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RESEARCH ARTICLE

Identification of a novel Bax–Cdk1 signalling complex that links activation of the mitotic checkpoint to apoptosis

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ABSTRACT

In eukaryotes, entry into and exit from mitosis is regulated, respectively, by the transient activation and inactivation of Cdk1. Taxol, an anti-microtubule anti-cancer drug, prevents microtubule–kinetochore attachments to induce spindle assembly checkpoint (SAC; also known as the mitotic checkpoint)-activated mitotic arrest. SAC activation causes mitotic arrest by chronically activating Cdk1. One consequence of prolonged Cdk1 activation is cell death. However, the cytoplasmic signal(s) that link SAC activation to the initiation of cell death remain unknown. We show here that activated Cdk1 forms a complex with the pro-apoptotic proteins Bax and Bak (also known as BAK1) during SAC-induced apoptosis. Bax- and Bak-mediated delivery of activated Cdk1 to the mitochondrion is essential for the phosphorylation of the anti-apoptotic proteins Bcl-2 and Bcl-x_L (encoded by *BCL2L1*) and the induction of cell death. The interactions between a key cell cycle control protein and key pro-apoptotic proteins identify the Cdk1–Bax and Cdk1–Bak complexes as the long-sought-after cytoplasmic signal that couples SAC activation to the induction of apoptotic cell death.

KEY WORDS: Spindle assembly checkpoint, Mitotic checkpoint, Cdk1, Bax, Bak, Apoptosis

INTRODUCTION

It is universally acknowledged that Cdk1–cyclin B [a complex between the cyclin-dependent kinase 1 (Cdk1) catalytic subunit and the cyclin B regulatory subunit] regulates mitosis in eukaryotic cells (Nurse, 1990; Nigg, 2001). Before mitosis, Cdk1–cyclin B is maintained in an inactive state by several mechanisms including inhibitory phosphorylation of Cdk1 at Thr14 and Tyr15 by the protein kinases Wee1 and Myt1, respectively (Leise and Mueller, 2002). At prophase, Cdk1 is activated by dephosphorylation of the inhibitory phosphates (Mochida et al., 2010) and initiates entry of the cell into mitosis. At the end of mitosis Cdk1 is inactivated by the proteolytic degradation of cyclin B (Zachariae and Nasmyth, 1999) Therefore, the temporal regulation of Cdk1 activation and inactivation is critical not only for the accurate transmission of genetic information to the daughter cells but also to ensure cell survival. Anti-mitotic anticancer drugs, such as Taxol, inhibit the function of the mitotic spindle by stabilising microtubules (Schiff and Horwitz, 1980; Wang et al., 2000) and suppressing microtubule–kinetochore attachments (Weaver, 2014). The presence of unattached kinetochores activates a well-characterised signalling pathway called the spindle assembly checkpoint (SAC) (Foley and Kapoor, 2013), which inhibits an E3 ubiquitin ligase called the anaphase-promoting complex or cyclosome (APC/C) (Yu, 2002). By preventing the ubiquitylation and degradation of cyclin B, the Taxol-activated SAC leads to the arrest of cells in mitosis owing to high Cdk1 kinase activity (Ibrado et al., 1998). One consequence of the Taxol-induced prolonged-mitotic arrest is the induction of cell death (also referred to as mitotic catastrophe) (Castedo et al., 2004; Vitale et al., 2011) through the mitochondrial pathway (Friesen et al., 2008). Mitochondrial apoptosis is also a well-characterised signalling pathway comprising Bcl-2 anti-apoptotic proteins, such as Bcl-2, Bcl-x_L (encoded by *BCL2L1*) and Mcl-1, whose function is antagonised by pro-apoptotic BH3-only proteins, such as Bad, Bim (also known as BCL2L11), Puma (BBC3) and Noxa (PMAIP1) (Llambi et al., 2011; Kutuk and Letai, 2010; Haschka et al., 2015). The anti-apoptotic function of the Bcl-2 proteins can also be inhibited by phosphorylation of Bcl-2 and Bcl-x_L (Terrano et al., 2010; Sakurikar et al., 2012), and by the Cdk1-mediated phosphorylation and subsequent degradation of Mcl-1 (Harley et al., 2010; Chu et al., 2012). Inhibition of the anti-apoptotic proteins allows the pro-apoptotic Bcl-2 proteins Bax and Bak (also known as BAK1) to permeabilise the outer mitochondrial membrane (OMM) (Youle and Strasser, 2008; Große et al., 2016; Salvador-Gallego et al., 2016), resulting in the release of intermembrane space proteins into the cytoplasm, such as cytochrome *c*, and subsequent activation of caspases and the progression of apoptosis. Although, the mechanism of the SAC and mitochondrial apoptosis signalling are relatively well defined, the identity of the apoptosis-inducing signal that accumulates in the cytoplasm following mitotic cell cycle arrest remains unknown. In this study, we demonstrate that Cdk1 and Bax form a complex in cells and that they can interact directly *in vitro*. In cells, the complex of activated Cdk1 and activated Bax is essential for the phosphorylation (and inactivation) of the mitochondrial pro-apoptotic proteins Bcl-2 and Bcl-x_L. The presence of Bax is both necessary and sufficient for Cdk1-dependent phosphorylation of Bcl-2 and Bcl-x_L and the induction of Taxol-induced mitotic cell death. Our data lead us to conclude that the activated Cdk1 is co-translocated to the OMM by activated Bax. We suggest that the Cdk1–Bax complex constitutes a major death signal that accumulates during mitotic cell cycle arrest and couples SAC activation to the initiation of cell death.

RESULTS

Identification of a novel Cdk1–Bax complex in mammalian cells

To understand the mechanism that leads to cell death following SAC activation and mitotic arrest, we identified Bax- and Bak-associated

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proteins. Using conformation-specific antibodies (Griffiths et al., 1999; Hsu and Youle, 1998), we immunoprecipitated activated Bax and Bak from the lysates of Taxol-arrested HeLa cells (SAC-activated) and identified the interacting proteins by in-gel trypsin digestion and mass spectrometry (MS). Following elimination of non-specific binding proteins, 36 Bax-associated and 43 Bak-associated proteins were identified (Table S1). Cdk1 was consistently identified in the Bax immunoprecipitates (IPs) of

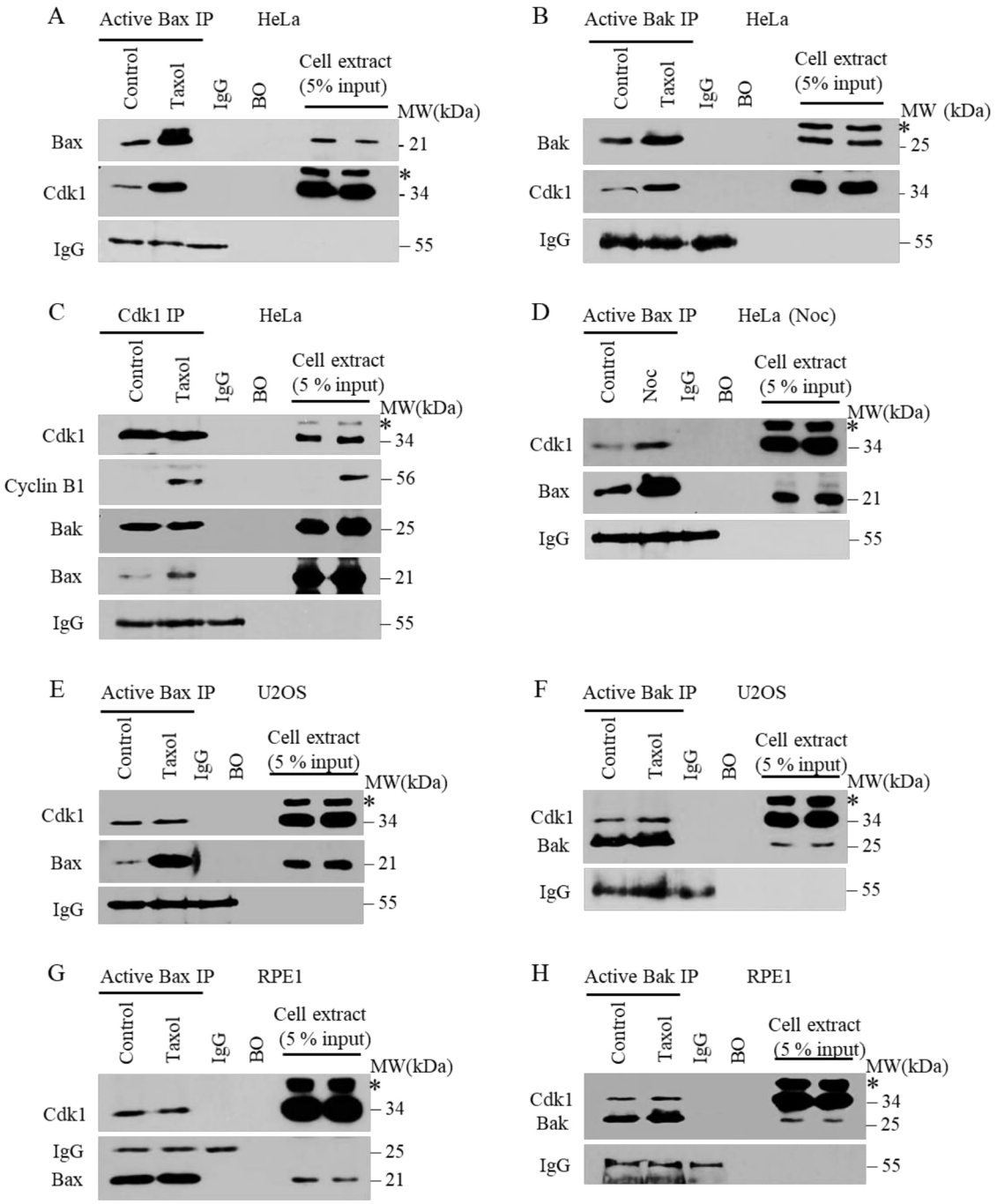

Fig. 1. Cdk1 is complexed with Bax and Bak in mammalian cells. (A) Immunoblot analysis of anti-active Bax (6A7) IPs and cell lysates (input) with the indicated antibodies. HeLa cells were either left untreated (control) or synchronised and treated with Taxol (60 nM) for 24 h prior to cell lysis and immunoprecipitation of Bax. Taxol treatment typically results in 75.3±3.8% (mean±s.d.; *n*=3) apoptotic cells compared to <1% in the untreated (control) cells as assessed by cytokeratin 18 cleavage (see Fig. 4B). Control cell lysates incubated with either a non-specific IgG (IgG) or with beads alone (BO) were used as negative controls. Positions of molecular mass (MW) markers (kDa) are indicated on the right. (B) Immunoblot analysis as in A but an anti-active Bak (N20) antibody was used for immunoprecipitation. (C) Immunoblot analysis as in A but an anti-Cdk1 [Cdc2 p34 (17)] antibody was used for immunoprecipitation. (D) Immunoblot analysis as in A except that HeLa cells were treated with Nocodazole (1 µM for 24 h, to produce an equivalent level of apoptotic cells as Taxol). (E,F) Immunoblot analysis as in A and B except that Bax and Bak, respectively, were immunoprecipitated from U20S cells. (G,H) Immunoblot analysis as in A and B except that Bax and Bak, respectively, were immunoprecipitated from RPE cells. * indicates a non-specific protein detected by the Cdk1 antibody. Each result (A–F) is representative of two independent experiments.

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Taxol-arrested cells. Therefore, in this study we have focused primarily on examining the role of the Bax–Cdk1 interaction during Taxol-induced cell death.

To confirm the results of the MS analysis, we immunoblotted the active-Bax IPs from control and Taxol-arrested HeLa cells with an anti-Cdk1 antibody. The result confirmed the presence of a Bax–Cdk1 complex (Fig. 1A). A low level of active Bax was associated with Cdk1 in the control cells, but the level of this complex was elevated in the Taxol-arrested cells, which is consistent with Bax activation during Taxol-induced apoptosis (Makin and Dive, 2001). Surprisingly, in parallel experiments, we also detected a Bak–Cdk1 complex in HeLa cells (Fig. 1B). In reciprocal IPs, we confirmed the existence of Bax–Cdk1 and Bak–Cdk1 complexes by demonstrating the presence of Bax and Bak in the Cdk1 immunoprecipitates (Fig. 1C). As Cdk1 is chronically activated in Taxol-arrested cells, we determined whether cyclin B was also complexed with Bax–Cdk1 and Bak–Cdk1. Our immunoblot analysis of the Cdk1 IPs indicated, as expected, that cyclin B was not associated with Cdk1 in the control cells but was associated with Cdk1, Bax and Bak in the Taxol-arrested mitotic cells (Fig. 1C). We concluded that a complex comprising activated Bax, Bak, Cdk1 and cyclin B accumulates in Taxol-arrested cells.

To investigate whether the activated Bax–Cdk1 complex formed specifically in response to Taxol-induced SAC activation and cell death, we induced SAC activation and cell death by treating a synchronised population of HeLa cells with Nocodazole (a

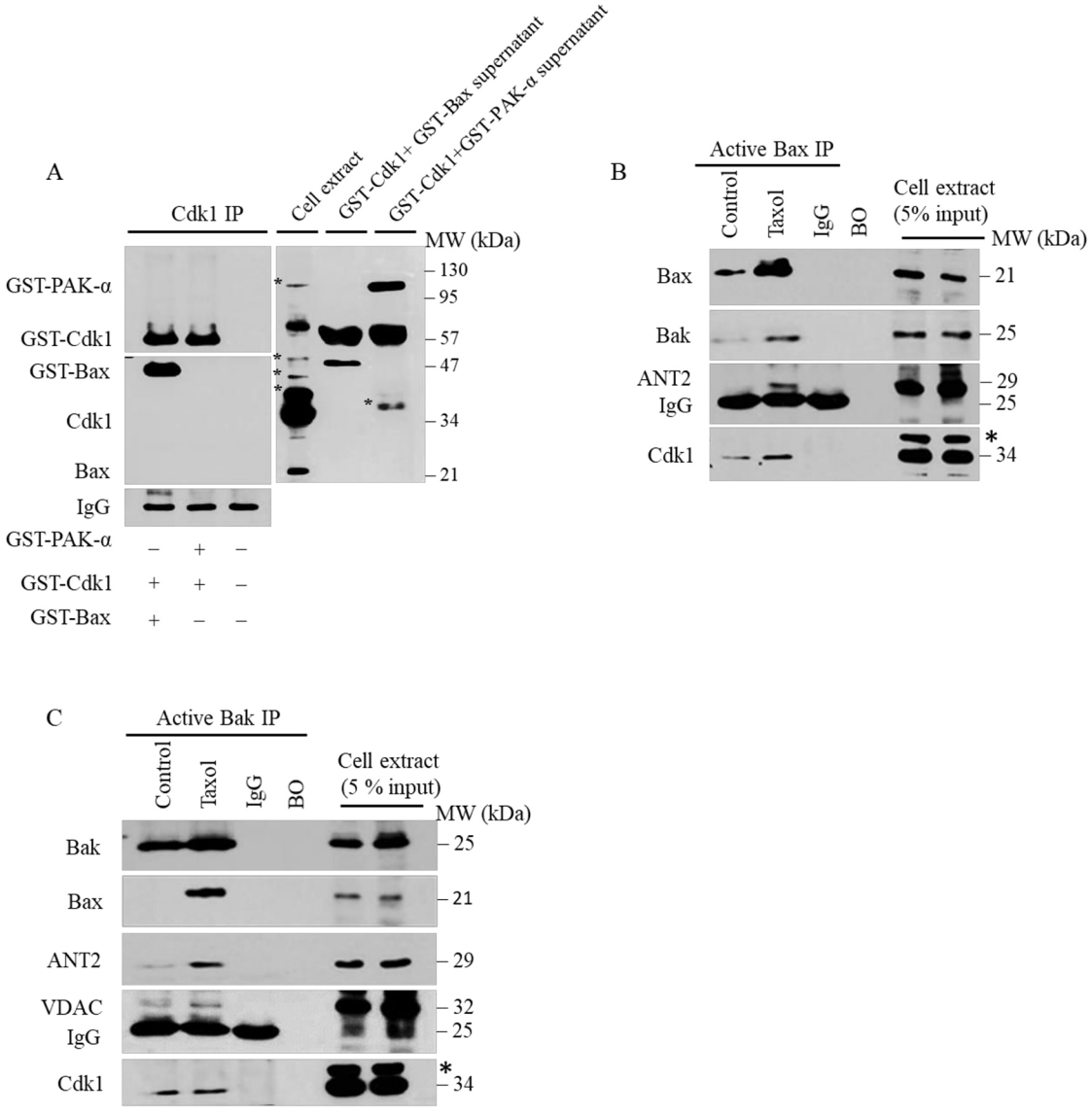

Fig. 2. Cdk1 binds directly to Bax *in vitro* and is co-translocated by Bax and Bak to mitochondria in Taxol-arrested cells. (A) Immunoblot analysis of Cdk1 [antibody used was Cdc2 p34 (17)] IPs with the indicated antibodies. Equimolar concentrations (50 µM) of GST–Cdk1 and GST–Bax were incubated in 1% CHAPS lysis buffer for 2 h at 4°C. GST–PAKα (50 µM) was incubated with GST–Cdk1 as a control. The post-IP supernatants (10% of total volume) and cell lysate were run as positive. Positions of molecular mass (MW) markers (kDa) are indicated on the right. * indicates non-specific proteins. (B) Immunoblot analysis of active Bax-IPs with the indicated antibodies. HeLa cells were either left untreated (control) or synchronised and treated with Taxol (60 nM) for 24 h. Control cell lysates incubated with either a non-specific IgG (IgG) or with beads alone (BO) and were used as negative controls. The doublet of Bax protein (seen in the Bax IP of Taxol-treated cells) was analysed by mass spectrometry to determine whether it was phosphorylated. We did not detect any phosphorylation of Bax but did find that it was oxidised. (C) As B, but an anti-active Bak (N20) antibody was used for immunoprecipitation. Each result (A–C) is representative of two independent experiments.

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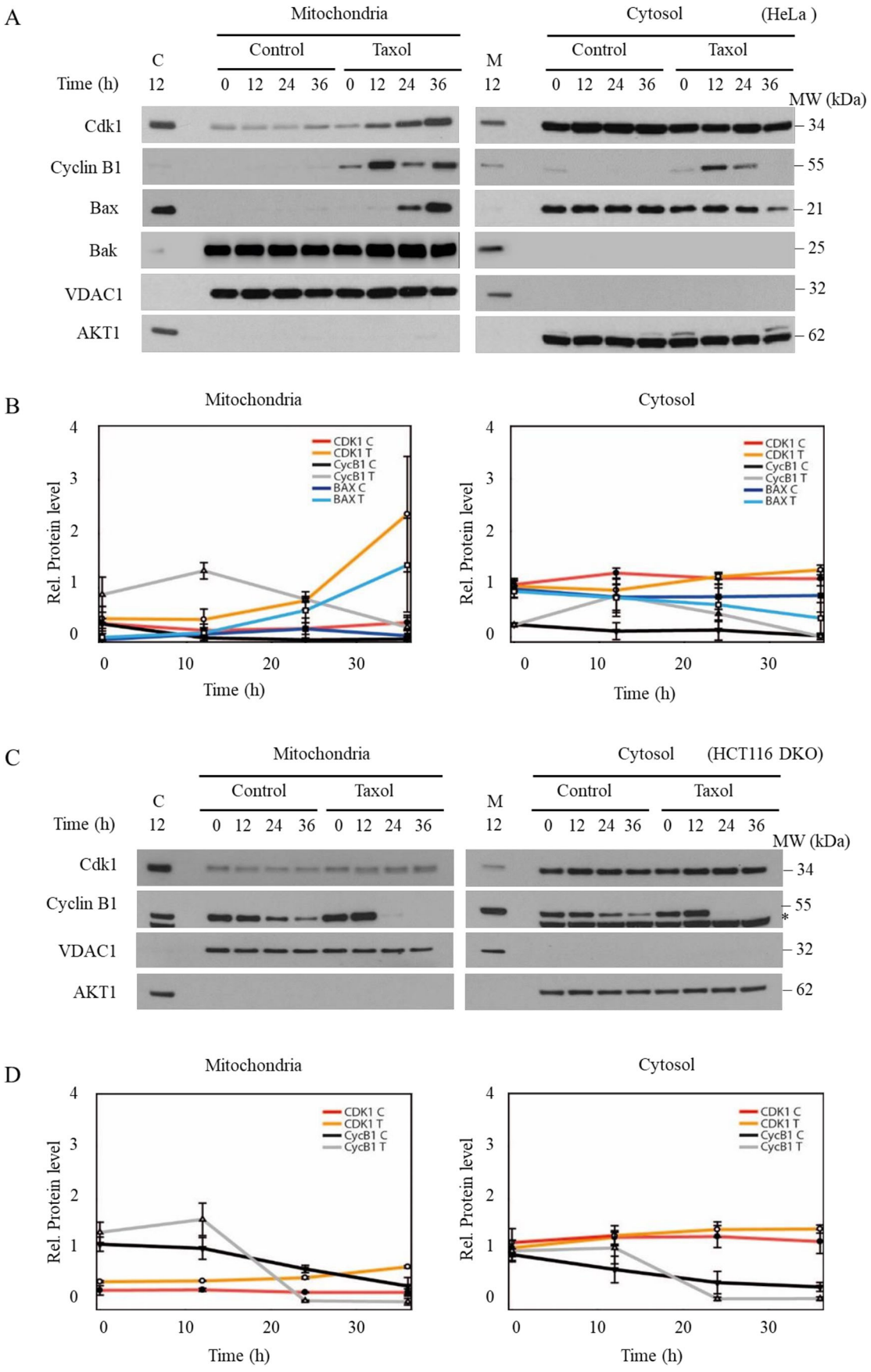

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Fig. 3. Taxol induces translocation of Cdk1 and Bax to mitochondria. (A) Western blot analysis of Cdk1, cyclin B and Bax localisation in the cytosol (C) and mitochondrial (M) fractions of a synchronised population of Hela cells following either no treatment (control) or Taxol addition (60 nM) for 0–36 h. VDAC1 and AKT1 serve as loading control for heavy membrane and cytosolic fractions, respectively. Representative of *n*=3. Positions of molecular mass (MW) markers (kDa) are indicated on the right. (B) Quantification of the data shown in A. (C) Western blot analysis performed as described in A using HCT115 Bax and Bak double knockout (DKO) cells. (D) Quantification of the data shown in C. B and D show the mean±s.d. of three independent experiments.

microtubule-depolymerising agent) (Deacon et al., 2003). Immunoblot analysis of the active Bax IPs indicated the presence of an active Bax–Cdk1 complex in the Nocodazole-arrested cells (Fig. 1D). We also wanted to eliminate the possibility that the Bax–Cdk1 and Bak–Cdk1 complexes were unique to HeLa cells. Therefore, we immunoprecipitated active Bax and Bak from control and synchronised Taxol-treated U2OS and retinal pigment epithelial (RPE) cells (a diploid cell line; Dunn et al., 1996). Consistent with our results in HeLa cells, the interaction between Bax–Cdk1 and Bak–Cdk1 was also observed in U2OS (Fig. 1E,F) and RPE cells (Fig. 1G,H). We note that the level of Cdk1 co-immunoprecipitating with active Bax in U2OS (Fig. 1E) and RPE1 cells (Fig. 1H) is not increased in mitotic cells. One possibility is that in both U2OS and RPE cells a basal level of Cdk1 is constitutively associated with Bax throughout the cell cycle. At mitosis, Cdk1 may be activated by cyclin B binding to the Cdk1–Bax complex. We conclude that inactive Cdk1 is constitutively associated with Bax and Bak in multiple cell types and that a complex of activated Cdk1, cyclin B and Bax or Bak is assembled in the SAC-activated cells, irrespective of the anti-microtubule agent used.

Cdk1 interacts directly with Bax *in vitro* and is targeted to the OMM by Bax and Bak in cells

We next determined whether there was a direct interaction between Bax and Cdk1. Purified recombinant GST–Cdk1 and GST–Bax (Fig. S1) were incubated together at equimolar concentrations. Purified recombinant GST–Pakα was used a control. Immunoprecipitation of Cdk1 resulted in the co-immunoprecipitation of GST–Bax but not of GST–Pak1 (Fig. 2A). A similar experiment using non epitope-tagged, recombinant Bax also co-immunoprecipitated with GST–Cdk1 (Fig. S2). We conclude that the *in vitro* interaction between GST–Cdk1 and GST–Bax is direct and specific and suggest that the Cdk1–Bax complex isolated in our co-IP experiments may result from a direct protein–protein interaction. Since Cdk1-dependent phosphorylation and inactivation of the anti-apoptotic proteins Bcl-2 and Bcl-x_L has been linked to Taxol-induced apoptosis (Terrano et al., 2010; Sakurikar et al., 2012; Scatena et al., 1998), we hypothesise that in the process of OMM-accumulation of activated Bax and Bak (Todt et al., 2013, 2015; Lauterwasser et al., 2019), activated Cdk1 may also be co-translocated to the OMM following SAC activation and mitotic arrest. To test this hypothesis, we immunoprecipitated activated Bax and Bak from the control and Taxol-arrested cells. Immunoblot analysis of the Bax and Bak IPs indicated that the Bax–Cdk1 complex was associated with the mitochondrial protein ANT2 specifically in the Taxol-arrested cells (Fig. 2B). In contrast, the Bak–Cdk1 complex was associated with both ANT2 (SLC25A5) and VDAC1 (VDAC), but only in the Taxol-arrested cells (Fig. 2C). We confirmed our hypothesis by directly assessing the levels of mitochondrial-associated Cdk1, cyclin B and Bax proteins at intervals following Taxol-addition to a synchronised population of HeLa cells. Our results (Fig. 3A,B) show that there was a time-dependent accumulation of Cdk1, cyclin B (0–12 h) and Bax, specifically in the mitochondrial fraction of the Taxol-treated cells. To further support our hypothesis that Bax is required to recruit Cdk1 to the OMM, we also assessed the levels of mitochondrial-associated Cdk1 and cyclin B at intervals following Taxol-addition to a synchronised population of Bax and Bak double knockout (DKO) HCT116 cells (Wang and Youle, 2012). A low level of Cdk1 was observed in the control and Taxol-treated mitochondrial fractions (Fig. 3C,D). Importantly, Cdk1 accumulation was not observed in the mitochondrial fraction of the DKO HCT116 cells after Taxol addition (Fig. 3C,D). We note that the mitochondrial fraction in DKO HCT116 cells, unexpectedly, contained high levels of cyclin B prior to Taxol addition. This finding was unexpected, and its significance remains to be determined. However, taken together, we conclude from these results that activated Bax mediates co-translocation of Cdk1 to the OMM following Taxol-induced SAC activation.

The Bax–Cdk1 signalling complex is necessary for Bcl-2 and Bcl-x_L phosphorylation

The phosphorylation and inactivation of the mitochondria-based, anti-apoptotic proteins Bcl-2 and Bcl-x_L is dependent on Cdk1 during Taxol-induced apoptosis (Terrano et al., 2010; Sakurikar et al., 2012; Scatena et al., 1998). We have reproduced this result in HeLa cells. Treatment of Taxol-arrested cells with the Cdk1 kinase inhibitor RO3306 (5 µM) (Kojima et al., 2009; Vassilev et al., 2006) inhibited both Cdk1 activity, as observed by the degradation of cyclin B (Vassilev et al., 2006), and the phosphorylation of both Bcl-2 and Bcl-x_L, as judged by a change in their gel mobility to the non-phosphorylated faster-migrating forms (Fig. 4A). Treatment of Taxol-arrested cells with R3306 was also accompanied by a significant reduction in apoptosis, as examined by both PARP-1 cleavage (Fig. 4A) and cytokeratin 18 cleavage (Fig. 4B). To confirm that R03306 inhibits the phosphorylation of Bcl-2 and Bcl-x_L, we performed a similar immunoblot analysis using phospho-Bcl-2 (Ser70) and phospho-Bcl-x_L (Ser62) antibodies, which recognise known Cdk1 phosphosites (Terrano et al., 2010; Dai et al., 2013). Our results indicate that the phospho-specific antibodies reacted with their target protein only in the Taxol-treated cells and not in the control cells or in the Taxol-arrested cells treated with R03306 (Fig. 4C). This result confirmed that the phosphorylation of Bcl-2 and Bcl-x_L in the Taxol-arrested cells is dependent on active Cdk1.

Next, we determined the requirement for Bax and Bak in the Cdk1-mediated phosphorylation of Bcl-2 and Bcl-x_L. We first depleted Bax and Bak in HeLa cells, either individually or in combination using siRNA. Immunoblot analysis of the lysates of siRNA-treated cells indicated that both Bax and Bax were efficiently depleted (Fig. 4D). We then assessed the effect of Bax and Bak-depletion on both Taxol-induced apoptosis and the phosphorylation state of Bcl-2 and Bcl-x_L. As expected, depletion of Bax and Bak either individually or in combination significantly reduced Taxol-induced apoptosis (Fig. 4E). Surprisingly, our immunoblot analysis indicated that the phosphorylation of both Bcl-2 and Bcl-x_L, in response to Taxol, was also inhibited when compared to the control siRNA-treated cells (Fig. 4F). We note that some residual phosphorylation of Bcl-2 was seen in our immunoblot (Fig. 4F). We attribute this to the presence of Bax in the Bak-depleted cells and vice versa. The low-level phosphorylation of Bcl-2 was completely eliminated when Bax and Bak were co-depleted.

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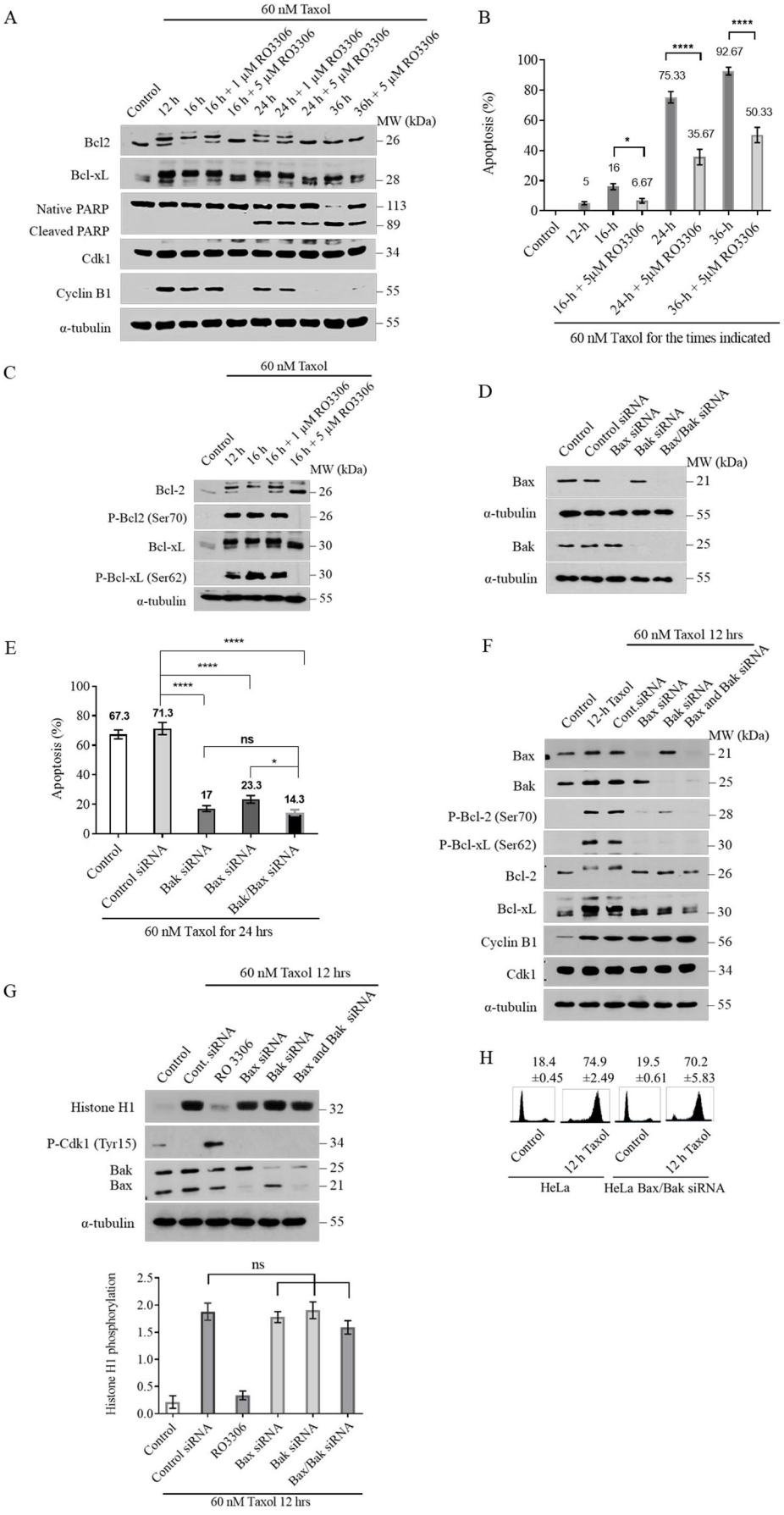

Fig. 4. siRNA depletion of Bax and Bak inhibits Cdk1 phosphorylation of Bcl-2 and Bcl-xl and Taxol-induced apoptosis. (A) Immunoblot analysis of HeLa cell lysates with the indicated antibodies following treatment with Taxol or a combination of Taxol and RO-3306. Cells were untreated (control) or treated with Taxol (60 nM for 12 h). Mitotic cells were collected by shake-off and incubated with Taxol or Taxol plus RO-3306 (1 and 5 μM) for the indicated times. Parallel cell samples were assayed for apoptosis by immunostaining with the M30 antibody. Positions of molecular mass (MW) markers (kDa) are indicated on the right. (B) Quantification of apoptosis for the experiment described in A. Bars represent the mean±s.d. of three independent experiments. **P*<0.05, *****P*<0.0001 (one-way ANOVA). A total of 300 cells were counted in the three independent experiments. The total number of M30-positive cells for each condition was as follows: control, 0; Taxol (12 h), 15; Taxol (16 h), 48; Taxol (24 h), 226; Taxol (36 h), 278; Taxol (16 h plus RO3306), 20; Taxol (24 h plus RO3306), 107; Taxol (36 h plus RO3306), 151. (C) Immunoblot analysis as in A. (D) Immunoblot analysis of HeLa cell lysates following siRNA depletion of Bax and Bak. Cells were untreated (control) or transfected with the indicated siRNAs (50 nM) for 48 h. Parallel siRNA-treated cells were incubated with Taxol (60 nM for 24 h) and apoptosis analysed with the M30 antibody. (E) Quantification of apoptosis described in D. Each bar represents the mean±s.d. of three independent experiments. **P*<0.05, *****P*<0.0001 (one-way ANOVA). (F) Immunoblot analysis of HeLa cell lysates with the indicated antibodies following siRNA-depletion of Bax and Bak and Taxol treatment. (G) Autoradiogram showing Cdk1 kinase activity in HeLa cells following the indicated treatment. Cells were untreated (control) or transfected with the indicated siRNAs (50 nM) for 48 h. Cells were then treated with Taxol (60 nM for 12 h) or with Taxol and RO-3306 (5 μM). Cdk1 IPs of the cell lysates were assayed for kinase activity using Histone H1 as substrate. Histogram shows quantification of Cdk1 kinase activity. Each bar represents the intensity (mean±s.d.) of three independent experiments. ns, not significant compared to control siRNA (one-way ANOVA). (H) FACS analysis of untreated (control) and Taxol-treated (60 nM for 12 h) HeLa cells following 48 h transfection with control or Bax/Bak siRNA. The percentage (mean±s.d. of three independent experiments) of cells in G2/M phase is indicated.

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We examined possible reasons for the loss of Taxol-induced phosphorylation of Bcl-2 and Bcl-x_L in the absence of Bax and Bak. These include the failure to activate Cdk1 or the failure of the Bax- and Bak-depleted cells to arrest in mitosis in response to Taxol. We know that these explanations are unsatisfactory. Measurement of Cdk1 kinase activity indicated that there was no significant difference in Cdk1 activity between the Taxol-arrested control and the Taxol-arrested Bax- or Bak-depleted cells, whereas Cdk1 kinase activity was efficiently inhibited by RO3306 (Fig. 4G). We also confirmed by FACS analysis that the Taxol-treated Bax- and Bak-depleted cells arrested in mitosis (G2/M-phase) (Fig. 4H). These results suggest that the activation of both Cdk1 and the SAC are not compromised in the Bax- and Bak-depleted cells. We concluded that cytoplasmic activated Cdk1 alone is insufficient to phosphorylate Bcl-2 and Bcl-x_L following SAC activation. Indeed, our results support our hypothesis that the targeted

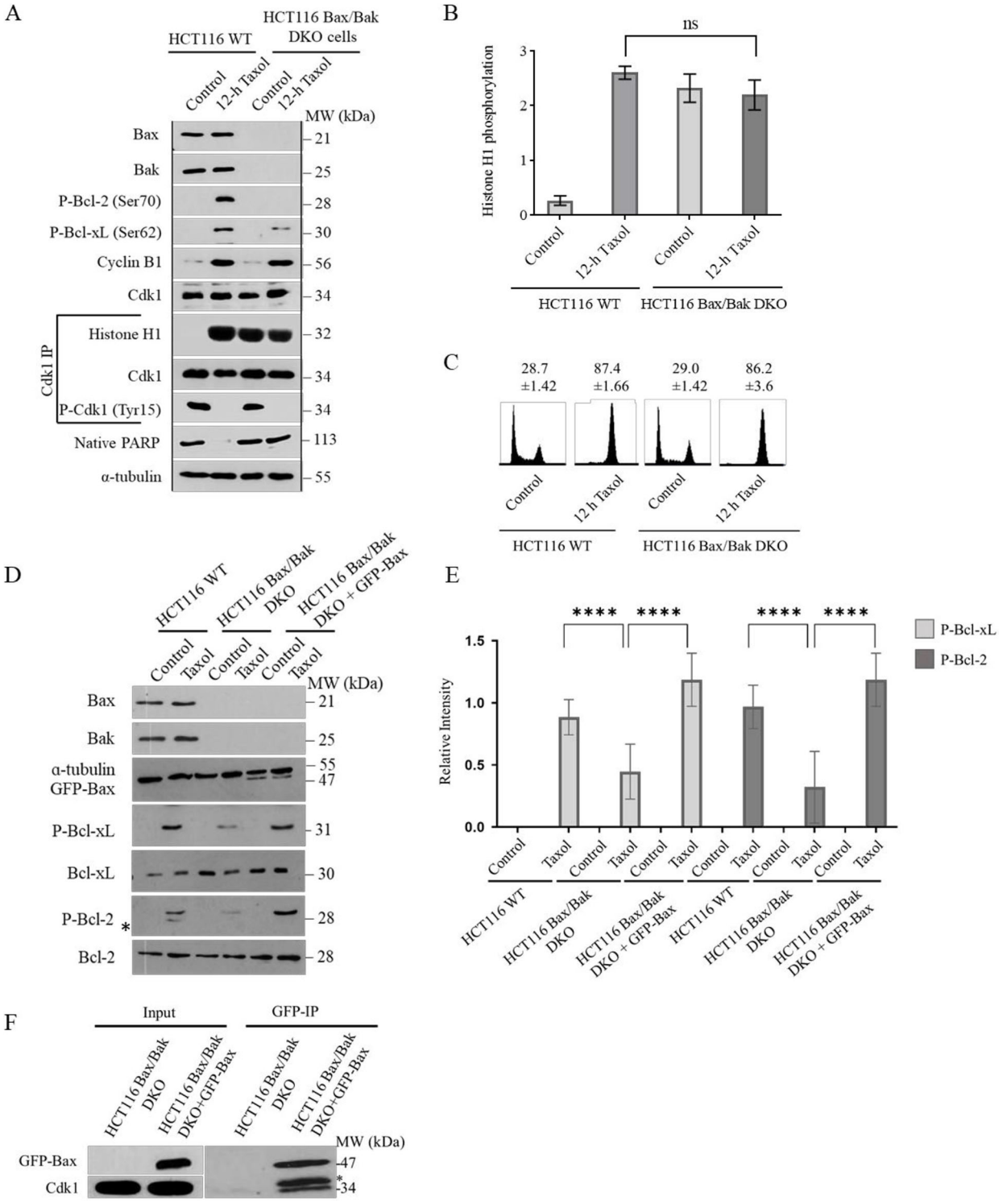

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Fig. 5. Taxol-induced Cdk1 activation does not lead to the phosphorylation of Bcl-2, Bcl-x_L and apoptosis in Bax/Bak DKO HCT116 cells. (A) Immunoblot analysis of HCT116 WT and Bax and Bak DKO cells with the indicated antibodies following Taxol treatment. Cells were untreated (control) or treated with Taxol (60 nM for 12 h). Parallel cell lysates were used for immunoprecipitation of Cdk1 with the Cdc2 p34 (17) antibody. The Cdk1 IPs were immunoblotted with the indicated antibodies or assayed for kinase activity using histone H1 as substrate (autoradiogram shown). Positions of molecular mass (MW) markers (kDa) are indicated on the right. The immunoblots are representative of two independent experiments.
(B) Quantification of the Cdk1 IP kinase assay shown in A. Each bar represents the intensity (mean±s.d. of three independent experiments) of phosphorylated histone H1 normalised to α-tubulin. ns, not significant when compared to Taxol-treated WT HCT116 cells (one-way ANOVA). (C) FACS analysis of untreated or Taxol-treated (1 µM for 12 h) HCT116 WT and HCT116 Bax and Bak DKO cells. The percentage (mean±s.d. of three independent experiments) of cells in G2/M phase is indicated. (D) Immunoblot analysis of HCT116 Bax and Bak DKO cell lysates with the indicated antibodies following transfection with GFP–Bax and Taxol treatment. Cells were either mock transfected or transfected with GFP–Bax for 24 h. The cells (including HCT116 WT cells as control) were then treated with Taxol (1 µM for 12 h) and then lysed. Positions of molecular mass (MW) markers (kDa) are indicated on the right.
(E) Histogram shows quantification of the phosphorylation of Bcl2 and Bcl-x_L shown in D. Each bar represents the normalised intensity (mean±s.d.) of three independent experiments. (F) Immunoblot analysis of GFP IPs with the Cdk1 antibody. HCT116 Bax and Bak DKO cells were either mock transfected or transfected with GFP–Bax for 24 h. The cells were then treated with Taxol (1 µM for 12 h) and then lysed. GFP–Bax was immunoprecipitated from the cell lysates using a GFP-Trap. Cell lysates (input) were run as controls. Positions of molecular mass (MW) markers (kDa) are indicated on the right. * indicates a non-specific protein detected by the Cdk1 antibody.

delivery of active Cdk1 to the OMM by activated Bax and Bak is necessary to phosphorylate Bcl-2 and Bcl-x_L.

To further substantiate our finding that siRNA-mediated depletion of Bax and Bak inhibits Cdk1-dependent phosphorylation of Bcl-2 and Bcl-x_L, we performed a similar analysis using Bax and Bak DKO HCT116 cells. Immunoblot analysis of the lysates of the wild-type (WT) and DKO HCT116 cells confirmed our results obtained with the siRNA analysis. As expected, both Bcl-2 and Bcl-xl were phosphorylated in the WT Taxol-treated cells but not in the Taxol-treated DKO cells (Fig. 5A). Consistent with the fact that phosphorylation of Bcl-2 and Bcl-x_L is required for their inactivation and the induction of cell death, the WT Taxol-treated cells underwent apoptosis as assessed by PARP-1 cleavage, whereas PARP-1 cleavage was not observed in the Taxol-treated DKO cells (Fig. 5A). As observed with our siRNA results, Cdk1 was also activated to an equivalent level in both the WT and DKO cells (Fig. 5A,B) and both cell types also arrested in mitosis (G2/M) following Taxol addition as assessed by flow cytometry (Fig. 5C). Interestingly, when we assayed histone H1 kinase activity in the Cdk1 IPs of the untreated DKO HCT116 cells, we consistently observed a high-level of histone H1 phosphorylation although neither Bcl-2 nor Bcl-xl was phosphorylated. We confirmed that Cdk1 was inactive in the DKO HCT116 cells as it was still phosphorylated on tyrosine 15 as assessed with an anti-phospho-Cdk1 (Tyr15) antibody. Therefore, we attribute the high level of H1 kinase activity to one or more unidentified protein kinases that co-IP with Cdk1.

Next, we determined whether the phosphorylation of Bcl-2 and Bcl-xl in the DKO cells could be rescued by reintroducing Bax. We transfected GFP–Bax into the DKO HCT116 cells and then treated them with Taxol. Immunoblot analysis of the cell lysates indicated that the Taxol-induced phosphorylation of both Bcl-2 and Bcl-x_L was restored by expression of GFP–Bax (Fig. 5D) to levels observed in the WT cells. We observed some residual phosphorylation of Bcl-2 and Bcl-x_L in the Taxol-treated DKO HCT116 cells (Fig. 5D,E). This may due to the activation of kinases, other than Cdk1, that have been reported to phosphorylate Bcl-2 and Bcl-x_L (Yamamoto et al., 1999; Basu and Haldar, 2003; Wang et al., 2011, 2012). However, it is important to note that this low-level phosphorylation of Bcl-2 and Bcl-x_L was not sufficient to induce cell death as assessed by the absence of PARP-1 cleavage. Therefore, the (GFP–Bax)–Cdk1 complex is primarily responsible for the phosphorylation of Bcl-2 and Bcl-x_L in the Taxol-arrested DKO HCT116 cells. As shown in Fig. 5F, our immunoblot analysis of the GFP-Bax IPs, from the GFP-Bax transfected DKO cells, indicated that the GFP-Bax was complexed with Cdk1 (Fig. 5F). Together, these results support our hypothesis that the Bax and Bak-mediated translocation of active Cdk1 to the OMM is a necessary step in the phosphorylation of Bcl-2 and Bcl-x_L and eventually the induction of cell death.

DISCUSSION

Anti-mitotic drugs, such as the Taxanes, cause prolonged activation of the SAC, which leads to chronic activation of Cdk1 and mitotic cell cycle arrest. One consequence of prolonged SAC activation is the induction of apoptotic cell death via the mitochondrial pathway (Yamada and Gorbsky, 2006; McGrogan et al., 2008; Singh et al., 2008; Dumontet and Jordan, 2010). However, the cytoplasmic signal(s) that relays SAC activation to the mitochondria has remained elusive. We show, in this study, that activated complexes of Bax–Cdk1 and Bak–Cdk1 accumulate at the OMM in Taxol-arrested cells and are likely to constitute the primary cytoplasmic signal that is delivered to the mitochondria to initiate cell death (Fig. 6). *In vitro*, the interaction between Bax and Cdk1 is direct, although the molecular details of this interaction require further analysis. As both Bax and Bak are known to shuttle between the cytoplasm and the mitochondria (Todt et al., 2013, 2015; Lauterwasser et al., 2019) in healthy cells, it is likely that the Cdk1–Bax and Cdk1–Bak complexes may also undergo similar futile cycles of translocation–retrotranslocation and will require further study. This is very much reminiscent of the shuttling of the inactive Cdk1–cyclin B complex between the cytoplasm and the nuclear membrane prior to prophase. Upon activation of the Cdk1–cyclin B complex, while the majority of the kinase remains in the cytoplasm, a subset of the complex is targeted into the nucleus to induce prophase (Gavet and Pines, 2010a).

Our findings indicate that, in SAC-arrested cells, both the Cdk1–Bax and Cdk1–Bak complexes are activated. The Bax and Bak components of the complex are activated through a conformational change (Griffiths et al., 1999; Hsu and Youle, 1998; Moldoveanu et al., 2006), and their retrotranslocation to the cytoplasm is suppressed (Todt et al., 2013, 2015; Lauterwasser et al., 2019). Cdk1 is activated by a series of well-defined post-translational modifications (Gavet and Pines, 2010b) and by binding to cyclin B (Jackman et al., 2003). Our finding that active Bax and Bak co-immunoprecipitate with Cdk1, VDAC1 and ANT2, supports the idea that there are Cdk1–Bax and Cdk1–Bak complexes at the OMM. In addition, we also used biochemical fractionation to demonstrate the accumulation of Cdk1, cyclin B and Bax at the OMM of Taxol-treated cells. These observations are clearly consistent with the activated Bax–Cdk1 signal initiating cell death following Taxol-induced SAC activation. The mitochondrial association of Bax and Bak subsequently induces OMM permeabilisation that permit the release of pro-apoptotic factors, such as cytochrome *c* (Salvador-Gallego et al., 2016; Czabotar et al., 2013; Bleicken et al., 2013; Cosentino and García-Sáez, 2017). The release of cytochrome *c* results in the activation of

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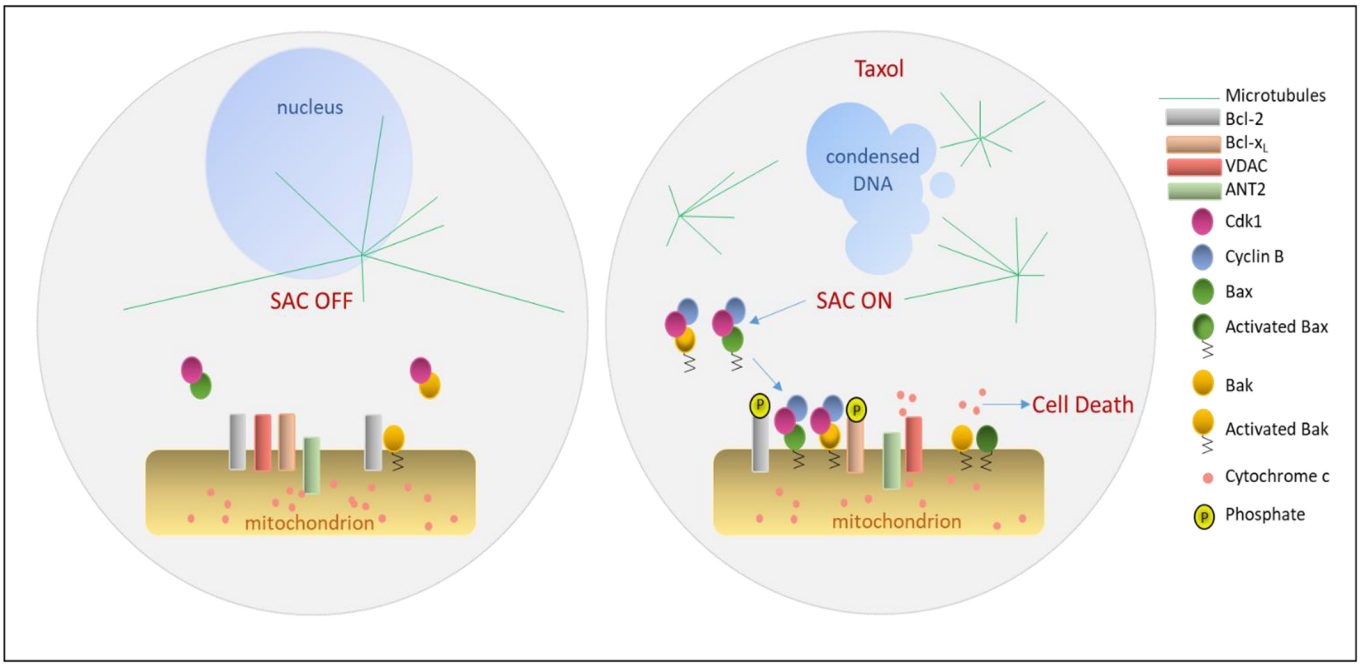

Fig. 6. Model of the roles of Cdk1–Bax and Cdk1–Bak during SAC activation and the induction of apoptosis. In healthy interphase cells (SAC Off) pro-apoptotic inactive Bax and a small pool of Bak (the majority of Bak is present in the OMM) are complexed with inactive Cdk1. The presence of anti-apoptotic Bcl-2 and Bcl-x_L at the OMM suppresses the release of mitochondrial pro-apoptotic factors, such as cytochrome *c*, by directly interacting with Bax and Bak or by sequestering Bax and Bak activators. Taxol, by stabilising microtubules, causes prolonged SAC activation and subsequent mitotic cell cycle arrest. During mitosis, Bax and Bak are activated and their associated Cdk1-binding partner is also activated through binding cyclin B1 (and other post-translational modifications). The activated Bax–Cdk1 (and activated Bak–Cdk1) complex is translocated to the OMM. The presence of activated Cdk1 at the OMM results in the phosphorylation of Bcl-2 and Bcl-x_L, inactivating their anti-apoptotic function, and results in the release of cytochrome *c* from pores formed in the OMM by Bax and Bak oligomers and/or the constitutive opening of the VDAC1–ANT2 pore complex. The release of cytochrome *c* results in the activation of the caspase cascade and cell death ensues.

apoptotic caspases which bring about cell death (Mikhailov et al., 2003). At the OMM the Cdk1-mediated phosphorylation of Bcl-2 and Bcl-x_L inhibits their anti-apoptotic function and is necessary for Taxol-induced apoptosis (Terrano et al., 2010; Sakurikar et al., 2012; Chu et al., 2012; Eichhorn et al., 2013). In addition, Mcl-1 has also been reported to be phosphorylated by Cdk1 on threonine 92 (T92) and undergo degradation during prolonged mitotic cell cycle arrest (Harley et al., 2010; Chu et al., 2012). A phospho-null Mcl-1 mutant (T92A) has been shown to inhibit apoptosis during mitotic cell cycle arrest (Harley et al., 2010), suggesting that the phosphorylation and subsequent degradation of Mcl-1 is another factor predisposing to cell death. Thus, our data demonstrate that the Bax- and Bak-mediated transport of activated Cdk1 to the OMM is necessary for the phosphorylation of the mitochondrial anti-apoptotic proteins Bcl-2 and Bcl-x_L and the induction of cell death. Activated, cytoplasmic Cdk1 is not sufficient. We conclude that the formation and delivery of the Bax–Cdk1 (and Bak–Cdk1) complex to the OMM, following Taxol-induced mitotic arrest, constitutes the primary signal that couples SAC activation to apoptotic cell death.

MATERIALS AND METHODS

Mammalian cell culture, synchronisation and transfection

HeLa cells were originally purchased from BioWhittaker (now Lonza, Slough, UK). U2OS cells and RPE1 cells were provided by Andrew Fry (University of Leicester, UK). HCT-116 (WT), HCT-116 $Bax^{-/-}/Bak^{-/-}$ double knockout (DKO) cells and the GFP–Bax plasmid were a gift from Dr Richard Youle (National Institute of Neurological Disorders and Stroke, MD, USA). All cell lines were routinely tested to ensure that they were mycoplasma free. HeLa and U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, UK). RPE1 cells were cultured in DMEM F12 (Invitrogen, UK). Both DMEM and DMEM F12 were supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin solution (100 IU/ml and 100 µg/ml, respectively). HCT-116 (WT) and DKO cells were cultured in McCoy's 5A (Sigma, Poole, UK) supplemented with 10% (v/v) foetal bovine serum. Cells were transfected with plasmid DNA or siRNA, for 24–48 h, when they reached 50–60% confluency using Fugene 6 (Promega, UK) or Interferin (Polyplus Transfection, NY, USA), respectively, using the manufacturer's instructions. The siRNAs were used at a concentration of 10–20 nM. Cells were synchronised in S-phase with thymidine (2 mM) for 24 h, washed with PBS and released into fresh medium containing 60 nM Taxol (Sigma, Poole, UK) for 0–36 h. For inhibition of Cdk1 kinase, synchronised cells that were arrested in mitosis by Taxol (60 nM for 12 h) were treated with RO3306 (Tocris Bioscience, Bristol, UK) at a concentration of 1 or 5 µM for 4 h.

Antibodies and recombinant proteins

Antibodies against the following proteins were used for immunoblotting (IB), immunoprecipitation (IP) or immunofluorescence (IF): Bak (N-20, Sc-1035, 1:500 for IP), Cdc2 p34 (C-19, Sc-954; 1:1000), Cdc2 p34 (17, Sc-54, 1:500 for IP), Bax (6A7, used for Bax IP only, 1:500 dilution) and αPAK (N-20, Sc-1035; 1:1000) were purchased from Santa Cruz Biotechnology (TX, USA); Bak (D4E4, 12105; 1:1000), phospho-Bcl-2 (Ser70, 2827; 1:1000), Bcl-xL (2762; 1:1000), cyclin B1 (4138; 1:1000), phospho-Cdc2 (Tyr15, 9111; 1:1000), ANT2/SLC25A5 (14671; 1:1000), VDAC (4866; 1:1000), and goat anti-rabbit-IgG, HRP-linked antibody and Bax (#2772, used for IB only; 1:1000) were purchased from Cell Signaling Technology; goat anti-mouse-IgG, HRP-linked antibody (1:5000 for IB) and rabbit IgG (1:500 for IP) were purchased from Bethyl; and also Bcl-xL (BD Transduction Laboratories, 610212; 1:1000), M30 CytoDEATH (Roche,

2140322, 1:10 for IF), poly-(ADP-Ribose) polymerase (PARP) (Roche, 11835238001; 1:2000), BcL-2 Clone 124 (Dako, M0887; 1:1000), phospho-Bcl-xL (Ser62, GTX79124; 1:1000) (Gene Tex), α-tubulin (Sigma, 37981, 1:5000 for IB) and Alexa Fluor 488 goat anti-mouse-IgG (Life Technologies, 1:1000 for IF). N-terminus-GST-Cdk1 was purchased from Sino Biological Inc (Beijing, China); N-terminus-GST-Bax was purchased from Novus Biologicals (CO, USA), GST–PAKα was purified as described previously (Deacon et al., 2003). GFP–Trap was purchased from Chromotek (Martinsreid, Germany). Recombinant, full-length active Bax was purified as described previously (Suzuki et al., 2000). Unless indicated, all antibodies were used at a dilution of 1:1000 for IB.

siRNAs

The following ON-Target Plus predesigned siRNA's were purchased from Dharmacon (now Horizon Discovery, Cambridge, UK) and used in this study: Bax, 5′-GUGCCGGAACUGAUCAGAA-3′, 5′-ACAUGUUUUC-UGACGGCAA-3′, 5′-CUGAGCAGAUCAUGAAGAC-3′ and 5′-UGG-GCUGGAUCCAAGACCA-3′; Bak, 5′-CGACAUCAACCGACGCUAU-3′, 5′-UAUGAGUACUUCACCAAGA-3′, 5′-GACGGCAGCUCGCCA-UCAU-3′ and 5′-AAUCAUGACUCCCAAGGGU-3′.

Preparation of cell extracts, subcellular fractionation, immunoprecipitation and western blotting

Cells were collected and lysed in lysis buffer [20 mM Tris HCl pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol and protease inhibitors] and whole-cell extracts prepared as described previously (Deacon et al., 2003). For immunoprecipitation of active Bax and Bak, the cells were lysed in lysis buffer supplemented with 1% (w/v) CHAPS. Immunoprecipitations were performed as described previously (Deacon et al., 2003) except that Protein A/G magnetic beads were used (Thermo Scientific, MA, USA) according to the manufacturer's protocol. Subcellular fractionation was performed as described previously (Todt et al., 2013). Protein concentrations were determined using a Bradford Assay (ThermoScientific, MA, USA) and SDS-PAGE and western blot analysis was performed as described previously (Deacon et al., 2003).

Cell death assays

Apoptotic cell death was detected either by immunoblotting to detect PARP-1 cleavage or by immunofluorescence microscopy using the M30 cytoDEATH antibody, which specifically detects caspase-cleaved cytokeratin 18 (Leers et al., 1999). A minimum of 200 cells were counted in three independent experiments when the M30 antibody was used.

Flow cytometry

Flow cytometry was performed as described previously (Deacon et al., 2003) using an Accuri C6 Plus Flow Cytometer (Becton Dickinson, UK). Data was analysed using FlowJo (Version 10.0.6).

Immunofluorescence microscopy

Immunofluorescence staining experiments were performed as described previously (Deacon et al., 2003). Confocal images were captured using a Leica Confocal SP5. *Z*-stacks comprising 30–50 0.3 µm sections were acquired and the images analysed as maximum intensity projections using ImageJ software.

Cdk1 kinase assay

Immunocomplex kinase assays for Cdk1 were performed as described previously (Deacon et al., 2003) using histone H1 as substrate. The autoradiograms were scanned and the intensity of bands quantified using ImageJ. The intensity of each band was normalised to the intensity of the α-tubulin, and the mean values (±s.d.) were calculated from three independent experiments.

Preparation of recombinant GST–Pakα

Recombinant GST-Pakα was prepared using methods described previously (Deacon et al., 2003).

Mass spectrometry

The trypsin-digested proteins were subjected to peptide mass fingerprint analysis (using an LTO-Orbitrap-Velos mass spectrometer; Thermo Fisher Scientific, MA, USA) by the Protein and Nucleic Acid Chemistry Laboratory (PNACL) at the University of Leicester. The results were analysed using Mascot software (Matrix Science).

Statistical analyses

All statistical analyses were performed using a one-way ANOVA on GraphPad Prism. The results are reported as mean±s.d. and $P<0.05$ was considered statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: F.E., R.R.P.; Methodology: O.D., E.A.-S., H.F., J.L., R.R.P.; Validation: O.D., E.A.-S., H.F., J.L.; Formal analysis: O.D., E.A.-S., H.F., J.L.; Investigation: O.D., E.A.-S., H.F., J.L.; Resources: F.E.; Data curation: O.D., E.A.-S., H.F., J.L.; Writing - original draft: R.R.P.; Writing - review & editing: F.E., R.R.P.; Visualization: O.D., E.A.-S., H.F., J.L.; Supervision: F.E., R.R.P.; Project administration: F.E., R.R.P.; Funding acquisition: F.E., R.R.P.

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Supplementary information

Table S1

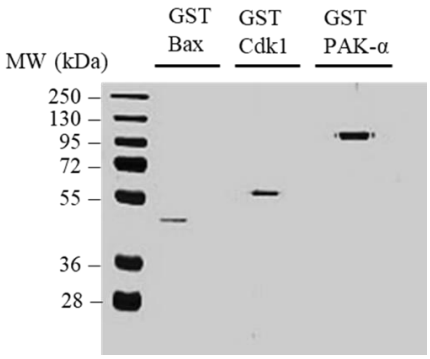
Protein	Bax IP		Bak IP	
	Score	Coverage (%)	Score	Coverage (%)
ADP/ATP translocase 2 OS=Homo sapiens	410	35	317	27
ADP/ATP translocase 3 OS=Homo sapiens	280	29	233	23
60 kDa heat shock protein, mitochondrial OS=Homo sapiens	426	23	256	12
14-3-3 protein zeta/delta OS=Homo sapiens	363	36	169	18
Apoptosis regulator BAX OS=Homo sapiens	776	55	459	35
Bcl-2 homologous antagonist/killer OS=Homo sapiens GN=BAK1	49	5.8	259	33
Peptidyl-prolyl cis-trans isomerase OS=Homo sapiens	370	37	139	30
ATP synthase subunit alpha, mitochondrial OS=Homo	169	26	386	22
ATP synthase subunit beta OS=Homo sapiens	196	13	210	23
L-lactate dehydrogenase A chain OS=Homo sapiens	236	26	98	16
Serpin H1 OS=Homo sapiens	250	15	310	36
Myosin regulatory light chain 12A OS=Homo sapiens	279	32	537	49
60S acidic ribosomal protein P0 OS=Homo sapiens	76	15	40	22
Cyclin-dependent kinase 1 OS=Homo sapiens	139	16		
Nucleophosmin OS=Homo sapiens	247	35		
Heterogeneous nuclear ribonucleoprotein M OS=Homo sapiens	345	18		
ATP-dependent RNA helicase DDX5 OS=Homo sapiens	146	9.9		
Triosephosphate isomerase OS=Homo sapiens	207	24		
Tropomyosin alpha-4 chain OS=Homo sapiens	117	14		
Clusterin OS=Homo sapiens	234	15		
Nucleoside diphosphate kinase OS=Homo sapiens	189	17		
Transgelin-2 OS=Homo sapiens	88	23		
Heterogeneous nuclear ribonucleoprotein K OS=Homo sapiens	74	10		
Protein disulfide-isomerase OS=Homo sapiens	160	17		
Receptor of-activated protein C kinase 1 OS=Homo sapiens	218	24		

Galectin-1 OS=Homo sapiens	66	19		
Reticulocalbin-1 OS=Homo sapiens	90	17		
DnaJ homolog subfamily A member 3, mitochondrial OS=Homo sapiens	70	11		
Drebrin OS=Homo sapiens	71	8.8		
Heterogeneous nuclear ribonucleoproteins C1/C2 OS=Homo sapiens	138	26		
10 kDa heat shock protein, mitochondrial OS=Homo sapiens	76	35		
Brain acid soluble protein 1 OS=Homo sapiens	52	28		
Plasminogen activator inhibitor 1 RNA-binding protein OS=Homo sapiens	87	9.3		
Heterogeneous nuclear ribonucleoprotein D0 OS=Homo sapiens	137	12		
Heterogeneous nuclear ribonucleoprotein A/B OS=Homo sapiens	110	11		
Filamin-A OS=Homo sapiens	867	15		
Tripartite motif-containing protein 47 OS=Homo sapiens			778	26
Probable ATP-dependent RNA helicase DDX41 OS=Homo sapiens			653	33
Erlin-2 OS=Homo sapiens			718	44
Erlin-1 OS=Homo sapiens			661	49
Voltage-dependent anion-selective channel protein 1 OS=Homo sapiens			545	34
Voltage-dependent anion-selective channel protein 2 OS=Homo sapiens			423	40
Phosphate carrier protein, mitochondrial OS=Homo sapiens			61	8.3
Stress-70 protein, mitochondrial OS=Homo sapiens			657	25
Mitochondrial carrier homolog 2			69	16
Transmembrane emp24 domain-containing protein 10 OS=Homo sapiens			150	22
Leucine-rich repeat-containing protein 59 OS=Homo sapiens			68	15
Surfeit locus protein 4 OS=Homo sapiens			199	15
Tropomyosin beta chain OS=Homo sapiens			237	17
Putative RNA-binding protein Luc7-like 2 OS=Homo sapiens			226	24
RNA-binding protein 39 OS=Homo sapiens			189	20
Lamin-B1 OS=Homo sapiens			139	14
Very-long-chain enoyl-CoA reductase OS=Homo sapiens			136	13
Sequestosome-1 OS=Homo sapiens			67	9.8
Calnexin OS=Homo sapiens			111	8.4
Transmembrane emp24 domain-containing protein 10 OS=Homo sapiens			150	22

4F2 cell-surface antigen heavy chain OS=Homo sapiens			60	6.5
Voltage-dependent anion-selective channel protein 3 OS=Homo sapiens			290	23
Surfeit locus protein 4 OS=Homo sapiens			199	15
Polypeptide N-acetylgalactosaminyltransferase 2 OS=Homo sapiens			69	5.1
Leucine-richrepeat-containing protein 59 OS=Homo sapiens			68	15
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit OS=Homo sapiens			101	6.4
Mitochondrial carrier homolog 2 OS=Homo sapiens			69	16
Bleomycin hydrolase OS=Homo sapiens			64	14
Serpin B3 OS=Homo sapiens			86	6.7
Desmocollin-1 OS=Homo sapiens			106	3.9

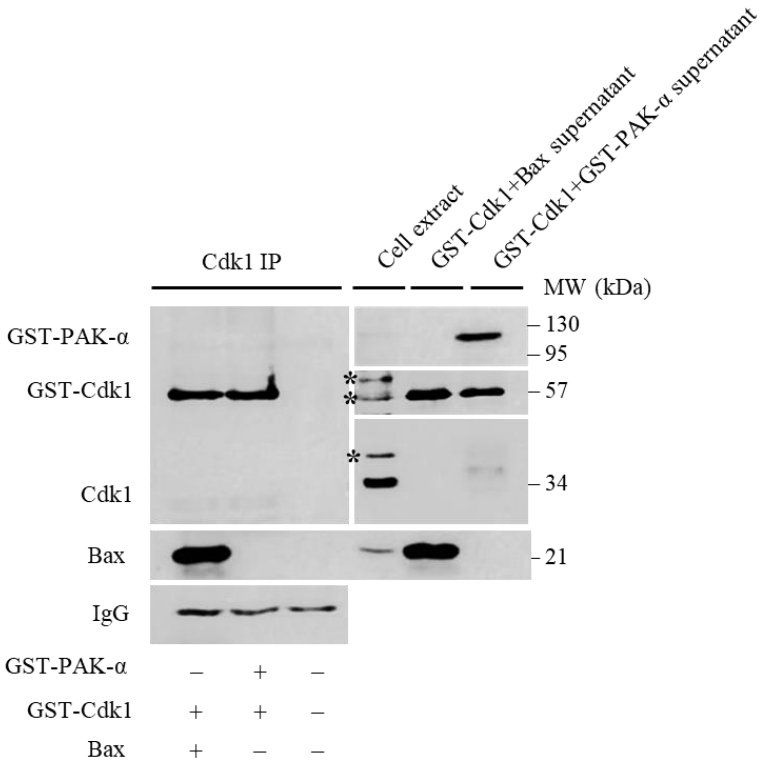
Identification of Bax and Bak interacting proteins. Bax and Bak were immunoprecipitated from synchronised HeLa cell mitotic cell lysates (60nM Taxol treatment for 24h) and the co-immunoprecipitating proteins identified by trypsin digestion and mass spectrometry (MS). Score=Mascot score, Cov= % sequence coverage.

Fig S1



Coomassie-stained SDS-PAGE gel of purified, recombinant GST-Bax, GST-Cdk1 and GST-Pakα used in the study. Molecular weight markers (kDa) are indicated on the left.

Fig S2



Immunoblot analysis of Cdk1[Cdc2p34(17)] IP's with the indicated antibodies Equimolar concentrations (50 µM) of GST-Cdk1 and Bax were incubated in 1% CHAPS lysis buffer for 2 hrs at 4 °C. GST-PAK alpha (50 µM) was incubated with GST-Cdk1 as a control. The post-IP supernatants (10 % of total volume) and cell lysate were run as positive controls. Molecular weight markers (kDa) are indicated on the right. * indicates non-specific proteins. This result is representative of 2 independent experiments.