1 ultimately halt the cell cycle at metaphase and induce transcription of DNA repair genes. The finding that *ulp2*Δ cells permanently arrested after a DNA DSB even when Chk1 was deleted implies that Ulp2 counters a signal transmitted by the Rad53 branch of the checkpoint pathway. It was therefore surprising to observe that the Pds1 deletion was also able to suppress *ulp2*Δ permanent arrest despite being located directly downstream of Chk1 in the checkpoint pathway. However, crosstalk between the two checkpoint branches has previously been observed, as both Rad53 and Chk1 can stabilize Pds1 by inhibiting Pds1 ubiquitination.[24] Our findings further this claim and suggest that Rad53 plays a more dominant role in checkpoint signaling than Chk1.

One hallmark of known recovery and adaptation mutants is that Rad53 persists in a hyperphosphorylated state in the presence of DNA damage and even after its repair. Rad53 phosphorylation is important for activating the DNA damage checkpoint, as mentioned above, and its dephosphorylation correlates with checkpoint turnoff.[25] Thus, persistent Rad53 phosphorylation in recovery and adaptation mutants indicates that the checkpoint remains activated in these

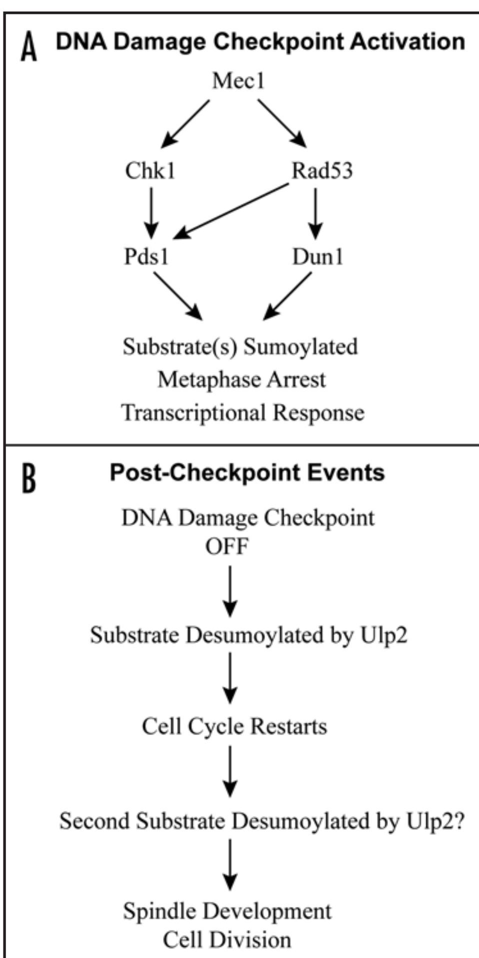

Figure 2. A model for sumoylation and desumoylation in the DNA damage checkpoint. (A) The activated DNA damage checkpoint signals through a bifurcated pathway to assert a metaphase arrest and induce transcription of DNA repair genes. A substrate(s) is most likely sumoylated in response to signaling generated by the Rad53 branch of the checkpoint pathway. (B) Following DNA damage checkpoint termination, Ulp2 desumoylates a substrate to restart the cell cycle. The requirement of this desumoylation step can occasionally be bypassed. Ulp2 may also be required during a later stage of mitosis to desumoylate a second substrate and promote successful spindle development.

cells, explaining why they remain arrested. Surprisingly, *ulp2*Δ cells did not show sustained hyperphosphorylated Rad53 in the presence of DNA damage.[21] Rather, they exhibited wildtype Rad53 phosphorylation and dephosphorylation kinetics. This finding suggests a unique role for Ulp2 in cell cycle resumption at a step after Rad53 dephosphorylation.

Potentially, Ulp2 might be a part of the recovery and adaptation mechanisms but might act at a very late step to turn off the DNA damage-signaling cascade. An alternative, but not mutually exclusive, possibility is that Ulp2 acts after turnoff of the damage-signaling cascade to promote a step in cell division following release from the metaphase arrest. In either case, the role of Ulp2 most likely involves the desumoylation of one or more protein substrates, as we found that the SUMO protease activity of Ulp2 was required for its function and that inhibiting protein-SUMO conjugation could suppress the permanent arrest of *ulp2*Δ cells after DNA damage.[21]

If the first model were true, desumoylation of a protein by Ulp2 would be the most terminal step in adaptation and recovery that has been identified to date. This hypothesis predicts that *ulp2*Δ cells bearing DNA damage should be unable to complete these processes and initiate anaphase. Thus, the *ulp2*Δ mutant should be permanently arrested at metaphase, the stage of initial arrest following DNA damage checkpoint activation. If the second model were true, wherein Ulp2 acts after checkpoint signaling has been turned off, *ulp2*Δ cells with DNA damage should terminally arrest in some later stage of mitosis. In actuality, closer examination of the cell division arrest of *ulp2*Δ cells after induction of an unrepairable DNA DSB revealed that approximately half of the cells remained arrested in metaphase whereas the other half terminated the cell cycle post-metaphase.[21]

This heterogeneous arrest lends itself to two interpretations (Fig. 2B). In the first, Ulp2 might desumoylate distinct substrates during adaptation/recovery and at a later mitotic stage. Substrate desumoylation by Ulp2 at the earlier stage might not be absolutely necessary for adaptation/recovery, allowing occasional breakthrough from the metaphase arrest, but the contribution of Ulp2 at the later mitotic stage cannot be bypassed. In the second interpretation, cell cycle restart might only require Ulp2 to desumoylate one specific substrate, during the adaptation or recovery process. Although this requirement may again be occasionally bypassed, enabling the cell cycle to restart and progress past metaphase, the persistent sumoylation of this substrate ultimately interferes with subsequent mitotic events.

Given the unique arrest phenotype of *ulp2*Δ cells, should *ulp2*Δ be considered a DNA damage checkpoint recovery or adaptation mutant? The evidence suggests that Ulp2 is required following activation of the DNA damage checkpoint and likely acts while cells are still arrested at metaphase. However, Ulp2 is not absolutely required for the cell cycle to resume, although it is needed subsequently for the successful completion of mitosis. When we compared the arrest phenotype of *ulp2*Δ cells to another recovery and adaptation mutant (*srs2*Δ), we found that *srs2*Δ cells also showed a limited ability to break through the metaphase arrest, although to a much lesser extent than *ulp2*Δ cells.[21] Srs2-deficient cells also demonstrate a more classical adaptation and recovery mutant profile than *ulp2*Δ cells: unlike *ulp2*Δ, *srs2*Δ cells arrest with hyperphosphorylated Rad53 and overexpression of the Rad53 phosphatase Ptc2 can suppress this arrest.[21,26] Other metaphase arrest-inducing conditions also cause a permanent block to cell division in *ulp2*Δ cells (see below).[10,19] Ulp2 can therefore be viewed either as a factor that operates outside of adaptation or recovery per se, or as an adaptation/recovery factor that functions in a cell cycle restart mechanism potentially common to different metaphase arrest-inducing events.

Potential substrates for Ulp2

Regardless of these ambiguities in definition, it is clear that Ulp2 plays an important role in regulating the cell cycle following termination of the DNA damage checkpoint. As mentioned above, this role most likely involves the desumoylation of one or more protein substrates. To gain a full understanding of how Ulp2 acts in restarting cell division, identification of the relevant substrate(s) will be required. Although this information is not currently at hand, several reasonable candidates can be suggested.

One possibility is that the sumoylation of a DNA repair protein coordinates cell cycle arrest and repair. For example, the repair

protein Rad52 has been shown to be sumoylated in response to DNA damage.[27] Sumoylated Rad52 could potentially serve as a signal to the cell that the DNA repair machinery has been engaged and thus the cell should halt division. Desumoylation could coincide with the removal of the repair proteins from the DNA site of damage as a signal that it is now appropriate for the cell to resume the cell cycle. Although reasonable, this particular scenario is unlikely. If a repair protein were the key Ulp2 substrate for cell cycle restart, it most likely would assert its cell cycle inhibitory effect by signaling to the DNA damage checkpoint to remain activated, as it has no other obvious means for inhibiting the cell cycle machinery. Consequently, we would expect to see persistent checkpoint signaling in *ulp2*Δ cells bearing DNA damage, similar to DNA damaged *srs2*Δ cells, which have defects in removing the repair machinery from DNA. However, as outlined above, the DNA damage checkpoint was deactivated in permanently arrested *ulp2*Δ cells. It is also difficult to imagine how a sumoylated DNA repair protein could interfere with mitotic progression in those cells that break out of metaphase arrest, thereby yielding the heterogeneous arrest phenotype observed for *ulp2*Δ cells. If a repair protein is a Ulp2 substrate, it most likely is not the only Ulp2 substrate regulating cell division following DNA damage checkpoint termination.

A more likely scenario is that the Ulp2 substrate(s) is a protein that is not only involved in the cell's response to DNA damage, but also plays roles in normal cell cycle progression. Along these lines, we found that roughly half of *ulp2*Δ cells that had permanently arrested following induction on an unrepairable DNA DSB showed evidence of a broken or otherwise aberrant mitotic spindle, whereas almost all of the remaining cells exhibited an intact metaphase/early anaphase spindle.[21] This finding implies that the persistent sumoylation (or polysumoylation) of some substrate interferes with mitotic spindle development, which results in a second block to cell cycle progression after metaphase breakthrough. This substrate is probably not sumoylated during a normal cell cycle when there is no DNA damage, enabling safe passage through mitosis. The fact that Ulp2 is not essential during normal cell division is consistent with this idea.

Several spindle-regulating kinetochore proteins are known to be sumoylated, including Ndc10, Bir1, Ndc80, and Cep3.[20] Preventing sumoylation of Ndc10 has been shown to lead to mislocalization from the spindle midzone, abnormally long anaphase spindles, and loss of Bir1 sumoylation. Interestingly, treatment of yeast cells with the microtubule-destabilizing drug nocodazole, which activates the spindle checkpoint, leads to Ulp2-mediated desumoylation of Ndc10, Bir1, and Cep3, but not Ndc80. These data reflect the opposite trend of what we saw in response to activation of the DNA damage checkpoint. Our experiments implied that persistent sumoylation (of an unknown protein) interfered with proper spindle development and that checkpoint activation promoted, rather than decreased, this sumoylation. Despite these differences, the combined data suggest a dynamic interplay among kinetochore proteins, SUMO, and Ulp2 in regulating both the mitotic spindle as well as each other. Cell cycle checkpoints might take advantage of this regulation and alter the sumoylation status of kinetochore proteins to enforce a halt on cell division. On the other hand, if the sumoylation status of any of these kinetochore proteins is inappropriate once the cell cycle has restarted, spindle development might be disrupted.

In addition to those proteins that are known to be sumoylated, several other potential substrates stand out. For instance, both *ULP2* and *SMT3* (encoding yeast SUMO) were identified as high-copy suppressors of the *mif2-3* kinetochore mutant.[28] This mutant exhibits broken spindles at restrictive temperature.[29] Another mutant that demonstrates morphologically aberrant spindles is *esp1-1*.[30] Esp1 (yeast separase) is responsible for the cleavage of the cohesin complex that occurs at the onset of anaphase and is involved in other mitotic exit-promoting activities as well.[31] Normally, Esp1 is kept inactive by Pds1 (securin), and this interaction is promoted by the DNA damage checkpoint. Sustained sumoylation of Mif2 or Esp1 in *ulp2*Δ cells could potentially disrupt their normal activities and result in spindle damage.

Yet another possibility is that the Ulp2 substrate(s) indirectly causes spindle damage. For example, one of the subunits of the anaphase promoting complex (APC) ubiquitin ligase, such as Cdc20 or Cdh1, might become aberrantly sumoylated in the *ulp2*Δ mutant; sumoylation has been linked previously to APC-mediated proteolysis.[32] Cdc20 and Cdh1 are specificity factors for the APC and dictate which proteins in the cell are ubiquitinated and targeted for degradation during various stages of the cell cycle.[33] Persistent (poly)sumoylation of the APC or one of these factors could alter the stability of spindle-regulating proteins and thus disrupt spindle development. Cdc20 in particular is an interesting Ulp2 substrate candidate, as it is inhibited upon activation of both the DNA damage and spindle checkpoints, albeit by different mechanisms.[34,35]

A broader role for Ulp2 following different metaphase arrests?

As mentioned at the beginning of this article, *ulp2*Δ cells have a pleiotropic phenotype and show sensitivities to a variety of DNA-damaging, replication-inhibiting, and spindle-damaging drugs. Many of these conditions induce a cell cycle arrest. One possibility, therefore, is that *ulp2*Δ is generally unable to survive a cell cycle arrest. Upon closer examination, however, it seems that some distinctions apply. Ulp2-depleted cells can divide normally following an α factor-induced G_1 arrest, and do not permanently arrest after an HU-induced S-phase arrest, although they are sick.[10,21] However, the permanent arrest of *ulp2*Δ cells observed following DNA damage might reflect a general inability of *ulp2*Δ cells to survive a prolonged metaphase arrest of any kind.

Several factors can trigger a metaphase arrest, including DNA damage, spindle damage, and APC inhibition. Cells lacking Ulp2 die in the presence of any of them, although it is presently unknown whether they exhibit the broken spindles observed after prolonged arrest of DNA damaged *ulp2*Δ cells.[10,19] Ulp2 is required to restart the cell cycle from metaphase arrest in response to DNA damage at a step after the checkpoint is turned off. Hence, it is plausible that Ulp2 fulfills the same role during the resumption of the cell cycle following metaphase arrests induced by other means. One possibility is that any mechanism of inducing metaphase arrest leads to a common substrate (such as an APC subunit or regulator) being sumoylated. This might pose an additional block to cell cycle progression and provide reinforcement to the activated checkpoint. Desumoylation by Ulp2 would later be required to release this secondary block and allow the successful completion of mitosis.

The metaphase-to-anaphase transition marks a point of no return in the cell cycle. Once daughter chromosomes separate, there is no known way to realign or redistribute them to correct

errors resulting from chromosomal or spindle lesions. It makes sense for cells to have multiple mechanisms in place to ensure that a metaphase arrest remains engaged until the cell is ready to segregate its duplicated genome to two daughter cells. Sumoylation of a cell cycle protein(s) provides a potential mechanism for inhibiting the metaphase-to-anaphase transition. Interestingly, we found that sumoylation was not required to assert a metaphase arrest in response to DNA damage.[21] However, if it occurred, Ulp2 was then needed to enable successful cell division. This implies that sumoylation is not an integral part of establishing a metaphase arrest, but rather may help reinforce or maintain such an arrest.

How cells restart the cell cycle following a checkpoint arrest has long been a mystery. The finding that Ulp2 is required for proper cell cycle progression after the DNA damage checkpoint has been terminated implies that a desumoylation event must occur for proper restart from a metaphase arrest. Moreover, the sensitivity of *ulp2*Δ cells to a range of metaphase arrest-inducing agents suggests that this desumoylation step may be shared among different metaphase-arrest pathways. A shared mechanism for restarting the cell cycle following differentially induced metaphase arrests is plausible since much of the same cell cycle machinery is engaged during the block to anaphase progression. A great deal still remains to be learned about how such a restart mechanism would operate.

Acknowledgements

We thank M. Kroetz and Y. Xie for comments on the manuscript. R.F. was supported in part by NIH training grant GM007223. This work was supported by NIH grant R01 GM053756 to M.H.

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