Restraint of apoptosis during mitosis through interdomain phosphorylation of caspase-2

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The apoptotic initiator caspase-2 has been implicated in oocyte death, in DNA damage- and heat shock-induced death, and in mitotic catastrophe. We show here that the mitosis-promoting kinase, cdk1–cyclin B1, suppresses apoptosis upstream of mitochondrial cytochrome *c* release by phosphorylating caspase-2 within an evolutionarily conserved sequence at Ser 340. Phosphorylation of this residue, situated in the caspase-2 interdomain, prevents caspase-2 activation. S340 was susceptible to phosphatase 1 dephosphorylation, and an interaction between phosphatase 1 and caspase-2 detected during interphase was lost in mitosis. Expression of S340A non-phosphorylatable caspase-2 abrogated mitotic suppression of caspase-2 and apoptosis in various settings, including oocytes induced to undergo cdk1-dependent maturation. Moreover, U2OS cells treated with nocodazole were found to undergo mitotic catastrophe more readily when endogenous caspase-2 was replaced with the S340A mutant to lift mitotic inhibition. These data demonstrate that for apoptotic stimuli transduced by caspase-2, cell death is prevented during mitosis through the inhibitory phosphorylation of caspase-2 and suggest that under conditions of mitotic arrest, cdk1–cyclin B1 activity must be overcome for apoptosis to occur. *The EMBO Journal* (2009) 28, 3216–3227. doi:10.1038/emboj.2009.253; Published online 3 September 2009
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Introduction

Tissue homeostasis relies on maintaining a precise balance between cell proliferation and death, yet much of the interplay between the cell division and cell death pathways remains to be elucidated. Indeed, although some typical features of mitotic cells (e.g. loss of contact with neighboring

cells) would typically cue cells to undergo apoptosis, cells don't initiate apoptotic programmes when undergoing normal, unperturbed mitoses.

When cells are arrested in mitosis by the activation of spindle assembly checkpoint (e.g. after treatment with microtubule poisons, such as nocodazole, taxol and others, or following accrual of chromosomal alterations that have escaped detection by G2/M checkpoint machinery), high cdk1 activity is maintained by delaying the anaphase-promoting complex/cyclosome (APC/C)-mediated ubiquitination and degradation of cyclin B1. Although this may effectively allow time for rectification of spindle defects, in cases in which the block to mitotic progression cannot be overcome, prolonged activation of the spindle assembly checkpoint ultimately leads to mitotic cell death, commonly referred to as 'mitotic catastrophe'. Although the term mitotic catastrophe has been used broadly to refer to multiple forms of mitotic cell death, prolonged activation of the spindle assembly checkpoint, specifically, is believed to lead to cell death through a process known as 'mitotic slippage', apparently caused by slow but eventual APC/C-mediated degradation of cyclin B1 (Tao *et al*, 2005; Brito and Rieder, 2006), allowing premature mitotic exit with chromosomal abnormalities. Despite the failure to complete normal cell division, cells that exit mitosis through mitotic slippage still undergo reassembly of the nuclear envelope, which forms around random groups of chromosomes, leading to the formation of genomically unstable multinucleated aneuploid cells (Castedo *et al*, 2004; Mansilla *et al*, 2006) and, ultimately, cell death. Thus, although cells are clearly not susceptible to death at each mitosis, prolonged mitotic arrest induces death. This suggests that mediators of mitotic catastrophe may be suppressed during normal mitosis, and pressed into action when spindle disruption is prolonged.

Although the precise pathways of death by mitotic catastrophe have yet to be elucidated, Ho *et al* (2008) reported that cytoskeletal-disrupting agents, such as vincristine and paclitaxel, which activate the spindle assembly checkpoint by disrupting microtubule stability, require the initiator caspase, caspase-2, for induction of cell death. Moreover, Castedo *et al* (2004) showed that mitotic cell death required caspase-2 at an apical step, upstream of cytochrome *c* release. These data suggested that there might be a mechanism for suppressing caspase-2 during each mitotic cycle, unless M phase arrest was prolonged.

A number of apoptotic pathways, including those triggered in response to stressors, such as heat shock and certain chemotherapeutic agents seem to be caspase-2 dependent (for review, see (Krumschnabel *et al*, 2009)). However, the only defect originally observed in caspase-2 knockout mice was an abundance of oocytes (Bergeron *et al*, 1998). Underscoring the oocyte defect in caspase-2^{−/−} mice, work from our laboratory also implicated caspase-2 in apoptosis of *Xenopus* oocytes and cell-free extracts prepared from these cells. Caspase-2 is believed to work upstream of the

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mitochondria to promote cytochrome *c* release (Guo *et al*, 2002; Lassus *et al*, 2002; Robertson *et al*, 2002). This effect of caspase-2 on the mitochondria is thought to require the Bcl-2 family member, Bid (Guo *et al*, 2002; Bonzon *et al*, 2006), which upon cleavage by caspase-2 forms tBid, translocates to the mitochondria to induce outer membrane permeabilization and release of cytochrome *c*. The prevailing model to explain caspase-2 activation involves caspase-2 binding to an adaptor protein such as RAIDD, although RAIDD-independent pathways for caspase-2 activation have been recently described (Manzl *et al*, 2009; Olsson *et al*, 2009). Once bound to RAIDD, monomeric caspase-2 becomes dimerized and activated in a multiprotein complex (that includes an additional protein, PIDD) known as the PIDDosome (Tinel and Tschopp, 2004).

In the oocyte system, we found that caspase-2 was, at least in part, under the control of cellular metabolism. Specifically, under conditions of nutrient abundance, caspase-2 was suppressed by high levels of NADPH, produced through the pentose phosphate pathway (PPP). This effect on caspase-2 was mediated by Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), which could trigger phosphorylation of Ser 135 (*Xenopus* numbering) within the caspase-2 prodomain to impede RAIDD recruitment and activation of caspase-2. These findings suggested that for cells to undergo caspase-2-dependent apoptosis, nutrients sufficient to drive the PPP must drop below a critical level.

We show here that caspase-2 can be suppressed during mitosis, by a mechanism distinct from that triggered by nutrient abundance. Specifically, we find that cdk1–cyclin B1 activity during mitosis exerts a block upstream of mitochondrial cytochrome *c* release by suppressing caspase-2 through phosphorylation at a conserved serine–proline motif (S308 in *Xenopus* and S340 in human). In contrast to S135 phosphorylation, which lies within the pro-domain required for adaptor recruitment and activation, S340 lies within well-conserved pro-caspase sequences situated between the small and large caspase subunits, in a region that is absent from the mature enzyme. This phosphorylation could be reversed by the action of protein phosphatase 1 (PP1). In the settings of both maturing oocytes and mitotic catastrophe of human cells in response to spindle poisons, mutation of Ser340 to Ala allowed caspase-2 to escape mitotic suppression, significantly lowering the threshold for apoptotic cell death. Thus, when both metabolic and cell cycle brakes are lifted, caspase-2 can trigger mitochondrial cytochrome *c* release and apoptosis.

Results

Cdk1–cyclin B1 activity confers resistance to apoptosis upstream of cytochrome *c* release in mitotic extracts

To delineate differences in the regulation of cell death during interphase and mitosis, we initially took advantage of the ability to produce controlled cell cycle transitions *in vitro* using a *Xenopus* egg extract system. *Xenopus* eggs are normally arrested in M phase, but when eggs are lysed, release of Ca^{2+} from internal stores promotes activation of the anaphase promoting complex, degradation of cyclin B1, and transition into interphase. These extracts can then be converted to a stable mitotic state by the addition of recombinant non-degradable cyclin B1, which activates

endogenous cdk1 and drives the extract into mitosis. To compare the induction of apoptosis in interphase and mitotic extracts, we incubated both extracts at room temperature to allow for activation of the endogenous apoptotic programme (Newmeyer *et al*, 1994) and monitored cleavage of the caspase 3 substrate DEVD-pNA. As shown in Figure 1A, caspase activation was completely suppressed in the mitotic extracts for the duration of the experiment, whereas caspase-3 activation occurred after approximately 4 h of incubation in interphase extracts (it should be noted that the timing of activation in interphase extracts varied from extract to extract, but caspase activation was consistently suppressed in mitotic extracts).

On the basis of a previous report in which extracellular signal-regulated kinase (ERK) was shown to be critical for the suppression of the apoptosome in egg extracts arrested in metaphase of meiosis II (Tashker *et al*, 2002), we postulated that MAPK/ERK activity induced by the upregulation of cdk1–cyclin B1 activity in mitotic extracts might also account for the suppression of caspase-3 that we observed. Therefore, in an attempt to reverse the suppression of caspase-3 activation, we added the ERK/MAPK inhibitor, U0126, and as an additional control the cdk1 inhibitor, roscovitine, to mitotic extracts. Although inhibition of ERK activity by U0126 (Figure 1B) had no effect on caspase activity, inhibition of cdk1 by the addition of roscovitine restored caspase-3 activation to near-normal kinetics (Figure 1A).

An important distinction between these experiments and those of Tashker *et al* (2002) in which ERK activation was found to be required for the suppression of caspase-3 in mitochondria-free metaphase II-purified cytosol supplemented with exogenous cytochrome *c*, is that the experiments here were carried out in unfractionated crude extracts containing mitochondria and a fully intact apoptotic pathway. Therefore, we reasoned that the lack of caspase-3 activity in mitosis might be due to a new ERK-independent locus of suppression upstream of mitochondria. As shown in Figure 1C, release of cytochrome *c* from mitochondria was suppressed in mitosis. Importantly, interphase and mitotic extracts did not show any significant differences in the release of cytochrome *c* in response to the addition of truncated Bid (tBid) (Figure 1D). These data indicate that mitotic extracts are capable of releasing mitochondrial cytochrome *c*, but that suppression of apoptosis most likely occurs first at an apical point in the apoptotic cascade, upstream of the mitochondria.

Caspase-2 is suppressed during mitosis

The observation that mitotic and interphase extracts were equally sensitive to the addition of tBid made it highly unlikely that regulation of Bax, Bcl-2, or other proteins at the level of mitochondria were responsible for the mitotic suppression of apoptosis we observed. This prompted us to look further upstream to explain the lack of cytochrome *c* release in mitosis. As caspase-2 had been implicated as the upstream activator of Bid, we first examined the processing of radiolabelled caspase-2 during spontaneous apoptosis in the extract. Although processing is not synonymous with activation (as it is dimerization, rather than cleavage that promotes activation), caspase-2 cleavage is required to stabilize caspase-2 and is generally indicative that caspase-2 activation has occurred (Baliga *et al*, 2004). As shown in Figure 2A,

Figure 1 Mitotic suppression of apoptosis occurs upstream of cytochrome *c* release. **(A)** Interphase and mitotic extracts were incubated at RT alone or in the presence of roscovitine (0.28 mM) or U0126 (200 μ M). Caspase activity was determined by a colorimetric measurement of DEVD-pNA cleavage. **(B)** The activity of the ERK inhibitor U0126 was verified by immunoblotting for phospho-ERK. **(C)** Interphase and mitotic extracts were incubated at RT. Samples were collected at indicated times, fractionated to make mitochondria-free cytosolic supernatants, and immunoblotted for cytochrome *c*, actin and VDAC (*P*=cell pellet containing mitochondria). **(D)** Mitotic and interphase extracts were incubated with caspase-8-cleaved Bid (tBid) at a concentration of 1 nM. Release of cytochrome *c* was measured as in panel (C).

Figure 2 Caspase-2 is suppressed in mitosis. **(A)** 35 S-labelled caspase-2 was incubated in interphase and mitotic extract. Samples collected at the indicated times were resolved by SDS-PAGE/phosphorimager. **(B)** 35 S-labelled Bid was incubated in interphase and mitotic extract. Samples collected at the indicated times were resolved as in panel (A). **(C)** NADPH levels from samples of interphase and mitotic extract, collected as in panel (A), were measured by spectrophotometric colour change (kit supplied by BioVision). **(D)** 35 S-labelled caspase-2 (WT or S135A) was incubated in mitotic extract and resolved by SDS-PAGE/film. **(E)** 35 S-labelled caspase-2 was incubated in interphase and mitotic extracts in the presence of GST-RAIDD (10 μ g). At the indicated times, GST-RAIDD was retrieved, washed once in PBS, and resolved as in panel (D).

caspase-2 processing occurred in interphase but not in mitotic extracts unless supplemented with the cdk1 inhibitor, roscovitine, suggesting that caspase-2 itself or a signalling

molecule(s) upstream of caspase-2 was inhibited during mitosis. In addition, cleavage of Bid, a known target of caspase-2, was also suppressed in mitotic extract unless

supplemented with roscovitine (Figure 2B). Given that our laboratory had previously reported that PPP activity, and consequent NADPH production, suppressed caspase-2 in interphase extracts, we speculated that lack of caspase-2 processing in mitosis might be due to a slower depletion of NADPH (i.e. lower metabolic activity). However, no significant differences in NADPH levels/depletion were observed between extracts arrested in interphase and mitosis (Figure 2C). Moreover, the S135A mutant variant of caspase-2, shown previously to escape suppression by metabolic (NADPH dependent) pathways, was suppressed equally compared with WT protein during mitosis (Figure 2D), suggesting that metabolic regulation did not underlie mitotic suppression of caspase-2.

To examine caspase-2 activation in a more targeted manner, we induced oligomerization and activation of radiolabelled caspase-2 by the overexpression of the caspase-2 adaptor protein, RAIDD (Nutt *et al*, 2005), and measured caspase-2 processing. Although RAIDD induced the processing of caspase-2 in interphase extracts, processing in mitosis was suppressed (Figure 2E). Importantly, suppression of RAIDD-induced processing of caspase-2 did not occur because of defective recruitment of caspase-2 to RAIDD, as RAIDD/caspase-2 binding did not differ appreciably in interphase and mitosis (Supplementary Figure S1). Therefore, we suspected that caspase-2 itself, and not RAIDD, was directly inhibited in mitosis.

Caspase-2 is regulated by phosphorylation at Ser308 (*Xenopus*) in mitosis

We postulated either that caspase-2 was bound to an inhibitor in mitosis, or that post-translational modification(s) in mitosis prevented caspase-2 activation. Our attempts to identify proteins bound to recombinant caspase-2 in mitosis did not reveal any differential binding of potential inhibitors. Therefore, we focused on other possible mechanisms of caspase-2 inhibition. To search for post-translational modifications of caspase-2 unique to mitosis, we carried out tandem mass spectrometry with recombinant caspase-2 that had been incubated in interphase or mitotic extract. As shown in Figure 3A, these analyses revealed a mitosis-specific mass shift in caspase-2 corresponding to a short sequence of amino acids encompassing Ser 308 (*Xenopus* numbering corresponding to human S340). Incubation of either WT GST-caspase-2, or mutant S308A GST-caspase-2 in interphase, or mitotic extract with [γ - 32 P]ATP, confirmed that Ser 308 was phosphorylated in mitosis, but not in interphase (Figure 3B). Moreover, as shown in Figure 3C, purified cdk1-cyclin B1 phosphorylated S308 *in vitro*.

Ser 308 of caspase-2 sits adjacent to a proline (forming the cdk1 'SP' minimal phospho site) and is conserved in humans, mice, and zebrafish (Figure 3D). To examine whether mitotic phosphorylation of caspase-2 could be observed in mammalian cells, we carried out two-dimensional gel electrophoresis of human U20S cell lysates, made from either cycling cells or cells arrested in mitosis by nocodazole treatment, and immunoblotted for endogenous caspase-2. Mitotic caspase-2 showed a shift to lower pH, indicative of phosphorylation (Figure 3E). To determine whether mammalian caspase-2 was phosphorylated at Ser 340 (human numbering, corresponding to S308 in *Xenopus*) during mitosis, we generated anti-phospho-S340 antibodies directed against human cas-

pase-2, and immunoblotted mitotic and asynchronous cycling cell lysates for comparison. As shown in Figure 3F, human caspase-2 was phosphorylated at Ser 340 during mitosis which correlated with a comparable increase in anti-phospho-histone H3 staining, strongly suggesting that endogenous caspase-2 in mitotic human cells was phosphorylated at Ser 340.

A recent study from our laboratory has shown that PP1 regulates caspase-2 activation in interphase (Nutt *et al*, 2009). Indeed, treatment of interphase extract with 10 μ M okadaic acid, a concentration that inhibits PP1, suppresses caspase-2 processing, whereas 1 μ M okadaic acid, specific to PP2A, has no effect (Figure 4A). Moreover, addition of the PP1-specific inhibitor, I2, to interphase extracts also inhibited caspase-2 processing (Figure 4B). We recently found that PP1 could bind physically to caspase-2 to promote Ser 135 dephosphorylation upon nutrient depletion (Nutt *et al*, 2009). Our data suggested that PP1 might also be capable of dephosphorylating Ser 308. As shown in Figure 4C, addition of PP1 to cdk1-cyclin B1-phosphorylated caspase-2 decreased Ser 308 phosphorylation, which was suppressed by the addition of I2. These data suggest that PP1 might antagonize phosphorylation at Ser 308 and that, during mitosis, PP1 activity towards caspase-2 might be negatively regulated. As shown in Figure 4D, PP1 is bound to caspase-2 during interphase but not in mitosis. In that we have recently reported that PP1 catalytic activity is suppressed in mitosis (Wu *et al*, 2009), the dual action of cdk1-mediated phosphorylation and inhibition of PP1 activity, and binding to caspase-2 ensures that caspase-2 will not be active during a normal mitosis.

Ser 308Ala mutation renders caspase-2 refractory to mitotic suppression

To show more definitively that phosphorylation at Ser 308 had a function in the suppression of caspase-2 in mitosis, we added radiolabelled full-length WT or S308A mutant caspase-2 to mitotic egg extracts and monitored caspase-2 processing. As shown in Figure 5A, the S308A mutation abrogated mitotic suppression, allowing processing of caspase-2 in mitosis. Importantly, this mutation had no notable effect during interphase, suggesting that this site is a locus of mitotic, but not interphase control of caspase-2 (it should be noted that this mutant is still subject to metabolic control through Ser 135 phosphorylation, so activation occurred at the same time as WT caspase-2). Although mitotic phosphorylation of caspase-2 did not affect its binding to RAIDD (Supplementary Figure S1), replacement of Ser 308 with glutamic acid (S308E), to mimic phosphorylation, resulted in the inhibition of caspase-2 autoprocessing induced by overexpression of RAIDD (Figure 5B). Importantly, cdk1-mediated phosphorylation of caspase-2 did not prevent caspase-3 'feedback' processing of caspase-2 (Supplementary Figure S2). Together, these data suggest that phosphorylation at Ser 308 blocks caspase-2 activation downstream of RAIDD-binding, probably at the level of autocatalytic processing, and that phosphorylation at Ser 308 is sufficient to suppress caspase-2 activation even in the absence of other suppressive mitotic factors. Moreover, addition of recombinant S308A mutant caspase-2 led to rapid mitochondrial cytochrome *c* release in mitotic extract, as compared with WT caspase-2 addition (Figure 5C), consistent with the suppression of

Figure 3 Caspase-2 is phosphorylated in mitosis at S340 (S308 in *Xenopus* numbering). **(A)** GST-caspase-2, incubated in interphase and mitotic extract, was retrieved, washed, and resolved by SDS-PAGE. Excised caspase-2 bands were analysed by tandem mass spectrometry. Red arrows point to the relevant shifted peaks. **(B)** GST-caspase-2 (WT and S308A) was incubated in interphase or mitotic extract in the presence of [γ - 32 P]ATP. Samples were resolved by SDS-PAGE/phosphorimager. **(C)** GST-caspase-2 (WT and S308A) was incubated in the presence of purified cdk1-cyclin B1. Samples were resolved by SDS-PAGE/film. **(D)** Sequence alignment of caspase-2 from different species. Letters highlighted in red indicate the conserved cdk1 phospho site. Asterisks denote the aspartate cleavage sites used to form processed active caspase-2. **(E)** Mitotic and cycling lysates from U2OS cells were subjected to 2D gel electrophoresis followed by immunoblotting for endogenous caspase-2. **(F)** Mitotic and cycling lysates from U2OS cells were immunoblotted with anti-phospho S340 caspase-2, anti-phospho histone H3, and actin antibodies.

mitochondrial cytochrome *c* release in mitosis through caspase-2 phosphorylation.

To compare the activity of S308A with WT caspase-2 in whole cells, we injected mRNA into oocytes that had been

treated with progesterone to induce oocyte maturation and cycling into M phase with high cdk1-cyclin B1 activity, as shown by pHistone H3 staining (Figure 5G). As shown in Figure 5D-F, expression of caspase-2 S308A induced rapid

Figure 4 PP1 activity is required for mitotic suppression of caspase-2. (A) Okadaic acid, at indicated concentrations, was incubated in interphase extract with ³⁵S-labelled caspase-2. Processing of caspase-2 was measured by SDS-PAGE/film. (B) ³⁵S-labelled caspase-2 was incubated with buffer or with the PP1-specific inhibitor I2. Processing of caspase-2 at various time points was measured as in panel (A). (C) GST-caspase-2 was incubated *in vitro* with the indicated combinations of purified cdk1-cyclin B1 (0.5 μM), PP1 (2 μM) and I2 (0.5 μM), in the presence of γ³²P. Samples were resolved by SDS-PAGE/phosphorimager. (D) Glutathione sepharose-bound caspase-2, or GST alone, were incubated in interphase or mitotic extract for 30 min, retrieved, washed, and subjected to immunoblotting for endogenous PP1.

killing of these oocytes as compared with equivalent levels of WT caspase-2.

Metabolic and mitotic factors contribute to the suppression of caspase-2 in *Xenopus* extract

We noted that processing of caspase-2 S308A in mitosis did not occur immediately, but rather occurred with roughly the same kinetics as WT caspase-2 in interphase. As our laboratory had previously shown that high levels of PPP activity stimulated phosphorylation and suppression of caspase-2 at Ser 135 (Nutt *et al*, 2005), we postulated that the underlying metabolic activity in mitosis prevented the rapid processing of caspase-2 S308A. In support of this idea, we had observed no significant differences in NADPH depletion in either interphase or mitosis (Figure 2C), implying that metabolic suppression of caspase-2 should endure with similar kinetics regardless of the cell cycle phase. If metabolic activity was responsible for the delay in mitotic processing of caspase-2 S308A, then expression of caspase-2 carrying mutations at both the metabolic phospho site (S135A) and the mitotic phospho site (S308A) should abrogate metabolic and mitotic suppression of caspase-2, and thus further accelerate proces-

sing. Indeed, as shown in Figure 6A, caspase-2 S135A/S308A rapidly processed in mitosis compared with caspase-2 carrying the S308A mutation alone. This result suggests that dephosphorylations at both Ser 135 and Ser 308 are important for efficient caspase-2 processing. It follows, therefore, that stimulation of PPP activity would be sufficient to block the processing of caspase-2 S308A in mitosis and, conversely, processing of caspase-2 S135A should be blocked by upregulation of cdk1-cyclin B1 activity (as shown in Figure 2D). Indeed, supplementation of mitotic extract with excess glucose-6-phosphate, to stimulate PPP activity, prevented processing of caspase-2 S308A (Figure 6B).

Replacement of endogenous human caspase-2 with caspase-2 Ser 340Ala mutant enhances sensitivity to mitotic cell death in human tissue culture cells

Previous studies from other laboratories has shown that caspase-2 was required for mitotic cell death caused by agents that disrupt mitotic spindle formation (Castedo *et al*, 2004; Ho *et al*, 2008), indicating that caspase-2 is a key regulator of mitotic catastrophe. We propose that phosphorylation on caspase-2 at Ser 308 safeguards mitotic

Figure 5 Phosphorylation of caspase-2 at S308 is required for mitotic suppression of apoptosis. (A) ^{35}S -labelled caspase-2 (WT and S308A) was incubated in interphase or mitotic extract. Processing of caspase-2 was resolved by SDS-PAGE/film. (B) ^{35}S -labelled caspase-2 (WT and S308E) were incubated with GST-RAIDD. Processing of caspase-2 was measured by SDS-PAGE/phosphorimager (C) GST-caspase-2 (WT and S308A) were incubated in mitotic extract. Samples were collected at indicated times, fractionated, and immunoblotted for cytochrome c. (D) Results of oocyte injection, in the presence of progesterone, with flag-tagged WT and S308A caspase-2. Apoptotic oocytes were scored as described previously by Nutt *et al* (2005). (E) Representative images of oocytes injected with the indicated RNA. (F) Oocytes injected with RNA were lysed, and expression of caspase-2 protein was determined by immunoblotting with anti-flag antibodies. (G) Oocytes with and without progesterone treatment were lysed and subjected to immunoblotting with anti-phospho histone H3 antibodies.

Figure 6 Metabolic and mitotic factors combine to suppress caspase-2 in *Xenopus* extract. (A) ^{35}S -labelled caspase-2 both WT and, S308 and S308A/S135A double mutant were incubated in mitotic extract. Processing of caspase-2 was measured SDS-PAGE/phosphorimager. (B) ^{35}S -labelled caspase-2 (S308A) was incubated in mitotic extract $\pm$ glucose-6-phosphate (5 mM). Processing of caspase-2 was measured by SDS-PAGE/film.

cells from aberrant caspase-2 activation in the absence of cellular stressors. We observed that mitotic phosphorylation of caspase-2 at Ser 308 (S340 in human) also occurred in human tissue culture cells (Figure 3E and F), and on the basis of our data from *Xenopus*, we predicted that expression of the phospho-mutant human caspase-2 S340A should lift the normal mitotic suppression of caspase-2, lower the threshold for mitotic catastrophe, and sensitize cells to death induced by microtubule poisons. To test this, we used a knockdown/addback strategy in which we targeted the 3' untranslated region of caspase-2 with siRNA, followed by transduction with lentivirus expressing either WT or mutant caspase-2 cDNA. The percentage of infected cells, as determined by flow cytometry was 47.4% and 45.5% for WT caspase-2 and caspase-2 S340A, respectively (Supplementary Figure S3). As shown in Figure 7A, expression of lentiviral caspase-2 cDNA was not affected by the presence of caspase-2 siRNA. As shown in Figure 7B, knockdown of caspase-2 suppressed cell death caused by nocodazole arrest, whereas addback of the WT caspase-2 restored cell death to near normal levels.

Figure 7 Loss of phosphorylation of human caspase-2 at S340 sensitizes cells to mitotic cell death. **(A)** U2OS cells were transfected with the indicated siRNA. At 24 h post-transfection, cells were infected with lentivirus expressing human caspase-2 cDNA (WT or S340A). At 36 h after viral infection, cells were collected and immunoblotted for caspase-2. **(B)** Cells treated as in panel (A) were placed in medium supplemented with nocodazole (starting 24 h after lentiviral infection). After 30 h of nocodazole treatment, cells were collected, incubated in PBS + propidium iodide and analysed by flow cytometry. **(C)** Representative images of cells from panel (B).

Expression of caspase-2 S340A more than doubled the sensitivity of cells to nocodazole-induced mitotic death compared with WT caspase-2. Figure 7C shows representative images of cells from Figure 7B. These data strongly suggest that phosphorylation of human caspase-2 at Ser 340 restrains cell death in response to mitotic insult, and suggests that pathways downstream of spindle poisons must abrogate S340 phosphorylation to allow for cell death in the face of irreparable spindle disruption.

Discussion

Caspase-2 has been implicated in multiple paradigms of cell death as an upstream regulator of mitochondrial cytochrome *c* release. Previous studies from our laboratory described caspase-2 as a target of metabolic regulation whose activation was suppressed by pro-domain phosphorylation. We show here that caspase-2 can also be suppressed by cdk1 through phosphorylation at a distinct site and that these pathways suppress caspase-2 non-redundantly. This suggests that the

criteria of nutrient depletion and loss of mitotic suppression must both be met if caspase-2 is to be activated.

Previous studies in both somatic cells and oocytes has shown that mitotic cells are resistant to cell death. Allan and Clarke (2007) showed that mitotic cells exhibited a block in apoptosis downstream of cytochrome *c* release through phosphorylation of caspase-9 at Thr 125, a site also phosphorylated by ERK/MAPK in growth factor-stimulated cells (Allan *et al*, 2003), and more recently shown as target of DYRK1A in retinal development (Laguna *et al*, 2008). In addition, work by Marash *et al* (2008) showed that IRES-mediated translation sustained cdk1 levels and promoted survival during mitosis. Our data show mitotic suppression of cell death at an apical point in the apoptotic pathway, upstream of cytochrome *c* release. In agreement with these previous studies, we have also observed the suppression of apoptosis downstream of cytochrome *c* release during mitosis (unpublished data and (Tashker *et al*, 2002)). Indeed, although the release of cytochrome *c* from mitochondria is clearly delayed in mitotic extract, once it is eventually released, the activation of downstream caspases is attenuated

compared with the kinetics of cytochrome *c*-induced caspase activation in interphase extract. Thus, survival pathways that exert pre-cytochrome and post-cytochrome suppression of apoptosis in mitosis seem to jointly contribute to protect a mitotic cell. Therefore, abrogation of cdk1-mediated phosphorylation of either caspase-9 or caspase-2 compromises the cell's ability to protect itself during mitosis. The data presented here, together with previous studies, suggest that cells use a variety of fail-safe mechanisms to ensure survival following commitment to division.

These data raise the question of why somatic cells suppress their apoptotic machinery so robustly during mitosis. One interesting possibility is that certain morphological aspects of mitosis may render the cell more sensitive to apoptosis. For example, mitochondrial fission, which occurs before apoptotic release of cytochrome *c*, also occurs during mitosis (Taguchi *et al*, 2007). Given the growing body of data implicating mitochondrial fusion/fission machinery as critical for cytochrome *c* release and apoptosis (for review, see (Suen *et al*, 2008)), it is possible that mitotic fragmentation of mitochondria heightens the cell's sensitivity to apoptotic stimuli. In support of this idea, we observed a moderate but reproducible increase in the sensitivity of mitotic extracts to tBid-induced release of cytochrome *c*. Alternatively, the loss of epithelial cell attachment that occurs during mitosis might otherwise, if not for suppression of caspase-2, induce an unintended anoikis response. Consistent with this idea, caspase-2 has been shown to regulate anoikis in airway epithelial cells exposed to the toxin mechlorethamine (Sourdeval *et al*, 2006).

Despite being one of the most evolutionarily conserved caspases, caspase-2 has been enigmatic in regards to its precise role in apoptosis. Studies examining the requirement for caspase-2 in apoptosis induced by chemotherapeutics have yielded conflicting results (Lassus *et al*, 2002; Robertson *et al*, 2002; Marsden *et al*, 2004; Werner *et al*, 2004), and the lack of overt developmental defects in mice lacking components of the PIDDosome (caspase-2, PIDD, RAIDD), aside from an over abundance of oocytes in caspase-2-deficient mice, has further confounded our understanding of caspase 2 (Bergeron *et al*, 1998; Berube *et al*, 2005). How do we reconcile these results with our findings that support a central role for caspase-2 in governing mitotic cell death? Although caspase-2-deficient mice did not develop any early developmental tumours, a subsequent study showed that embryonic fibroblasts from these mice show accelerated proliferation after oncogenic transformation and that tumourigenesis driven by *c-myc* expression (*E mu-myc*) is accelerated with the loss of caspase-2 (Ho *et al*, 2009). In addition, caspase-2-deficient mouse embryonic fibroblasts were shown to be less prone to apoptosis (compared with wild type) in response to cytoskeleton-disrupting agents that cause mitotic arrest (Ho *et al*, 2008). Previous data have also implicated caspase 2 in heat shock-induced apoptosis and our own study suggests a role for caspase 2 in cell death after nutrient depletion (Nutt *et al*, 2005; Tu *et al*, 2006). Taken together with our data, we speculate that in the absence of cellular stressors, caspase 2 may be silent. Indeed, in our experiments neither transient silencing through siRNA nor overexpression of caspase-2 seemed to appreciably affect cell proliferation/death until the addition of an apoptotic stimulus. Additionally, in retrospect, the fact that the only overt

developmental defect reported in caspase-2 deficient mice was confined to oocytes may not be surprising given the role of caspase-2 in metabolically triggered cell death, and that oocytes are the only cells known to rely completely on internal energy stores.

Although regulation of caspase activation through prodomain phosphorylation has been reported previously (including caspase-2 (Nutt *et al*, 2005; Shi *et al*, 2009)), this is the first indication of caspase-2 regulation through phosphorylation in the linker region between the large and small catalytic subunits. Interestingly, Cursi *et al* (2006) showed that caspase-8 is also suppressed through interdomain phosphorylation (at Tyr380), which occurs in tumour cells with high levels of Src kinase activity. In a manner similar to caspase-8, caspase-2 activation occurs through dimerization, followed by an initial processing event to generate large and small subunits that form the active p37/p14 conformation (Baliga *et al*, 2004). The initial processing of caspase-2 is followed by a subsequent cleavage that excises the linker region. The position of this phosphorylation suggests that excision of the linker region during maturation of caspase-2, to form the p19/p12 active heterodimer, may be an important step in caspase-2 activation. However, the fact that we observed no processing of caspase-2 in mitotic extracts suggests that the block in caspase-2 activation occurs earlier, possibly by preventing the initial auto-processing to form the p37/p14 active heterodimer. It is important to note that Baliga *et al* (2004) reported no difference in activity between the p37/p14 (containing the linker) and the p19/p12 heterodimers when analysed *in vitro*. However, a difference in activity may only be observed under phosphorylating conditions that would not be achieved *in vitro* unless exogenous kinase were added.

After scanning human caspase sequences for potential cdk consensus "S/T-P" phosphorylation sites, we found that caspases that contain a linker region between their large and small subunits (caspase-1, 2, 4, 5, 8, and 9) possessed an SP site within the linker region or within a few amino acids of the aspartate cleavage site (Figure 8). In contrast, the effector caspases 3, 7, and 6 do not contain an SP or TP site. This lack of S/T-P sites within executioner caspases raises the possibility that they may be positioned too far downstream in

Figure 8 Comparison of caspases with cdk1 phospho sites within or adjacent to the interdomain. Human caspase sequences were scanned for S/T-P sites, denoted by red arrowheads.

the apoptotic cascade (i.e. beyond the 'decision' phase of apoptosis) to be responsive to this mode of regulation. The presence of SP sites within the linker region of other caspases suggests that it may be a more general mode of regulation (notably, however, not all SP sites show complete evolutionary conservation; for example, the Ser 307 site in caspase-9 is present in human and dog but not mouse, although a TP site exists in mouse caspase-9 at Thr 375, near the Asp 368 interdomain cleavage site). However, the possible contribution of S/T-P site-directed kinases (e.g. cdks and certain MAPKs) to non-apoptotic, inflammasome-associated caspase regulation (e.g. caspase-1) is less clear.

Together, these data show that the activity of cdk1-cyclin B1 is critical for suppressing cell death during mitosis, and that phosphorylation of caspase-2 modulates the sensitivity of mitotic cells to death. In agreement with a 'mitotic slippage' model for mitotic catastrophe, our data suggest that cdk1 activity, conferred by sustained cyclin B1 translation during mitotic arrest, must be overcome (or lost) for apoptosis to occur. As our data predict that caspase-2 Ser 340 would have to be dephosphorylated to initiate apoptosis, this suggests either that prolonged spindle checkpoint activation must alleviate mitotic suppression of PP1 to dephosphorylate that site (perhaps through slow degradation of cyclin B1 and loss of cdk1-cyclin B1-mediated PP1 suppression and/or through factors that regulate PP1-caspase-2 interactions), or that one or more additional phosphatases that are activated during prolonged M phase arrest can promote Ser 340 dephosphorylation, caspase-2 activation, and apoptosis.

Materials and methods

Preparation of *Xenopus* oocytes and extracts

Xenopus egg interphase extracts were prepared by using the protocol described by Smythe and Newport (1991). Mitotic extracts were generated by supplementing interphase extract with non-degradable cyclin B1.

For oocyte injection studies, stage VI oocytes were collected from ovaries that had been excised from PMSG-injected frogs, digested using 2.8 U of liberase dissolved in OR-2 buffer (1 mM MgCl₂, 82.5 mM NaCl, 2 mM KCl, 5 mM HEPES (pH 7.5)) for 1.5 h at room temperature. Following liberase digestion, oocytes were washed in OR-2 buffer and stored in OR-2 buffer supplemented with 1% fetal bovine serum and 0.2% gentamicin. Before injection, oocytes were transferred to buffer supplemented with 200 µM progesterone.

Apoptosis assays

For extract experiments, caspase activity was measured by transferring 5-µl aliquots of extract to 85 µl DEVDase buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, and 10% glycerol) and 10 µl Ac-DEVD-pNA (200 µM) at 37°C for 30–60 min. Absorbance was measured at 405 nm. Release of cytochrome *c* was measured by filtering 30 µl of extract through a 0.1-µm ultrafree-MC filter spin-column (Millipore, Billerica, MA) at 10 000 *g* for 10 min. Flow-through was collected and immunoblotted for cytochrome *c*. NADPH levels were measured using the NADP/NADPH quantitation kit (BioVision, Mountain View, CA). For flow cytometric analysis of cell death, U2OS cells were collected, washed with PBS, re-suspended in 1 µg/ml propidium iodide (dissolved in PBS plus 2% FBS), and kept on ice until analysis. Oocytes were scored visually for apoptosis as described previously (Nutt *et al*, 2005).

Cloning and protein expression

Caspase-2 cDNA (Origene, Rockville, MD) was inserted by restriction cloning into pGEX-KG. Expression and purification of GST-tagged caspase-2 and RAIDD proteins were carried out as described previously (Nutt *et al*, 2005). ³⁵S-labelled caspase-2 proteins were synthesized using the TNT SP6 quick-coupled

transcription/translation system (Promega, Madison, WI). Mutagenesis was carried out by using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Protein analysis

Antibodies used were anti-cytochrome *c* (Becton Dickinson, Franklin Lakes, NJ), anti-caspase-2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-ERK (Cell Signaling Technology, Danvers, MA), and anti-protein phosphatase-1. Where indicated, protein bands were visualized with infrared fluorescent secondary antibodies and analyzed/quantified using the Odyssey infrared scanner (Licor, Lincoln, NE). To examine phosphorylation of GST-tagged caspase-2, glutathione sepharose-bound GST-caspase-2 proteins were incubated in mitotic or interphase extract in the presence of 5 µCi [γ -³²P]ATP at room temperature for 30 min. Beads were washed in PBS plus 0.1% Triton X-100 and 300 mM NaCl, and eluted with SDS-PAGE sample buffer. *In vitro* cdk1 kinase assays were carried out as described above except in the presence of buffer (10 mM Tris (pH 7.5), 10 mM MgCl₂, 1 mM DTT, and 0.1 mM ATP) and purified cdk1-cyclin B1. Dephosphorylation assays were carried out by incubating pre-phosphorylated glutathione sepharose-bound caspase-2 in buffer (50 mM HEPES (pH 7.7), 1 mM DTT, 0.025% Tween, 0.1 mM EGTA, and 100 mM NaCl) supplemented with recombinant PP1 or PP1 plus I2, and incubated for 30 min at room temperature.

Analysis of post-translational modification of endogenous human caspase-2 by two-dimensional gel electrophoresis was carried out as described previously (Keaton *et al*, 2007). In short, 300 µg of mitotic/interphase cell lysate (in 100 µl volume) was subjected to a TCA-like precipitation using the ReadyPrep 2D cleanup kit (Bio-Rad, Hercules, CA). Purified samples were re-suspended in 200 µl of rehydration buffer (8.5 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 2 mM tributyl phosphine, and 0.2% pH 3–10 carrier ampholyte) and applied to ReadyStrip IPG strips (11 cm; pH 3–10; Bio-Rad) followed by active rehydration at 50 V for 12 h at 20°C. Samples were focused at 250 V for 20 min, gradually ramped up to 8000 V for 2.5 h, and maintained at 8000 V for a total of 35 000 V-h per gel. Focused IPG strips were incubated in 2.5 ml of equilibration buffer (6 M urea, 2% SDS, 0.05 M Tris-HCl, and 20% glycerol) with 2% DTT for 20 min with agitation, followed by a 20-min incubation in equilibration buffer with 2.5% iodoacetamide. Equilibrated strips were rinsed in Tris-glycine SDS running buffer, inserted into the IPG well of a precast 10% Tris-HCl Criterion Gel (Bio-Rad), and covered with low-melting point agarose (0.5%). Gels were run at 200 V for 60 min and prepared for western transfer. For analysis of caspase-2 by mass spectrometry, GST-tagged caspase-2 was incubated for 30 min in mitotic or interphase extract, then subjected to SDS-PAGE gel electrophoresis. Caspase-2 bands were visualized through Coomassie blue staining, excised, and submitted for analysis through tandem mass spectrometry.

Analysis of protein processing

³⁵S-labelled proteins were made using the SP6-coupled reticulocyte lysate system (Madison, WI) and were assayed by SDS-PAGE followed by detection by either exposure to film or phosphorimager (Molecular Dynamics). Caspase-3 processing of caspase-2 was carried out in following buffer: 50 mM HEPES (pH 7.2), 50 mM NaCl, 0.1% CHAPS, 10 mM EDTA, 5% Glycerol, and 10 mM DTT.

Cell culture, siRNA, and drug treatments

Custom caspase-2 oligos targeted to the 3' untranslated region of caspase-2 were ordered from Dharmacon, Lafayette, CO. The sequence for the caspase-2 siRNA used was UGGAAGUAUUUGAGA GAGAUU. Exponentially growing U2OS cells were grown in Dulbecco minimal essential medium (DMEM) supplemented with 10% fetal bovine serum. Nocodazole was diluted in DMEM at a concentration of 40 µg/ml and added to cells for the duration of the experiment. For apoptosis experiments, cells were collected after 24–30 h in nocodazole. To generate mitotic lysates, cells were incubated in nocodazole for 16 h and collected by agitating flasks to dislodge loosely attached mitotic cells. Interphase and mitotic cells were incubated on ice in lysis buffer (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1% CHAPS, 1 mM PMSF, and protease inhibitor cocktail. The inhibitor cocktail is made up of 1 µg/ml aprotinin and leupeptin) for 15 min, followed by dounce homogenization.

Lentivirus vectors

Lentivirus vectors were produced by transient transfection of HEK293T cells using plasmids from the Plenti7.3/V5-DEST Gateway vector kit (Invitrogen, Carlsbad, CA), which generates lentivirus expressing the gene of interest and GFP. Virus supernatants were collected at 24, 48, and 72 h post-transfection. Collected supernatants were cleared by centrifugation (828 g), then concentrated by ultracentrifugation at 115 889 g for 1.5 h at 4°C. Concentrated virus was incubated overnight at 4°C. Viral stocks were stored at -80°C. Viral titres were determined by infecting U2OS cells and determining the percentage of GFP-positive cells by flow cytometry. Vector titers were determined using the equation $[(F \times C_0)/V] \times D$, where F is the frequency of GFP-positive cells, C_0 is the total number of cells at the time of infection, V is the volume of inoculum, and D is the virus dilution factor. For all experiments, infections were carried out at a multiplicity of infection of 2.5 with 10 µg/ml polybrene for 3 h. For addback experiments, cells were infected 24 h after siRNA transfection.

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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