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ERK1/2-dependent phosphorylation of Bim_{EL} promotes its rapid dissociation from Mcl-1 and Bcl-x_L

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The proapoptotic protein Bim is expressed *de novo* following withdrawal of serum survival factors. Here, we show that Bim−/− fibroblasts and epithelial cells exhibit reduced cell death following serum withdrawal in comparison with their wild-type counterparts. In viable cells, Bax associates with Bcl-2, Bcl-x_L and Mcl-1. Upon serum withdrawal, newly expressed Bim_{EL} associates with Bcl-x_L and Mcl-1, coinciding with the dissociation of Bax from these proteins. Survival factors can prevent association of Bim with pro-survival proteins by preventing Bim expression. However, we now show that even preformed Bim_{EL}/Mcl-1 and Bim_{EL}/Bcl-x_L complexes can be rapidly dissociated following activation of ERK1/2 by survival factors. The dissociation of Bim from Mcl-1 is specific for Bim_{EL} and requires ERK1/2-dependent phosphorylation of Bim_{EL} at Ser^{65}. Finally, ERK1/2-dependent dissociation of Bim_{EL} from Mcl-1 and Bcl-x_L may play a role in regulating Bim_{EL} degradation, since mutations in the Bim_{EL} BH3 domain that disrupt binding to Mcl-1 cause increased turnover of Bim_{EL}. These results provide new insights into the role of Bim in cell death and its regulation by the ERK1/2 survival pathway.

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Introduction

The pro-survival Bcl-2 proteins, such as Bcl-2 and $Bclx_L$, typically contain four Bcl-2 homology (BH) domains, while the pro-death proteins include those with the BH1, 2 and 3 domains (Bax and Bak) and the 'BH3-only proteins' (BOPs), including Bim, Hrk/DP5, Bmf, Puma, Bid and Bad (Cory and Adams, 2002). Bax and Bak proteins promote the release of apoptogenic factors from the mitochondria, but in viable cells are restrained by their binding to pro-survival Bcl-2 proteins. A variety of studies suggest that the BOPs bind to the pro-survival Bcl-2 proteins and neutralise them, thereby allowing Bax and/or Bak to initiate cell death (Puthalakath and Strasser, 2002). Bim and Puma are the most effective BOPs for cell killing, probably because they can engage with all the pro-survival Bcl-2 proteins (Chen *et al*, 2005). The different BOPs respond to different forms of cellular stress and are subject to regulation at both the transcriptional and post-translational level (Puthalakath and Strasser, 2002). For example, Puma is a transcriptional target of p53 (Nakano and Vousden, 2001), while Bad is phosphorylated and sequestered by 14-3-3 proteins so that it cannot bind to Bcl-2 proteins (Zha *et al*, 1996; Scheid *et al*, 1999; Datta *et al*, 2000).

Bim is expressed *de novo* following withdrawal of survival factors (Dijkers *et al*, 2000; Whitfield *et al*, 2001; Reginato *et al*, 2003; Weston *et al*, 2003; Wang *et al*, 2004). Bim mRNA increases due to inactivation of the protein kinase B (PKB) (Dijkers *et al*, 2000; Gilley *et al*, 2003) or ERK1/2 pathways (Weston *et al*, 2003), or due to activation of JNK (c-Jun N-terminal kinase) (Whitfield *et al*, 2001). Alternative splicing of the *Bim* gene gives rise to multiple isoforms that differ in proapoptotic potency (O'Connor *et al*, 1998) due to differences in their post-translational regulation (Ley *et al*, 2005b). Of the short, long and extra-long Bim proteins (Bim_S, Bim_L and Bim_{EL}), Bim_S is the most effective killer and consists of the pro-death BH3 domain and a C-terminal membrane-tethering domain. Bim_L and Bim_{EL} contain a dynein light chain 1 (DLC1) interacting domain, allowing their sequestration at microtubules in viable cells (Puthalakath *et al*, 1999; Lei and Davis, 2003). Bim_{EL}, the least effective cell killer, contains a unique exon that encodes an ERK1/2 docking domain (Ley *et al*, 2005a) and ERK1/2 phosphorylation sites that control the proteasomal turnover of Bim_{EL} (Ley *et al*, 2003; Luciano *et al*, 2003; Ley *et al*, 2004; Marani *et al*, 2004; Ley *et al*, 2005a).

Here, we define a new role for ERK1/2-dependent phosphorylation of Bim_{EL}. We find that Bim is required for optimal cell death upon serum withdrawal and associates with Mcl-1 and Bcl-x_L; survival factors prevent this association by preventing Bim expression. However, we now show that survival factors can also promote the rapid dissociation of preformed Bim_{EL}/Mcl-1 and Bim_{EL}/Bcl-x_L complexes by ERK1/2-dependent phosphorylation of Bim_{EL}. Dissociation is not a consequence of Bim_{EL} degradation, since proteasome inhibitors and mutations that prevent Bim_{EL} ubiquitylation do not impair dissociation of Bim_{EL} from pro-survival proteins; indeed, dissociation may contribute to Bim_{EL} degradation. Our results provide new insights into the regulation of Bim_{EL} and cell survival by the ERK1/2 pathway.

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[3]SC dedicates this study to Peter Lockyer who passed away December 28, 2006 and is sorely missed by friends and colleagues alike

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Results

Bim expression determines the rate and magnitude of cell death following serum withdrawal

CCl39 fibroblasts die by apoptosis following withdrawal of serum survival factors (Chalmers *et al*, 2003; Weston *et al*, 2003). Bim has been implicated in cell death in neurons (Whitfield *et al*, 2001), epithelial cells (Reginato *et al*, 2003; Wang *et al*, 2004) and haematopoietic cells deprived of trophic support (Bouillet *et al*, 1999). Indeed, following serum starvation of CCl39 cells we observed a rapid increase in Bim_{EL}, followed by a slower increase in expression of Bim_L, whereas the expression of Bmf and Bax did not change (Figure 1A). Of the pro-survival proteins, Bcl-2 and Bcl-x_L levels remained unchanged, but there was a gradual decrease in the expression of Mcl-1 (Figure 1A), although the kinetics and magnitude of this varied between experiments.

Death following withdrawal of cytokines is thought to proceed through the Bcl-2-inhibitable, cell intrinsic pathway. To test this, we transfected CCl39 cells with expression plasmids for Bcl-2 or Mcl-1 (or empty vector, pcD) together with a plasmid encoding EGFP-spectrin to identify transfected cells. Overexpression of Bcl-2 or Mcl-1, confirmed by Western blotting (Figure 1B), caused a reduction in cell death (Figure 1C). Thus, serum withdrawal-induced apoptosis in CCl39 cells proceeds through the cell intrinsic pathway since it is inhibited by Bcl-2 or Mcl-1. It was notable that expression of Bcl-2 or Mcl-1 did not block the increase in Bim expression under these conditions (Figure 1B). Increased Bim expression following serum withdrawal was highly reproducible and was also seen in Rat-1 cells, immortalised mouse embryo fibroblasts (iMEFs) and immortalised baby mouse kidney epithelial (iBMK) cells (Figure 1D), albeit with distinct kinetics in each cell line.

To address directly if Bim was involved in promoting cell death following serum withdrawal, we examined iMEFs derived from wild-type (WT) and Bim−/− mice (Figure 2A). Bim−/− iMEFs exhibited an approximate 90% reduction in peak caspase activation in comparison to their WT counterparts (Figure 2B). The Bim−/− iMEFs also exhibited a delay and reduction in the accumulation of dead cells with sub-G_1 DNA (Figure 2C), but this was not persistent, and Bim−/− iMEFs clearly started to die at later time points (72–96 h). Furthermore, there was no difference between WT and Bim−/− iMEFs in long-term (7 day) clonogenic survival assays (K Ewings, K Balmanno and S Cook, unpublished observations). Similar results were obtained by comparing WT and Bim−/− BMK cells (Figure 2D); the Bim−/− cells again exhibited a reduction, but not a complete inhibition, of cell death (Figure 2E). As a comparison, we confirmed that loss of Bim protected iBMK cells for up to 48 h of paclitaxel exposure (Supplementary Figure 1; Tan *et al*, 2005). In contrast, loss of Bim provided some resistance to serum withdrawal at early time points, but by 48 h there was no difference in viability between WT and Bim−/− BMK cells. These results, in two different cell systems, suggest that there is a major role for Bim in the initiation of caspase activation and cell death following serum withdrawal, but other redundant BOPs or alternative death pathways can substitute for Bim in promoting cell death at later time points.

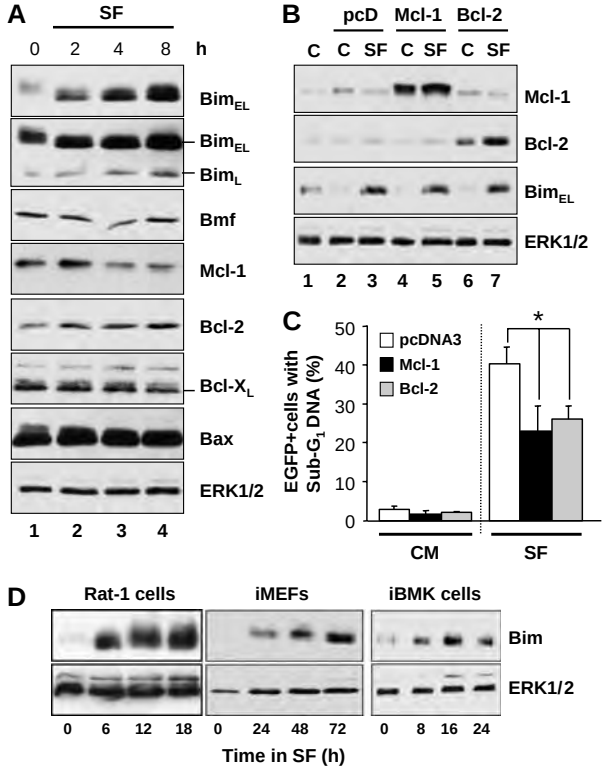

Figure 1 Expression of Bim is an early event during serum withdrawal-induced death. (**A**) CCl39 cells in complete medium (0 h) were serum starved for 2, 4 or 8 h. Cell extracts were fractionated by SDS–PAGE and immunoblotted with the indicated antibodies. The blot for Bim_{EL} (top blot) was overexposed (second blot) to reveal the expression of Bim_L. (**B, C**) Cycling CCl39 cells were transfected with EGFP-spectrin and pcDNA3, pcDNA3-Mcl-1 or pcDNA3-Bcl-2; 24 h later cells were switched to serum-free medium (SF) or fresh complete medium (C). (B) Expression of Bcl-2, Mcl-1 and Bim_{EL} was confirmed by Western blotting. (C) The percentage of EGFP-positive cells with sub-G_1 DNA was determined by flow cytometry from triplicate cell samples (mean±s.d.). The asterisk indicates expression of Bcl-2 or Mcl-1 afforded significant protection against serum withdrawal, by the Student's *t*-test ($P<0.05$). (**D**) Rat-1 cells, iMEFs or iBMK cells were serum starved as indicated and whole-cell extracts were analysed or expression of Bim and total ERK1/2 as a loading control.

Bim expressed following serum withdrawal associates with Bcl-x_L and Mcl-1 but not Bax

Bim promotes apoptosis by binding to pro-survival Bcl-2 proteins and neutralising their protective effect or, in the case of Bim_S, perhaps by binding directly to Bax (Marani *et al*, 2002). We examined which Bcl-2 proteins Bim interacted with by immunoprecipitation of Bcl-x_L or Mcl-1 from CCl39 whole cell extracts (WCE) (Figure 3A and B). Serum starvation increased Bim_{EL} expression and the newly expressed Bim_{EL} associated with Mcl-1 and Bcl-x_L (Figure 3A and B). While Bim also binds to Bcl-2 (O'Connor *et al*, 1998), we failed to identify an antibody that could immunoprecipitate Bcl-2 complexes from CCl39 cells.

During these studies we found that Bax associated with Bcl-x_L and Mcl-1 in viable cells (Figure 3A and B). Serum withdrawal (SF) caused a small decrease in the amount of Bax bound to Mcl-1 (Figure 3A), but a very pronounced dissociation of Bax from Bcl-x_L complexes, commensurate with the binding of Bim_{EL} (Figure 3B). To investigate this

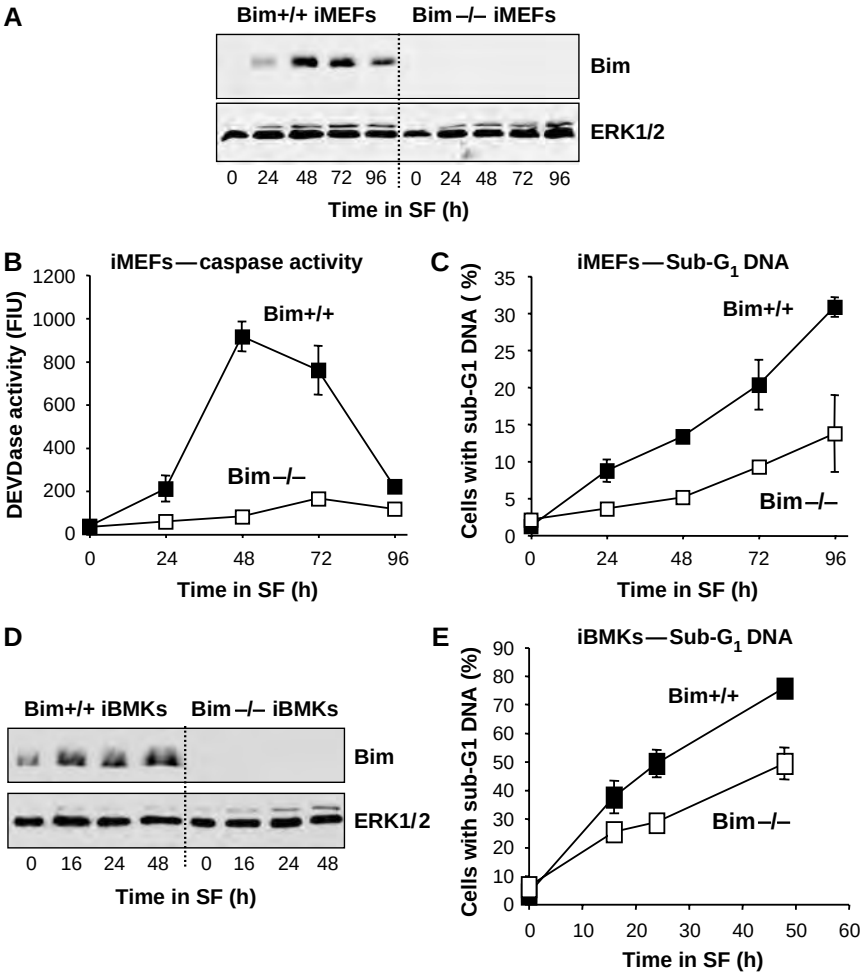

Figure 2 Bim contributes to cell death following serum withdrawal in iMEFs and iBMK epithelial cells. (**A–C**) WT or Bim−/− iMEFs were serum starved as indicated. (A) Cell extracts were immunoblotted with antibodies to Bim and ERK1/2. (B) Cell extracts were assayed for caspase activity using a DEVDase assay. (C) The percentage of cells with sub-G_1 DNA was determined by flow cytometry. (**D, E**) WT or Bim−/− iBMK cells were serum starved for the times indicated. (D) Whole-cell extracts were immunoblotted with antibodies to Bim and ERK1/2. (E) Cells were fixed, stained with PI and the percentage of cells with sub-G_1 DNA was determined by flow cytometry. In panels B, C and E each data point represents the mean ± s.d. of triplicate dishes of cells from a single experiment representative of three.

further, we immunoprecipitated Bax using the N-20 N-terminal antibody. The N-20 epitope is exposed by a conformational change in response to apoptotic insults, but the presence of Triton X-100 in the lysis buffer ensured that all the Bax had undergone this conformational change (Marani *et al*, 2002). Bax immunoprecipitates (IPs) from viable cells contained Bcl-2, Bcl-x_L and Mcl-1 but no Bim_{EL} (Figure 3C). Upon serum starvation, a treatment that results in Bim binding to pro-survival proteins (Figure 3A and B), the amount of Bcl-2, Bcl-x_L and Mcl-1 bound to Bax decreased (Figure 3C). In addition, while serum starvation increased Bim expression, we failed to detect Bim_L or Bim_{EL} in Bax IPs (Figure 3C); we have been unable to reproducibly detect the low levels of Bim_S in CCl39 cells. Interestingly, these results indicate that changes in the N-terminal conformation of Bax (monitored by the N-20 antibody) are not sufficient to cause its dissociation from pro-survival Bcl-2 proteins. These results demonstrate that following serum starvation, newly expressed Bim binds to pro-survival proteins but fails to associate with Bax; indeed, the binding of Bim to Bcl-x_L and Mcl-1 coincides with a reduction in the binding of Bax to these proteins.

Survival factors promote the rapid dissociation of Bim_{EL} from Mcl-1

Thrombin is a survival factor for CCl39 cells (Chalmers *et al*, 2003; Figure 4A). When added to cells at the time of serum withdrawal, thrombin inhibited Bim expression (Figure 4B, input WCE) and so prevented its recruitment into Mcl-1 complexes (Figure 4B, IP Mcl-1); addition of fresh FBS was equally effective. Quantification of these blots (Figure 4C) confirmed that thrombin blocked Bim expression and so prevented its binding to Mcl-1.

In the course of these studies, we noted that addition of thrombin to cells that had been serum starved for 6 h was still able to protect cells from cell death (Figure 4D). Since assembly of Bim_{EL}/Mcl-1 complexes was already apparent after 6 h of serum withdrawal (Figure 4B and E), we examined the effect of acute thrombin stimulation on the preassembled Bim_{EL}/Mcl-1 complex. CCl39 cells were serum starved for 6 h to induce the assembly of a preformed Bim_{EL}/Mcl-1 complex. Stimulation with thrombin for 15 min had no impact on the turnover of Bim_{EL} (Ley *et al*, 2003) or the total amount of Bim_{EL} in whole-cell extracts (Figure 4E, input WCE, quantified in Figure 4F), but pro-

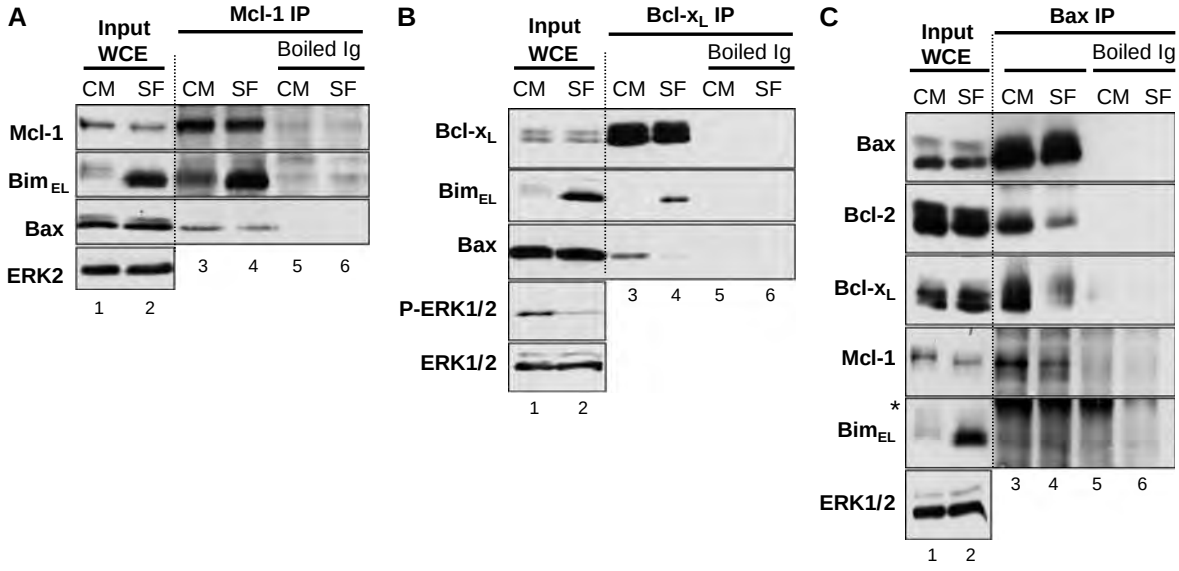

Figure 3 Newly expressed Bim_{EL} associates with the pro-survival proteins Bcl-x_L and Mcl-1 following serum withdrawal. CCl39 cells in complete medium (CM) were serum starved (SF) for 6 h. Cell extracts (input) were used for immunoprecipitation with antibodies to (**A**) Mcl-1 or (**B**) Bcl-x_L or (**C**) Bax (N-20). Antibodies denatured before immunoprecipitation (boiled Ig) served as a control. Input and IP samples were immunoblotted with the indicated antibodies. The asterisk in panel (C) indicates cross-reactivity with the antibody light chain used in the IP. Results are taken from a single experiment representative of three giving similar results.

moted the phosphorylation of Bim_{EL} (Figure 4E, input WCE) and caused a clear reduction in the amount of Bim_{EL} in Mcl-1 IPs (Figure 4E, IP Mcl-1, quantified in Figure 4F). Stimulation with FBS for 15 min was also effective at promoting the phosphorylation and rapid dissociation of Bim_{EL} from Mcl-1 (Supplementary Figure 2A). Furthermore, dissociation of Bim_{EL} from Bcl-x_L complexes was also seen in starved WT iMEFs re-stimulated with FBS, confirming that this was not unique to CCl39 cells (Supplementary Figure 2B).

Activation of ERK1/2 is necessary and sufficient to promote the dissociation of Bim_{EL} from Mcl-1 and Bcl-x_L

The ERK1/2 and PKB pathways promote cell survival and regulate Bim (Dijkers *et al*, 2000; Gilley *et al*, 2003; Weston *et al*, 2003), so we used selective inhibitors to determine which pathway was responsible for the dissociation of Bim_{EL} from Mcl-1. Stimulation of serum-starved CCl39 cells with thrombin caused dissociation of Bim_{EL} from Mcl-1 (Figure 5A, IP Mcl-1) and U0126, an inhibitor of MEK1/2, blocked ERK1/2 phosphorylation and Bim_{EL} phosphorylation (Figure 5A, input WCE) and prevented the thrombin-stimulated dissociation of Bim_{EL} from Mcl-1 (Figure 5A, IP Mcl-1). In contrast, LY294002, a PI3′-kinase inhibitor, prevented PKB phosphorylation, but did not prevent Bim_{EL} dissociation from Mcl-1 (Figure 5A).

To determine if the ERK1/2 pathway was sufficient to dissociate Bim_{EL}, we used CR1-11 cells (Weston *et al*, 2003), a clone of CCl39 cells expressing the conditional protein kinase ΔRaf-1:ER*, which selectively activates ERK1/2 in response to treatment with 4-hydroxytamoxifen (4-HT). A 1 h stimulation with 4-HT promoted ERK1/2, phosphorylation of Bim_{EL} (Figure 5B, input WCE) and resulted in dissociation of Bim_{EL} from preformed Bim_{EL}/Mcl-1 complexes (Figure 5B, IP Mcl-1), although the total amount of Bim in whole-cell extracts was not affected (Figure 5B, input WCE). All these effects were reversed by treatment with U0126. ΔRaf-1:ER* also promoted the ERK1/2-dependent dissociation of preformed Bim_{EL}/Bcl-x_L complexes (Supplementary Figure 2C). Thus, activation of the ERK1/2 pathway is necessary and sufficient to promote dissociation of endogenous Bim_{EL} from endogenous Mcl-1 or Bcl-x_L, two major pro-survival proteins.

ERK1/2-dependent dissociation of Bim_{EL}/Mcl-1 complexes is not a consequence of Bim_{EL} degradation

Phosphorylation of Bim_{EL} by ERK1/2 promotes its proteasome-dependent destruction (Ley *et al*, 2003), so it was important to establish the relationship between dissociation and destruction. Dissociation of Bim_{EL} from Mcl-1 was readily apparent within 15 min of FBS stimulation (Supplementary Figure 2A), whereas emetine chase experiments revealed that significant turnover of endogenous Bim_{EL} only occurred between 20 min and 1 h (Supplementary Figure 3A and B). However, to be sure, we devised a series of experiments to determine whether the dissociation of Bim_{EL} from Mcl-1 was simply a consequence of ERK1/2-dependent Bim_{EL} destruction. First, CR1-11 cells were serum starved for 6 h to assemble the Bim_{EL}/Mcl-1 complex and then re-stimulated with 4-HT in the presence of the proteasome inhibitor MG132. Following activation of ΔRaf-1:ER*, phosphorylated forms of Bim_{EL} accumulated due to the presence of MG132, and were detected in the whole-cell extracts (Figure 6A, input WCE). This phosphorylation of Bim_{EL} caused a 60–70% reduction in the amount of Bim_{EL} associated with Mcl-1 (Figure 6A and B), similar to that observed in response to thrombin (Figure 4F); dissociation of Bim_{EL} was apparent within 30 min, maximal by 1 h and persisted for as long as ΔRaf-1:ER* remained active. These results, using a pharmacological inhibitor of the proteasome, show that dissociation of Bim_{EL} takes place even when total Bim_{EL} levels are constant and so is not a consequence of it being degraded by the proteasome. In addition, we observed that a mutant

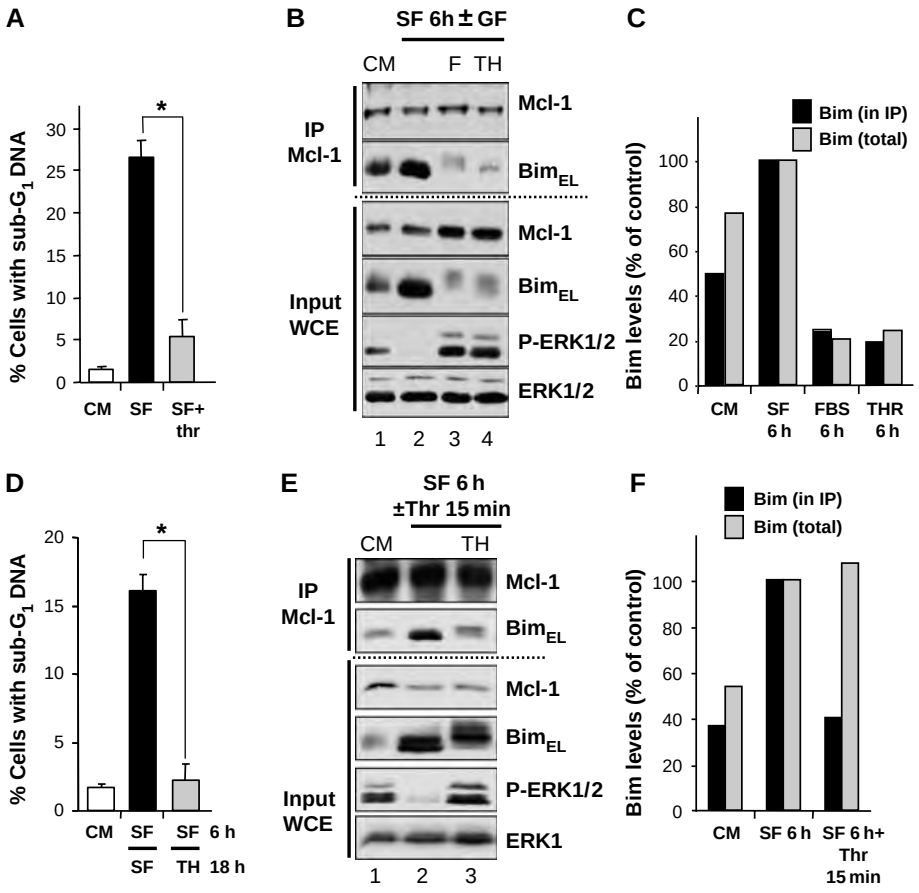

Figure 4 Survival factors block Bim expression but can also promote the dissociation of preformed Bim/Mcl-1 complexes. (**A–C**) CCl39 cells were subjected to the following condition: serum starvation ± 10 nM thrombin, which was added at the time of serum withdrawal. (A) Thrombin protected against serum withdrawal-induced cell death (*$P<0.01$), as judged by cells with sub-G_1 DNA after 24 h. (B) Cell extracts (input) were prepared after 6 h and used for immunoprecipitation of Mcl-1 and samples were immunoblotted with antibodies to Mcl-1, Bim, P-ERK1/2 and ERK1/2. (C) The amount of Bim in the input (total) and the Mcl-1 IP was quantified by densitometry. (**D–F**) CCl39 cells were serum starved for 6 h to allow the expression of Bim and the assembly of Bim_{EL}/Mcl-1 complexes. (D) Cells were re-stimulated with 10 nM thrombin for a further 18 h and cell death was assayed by the accumulation of cells with sub-G_1 DNA. (E) Cells were re-stimulated with 10 nM thrombin for 15 min. Whole-cell extracts (input) were used for immunoprecipitation of Mcl-1 and samples were immunoblotted with the indicated antibodies. (F) The amount of Bim in the input WCE (total) and the Mcl-1 IP was quantified by densitometry and revealed that a 15 min treatment with thrombin promoted the dissociation of Bim_{EL}/Mcl-1 complexes without reducing total Bim_{EL} levels.

version of Bim_{EL}, in which both lysines were mutated to arginine (KK/RR), dissociated from Mcl-1 to the same degree as WT Bim_{EL} following activation of ΔRaf-1:ER* in HR1 cells (HEK293 cells expressing ΔRaf-1:ER*; Boughan *et al*, 2006) (Figure 6C). Since this mutant cannot be ubiquitylated (Akiyama *et al*, 2003), this provides further molecular genetic evidence that ERK1/2-dependent dissociation of Bim_{EL} was not a consequence of its ubiquitylation or proteasomal degradation. Finally, growth factor-dependent dissociation of Bim_{EL} from Mcl-1 proceeded in the presence of cycloheximide and MG132, indicating that ERK1/2 was promoting Bim_{EL} dissociation from pre-existing complexes rather than reducing the stability of newly expressed Bim_{EL} before it has a chance to bind (Supplementary Figure 3C). Together, these results indicate that dissociation of Bim_{EL} from pro-survival proteins does not result from its destruction.

We also performed the reciprocal experiment by examining the kinetics of Bim_{EL}/Mcl-1 association following inhibition of the ERK1/2 pathway. CCl39 cells growing in complete medium (10% FBS) were serum starved in the presence of the ERK1/2 pathway-selective inhibitor PD184352 for between 20 and 120 min. This treatment led to the immediate inactivation of ERK1/2, dephosphorylation of Bim_{EL} and an increase in the amount of Bim_{EL} bound to Mcl-1 (Figure 6D). Significantly, while serum withdrawal (with or without ERK1/2 inhibition) can increase Bim_{EL} expression, this is not apparent until approximately 2 h (Ley *et al*, 2003; Weston *et al*, 2003), whereas the increase in association of Bim_{EL} with Mcl-1 was apparent within 20 min, a time point at which total Bim_{EL} and Mcl-1 levels were unchanged (Figure 6D). These results indicate that inhibition of ERK1/2 causes the rapid dephosphorylation of Bim_{EL} and its association with its pro-survival target proteins.

Bim_{EL} dissociation is reversible upon inhibition of the ERK1/2 pathway

When degradation of Bim_{EL} was blocked, we found that the dissociation of Bim_{EL} from Mcl-1 was readily reversible. For example, when serum-starved CR1-11 cells were stimulated with 4-HT for 2 or 4 h in the presence of MG132 to prevent Bim_{EL} degradation, we observed a 70% reduction in the amount of Bim_{EL} bound to Mcl-1, even though total Bim_{EL}

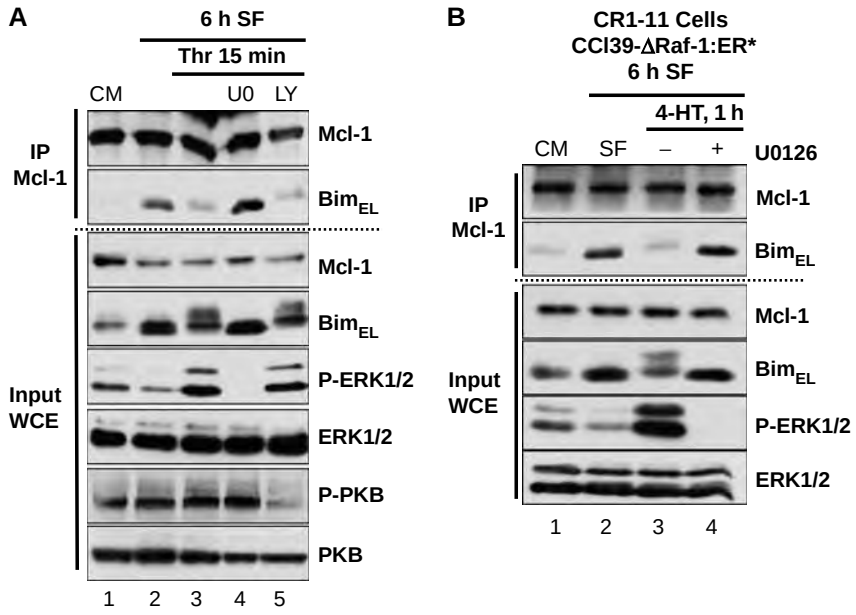

Figure 5 Activation of ERK1/2 pathway is necessary and sufficient to promote the release of Bim_{EL} from Mcl-1. (**A**) Cycling CCl39 cells (CM) were serum starved (SF) for 6 h to induce formation of Bim_{EL}/Mcl-1 complexes. Cells were then stimulated for 15 min with 10 nM thrombin, with or without 20 μM U0126 or 30 μM LY294002. Whole-cell extracts (input) were used for immunoprecipitation of Mcl-1 and samples were immunoblotted with the indicated antibodies. (**B**) CR1-11 cells (CCl39 cells expressing ΔRaf-1:ER*) in complete medium (CM) were serum starved (SF) for 6 h to induce formation of Bim_{EL}/Mcl-1 complexes. Cells were then stimulated for 1 h with 100 nM 4-HT ± 20 μM U0126. Whole-cell extracts (input) were used for immunoprecipitation of Mcl-1 and samples were then immunoblotted with the indicated antibodies. Results are taken from a single experiment representative of three.

levels did not change (Supplementary Figure 4A and B). In contrast, if cells were treated with 4-HT for 2 h to phosphorylate and dissociate Bim_{EL} and then treated with U0126 for a further 2 h to inhibit ERK1/2, Bim_{EL} was dephosphorylated and a significant proportion was found reassociated with Mcl-1 (Supplementary Figure 4A and B). A potential explanation for this came when we subjected these cell extracts to a crude subcellular fractionation. Regardless of its phosphorylation status, Bim_{EL} remained in the same COX-IV containing heavy membrane fraction as Mcl-1 rather than being released into the cytosol (Supplementary Figure 4C). These results suggest that when the proteasome is inhibited, both Bim_{EL} and Mcl-1 are retained on the mitochondria following their dissociation and so can readily reassociate when Bim_{EL} is dephosphorylated following ERK1/2 inhibition. This may explain why we have been unable to visualise the dissociation of Bim_{EL} from Mcl-1 using immunofluorescence microscopy. It also demonstrates the dynamic and reversible nature of ERK1/2-dependent Bim_{EL} dissociation.

ERK1/2-dependent phosphorylation of Bim_{EL} is required for dissociation of the Bim/Mcl-1 and Bim/Bcl-x_L complex

The dissociation of Bim_{EL} from Mcl-1 could be due to ERK1/2-dependent phosphorylation of Bim_{EL} (Luciano *et al*, 2003; Ley *et al*, 2004; Marani *et al*, 2004), Mcl-1 (Domina *et al*, 2004) or both. To address this, we expressed FLAG-tagged Puma (Nakano and Vousden, 2001) in HR1 cells and examined its binding to Mcl-1. Puma bound to endogenous Mcl-1 but was not phosphorylated and did not dissociate following activation of ΔRaf-1:ER* (Supplementary Figure 5). Furthermore, while Bim_S, Bim_L and Bim_{EL} (Figure 7A) were all able to bind to Mcl-1 in HR1 cells (Figure 7B), only HA-Bim_{EL} dissociated from Mcl-1 following activation of ΔRaf-1:ER* (Figure 7B, IP Mcl-1). Thus, ERK1/2 can promote the dissociation of Bim_{EL}/Mcl-1 and Bim_{EL}/Bcl-x_L complexes but not Puma/Mcl-1 complexes; this is a property unique to Bim_{EL} of the three canonical Bim splice variants and is observed in HEK293 cells, CCl39 cells and iMEFs. We have also seen similar effects in human colorectal cancer cells (J Wickenden and S Cook, unpublished observations), so this is a widespread mode of regulation.

Phosphorylation of Bim_{EL} at Ser^{65} is required for dissociation from Mcl-1

Bim_{EL}, but not Bim_S or Bim_L, is a substrate for ERK1/2, a proline-directed protein kinase. Bim_{EL} possesses six Ser–Pro or Thr–Pro motifs (p1–p6 in Figure 7A), one of which (Ser^{65}, p2) is an *in vitro* and *in vivo* ERK1/2 phosphorylation site (Luciano *et al*, 2003; Ley *et al*, 2004; Marani *et al*, 2004). To determine the role of proline-directed phosphorylation of Bim_{EL}, we analysed a mutant (HA-Bim_{EL}6Ala) in which all six Ser–Pro or Thr–Pro motifs had been mutated to non-phosphorylatable residues. As a control, we observed that Bim_{EL} with three mutations in the BH3 domain (HA-Bim_{EL}ΔBH3) exhibited greatly reduced binding to Mcl-1 in HR1 cells, despite expressing at the highest level of all three constructs (Figure 7C). HA-Bim_{EL}6Ala expressed at much higher levels than HA-Bim_{EL} (Figure 7C, input WCE), but was not phosphorylated and did not dissociate in response to ERK1/2 activation, whereas HA-Bim_{EL} did (Figure 7C, Mcl-1 IP), demonstrating that phosphorylation of proline-directed sites on Bim_{EL} is required for its ERK1/2-dependent dissociation from Mcl-1.

Since phosphorylation-dependent dissociation from Mcl-1 was only observed for Bim_{EL}, we focused on the three sites

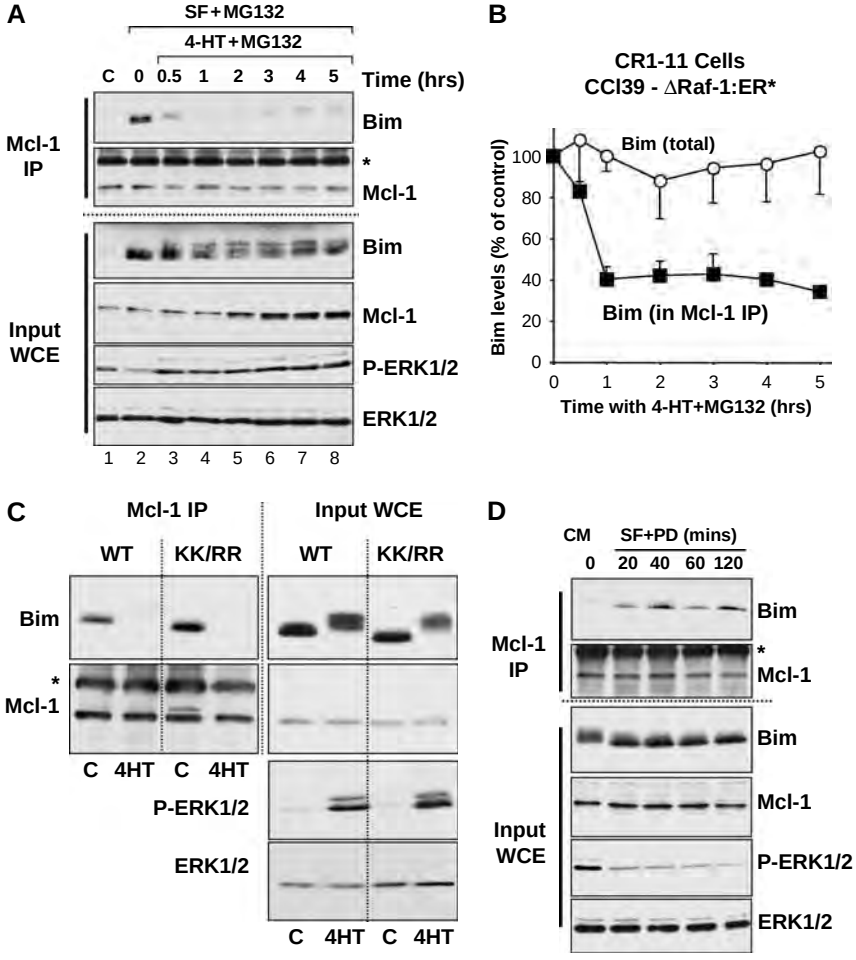

Figure 6 ERK1/2-dependent dissociation of Bim_{EL}/Mcl-1 complexes is not a consequence of Bim_{EL} degradation. (**A, B**) CR1-11 cells were serum starved to induce the assembly of Bim_{EL}/Mcl-1 complexes. Cells were then re-stimulated with 100 nM 4-HT in the presence of 10 μM MG132, as indicated. Whole-cell extracts (input) were prepared, used for immunoprecipitation of Mcl-1 and samples were then immunoblotted with the indicated antibodies. (A) Results from a single experiment representative of three yielding identical results. (B) Total and Mcl-1-associated Bim_{EL} were quantified from live ECL images; data represent the mean ± s.d. from three independent experiments. (**C**) HR1 cells (HEK293 cells expressing ΔRaf-1:ER*) were transfected with wild-type HA-BimEL (WT) or a mutant in which both lysines had been mutated to arginine (KK/RR). Cells were stimulated with 4-HT for 1 h. Whole-cell extracts (input) were used for immunoprecipitation of Mcl-1 and samples were then immunoblotted with the indicated antibodies. (**D**) CCl39 cells maintained in 10% FBS were serum starved in the presence of 5 μM PD184352 for the times indicated. Whole-cell extracts (input) were used for immunoprecipitation of Mcl-1 and samples were then immunoblotted with the indicated antibodies. The asterisk in panels (A, C, D) indicates cross-reactivity with the antibody light chain used in the IP.

(p1–p3) in exon 3 by analysing the effect of individual point mutations in the p1 (Ser^{55}Ala), p2 (Ser^{65}Ala) or p3 (Ser^{73}Ala) sites. All mutants associated with Mcl-1 normally in HR1 cells and the HA-Bim_{EL} protein was phosphorylated and dissociated from Mcl-1 following activation of the ERK1/2 pathway (Figure 8A). Mutation of the p1 (Ser^{55}) or p3 (Ser^{73}) sites had a partial effect on the ERK1/2-dependent phosphorylation and dissociation of Bim_{EL}. In contrast, mutation of the p2 site (Ser^{65}) strongly inhibited phosphorylation of Bim_{EL} and completely prevented its ERK1/2-dependent dissociation from Mcl-1. Mutation of the proline-directed sites (p4–p6) had no effect on the dissociation of Bim_{EL} from Mcl-1 (K Ewings and S Cook, unpublished observations), consistent with the fact that Bim_L did not dissociate upon ERK1/2 activation (Figure 7B). These results demonstrate that phosphorylation of Bim_{EL} at Ser^{65} is absolutely required for ERK1/2-dependent dissociation of Bim_{EL} from Mcl-1.

A logical prediction of these experiments is that phosphomimetic forms of Bim_{EL}, in which the p2 or p1–p3 sites are mutated to acidic residues (Asp or Glu), should bind poorly to Mcl-1. However, we found that the binding of such mutant forms of Bim_{EL} to Mcl-1 was highly variable so that it was not possible to draw any reliable conclusions. It would appear that acidic substitutions at Ser^{65}, either alone or in combination with mutation of Ser^{55} and Ser^{73}, cannot faithfully mimic the phosphorylation events *in vivo*. As an alternative, we examined the distribution of Bim_{EL} phosphorylated at Ser^{65} using a phospho-Ser^{65}-specific antibody. HR1 cells were transfected with HA-Bim_{EL} and then serum starved before stimulating with 4-HT in the presence or absence of U0126. Extracts were divided and either Mcl-1 or HA-Bim_{EL} was immunoprecipitated. Both the HA-Bim_{EL} and the endogenous Bim_{EL} underwent an ERK1/2-dependent phosphorylation (Figure 8B, input WCE) and dissociated from Mcl-1 (Figure 8B, Mcl-1 IP). We were unable to detect phospho-Ser^{65} Bim_{EL} in whole-cell extracts, but the antibody worked well when we immunoprecipitated HA-Bim_{EL} from these cell extracts; this allowed us to demonstrate 4-HT-dependent

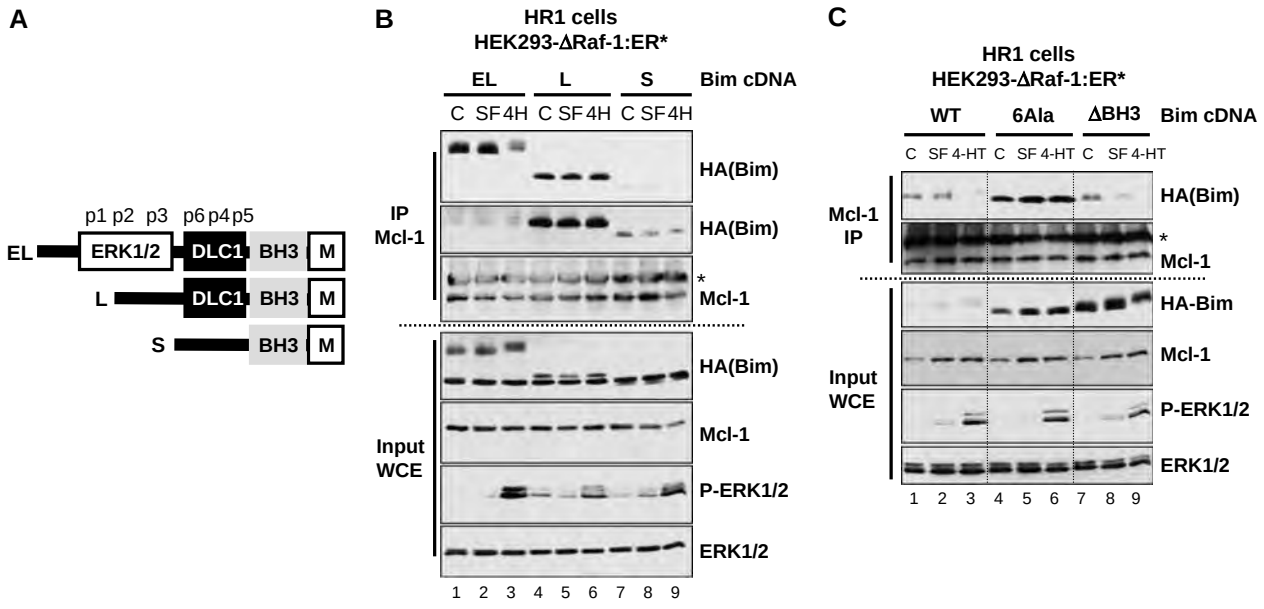

Figure 7 ERK1/2-dependent regulation of Bim_{EL}/Mcl-1 interactions is unique to the Bim_{EL} isoform. (**A**) Diagram of the structure of Bim_S, Bim_L and Bim_{EL} indicating the six proline-directed phosphorylation sites (p1–p6), the ERK1/2 docking domain and phosphorylation sites, the DLC1 binding site, BH3 domain and membrane binding motif. (**B**) HR1 cells were transfected with HA-Bim_{EL} (EL), HA-Bim_L (L) or HA-Bim_S (S). After 24 h, cells were serum starved (SF) for 6 h. Finally, one set of cells in serum-free medium was stimulated with 100 nM 4-HT for 15 min to activate ERK1/2 (4H). Cell extracts (input) were used to immunoprecipitate Mcl-1 and samples were subjected to immunoblotting. The anti-HA blot showing the binding of Bim_{EL} and Bim_L to Mcl-1 (top panel) has also been overexposed to reveal the binding of Bim_S (second panel from top). (**C**) HR1 cells were transfected with HA-Bim_{EL} (WT), HA-Bim_{EL}6Ala (6Ala) or HA-Bim_{EL}ΔBH3 (ΔBH3) in complete medium (CM). After 24 h, cells were serum starved (SF) for 6 h. Finally, one set of cells was stimulated with 100 nM 4-HT for 15 min to activate ERK1/2. Whole-cell extracts (input WCE) were used for immunoprecipitation of Mcl-1 and samples were then immunoblotted with antibodies to Mcl-1, HA(Bim), P-ERK1/2 and ERK1/2. The asterisk in panels (B, C) indicates cross-reactivity with the antibody light chain used in the IP.

increases in phospho-Ser^{65} Bim_{EL}, which were inhibited by U0126 treatment, confirming that Ser^{65} is a target for ERK1/2 *in vivo* (Figure 8B, HA IP). Significantly, while we could readily detect 4-HT-stimulated, ERK1/2-dependent phosphorylation of HA-Bim_{EL} at Ser^{65}, we always failed to detect any phospho-Ser^{65} Bim_{EL} in Mcl-1 IPs; the appearance of phospho-Ser^{65} Bim_{EL} always correlated with the dissociation of Bim_{EL} from Mcl-1 (Figure 8B, HA IP versus Mcl-1 IP). This result alone does not prove that Ser^{65} phosphorylation is sufficient for dissociation of Bim_{EL} from Mcl-1; however, together with Figure 8A in which Ser^{65} phosphorylation is shown to be required for dissociation from Mcl-1, our results suggest that phosphorylation of Bim_{EL} at Ser^{65} is not compatible with binding to Mcl-1.

Bim_{EL} mutants that fail to bind to pro-survival proteins exhibit accelerated turnover

Finally, we investigated whether the dissociation of Bim_{EL} was linked to its proteasomal degradation. For example, although phosphorylation might be the direct signal for degradation of Bim_{EL}, an alternative possibility was that Bim_{EL} that was not bound to a pro-survival Bcl-2 protein was the primary target for degradation, with phosphorylation simply serving to 'liberate' Bim_{EL} from Mcl-1 or Bcl-x_L. This possibility was further supported by our observation that phosphorylation of Ser^{65}, a site required for ERK1/2-dependent degradation of Bim_{EL} (Ley *et al*, 2004; Luciano *et al*, 2003), was also required for its dissociation from Mcl-1 (Figure 8). A logical prediction from this model is that a Bim_{EL} mutant defective for binding to pro-survival Bcl-2 proteins should exhibit a shorter half-life than the WT protein, so we compared the half-life of WT HA-Bim_{EL} with that of the HA-Bim_{EL}ΔBH3 mutant, which exhibits greatly reduced binding to pro-survival Bcl-2 proteins including Mcl-1 (Figure 7C). These constructs were expressed in HEK293 cells, which were then serum starved and subjected to an emetine chase protocol to examine their turnover. Immunoblotting for total ERK1/2, a relatively stable protein under these conditions, confirmed equal loading. Under serum-free conditions, HA-Bim_{EL} was stable in the presence of emetine for up to 12 h, whereas the HA-Bim_{EL}ΔBH3 mutant clearly turned over in the presence of emetine (Figure 9). A trivial explanation for the enhanced turnover of the HA-Bim_{EL}ΔBH3 protein was that mutations in the BH3 domain of Bim_{EL} compromised protein folding, so that the mutant protein did not express well. However, we reproducibly observed that HA-Bim_{EL}ΔBH3 actually expressed at considerably higher levels than the WT HA-Bim_{EL} (Figures 7C and 9). Furthermore, HA-Bim_{EL}ΔBH3 was still able to undergo ERK1/2-dependent phosphorylation (Figure 7C), suggesting that the mutant protein was folding normally. These results indicate that dissociation of Bim_{EL} from pro-survival proteins is sufficient to promote its degradation.

Discussion

Bim is involved in promoting cell death following serum withdrawal

The initiation of CCl39 cell death following serum withdrawal is a caspase-dependent process that requires new gene expression (Chalmers *et al*, 2003; Weston *et al*, 2003) and proceeds through the mitochondrial death pathway

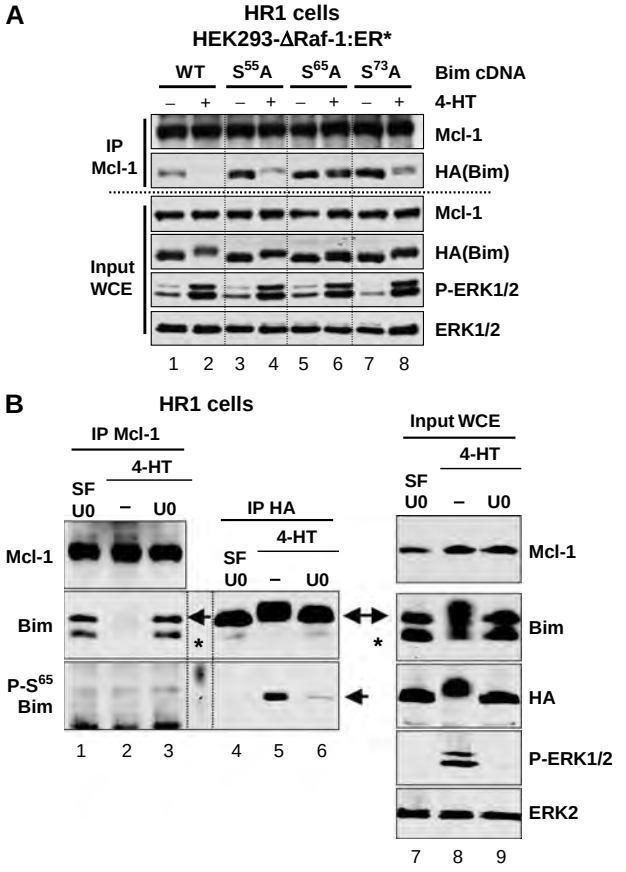

Figure 8 ERK1/2-dependent phosphorylation of Bim_{EL} at Ser^{65} is required for dissociation of Bim_{EL} from Mcl-1. (**A**) HR1 cells were transfected with plasmids encoding HA-Bim_{EL} (WT), or HA-Bim_{EL} with individual point mutations at Ser^{55}Ala, Ser^{65}Ala or Ser^{73}Ala. After 24 h, cells were serum starved for 6 h before treating for a further 15 min with 100 nM 4-HT to activate ERK1/2. Whole-cell extracts (input WCE) were used for immunoprecipitation of Mcl-1 and samples were then immunoblotted with antibodies to Mcl-1, HA(Bim), P-ERK1/2 and ERK1/2. (**B**) HR1 cells were transfected with HA-Bim_{EL}. Twenty-four hours later, cells were serum starved for 1 h in the presence of 20 μM U0126 to inactivate ERK1/2. Cells were then washed thoroughly and stimulated with 100 nM 4-HT ± 20 μM U0126 for 1 h. Whole-cell extracts (input WCE) were used to immunoprecipitate Mcl-1 or HA-Bim_{EL}. Samples were then immunoblotted with antibodies to Mcl-1, HA(Bim), Bim, Phospho-Ser^{65}-Bim, P-ERK1/2 and ERK1/2. The arrowheads indicate the position of the HA-Bim_{EL}, while the asterisk indicates the position of the endogenous Bim_{EL}. Note that ΔRaf-1:ER* induces phosphorylation of Bim_{EL} at Ser^{65}, but phospho-Ser^{65}-Bim_{EL} is not detected in Mcl-1 IPs.

(Figure 1). The increase in expression of Bim in CCl39 cells, Rat-1 cells, iMEFs and iBMKs following serum withdrawal implied a potential role for Bim in promoting cell death. By comparing WT and Bim−/− iMEFs and iBMK epithelial cells we have now demonstrated that Bim expression is rate-determining for cell death following serum withdrawal; indeed, Bim appears to play a major role in initiating caspase activation in iMEFs following serum withdrawal. Taken together with other recent studies, it appears that the *de novo* expression of Bim is involved in initiating apoptosis following withdrawal of trophic support in a variety of cell types (Bouillet *et al*, 1999; Dijkers *et al*, 2000; Whitfield *et al*, 2001; Reginato *et al*, 2003; Wang *et al*, 2004). However, in iMEFs and especially iBMKs, the protection afforded by loss

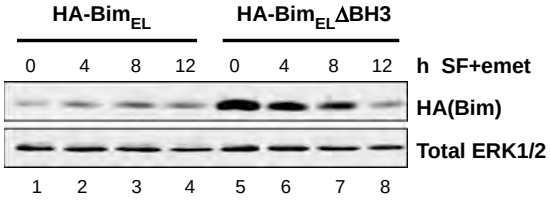

Figure 9 Dissociation from pro-survival proteins is sufficient to promote turnover of Bim_{EL}. HEK293 cells were transfected with HA-Bim_{EL} or HA-Bim_{EL}ΔBH3. After 24 h, cells were serum starved in the presence of 10 μM emetine for 4, 8 or 12 h. Whole-cell extracts were subjected to SDS–PAGE and immunoblotted with antibodies to HA(Bim) and total ERK1/2.

of Bim was transient; this is consistent with the role of Bim in promoting apoptosis being dependent on the death stimulus, tissue type and the presence of other redundant BOPs. A likely alternative to Bim in these studies is Bad, which is normally negatively regulated by RSK- and PKB-dependent phosphorylation (Datta *et al*, 1997, 2000; Bonni *et al*, 1999; Lizcano *et al*, 2000). While Bad−/− MEFs are not protected against serum withdrawal-induced death (Ranger *et al*, 2003), presumably reflecting the prominent role for Bim in MEFs (Figure 2), Bad−/− mammary epithelial cells are protected against withdrawal of EGF (Ranger *et al*, 2003). Similarly, loss of Bim only affords partial protection against NGF withdrawal in neurons (Whitfield *et al*, 2001) and this may reflect a role for Hrk/DP5 (Harris and Johnson, 2001). Alternatively, while Bim may regulate a classical caspase-dependent apoptosis at early times following serum withdrawal, other caspase-independent pathways (Chipuk and Green, 2005) may ensure cell death in the long term.

Newly expressed Bim associates with pro-survival proteins following serum withdrawal

In viable CCl39 cells maintained in complete medium, Bax associated with Bcl-2, Bcl-x_L and Mcl-1. Following serum withdrawal, newly expressed Bim_{EL} associated with Bcl-x_L and Mcl-1, and this was accompanied by the dissociation of Bax from the pro-survival proteins, especially Bcl-x_L. These results fit well with the features of the 'displacement' model (Willis and Adams, 2005), since we failed to observe an association between Bax and Bim_{EL} (or Bim_L). However, it is important to point out that only Bim_S has been proposed to bind directly to Bax (Marani *et al*, 2002), and we struggled to detect the very low levels of Bim_S in CCl39 cells. Furthermore, we cannot rule out the possibility that the Bax N-20 antibody disrupts interactions with Bim_{EL}, although this seems unlikely since Bax binding to Bcl-x_L or Mcl-1 was readily detectable in N-20 immune complexes.

Exposure of an occluded N-terminal region in Bax, including the N-20 epitope, occurs in response to stress but may not be sufficient for commitment to cell death (Makin *et al*, 2001). Subsequent changes towards the C-terminus occur commensurate with dissociation of Bax from pro-survival Bcl-2 proteins and may be more relevant to the initiation of cell death. A similar multistep process seems to operate for Bak activation (Griffiths *et al*, 2001). We previously showed that serum withdrawal caused the initial N-terminal change in Bax (Weston *et al*, 2003). Since this change is reversible (Makin *et al*, 2001), we studied changes in the repertoire of Bax complexes associated with the later conformational change(s) by

using the N-20 antibody to immunoprecipitate Bax from Triton X-100 cell lysates, thereby focusing on the Bax that had already undergone the initial N-terminal conformational change. We could readily detect complexes between N-20-reactive Bax and all three pro-survival proteins (Bcl-2, Bcl-x_L and Mcl-1) in viable cells and similar results have been reported in viable cells lysed in the presence of CHAPS (Gomez-Bougie *et al*, 2005). Thus, while changes at the N-terminus of Bax may be stress responsive (Makin *et al*, 2001; Marani *et al*, 2002; Weston *et al*, 2003), they are not sufficient to release Bax from pro-survival proteins. Rather, even when the N-terminus is exposed, serum starvation provides an additional signal to promote release of Bax; our results are consistent with Bim providing this additional signal to displace Bax from pro-survival proteins following serum withdrawal. The mechanism by which serum starvation induces early changes at the N-terminus and their precise role remains to be defined.

ERK1/2-dependent phosphorylation of BimEL at Ser^{65} is required for dissociation of BimEL from pro-survival Bcl-2 proteins

Survival factors such as thrombin can prevent the assembly of Bim_{EL}/Bcl-x_L or Bim_{EL}/Mcl-1 complexes by preventing Bim expression (Figure 4A–C). However, we have now shown that the association between Bim_{EL}/Bcl-x_L or Bim_{EL}/Mcl-1 is actually subject to dynamic, post-translational regulation by survival factors. Specifically, even an acute, 15 min stimulation with thrombin or FBS promoted the dissociation of preassembled Bim_{EL}/Mcl-1 complexes (Figure 4E and F). Since the total expression level of Bim_{EL} or Mcl-1 did not change over these short time points, the dissociation of the Bim_{EL}/Mcl-1 complex could not be explained by turnover of Bim_{EL}. Indeed, dissociation of Bim_{EL}/Mcl-1 proceeded in the presence of the proteasome inhibitor MG132 (Figure 6A and B) and a Bim_{EL} mutant that could not be ubiquitylated (Akiyama *et al*, 2003) underwent ERK1/2-dependent dissociation normally (Figure 6C); thus, dissociation of Bim_{EL} is not a consequence of its proteasome-dependent degradation. We also observed that inhibition of ERK1/2 for as little as 20 min was sufficient to promote the binding of Bim_{EL} to Mcl-1 without changing total Bim_{EL} or Mcl-1 expression (Figure 6D). Since Bim must interact with pro-survival proteins to promote cell death (O'Connor *et al*, 1998; Willis *et al*, 2007), these results describe a completely novel mechanism by which ERK1/2 can prevent Bim_{EL}-dependent cell death; namely, by reducing the binding of Bim_{EL} to its target pro-survival proteins. While it has previously been shown that preassembled Bad/Bcl-x_L complexes can be dissociated by phosphorylation of Bad (Scheid *et al*, 1999), this is the first such demonstration for Bim, or other BOPs, and may explain reports that ERK1/2 can inhibit the activity of Bim_{EL}, without apparent changes in its abundance (Wang *et al*, 2004; Reginato *et al*, 2005).

ERK1/2-dependent dissociation was unique to Bim_{EL}, consistent with exon 3 (unique to Bim_{EL}) harbouring the ERK1/2 docking domain and phosphorylation sites (Ley *et al*, 2005b). Furthermore, two lines of evidence strongly suggest that Ser^{65} (Ser^{69} in human Bim_{EL}) is the major site controlling dissociation. First, a Ser^{65}Ala Bim_{EL} mutant failed to dissociate, indicating that phosphorylation at Ser^{65} is absolutely required; mutation of the other ERK1/2 sites had only a moderate effect. Second, while we could detect ERK1/2-dependent phosphorylation of Bim_{EL} at Ser^{65} using a phospho-specific antibody, we could not detect phospho-Ser^{65} Bim_{EL} bound to Mcl-1, suggesting that phosphorylation at this site is not compatible with binding to Mcl-1. It is unclear if phosphorylation at Ser^{65} is sufficient to promote dissociation and so-called phospho-mimetic acidic substitutions at this site were not informative. Ser^{65} is outside the BH3 domain, so phosphorylation may not interfere with binding to pro-survival proteins by simple charge repulsion; indeed, access to the Bim BH3 domain might be expected to be limited in a preassembled Bim_{EL}/Mcl-1 complex. Alternatively, phosphorylation may promote binding of accessory proteins that facilitate dissociation of Bim_{EL}, as is seen with phosphorylation-dependent dissociation of Bad/Bcl-x_L complexes, which requires 14-3-3 (Datta *et al*, 1997, 2000; Bonni *et al*, 1999; Lizcano *et al*, 2000); in this case, simple acidic substitutions may fail to faithfully mimic phosphorylation-dependent interactions with such proteins. Dissociation of Bim_{EL} from Mcl-1 may require multiple phosphorylation events. In addition to Ser^{65} (so-called p2 site), two additional sites are phosphorylated in an ERK1/2-dependent manner *in vivo*, and one of these requires prior phosphorylation at Ser^{65} (Ley *et al*, 2004). Ser^{55} (p1) and Ser^{73} (p3) are likely sites, since their mutation reduces the ERK1/2-dependent mobility shift of Bim_{EL} and partially reverses its dissociation from Mcl-1 (Figure 8A).

Phosphorylation-dependent dissociation of Bim_{EL} from Bcl-x_L and Mcl-1 may be linked to its degradation. For example, while WT Bim_{EL} could bind to Mcl-1 and Bcl-x_L, and was stable under serum-free conditions, mutation of the Bim_{EL} BH3 domain greatly reduced interactions with pro-survival proteins and promoted Bim_{EL} degradation under the same conditions. These results are consistent with a model in which in serum-starved cells, Bim_{EL} is dephosphorylated, associates with pro-survival proteins, is stabilised and promotes cell death, whereas following activation of ERK1/2, Bim_{EL} is phosphorylated, dissociates from pro-survival proteins and in this unbound state is targeted for degradation. A recent study has provided some support for such a model by showing that Bim_{EL} belongs to the class of intrinsically unstructured proteins (Hinds *et al*, 2006); such proteins are extremely sensitive to proteolysis, are more efficiently processed by the proteasome and may be stabilised by interactions with binding partners (Dyson and Wright, 2005). Thus, it may not be ERK1/2-dependent phosphorylation *per se* that is the signal for degradation but rather the release of Bim_{EL} from pro-survival proteins that enhances its turnover, with ERK1/2 serving simply to promote this dissociation. One potential advantage of linking Bim_{EL} dissociation to its degradation is that it may prevent Bim_{EL} from reassociating with pro-survival proteins to promote apoptosis. Indeed, when degradation of Bim_{EL} was blocked by MG132, we found that inhibition of ERK1/2 resulted in dephosphorylation of Bim_{EL} and its reassociation with Mcl-1. The reason for this may be that both Bim_{EL} and Mcl-1 remain in the mitochondrial membrane fraction after dissociation, probably due to their own autonomous membrane targeting motifs, and so may remain in close proximity to facilitate binding and reassociation following Bim_{EL} dephosphorylation. This reversibility further underlines the dynamic nature of the ERK1/2-dependent dissociation of Bim_{EL} from pro-survival proteins.

In summary, we have shown that expression of Bim contributes to fibroblast and epithelial cell death following withdrawal of serum survival factors. Furthermore, by demonstrating that ERK1/2-dependent phosphorylation of Bim_{EL} causes the rapid dissociation of Bim_{EL}/Mcl-1 and Bim_{EL}/Bcl-x_L complexes, we have defined a completely new mechanism by which survival factors can antagonise Bim_{EL} function, providing a further explanation for the reduced efficacy of Bim_{EL} relative to Bim_S and Bim_L (O'Connor *et al*, 1998). Mutation of Ser^{65}Ala has been shown to enhance the toxicity of Bim_{EL} and it was assumed that this was due to stabilisation of the protein (Luciano *et al*, 2003; Ley *et al*, 2004). While this may well be the case, our results suggest that an additional effect of this mutation is to prevent growth factor-dependent dissociation of Bim_{EL} from its target pro-survival proteins. This may prove important in a variety of physiological and pathophysiological settings in which survival signals are limiting, including the developing CNS, immune system and in tumour cells.

Materials and methods

Antibodies

The following antibodies were used for Western blotting: Bax N-20, Santa Cruz (sc-493); Bcl-2, Santa Cruz (sc-7382); Bcl-x_L, Pharmingen (66491A); Bim, Chemicon (AB17003); phospho-Ser^{65} Bim, Upstate Biotech; Bmf, Alexis Biochemicals (210-831-R100); ERK1/2 (9102) and ERK1/2 phosphospecific (9106), Cell Signalling Technology; HA, Babraham Institute Mab Facility; Mcl-1, Santa Cruz (sc-819); PKB (9272) and PKB S473 phosphospecific (9271), Cell Signalling Technology.

Cells and cell culture

Culture of CCl39 and CR1-11 (Weston *et al*, 2003), HEK293 (Ley *et al*, 2003), HR1 (Boughan *et al*, 2006) and WT and Bim−/− E1A-immortalised iBMK cells (Tan *et al*, 2005) have been described previously. WT and Bim−/− iMEFs were provided by David Huang, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. MEFs were generated from E13-14.5 embryos and immortalised (at passages 2–4) with SV40 large T antigen. The mice used had been backcrossed (>10 generations) to the C57BL/6 genetic background and their genotype determined as described previously (Bouillet *et al*, 1999).

Assay of cell death

Assay of caspase activity by DEVDase assay and cell death by propidium iodide staining were described previously (Weston *et al*, 2003; Austin and Cook, 2005).

Cell extracts and immunoprecipitation

Following stimulation, cells were lysed in TG lysis buffer, as described previously (Weston *et al*, 2003). TG lysates were used for immunoprecipitations with the following antibodies: Bax N-20, Santa Cruz (sc-493) with protein G sepharose beads; Bcl-x_L, Cell Signalling Technology (2762) and Mcl-1, Santa Cruz (sc-819) with protein A sepharose beads.

Supplementary data

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

Acknowledgements

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