Rsk-mediated phosphorylation and 14-3-3 ϵ binding of Apaf-1 suppresses cytochrome c-induced apoptosis

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Many pro-apoptotic signals trigger mitochondrial cytochrome *c* release, leading to caspase activation and ultimate cellular breakdown. Cell survival pathways, including the mitogen-activated protein kinase (MAPK) cascade, promote cell viability by impeding mitochondrial cytochrome *c* release and by inhibiting subsequent caspase activation. Here, we describe a mechanism for the inhibition of cytochrome *c*-induced caspase activation by MAPK signalling, identifying a novel mode of apoptotic regulation exerted through Apaf-1 phosphorylation by the 90-kDa ribosomal S6 kinase (Rsk). Recruitment of 14-3-3 ϵ to phosphorylated Ser268 impedes the ability of cytochrome *c* to nucleate apoptosome formation and activate downstream caspases. High endogenous levels of Rsk in PC3 prostate cancer cells or Rsk activation in other cell types promoted 14-3-3 ϵ binding to Apaf-1 and rendered the cells insensitive to cytochrome *c*, suggesting a potential role for Rsk signalling in apoptotic resistance of prostate cancers and other cancers with elevated Rsk activity. Collectively, these results identify a novel locus of apoptosomal regulation wherein MAPK signalling promotes Rsk-catalysed Apaf-1 phosphorylation and consequent binding of 14-3-3 ϵ , resulting in decreased cellular responsiveness to cytochrome *c*.

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Introduction

Apoptosis is characterized by the death and elimination of individual cells within an organism while preserving the overall structure of the surrounding tissue (Zimmermann *et al*, 2001; Danial and Korsmeyer, 2004); deregulation

of apoptosis is associated with degenerative disorders (excessive apoptosis) and neoplasia (too little apoptosis) (Thompson, 1995). Under apoptotic conditions, cells activate a family of cysteine proteases known as caspases, which cleave a host of proteins to facilitate cellular breakdown. Apoptosis can be initiated by two main pathways, extrinsic or intrinsic (Danial and Korsmeyer, 2004); prodeath stimuli triggering the intrinsic pathway converge on the mitochondria to induce release of cytochrome *c* to the cytoplasm. After translocation to the cytosol, cytochrome *c* is incorporated into a cell death complex known as the apoptosome, which serves as a platform for activation of the initiator caspase, caspase-9 (Liu *et al*, 1996; Kluck *et al*, 1997; Ow *et al*, 2008). To nucleate apoptosome formation, cytosolic cytochrome *c* binds the adaptor protein Apaf-1, inducing a conformational change and dATP hydrolysis on Apaf-1. Subsequent nucleotide exchange allows Apaf-1 to oligomerize into a large heptameric structure, which can then recruit and bind the zymogenic form of caspase-9 (Kim *et al*, 2005, 2008). This interaction, mediated by binding between caspase recruitment domains (CARDs) on caspase-9 and Apaf-1, promotes dimerization of caspase-9, leading to its activation by induced proximity (Pop *et al*, 2006; Riedl and Salvesen, 2007). The downstream effector caspases-3 and -7 are cleaved by active caspase-9, leading to their activation and the cleavage of a host of cellular substrates (Li *et al*, 1997; Inoue *et al*, 2009), which ultimately results in the dismantling of the dying cell.

Cytochrome *c*-induced caspase activation is regulated by post-translational modification of apoptosome-associated proteins, and these can be both inhibitory and activating (Schafer and Kornbluth, 2006; Kurokawa and Kornbluth, 2009). The most intensively studied post-translational modifications occur on caspase-9 where phosphorylation is known to reduce its activation and/or cleavage. Phosphorylation of T125 on caspase-9, which was first identified as an Erk-catalysed modification, decreases caspase-9 activation and processing (Allan *et al*, 2003). More recently, the cyclin-dependent kinase cdk1, DYRK1A and p38 α have been shown to phosphorylate caspase-9 at this site, reducing its activity and the downstream activation of caspase-3 (Allan and Clarke, 2007; Laguna *et al*, 2008; Seifert *et al*, 2008; Seifert and Clarke, 2009). Phosphorylation of cytochrome *c*, another core apoptosome component, has been reported to occur at Y48 and Y97, though the consequences of these modifications remain unclear (Lee *et al*, 2006; Yu *et al*, 2008). It has been reported that active protein kinase A (PKA) can inhibit caspase-9 activation and that PKA can directly phosphorylate Apaf-1 at least *in vitro* (Martin *et al*, 2005). Nevertheless, the physiological relevance of this phosphorylation has yet to be elucidated.

The mitogen-activated protein kinase (MAPK) pathway promotes cell survival in response to various stimuli, including growth factors, serum and phorbol esters (Anjum and

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Blenis, 2008). Activation of the Raf-MEK pathway leads to activation of Erk1/2, which, in turn, results in direct phosphorylation and activation of the effector kinase, the p90 kDa ribosomal S6 kinase (Rsk). Rsk was first identified as a kinase that phosphorylated rpS6 in unfertilized *Xenopus laevis* eggs, and the MAPK/Rsk pathway is highly active during *Xenopus* oocyte maturation and mitosis (Erikson and Maller, 1985, 1989). Two Rsk isoforms (Rsk1 and Rsk2) have been characterized in *Xenopus* eggs, while there are four known human isoforms (Rsk1–4).

Both Erk and Rsk have direct substrates that are involved in the regulation of cell survival; Rsk1 can phosphorylate the pro-apoptotic Bcl-2 family member, Bcl-xL/Bcl-2-associated death promoter (Bad) at S112, inhibiting Bad-mediated apoptosis. Both Rsk1 and Rsk2 have been shown to suppress the pro-apoptotic activity of DAPK through phosphorylation at S289 (Shimamura *et al*, 2000; Anjum *et al*, 2005). More recently, it was demonstrated that Rsk1 and Rsk2 promote proteasomal degradation of the pro-apoptotic BH3 protein, Bim, in cooperation with Erk 1/2 (Dehan *et al*, 2009).

In the current study, we report a novel mode of apoptosome regulation, wherein Rsk kinase can directly phosphorylate Apaf-1 at two sites. Binding of the small adaptor protein 14-3-3 ϵ at one of the sites (phospho-S268) impeded the interaction between cytochrome *c* and Apaf-1, the initial step of apoptosome formation, thereby inhibiting Apaf-1 oligomerization and caspase activation. Accordingly, mutation of S268 to Ala diminished the interaction between 14-3-3 ϵ and Apaf-1, restoring the ability of Apaf-1 to interact with cytochrome *c* even in the presence of high Rsk activity. Moreover, knockdown of 14-3-3 ϵ using RNAi overrode the inhibition of cytochrome *c*-mediated caspase activation induced by Rsk. Collectively, these data suggest a novel mechanism of apoptosome inhibition mediated by Rsk and 14-3-3 ϵ , reducing cytochrome *c*-mediated apoptosome activation and apoptosis.

Results

Defective apoptosome formation in *Xenopus* mitotic extracts is mediated by Rsk phosphorylation of Apaf-1

Given the conserved nature of the apoptotic pathway and the ease of obtaining cytosolic extract without mitochondrial disruption, the *Xenopus* egg extract system has served as a powerful tool for probing underlying mechanisms of apoptotic regulation (Kluck *et al*, 1997). By utilizing *Xenopus* egg extracts, our laboratory has previously shown that extracts arrested at the metaphase of second meiosis (also known as cytostatic factor-arrested extracts) are refractory to cytochrome *c*, compared with interphase extracts. This cytochrome *c* resistance was traced to high activity of MAPK signalling pathways as cytochrome *c* sensitivity was restored by depletion of MEK or by treatment of extracts with the MEK inhibitor U0126 (Tashker *et al*, 2002). We noted that the inhibition of cytochrome *c*-induced caspase activation was also observed in mitotic egg extracts generated by addition of non-degradable cyclin B to interphase extracts; when exogenous cytochrome *c* was added, caspase activation was significantly lower in mitotic extracts than in interphase extracts prepared from the same eggs (Figure 1A). Moreover, both the cdk inhibitor, roscovitine (ROS) and the MEK inhibitor, U0126 relieved the mitotic inhibition of cas-

pase activation (Figure 1A), suggesting that a kinase downstream of Cdc2 and MEK1/2 might be involved in the observed resistance to cytochrome *c*-induced caspase activation in mitotic extracts. Seeking a mechanistic explanation for these observations, we analysed specific features of apoptosome formation in interphase and mitotic extracts. To examine the critical interaction between Apaf-1 and cytochrome *c*, we incubated agarose-bound cytochrome *c* (hereafter, referred to as cytochrome *c* beads) in either interphase or mitotic extract and found that significantly less Apaf-1 was affinity precipitated from the mitotic extract than from interphase extract (Figure 1B); interphase and mitotic extracts contain comparable amounts of Apaf-1 (Figure 1C).

If the interaction between cytochrome *c* and Apaf-1 is impaired in mitotic egg extract, we would also expect to observe reduced apoptosome formation. To evaluate this, we examined Apaf-1 oligomerization by performing gel filtration chromatography on cytosolic extract. In the absence of cytochrome *c*, both interphase and mitotic extracts contained Apaf-1 in lower molecular-weight fractions where it exists as a monomer (Figure 1D). However, upon addition of cytochrome *c* to the interphase extract, a proportion of the Apaf-1 shifted into higher molecular-weight fractions (fractions 21–24). This recruitment of Apaf-1 into the apoptosomal fractions was not detected in the mitotic extract, implying that Apaf-1 fails to efficiently oligomerize during mitosis even in the presence of cytochrome *c* (Figure 1D). Importantly, when ³⁵S-labelled *in-vitro* translated procaspase-9 T125A (refractory to Erk phosphorylation) was added to each fraction following gel filtration, cleavage products of *in-vitro* translated procaspase-9 were only detectable in the interphase extract supplemented with cytochrome *c*, indicating the formation of functional Apaf-1 oligomers (Supplementary Figure S1).

Since both ROS and U0126 restored the sensitivity of mitotic *Xenopus* egg extract to cytochrome *c* (Figure 1A), we wished to determine whether these two kinase inhibitors could restore the interaction between Apaf-1 and cytochrome *c* in mitotic extracts. As shown in Figure 1E, in comparison with mitotic extract alone, treatment with either ROS or U0126 enhanced the amount of Apaf-1 affinity precipitated by cytochrome *c* beads. It thus seemed likely that kinase activity downstream of Cdc2 and MEK1/2 was required to inhibit apoptosome formation in mitotic extract, leading us to examine whether the phosphorylation of Apaf-1 might be involved. His-tagged Apaf-1 recombinant protein incubated in egg extracts with γ -³²P-ATP was well phosphorylated in mitotic, but not in interphase extract (Figure 1F). We next investigated whether Apaf-1 could be directly phosphorylated by Erk and/or Rsk since they are common kinases downstream of Cdc2 and MEK1/2 in *Xenopus* mitotic signalling (Yue and Ferrell, 2004). As shown in Figure 1G, Rsk, but not its activator, Erk, was able to directly phosphorylate Apaf-1, suggesting that Rsk phosphorylation of Apaf-1 might be responsible for the decreased apoptosome formation in mitotic extract. In support of this hypothesis, treating mitotic extract with an Rsk inhibitor (SL0101 or BI-D1870) restored sensitivity of the extract to cytochrome *c* (Figure 1H). Furthermore, immunodepleting Rsk from extracts enhanced recruitment of endogenous Apaf-1 to cytochrome *c* beads (Supplementary Figure S2). Since Rsk is a member of AGC kinase family and many AGC kinases have redundant functions, we wished to determine if other AGC kinase(s) could

Figure 1 Rsk phosphorylation of Apaf-1 delays apoptosome formation in mitotic *Xenopus* egg extract. **(A)** Cytosolic interphase (S) or mitotic (M) extract was made from *Xenopus* eggs, and the inhibitors roscovitine (ROS) and U0126 were used to treat cytosolic extract at 1.2 mM and 200 μ M, respectively. Purified exogenous cytochrome *c* was added to the extracts at 2 ng/ μ l and incubated for 60 min. The colorimetric substrate Ac-DEVD-pNA was used to quantitate caspase activity. Data shown are the mean $\pm$ s.e.m. of four independent experiments. **(B)** Agarose-bound cytochrome *c* (cytochrome *c* beads) was incubated in S or M extract for 20 min. Bead-bound proteins were resolved by SDS-PAGE and immunoblotted with anti-Apaf-1 and anti-cytochrome *c* antibodies. **(C)** The indicated volumes of S or M *Xenopus* egg extract were subjected to SDS-PAGE and immunoblotted with anti-Apaf-1 or anti-actin antibodies. **(D)** Cytosolic S or M extract was incubated with cytochrome *c* (0 or 2 ng/ μ l) and incubated for 45 min at 25°C. Gel filtration chromatography was then performed, and one tenth of each fraction was resolved by SDS-PAGE and immunoblotted for Apaf-1. **(E)** Cytochrome *c* beads were used to affinity precipitate Apaf-1 from S or M extract pretreated with DMSO, 1.2 mM ROS or 200 μ M U0126. Bead-bound proteins were resolved by SDS-PAGE, and immunoblotted with anti-Apaf-1 and anti-cytochrome *c* antibodies (top). Extracts were analysed by immunoblotting for Rsk2, phospho-Erk (pErk), and actin (bottom). **(F)** His-Apaf-1 linked to nickel agarose (His-Apaf-1) was incubated in S or M extract with γ - 32 P-ATP. Beads were then washed and resolved by SDS-PAGE. Incorporation of 32 P was assessed by autoradiography. Note that all lanes come from the same gel. **(G)** His-Apaf-1 was subjected to an *in-vitro* kinase assay with active Erk, Rsk2 or heat-inactivated Rsk2 (Rsk-HI). Proteins were incubated with γ - 32 P-ATP, and the incorporation of 32 P was detected by autoradiography. **(H)** Cytochrome *c*-induced caspase activation was analysed as in **(A)** using cytosolic S or M extract pretreated with DMSO, 200 μ M U0126, 4 mM SL0101 or 100 μ M BI-D1870. Data shown are the mean $\pm$ s.e.m. of three independent experiments. Figure source data can be found in Supplementary data.

also be involved in inhibiting the Apaf-1 and cytochrome *c* interaction. As shown in Supplementary Figure S3, we used a panel of AGC kinase inhibitors to assess binding of Apaf-1 to cytochrome *c* beads. Most of the inhibitors (except for the PKC inhibitor Ro-31-8220, which has been shown to also function as an Rsk inhibitor) could not restore the binding of

Apaf-1 to cytochrome *c* (Alessi, 1997). This finding implies that within the AGC kinase family, the Rsk kinase is most likely to be the relevant modulator of Apaf-1-cytochrome *c* binding.

It has been reported that T125 on caspase-9 is modified by multiple kinases including Erk; phosphorylation at this site

leads to inhibition of caspase-9 activity and/or processing. To examine whether Rsk can also phosphorylate caspase-9, GST-tagged recombinant wild type or T125 mutant (T125A) caspase-9 prodomain was incubated *in vitro* with Rsk1, Rsk2 or Erk in the presence of γ -³²P-ATP. As shown in Supplementary Figure S4, Rsk was unable to phosphorylate the caspase-9 prodomain *in vitro*, suggesting that Rsk-mediated inhibition of apoptosome formation in *Xenopus* mitotic extracts likely results from Rsk phosphorylation of Apaf-1, rather than that of caspase-9.

Rsk phosphorylates Apaf-1 and inhibits its binding to cytochrome *c* in mammalian cells

Rsk kinase is activated in a subset of cancers, either through activation of the MAPK pathway or through Rsk gene amplification (Berns *et al*, 2004; Clark *et al*, 2005; Smith *et al*, 2005; Thakur *et al*, 2007; Kang *et al*, 2010). Thus, we next examined whether Rsk phosphorylation of Apaf-1 might contribute to the inhibition of cytochrome *c*-induced caspase activation in mammalian cells under circumstances where Rsk kinase activity is upregulated. After overexpressing constitutively active myristoylated-Rsk1 (myr-Rsk1) or vector alone, 293T cells were serum starved to minimize the activation of the endogenous MAPK pathway. Cytosolic lysates from these cells were then utilized for a kinase assay with recombinant His-Apaf-1 protein as a substrate. As shown in Figure 2A, recombinant Apaf-1 was well phosphorylated in the myr-Rsk1 expressing lysates. This constitutive Rsk activity also decreased the sensitivity of the lysates to cytochrome *c*, consistent with our data in *Xenopus* egg extracts (Figure 2B).

Phorbol 12-myristate 13-acetate (PMA) is known to activate the MAPK pathway. When recombinant His-tagged Apaf-1 protein was incubated in lysates from PMA-treated 293T cells, Apaf-1 phosphorylation was markedly increased, as measured by γ -³²P-ATP incorporation (Figure 2C). More importantly, the Rsk inhibitor SL0101 reduced the PMA-induced phosphorylation of Apaf-1, suggesting that this phosphorylation is at least in part dependent on Rsk activation (Figure 2C). Caspase activity of cytosolic lysates from these cells demonstrated that PMA treatment also reduced the responsiveness of the lysates to cytochrome *c* (Figure 2D). In agreement with this finding, cytochrome *c* beads precipitated less Apaf-1 from the PMA-treated lysates (Figure 2E).

To further confirm that Rsk kinase activity was required for the PMA-induced inhibition of the apoptosome, RNAi knockdown of Rsk1 and 2, two prominent isoforms of the kinase in mammalian cells, was performed. As shown in Figure 2F, silencing Rsk1 and 2 ablated phosphorylation of recombinant His-Apaf-1 protein added to cell lysates. More importantly, Rsk knockdown restored responsiveness to cytochrome *c* in lysates from the PMA-treated cells (Figure 2G; compare with Figure 2D where PMA-treated cells were less sensitive to cytochrome *c* than controls, while knockdown of Rsk rendered them *more* sensitive than controls). These data were consistent with an observed increased interaction between Apaf-1 and cytochrome *c* under these conditions (Figure 2H). The MAPK-Rsk pathway is elevated in many cancers as a result of constitutively active Ras. Thus, we examined whether Rsk is responsible for reduced caspase activation in cells stably expressing oncogenic Ras12V. As shown in Supplementary Figure S5, RNAi knockdown of Rsk1 and 2 in Ras12V cells substantially increased responsiveness of

cytosolic lysates to cytochrome *c*. Taken together, our results suggest that in mammalian cells, as we observed in *Xenopus* egg extracts, Rsk can phosphorylate Apaf-1 and inhibit its ability to bind to cytochrome *c*.

Apaf-1 is phosphorylated in PC3 prostate cancer cells

It has been reported that Rsk protein levels are elevated in some prostate cancers, where it can play a role in proliferation and prostate cancer progression. Although Apaf-1 and caspase-9 levels were the same in 293T cells and the prostate cancer cell line PC3 (ABP, JK and SK, unpublished observations), recombinant His-Apaf-1 protein was phosphorylated more robustly upon incubation in PC3 cell lysates than in lysates from 293T cells (Figure 3A). Interestingly, the phosphorylation levels of Apaf-1 in lysates from PMA-treated PC3 cells remained unchanged even following serum starvation. This result is consistent with the previous reports that in PC3 cells, Rsk is highly active regardless of Erk activation (Clark *et al*, 2005). Importantly, compared with lysates prepared from 293T cells, lysates from PC3 cells exhibited marked resistance to caspase activation upon addition of exogenous cytochrome *c* (Figure 3B). Treatment of PC3 cells with the Rsk inhibitor, SL0101, was able to enhance the sensitivity of the resulting cell lysates to cytochrome *c*, allowing for caspase activation (Figure 3C). A similar (but even more significant) result was observed following suppression of both Rsk1 and 2 in PC3 cells using RNAi; Apaf-1 phosphorylation was reduced following Rsk knockdown, and caspase activation in response to cytochrome *c* was restored (Figure 3D and E). Since the Rsk inhibitor SL0101 has been proposed as a potential chemotherapeutic (Clark *et al*, 2005; Smith *et al*, 2005), we also wished to determine whether pharmacological Rsk inhibition might induce basal cell death levels and/or boost the efficacy of chemotherapeutic agents (here, docetaxel) in PC3 cells. To this end, we treated PC3 cells with SL0101 alone or in combination with docetaxel (DTX). While there was a moderate increase in apoptotic cell death induced by SL0101 alone, we observed a significant enhancement of cell death when cells were co-treated with docetaxel and SL0101 (Figure 3F). Importantly, Apaf-1 knockdown using RNAi markedly reduced the synergy of DTX and SL0101 in inducing cell death, suggesting that the suppression of cell death by Rsk was exerted at least in part through Apaf-1 phosphorylation in PC3 cells (Figure 3F and G). Collectively, these data suggest that Rsk is responsible for both phosphorylation of Apaf-1 and resistance to cytochrome *c*-induced caspase activation, conferring a significant degree of protection from death stimuli in PC3 prostate cancer cells.

Rsk phosphorylates Apaf-1 at S268

Rsk recognizes a consensus motif defined as RXRXXS*/T* (phosphorylation sites are indicated with asterisks). Analyses of Rsk-consensus motifs on Apaf-1 using protein sequence annotation databases (GPS 2.1: gps.biocuckoo.org, ELM: elm.eu.org and Scansite: scansite.mit.edu) identified two target residues of particular interest, S268 and S357 (Figure 4A). To determine whether either of these sites was phosphorylated by Rsk, we mutated each of these two serines to alanine in full-length Apaf-1 and examined phosphorylation of the mutant recombinant proteins, Apaf-1 (S268A) and Apaf-1 (S357A). Lysates from 293T cells overexpressing myr-Rsk1 and the constitutively active Rsk2 mutant (K707A: Rsk2

Figure 2 Rsk phosphorylates Apaf-1 in mammalian cells and reduces sensitivity to cytochrome *c*. (**A**, **B**) 293T cells were transfected with expression plasmids encoding constitutively active myristoylated-Rsk1 (myr-Rsk) or empty vector as control. Cells were incubated in complete media for 24 h and then serum starved for additional 24 h. Cytosolic lysates were then prepared. (**A**) His-Apaf-1 was incubated with γ - 32 P-ATP in cytosolic lysates. Samples were resolved by SDS-PAGE, and incorporation of 32 P was detected by autoradiography (top). Lysates were analysed by immunoblotting for total Rsk1, phospho-S6 (pS6) and actin (bottom). (**B**) Exogenous cytochrome *c* (10 ng/ μ l) and dATP (1 mM) were added to cytosolic lysates. Samples were incubated for 30 min, and caspase activity was assayed as previously described; the experiment was repeated at least three times and shown is a representative assay profile. (**C**–**E**) 293T cells were left untreated or treated with PMA (25 ng/ml) and/or SL0101 (100 μ M) for 30 min at 37°C as indicated. Cytosolic lysates were prepared following 24 h of serum starvation. (**C**) His-Apaf-1 was incubated with γ - 32 P-ATP in cytosolic lysates, and the incorporation of 32 P was assessed by autoradiography. (**D**) Cytochrome *c*-induced caspase activation was measured following incubation of cytosolic lysates with cytochrome *c* (10 ng/ μ l) and dATP (1 mM); the experiment was repeated at least three times and shown is a representative assay profile. (**E**) Cytochrome *c* beads were incubated in cytosolic lysates. Bead-bound proteins were resolved by SDS-PAGE and immunoblotted with anti-Apaf-1 and cytochrome *c* antibodies. (**F**–**H**) 293T cells were transfected with control siRNA or Rsk1 and 2 targeted siRNA. Forty-eight hours post transfection, cells were serum starved for 24 h prior to PMA treatment. Cytosolic lysates were then prepared. (**F**) His-Apaf-1 was incubated with γ - 32 P-ATP in lysates to examine phosphorylation of Apaf-1 by autoradiography (top). Lysates were analysed by immunoblotting for Rsk1, Rsk2 and actin (bottom). (**G**) Caspase activity was measured as described in (**B**) for control and Rsk1 and 2 knockdown (Rsk KD) cells; data shown are representative profiles ($n=3$). (**H**) Cytosolic lysates were incubated with cytochrome *c* beads, and the amount of bound Apaf-1 was examined by immunoblotting. Figure source data can be found in Supplementary data.

CA) showed that Apaf-1 phosphorylation was decreased by mutating either residue (Figure 4B). To further evaluate whether these residues might be direct targets of Rsk, we examined Apaf-1 phosphorylation *in vitro*. HA-tagged wild-type Rsk (Rsk1-WT), but not kinase inactive mutant (Rsk1 KD), immunoprecipitated from PMA-treated, Rsk-overexpres-

sing 293T cells effectively phosphorylated wild-type Apaf-1. Phosphorylation was compromised by mutation of S268 or S357 (Supplementary Figure S6), suggesting that both S268 and S357 can be directly phosphorylated by Rsk. Since Rsk phosphorylation of Apaf-1 attenuates Apaf-1 binding to cytochrome *c* and subsequent caspase activation, abrogation

Figure 3 Reduced caspase activation in prostate cancer cells is associated with strong phosphorylation of Apaf-1 by Rsk. (A) 293T and PC3 cells were serum starved for 24 h, and PC3 cells were either treated, or left untreated, with PMA. 293T and PC3 cytosolic lysates were prepared, and His-Apaf-1 was added with γ -³²P-ATP to examine the phosphorylation status of Apaf-1. Incorporation of ³²P was assessed by autoradiography. (B) Exogenous cytochrome *c* (10 ng/ μ l) and dATP (1 mM) were added to the cytosolic lysates prepared in (A) for 20 min, and caspase activity was assayed as previously described; the experiment was repeated at least three times and shown is a representative assay profile. (C) PC3 cells were incubated with DMSO or SL0101 (100 μ M) for 3 h. Cytosolic lysates were prepared, and cytochrome *c*-induced caspase activation was analysed; the experiment was repeated at least three times and shown is a representative assay profile. (D) Rsk1 and 2 were silenced by a consecutive RNAi transfection in PC3 cells. PC3 cells were replated 24 h after the first transfection for the second transfection. Forty-eight hours after the second transfection, cells were harvested, and cytosolic lysates were prepared. His-Apaf-1 was incubated in these lysates with γ -³²P-ATP to examine the phosphorylation of Apaf-1 by autoradiography (left). Immunoblotting was performed for Rsk1, Rsk2 and actin (right). (E) Cytosolic lysates from PC3 cells, transfected as in (D), were incubated with exogenous cytochrome *c* to examine caspase activity; the experiment was repeated at least three times and shown is a representative assay profile. (F) Apaf-1 expression in PC3 cells was silenced by siRNA transfection. Twenty-four hours post transfection, cells were treated with 50 μ M SL0101 or DMSO for 24 h then treated with 100 nM docetaxel (DTX) or DMSO for an additional 24 h. Apoptotic cell death was assayed by Annexin V staining and FACS analysis. Results shown are mean $\pm$ s.e.m. of four independent experiments (**P* < 0.05; NS, not significant). (G) Knockdown of Apaf-1 was confirmed by immunoblotting for Apaf-1 and actin as control. Figure source data can be found in Supplementary data.

of phosphorylation should restore Apaf-1's responsiveness to cytochrome *c*, even upon Rsk activation. To address this question, we co-transfected 293T cells with myc-tagged wild-type Apaf-1(WT), Apaf-1(S268A) or Apaf-1(S357A) together with either vector or myr-Rsk1. We prepared cytosolic lysates from those cells and analysed binding between myc-Apaf-1 and cytochrome *c* beads. As shown in Figure 4C, expression of myr-Rsk1 resulted in a notable decrease in the binding between wild-type myc-Apaf-1 and cytochrome *c*. Similarly to wild-type myc-Apaf-1, Rsk diminished binding between myc-Apaf-1(S357A) and cytochrome *c* (albeit not completely) but importantly, myr-Rsk1 was unable to impede binding between myc-Apaf-1 (S268A) and cytochrome *c* (Figure 4C). In addition, S268A mutant Apaf-1, but not S357A Apaf-1 fully restored the responsiveness to cytochrome *c* in the presence of myr-Rsk (Figure 4D), suggesting

that S268, but not S357, may be the functionally relevant Rsk phosphorylation site. If disrupting phosphorylation by Rsk sensitized Apaf-1 to cytochrome *c*, we would expect that mimicking Rsk phosphorylation could impair the interaction between Apaf-1 and cytochrome *c*. Indeed, a substantially lower amount of Apaf-1(S268D) was affinity precipitated by cytochrome *c* beads than by wild-type Apaf-1 when expressed in 293T cells (Figure 4E); Apaf-1(S357D) showed an intermediate effect, consistent with the relative importance of these sites suggested by analysis of the non-phosphorylatable mutants (Figure 4C).

14-3-3 is required for Rsk-mediated inhibition on Apaf-1

Even though Rsk appeared to block the binding between Apaf-1 and cytochrome *c* by phosphorylating Apaf-1 at S268, we were unable to reconstitute this event *in vitro*; recombi-

Figure 4 Rsk phosphorylates Apaf-1 at S268 and S357, but S268 is functionally important. (A) Rsk phosphorylation sites in Apaf-1 are shown. Evolutionarily conserved Rsk-consensus motifs are boxed, and target residues are indicated in red. (B) Apaf-1 was mutated at S268 or S357, and the corresponding His-Apaf-1 proteins, as well as wild-type Apaf-1, were subjected to an *in-vitro* kinase assay in the absence or presence of active Rsk1. Proteins were incubated with γ - 32 P-ATP in cytosolic lysates from 293T cells transfected with plasmids encoding active Rsk1 and 2 or empty vector control. Incorporation of 32 P was assessed by autoradiography. (C) 293T cells were transfected with wild-type or mutant Apaf-1 in the presence or absence of myr-Rsk1. Twenty-four hours post transfection, cells were serum starved for an additional 24 h, and cytosolic lysates were made. Cytochrome *c* beads were added, and the amount of bound, transfected Apaf-1 was examined by immunoblotting (cyt.*c* PD). Lysates were analysed by immunoblotting for the indicated proteins (input). (D) Cytosolic lysates from 293T cells transfected as in (C) were incubated with exogenous cytochrome *c* (2 ng/ μ l) and dATP (1 mM) for 15 min. Caspase activity was assayed as previously described; the data shown are representative profiles ($n = 6$). (E) 293T cells were transfected with wild-type or mutant forms (S268D and S357D) of Apaf-1. Twenty-four hours post transfection, cells were serum starved for an additional 24 h, and cytosolic lysates were made. Lysates were then incubated with cytochrome *c* beads and the amount of Apaf-1 precipitated with cytochrome *c* was examined by immunoblotting (cyt.*c* PD). Lysates were immunoblotted for the indicated proteins (input). Figure source data can be found in Supplementary data.

nant Rsk kinase alone could not diminish the binding of recombinant Apaf-1 to cytochrome *c* despite phosphorylation at S268 (ABP, JK and SK, unpublished observation). These observations raised the possibility that an additional step/factor(s) dependent on S268 phosphorylation might be involved in Rsk-mediated inhibition of Apaf-1. Based on the protein sequence analysis, we noted that phosphorylated S268 and the flanking amino acids formed a potential docking site for the adaptor protein, 14-3-3 (Figure 5A). We determined that His-tagged recombinant Apaf-1 interacted with endogenous 14-3-3 when incubated in lysates from 293T cells (Figure 5B). Importantly, mutation of S268, but not S357, to

Ala abolished binding (Figure 5B). Conversely, recombinant GST-14-3-3 interacted with endogenous Apaf-1 when incubated in lysates prepared from 293T cells (Figure 5C). Interestingly, we found that the ϵ isoform of 14-3-3 interacted with Apaf-1 much more strongly than the ζ isoform in both 293T lysate and *Xenopus* egg extract (Figure 5C; Supplementary Figure S7).

Our ability to detect Apaf-1/14-3-3 binding in 293T cell lysates suggested that low levels of Rsk activity (or another kinase capable of phosphorylating S268) allowed for some basal level of interaction between Apaf-1 and 14-3-3. However, we hypothesized that increasing S268 phosphory-

Figure 5 Apaf-1 binds 14-3-3 ϵ at S268. (A) Putative 14-3-3 binding motifs in Apaf-1 are shown; [RHK][STALV]p[ST][PESRDIF] 14-3-3 binding motifs based on the reported natural interacting proteins (data obtained from databases: ELM: elm.eu.org and Scansite: scansite.mit.edu). 14-3-3 consensus motifs are boxed, and target residues are indicated in red. (B) His-Apaf-1, either wild-type (WT) or mutant (S268A, S357A) protein was incubated in 293T whole cell lysate, and bead-bound proteins were resolved by SDS-PAGE and immunoblotted with anti-pan-14-3-3 and anti-His antibodies. (C) Glutathione sepharose-bound GST, GST-14-3-3 ϵ or GST-14-3-3 ζ was incubated with 293T whole cell lysates. Beads were recovered, resolved by SDS-PAGE and immunoblotted for Apaf-1 and GST. (D) 293T cells were transfected with control siRNA or Rsk1 and 2 targeted siRNAs. Forty-eight hours after transfection, cells were serum starved for an additional 24 h prior to PMA treatment. Whole cell lysates were made and incubated with His-Apaf-1. Samples were resolved by SDS-PAGE and immunoblotted for 14-3-3 and Apaf-1 (His-PD). Lysates were analysed by immunoblotting for the indicated proteins (input). (E) 293T cells were transfected with plasmids encoding HA-14-3-3 ϵ or HA-14-3-3 ζ . Twenty-four hours after transfection, the cells were serum starved for 24 h. Cells were then treated with PMA or left untreated (Ctrl) and subjected to immunoprecipitation (IP) with an anti-HA antibody. Immune complexes were analysed by immunoblotting with anti-Apaf-1 and anti-HA antibodies (HA-IP). Cell lysates were immunoblotted for the indicated proteins (input). (F) COS-7 cells were co-transfected with plasmids encoding active Rsk1 and 2 or empty vector. After 24 h in complete media, cells were serum starved for 24 h, and whole cell lysates were prepared. His-Apaf-1 protein (WT, S268A and S357A) was incubated in lysates. Samples were analysed by SDS-PAGE and immunoblotted for His and 14-3-3 ϵ (His-PD). Lysates were immunoblotted for the indicated proteins (input). (G) Glutathione sepharose-bound GST-14-3-3 ϵ was incubated with whole cell lysates from 293T cells overexpressing wild-type or phosphomimetic mutants of myc-Apaf-1 (S268D and S357D), and sepharose-bound proteins were resolved by SDS-PAGE and immunoblotted for Apaf-1 (GST-PD). Lysates were analysed by immunoblotting for the indicated proteins (input). Figure source data can be found in Supplementary data.

lation in the presence of active Rsk would further enhance this interaction to effectively inhibit apoptosome formation. We thus examined the interaction between 14-3-3 and Apaf-1 in the presence or absence of Rsk activation in 293T cells.

As shown in Figure 5D, PMA treatment resulted in a marked increase in binding between Apaf-1 and 14-3-3. Importantly, this binding was significantly diminished when both Rsk1 and Rsk2 were silenced by RNAi. We further determined

that this increased binding between 14-3-3 and Apaf-1 is specific for the ϵ isoform of 14-3-3 (Figure 5E; Supplementary Figure S8).

It has been reported that Apaf-1 is expressed at extremely low levels in COS-1 and -7 cells (Hausmann *et al*, 2000). To minimize the effects of endogenous Apaf-1 protein, we employed COS cells to analyse the effect of Rsk on the interaction between 14-3-3 ϵ and Apaf-1. Recombinant His-Apaf-1, either wild type or mutant (S268A or 357A), was incubated in lysates from COS-7 cells overexpressing active Rsk1 and 2. As shown in Figure 5F, the interaction between WT Apaf-1 or Apaf-1 S357A and 14-3-3 ϵ was enhanced in the presence of the active Rsk kinases, but this increase was not observed for the Apaf-1 S268A mutant. Although phosphomimetic residues often cannot confer 14-3-3 binding, there are reports of enhanced interactions between 14-3-3 and phosphomimetic residues in some 14-3-3 binding partners (Faul *et al*, 2005; Kim *et al*, 2007; Maheswaranathan *et al*, 2011). Surprisingly, we found that phosphomimetic S268D Apaf-1 mutant interacted with 14-3-3 ϵ more robustly than wild-type Apaf-1 (Figure 5G; in comparison with wild type, S357D mutant showed a modest increase in 14-3-3 ϵ binding). We note, however, that the observed decrease in cytochrome *c* binding to S268D Apaf-1 (relative to wild-type Apaf-1) seen in Figure 4E appeared to be more striking than the increase in 14-3-3 binding. Thus, the decreased interaction of Apaf-1 S268D with cytochrome *c* might have resulted from a direct inhibitory effect of the charged phosphomimetic residue on Apaf-1–cytochrome *c* interactions and/or from inhibition of these interactions by S268D-bound 14-3-3 protein.

If 14-3-3 ϵ is required for Rsk-mediated inhibition of the Apaf-1–cytochrome *c* interaction, we expect that knockdown of 14-3-3 ϵ would alleviate this suppression and restore Apaf-1 sensitivity to cytochrome *c*. Consistent with this idea, the ability of PMA to suppress Apaf-1–cytochrome *c* interactions was diminished by knockdown of 14-3-3 ϵ (Figure 6A). Accordingly, PMA treatment could no longer effectively suppress cytochrome *c*-induced caspase activation following knockdown of 14-3-3 ϵ (Figure 6B). Similarly, overexpression of Rsk1 or Rsk2 enhanced recruitment of 14-3-3 ϵ to Apaf-1 and abolished Apaf-1 binding to cytochrome *c*, which were both reversed by 14-3-3 ϵ knockdown (Figure 6C and D). Finally, we performed a similar experiment using PC3 cells where endogenous Rsk is highly activated. When cytochrome *c* beads were incubated in cytosolic lysates from PC3 cells that had been treated with either control, or 14-3-3 ϵ targeted siRNA, significantly more Apaf-1 was affinity precipitated from the lysates depleted of 14-3-3 ϵ (Figure 6E). These data demonstrate that 14-3-3 ϵ is crucial for Rsk-mediated apoptosome inhibition stemming from a reduced interaction between Apaf-1 and cytochrome *c*.

Discussion

MAPK signalling is connected, either directly or indirectly, to many components of the core cellular machinery that direct cell proliferation and cell death. Here, we have identified a novel mode of apoptosome regulation involving the phosphorylation of Apaf-1 by Rsk, a terminal kinase in MAPK signalling. Rsk-mediated Apaf-1 phosphorylation promoted recruitment of the adaptor protein 14-3-3, suppressing the ability of cytochrome *c* to nucleate apoptosome formation.

Moreover, we have demonstrated that cells with high Rsk kinase activity exhibit Apaf-1 phosphorylation and resistance to cytochrome *c* in a manner dependent on 14-3-3, which is consistent with the ability of Rsk to enhance cell survival through phosphorylation of Apaf-1.

14-3-3 and control of apoptosis

14-3-3 is an adaptor protein that regulates various cellular functions by sequestering its target proteins and/or preventing protein–protein interactions (Nomura *et al*, 2003; Dong *et al*, 2007; Vander Haar *et al*, 2007). Previous studies have demonstrated that various pro-apoptotic proteins acting upstream of mitochondrial cytochrome *c* release can interact with 14-3-3 proteins, which negatively regulates protein function in most cases. For example, in healthy cells, 14-3-3 binding to the pro-apoptotic Bcl-2 family member Bad sequesters it in the cytoplasm, where it cannot antagonize prosurvival proteins such as Bcl-xL/Bcl-2 localized at the mitochondria (Zha *et al*, 1996). Recently, we have reported that the apical caspase-2 is complexed to 14-3-3 ζ in a manner dependent on cellular nutrient status; in this case, 14-3-3 binding prevents dephosphorylation of a suppressive residue within the caspase-2 prodomain that reduces its binding to dimerizing adaptor proteins (Nutt *et al*, 2009).

It has also been reported that Raf, an upstream kinase in the MAPK pathway, is associated with 14-3-3 proteins and that this is critical for Raf kinase activity (Fu *et al*, 1994; Tzivion *et al*, 1998). Indeed, careful examination of the data in Figure 5E (phospho-S6 levels) suggests that overexpression of 14-3-3 may have increased Rsk kinase activity; RNAi-mediated knockdown of 14-3-3 ϵ also reduced PMA-induced Rsk activation (phospho-S6 levels in Figure 6A). In analysing the Rsk sequence, we have noted several potential 14-3-3 binding sites. Therefore, 14-3-3 ϵ may modulate Rsk activity as well as controlling the Rsk target, Apaf-1.

Recent studies from several laboratories have elucidated specific phenotypic consequences of altering the levels of individual 14-3-3 isoforms, suggesting that individual isoforms can bind specific targets and play specialized roles in particular signalling pathways. For example, 14-3-3 τ is specifically induced in cancer cells grown on tenascin-C, and overexpression of this 14-3-3 isoform can lead to enhanced cell adhesion (Martin *et al*, 2003). Downregulation of the ζ isoform of 14-3-3 has been reported to sensitize cultured lung cancer cells to anoikis, thereby impeding their ability to grow in an anchorage-independent manner (Li *et al*, 2008). Knockdown of 14-3-3 β in renal cortical collecting duct cells diminished multiple aspects of the aldosterone response, even though other 14-3-3 isoforms were present in these cells (Liang *et al*, 2008). Our study provides evidence for a specific function of the 14-3-3 ϵ isoform in inhibiting apoptosis upon activation of the MAPK pathway in that 14-3-3 ϵ , but not the ζ isoform, was recruited to Apaf-1 upon Rsk activation. That said, we have examined only 14-3-3 ϵ and 14-3-3 ζ , the two most abundant isoforms of 14-3-3 in *Xenopus* eggs. Thus, it remains possible that additional 14-3-3 isoforms can also bind to S268 to modulate Apaf-1 function. The basis for the specificity of 14-3-3 ϵ –Apaf-1 interactions requires further investigation. As several different sequence motifs have been implicated in 14-3-3 binding, it may be that small variations in the motifs or combinations of motifs that confer binding of 14-3-3 dimers can result in the observed specificity (Muslin

Figure 6 14-3-3 ϵ is required for Rsk-mediated inhibition of Apaf-1 binding to cytochrome *c*. (A, B) 293T cells were transfected with control siRNA or siRNA targeted against 14-3-3 ϵ . Twenty-four hours post transfection, cells were serum starved for an additional 24 h, and treated (or untreated) with PMA. Cytosolic lysates were prepared as previously described. (A) Cytochrome *c* beads were used to affinity precipitate endogenous Apaf-1 from the indicated lysates. Bead-bound proteins were resolved by SDS-PAGE, and immunoblotted for cytochrome *c* and Apaf-1 (cyt.*c* PD). Lysates were analysed for the indicated proteins (input). (B) Exogenous cytochrome *c* (5 ng/ μ l) and dATP (1 mM) were added to cytosolic lysates for 60 min, and caspase activity was assayed as previously described; the experiment was repeated at least three times and shown is a representative assay profile. (C) 293T cells were transfected with control siRNA or siRNA targeted against 14-3-3 ϵ . Twenty-four hours post transfection, the cells were replated for DNA transfection with plasmids encoding active Rsk1, 2 or empty vector. Forty-eight hours after DNA transfection, the cells were harvested for cytosolic lysate preparation. Cytochrome *c* beads were used to precipitate endogenous Apaf-1 as in (A) (cyt.*c* PD). Immunoblotting was performed on the indicated proteins (input). (D) His-Apaf-1 was incubated in cytosolic lysates as described in (C), and immunoblotting with an anti-14-3-3 ϵ antibody was performed to examine the amount of endogenous 14-3-3 ϵ precipitating with Apaf-1 beads. (E) PC3 cells were transfected with control siRNA or siRNA targeted against 14-3-3 ϵ . Forty-eight hours post transfection, cytosolic lysates were made, and cytochrome *c* beads were used to affinity precipitate Apaf-1 from the lysates. Bead-bound proteins were resolved by SDS-PAGE, and immunoblotting was performed with anti-Apaf-1 and anti-cytochrome *c* antibodies (cyt.*c* PD). Lysates were analysed for the indicated proteins (input). Figure source data can be found in Supplementary data.

et al, 1996; Yaffe *et al*, 1997; Coblitz *et al*, 2005; Gardino *et al*, 2006). It is conceivable that pS268 on Apaf-1, possibly collaborating with pS357, provides a preferential docking site for 14-3-3 ϵ dimers upon Rsk activation. It is also possible that 14-3-3 ϵ can heterodimerize with other isoforms in cells to regulate Apaf-1 function.

In aggregate, our data demonstrate that 14-3-3 can modulate apoptosis downstream of mitochondrial cytochrome *c* release as well as upstream, controlling apoptosome formation through binding to Apaf-1.

The Apaf-1–cytochrome *c* interaction

It has been previously reported that T125 of caspase-9 is a target of Erk kinase, contributing to apoptosome inhibition by the MAPK pathway (Allan *et al*, 2003). Our data show that Apaf-1 is also a target of the MAPK pathway. It may be that modification of both of these targets contributes to full inhibition of cytochrome *c*-induced apoptosis. Interestingly, Rsk-mediated phosphorylation alone does not appear to be sufficient to efficiently prevent Apaf-1–cytochrome *c* interactions; rather, 14-3-3 binding is required.

The specific locus of 14-3-3 apoptosome control appears to be modulation of the Apaf-1–cytochrome *c* interaction.

Although we have not completely ruled out an effect of Rsk on oligomerization of Apaf-1 (due to the locations of the phosphorylation sites and 14-3-3 binding), the numerous experiments using cytochrome *c* beads to probe the interaction between Apaf-1 and cytochrome *c* support our proposed model (Figure 7). We would note that other factors reported to modulate the Apaf-1–cytochrome *c* interaction appear to act at the level of cytochrome *c*. For example, Bruey *et al* revealed that the chaperone, hsp27, interacts with cytochrome *c*, inhibiting its ability to activate the apoptosome (Bruey *et al*, 2000; Schafer and Kornbluth, 2006). Additionally, physiological levels of nucleotides, such as ATP and dATP, can directly bind to cytochrome *c*, inhibiting its interaction with Apaf-1 (Chandra *et al*, 2006). Though physiological concentrations of ions, such as calcium and potassium have been reported to control Apaf-1 function (Cain *et al*, 2001; Bao *et al*, 2007), S268 phosphorylation/14-3-3 ϵ binding is the first post-translational modification of Apaf-1 known to affect the ability of cytochrome *c* to nucleate apoptosome formation.

The precise way in which cytochrome *c* interacts with Apaf-1 to initiate apoptosome formation is currently unclear. Structural analysis of the apoptosome using cryoelectron

Figure 7 Model of Rsk-mediated inhibition of apoptosome formation. Rsk phosphorylates Apaf-1 at S268 and S357. The adaptor protein, 14-3-3 ϵ , can then bind to Apaf-1 at phosphorylated S268, reducing cytochrome *c*-induced apoptosome formation and subsequent caspase activation. It is still unclear exactly how 14-3-3 ϵ blocks Apaf-1 binding to cytochrome *c* although some possibilities are discussed in the text. Apaf-1, cytochrome *c*, Rsk and 14-3-3 ϵ are indicated.

microscopy has suggested that one cytochrome *c* molecule interacts with Apaf-1 between two β propeller structures formed by the 15 WD-40 repeats that comprise the auto-inhibitory C-terminal domain of Apaf-1 (Yuan *et al*, 2010). This analysis is consistent with data showing that Apaf-1 lacking the WD-40 repeats is constitutively active in binding and activating caspase-9 (Srinivasula *et al*, 1998). Since the 14-3-3 ϵ binding site, pS268, is not located within the propeller domains, but rather is within the nucleotide binding domain (NBD) of Apaf-1, the effect of 14-3-3 ϵ binding may not directly block cytochrome *c* binding. Instead, recruitment of 14-3-3 to Apaf-1 may induce a conformational change in monomeric Apaf-1 that renders the relevant cytochrome *c* binding sites on Apaf-1 less accessible.

In addition to S268 phosphorylation, we observed Rsk-mediated phosphorylation of S357 on Apaf-1. We have not observed a clear functional consequence of S357 phosphorylation on downstream caspase activation. Mutation of S357A did have a slight effect on the binding of Apaf-1 to cytochrome *c* and concomitantly slightly decreased binding to 14-3-3 ϵ , but this did not appear to translate into a significant effect on the kinetics of caspase activation (Figures 4C, D and 5F). It is possible that this residue plays some role(s) in optimal binding of Apaf-1 to cytochrome *c*, potentially by influencing binding of 14-3-3 ϵ to S268.

The MAPK pathway and cancer

Rsk is a well-known effector of Ras-MAPK signalling, and this pathway is one of the most important for promoting cell survival in response to extracellular stimuli; oncogenic Ras mutations occur in about one third of all cancers (Bos, 1989). Additional roles for many Ras effectors, including Rsk, have been reported in cancer. For example, Rsk1 and Rsk2 are overexpressed in breast and prostate cancers (Clark *et al*, 2005; Smith *et al*, 2005). Rsk3 has been shown as a possible tumour suppressor in ovarian cancer (Bignone *et al*, 2007).

Rsk4 is abnormally expressed in breast cancer, and it has been implicated in p53-dependent cell growth arrest (Berns *et al*, 2004; Thakur *et al*, 2007).

Our findings demonstrate an additional layer of apoptosis regulation provided by the Ras-MAPK-Rsk pathway. Under conditions with constitutively active Rsk, Apaf-1 is phosphorylated (Figures 2A and 3A), and the cells are less responsive to cytochrome *c*-induced cell death (Figures 2B and 3B). It would be of interest to evaluate more systematically the phosphorylation status of Apaf-1 in tumours with activated Rsk to determine whether this correlates with chemosensitivity. While Apaf-1 phosphorylation is certainly not the only mode of apoptotic suppression in tumours with an active Ras-MAPK pathway, the post-translational modification of this crucial apoptosome component appears to represent another layer of apoptotic resistance, which could potentially be targeted to develop more successful treatments.

Materials and methods

Cell culture and extract preparation

293T, PC3 and COS-7 cells were obtained from the American Type Culture Collection (ATCC). HEK-HT Ras12V cells were a kind gift from Dr C Counter (Duke University Medical Center). Cells were maintained according to the ATCC's specifications. Fugene 6 (Roche) was used for DNA transfection. Cytosolic lysates were made as previously described (Kurokawa *et al*, 2008). Whole cell lysates were prepared by incubating cells in extraction buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM EDTA, 0.5% Triton X-100, 12.5 mM β -glycerophosphate, 20 μ M aprotinin/leupeptin, 1 mM Na₃VO₄ and 1 mM PMSF).

Xenopus egg extract

To induce egg laying, mature female frogs were injected as described previously (Smythe and Newport, 1991). Interphase extracts were made as previously described (Nutt *et al*, 2009). Mitotic extracts were prepared by adding non-degradable cyclin B1 to interphase extracts. Cytosolic interphase and mitotic extracts were made by centrifuging crude extract at 55 000 r.p.m. (250 000 g) for 70 min in the TL-100 ultracentrifuge (Beckman Instruments). The cytosolic fraction was removed and recentrifuged at 55 000 r.p.m. for 30 min.

Kinase assays

For kinase assays in *Xenopus* egg extract and mammalian cell lysates, nickel-agarose bound His-Apaf-1 was incubated with 2 μ Ci γ -³²P-ATP in interphase/mitotic *Xenopus* egg extract or in the indicated cytosolic lysates from mammalian cells. After 1 h at 30°C, beads were removed and washed three times in washing buffer (1 mM Hepes-KOH, pH 7.5, 1 mM β -glycerophosphate, 15 mM KCl, 0.1% NP-40, 14.8 mM imidazole, 500 mM NaCl, 1 mM NaVO₃ and 5 μ g/ml aprotinin/leupeptin).

For the *in-vitro* kinase assay, His-Apaf-1 protein was incubated in kinase buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM ATP, 10 mM MgCl₂, 1 mM DTT, pH 7.2) with 2 μ Ci γ -³²P-ATP and the appropriate kinase. The reaction proceeded for 30 min at 30°C, at which point, the beads were washed with washing buffer. Analysis for all kinase assays was performed using SDS-PAGE and autoradiography.

Caspase assays

Xenopus and mammalian caspase assays were performed as previously described (Deming *et al*, 2004). Briefly, mammalian lysates at 10 mg/ml were incubated at 37°C with cytochrome *c* and 1 mM dATP; samples were added to caspase assay buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA and 10% glycerol) with the colorimetric substrate, Ac-DEVD-pNA (BioMol). All samples were read at 405 nm on a Bio-Rad Plate Reader (Bio-Rad Microplate Reader, Model 680).

Flow cytometry

PC3 cells were transfected with a specific siRNA targeting Apaf-1. Twenty-four hours post transfection, cells were treated for 24 h with 50 μ M SL0101 or DMSO as control and then treated with 100 nM docetaxel (DTX) or DMSO for another 24 h. Cells were then trypsinized and stained with Annexin V, Alexa Fluor 488 conjugate (Invitrogen), according to manufacturer's instructions. Apoptotic cell death was analysed by flow cytometry (FACS Vantage SE). Statistical analysis represents paired *t*-test, and null hypotheses were rejected at 0.05.

siRNA knockdown

siRNA oligonucleotides were introduced into cells using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. Rsk1 and 2 siRNA oligo sequences were previously described (Anjum *et al*, 2005). Apaf-1 siRNA (sense: 5'-AAUUGUGCA CUUUUACGUGA-3'), Rsk1, Rsk2 and control siRNA (sense: 5'-GUCAGUAUGACACACUGU-3') were purchased from Sigma, and 14-3-3 ϵ siRNA (sense: 5'-UGUUCUAUACUGCUGAUUAUUCUCC-3') was designed and synthesized by Invitrogen.

Protein affinity binding assay

To examine the recruitment of Apaf-1 to cytochrome *c*, *Xenopus* extract or mammalian cytosolic lysate was added to 20 μ l of packed agarose-bound cytochrome *c* and incubated at 25 or 37°C, respectively. Beads were then washed three times in extraction buffer containing 500 mM NaCl. To assay endogenous 14-3-3 binding to Apaf-1, nickel agarose-bound His-Apaf-1 was incubated in egg extract or cell lysates for 1 h at 25°C. Beads were then washed three times in PBS. Glutathione sepharose-bound GST-14-3-3 ϵ and ζ were used for reverse binding assays under similar conditions.

Immunoprecipitation

Appropriate antibody was incubated at a ratio of 1 μ g per 500 μ g of lysate for 2 h at 4°C. The antibody was then retrieved with sepharose-bound protein G preblocked in 10% BSA for 1 h at 4°C. The beads were washed in extraction buffer and prepared for immunoblotting.

Gel filtration chromatography

Xenopus cytosolic lysates were incubated in the presence of 2 ng/ μ l cytochrome *c* at 25°C for 45 min. Apaf-1 protein purification buffer described above was then added to a final volume of 300 μ l. The reaction mixture was loaded onto a Superdex 200 column at a flow rate of 0.3 ml/min. Fractions were collected and analysed by immunoblotting.

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Reagents and antibodies

Purified, soluble cytochrome *c* was purchased from Sigma. Active Rsk1 and Rsk2 were obtained from Cell Sciences and Upstate/Millipore, respectively. Roscovitine, cytochalasin B and active Erk were from Calbiochem. Cytochrome *c*-agarose beads, DMSO and PMA were purchased from Sigma. U0126 was from Biosource. Ro-31-8220 and LY294002 were kind gifts from J Rathmell (Duke University). SL0101 was obtained from Toronto Research Chemicals, Inc., and BI-D1870 was from Symansis.

The following antibodies were used: anti-Apaf-1 (AAP-300) from Stressgen; anti-Apaf-1 (ALX-804-348-C100) from Alexis Biochemicals; anti-cytochrome *c* (556433) from PharMingen; anti-pan-14-3-3 (ab14112) from AbCam; anti-Myc (9E10:SC-40), anti-GST (B-14:SC-138), anti-His (H-15:SC-803), anti-HA (F-7:SC-7392), anti-Rsk1 (C-21:SC-231), anti-Rsk2 (C-19:SC-1430), anti-14-3-3 ϵ (T-16:SC-1020) from Santa Cruz; anti-Flag (F-3165) and anti- β -actin (A1978) from Sigma; and anti-phospho-S6 Ribosomal protein (2211) from Cell Signaling.

Supplementary data

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

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Author contributions: JK, ABP, JLA and SAK designed research; JK, ABP and MK performed the experiments; KM performed and analysed flow cytometry; CDF and CEJ helped writing manuscript and preparing figures; JK, ABP and SAK wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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