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News and Commentary

Regulatory phosphorylation of Bim: sorting out the ERK from the JNK

R Ley[1], KE Ewings[1], K Hadfield[1] and SJ Cook*,[1]

[1] Laboratory of Molecular Signalling, The Babraham Institute, Babraham Research Campus, Cambridge CB2 4AT, UK
* Corresponding author: SJ Cook, Laboratory of Molecular Signalling, The Babraham Institute, Babraham Research Campus, Cambridge CB2 4AT, UK. Tel: 44-1223-496453; Fax: 44-1223-496043; E-mail: simon.cook@bbsrc.ac.uk

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Activation of Bax, Bak and the mitochondrial cell death pathway is regulated by the balance between prosurvival Bcl-2 proteins and the expression level or activation state of the proapoptotic 'BH3-only proteins'.[1,2] This life–death balance is exemplified by the genetic interactions between Bcl-2 and the BH3-only protein Bim.[3] Loss of Bcl-2 in the mouse undermines the lymphoid system and mice become runted, turn grey and succumb to a form of polycystic kidney disease. Strikingly, most of these phenotypes are ameliorated by the loss of only a single Bim allele, underlining the importance of Bim expression level in determining cell death. Bim expression is regulated by both transcriptional and post-translational mechanisms. Here we consider recent evidence that Bim proteins are regulated by phosphorylation and discuss the kinases responsible, the phosphorylation sites and effect of phosphorylation on Bim protein function, seeking to resolve some of the contradictions.

Bim Splice Variants

Bim (Bcl-2 interacting mediator of cell death) was identified by its binding to Bcl-2 in a cDNA expression cloning screen[4] and, independently, as BOD (Bcl-2-related ovarian death gene) in a yeast two-hybrid screen;[5] the name Bim is established in the literature. The Bim locus encodes three major isoforms: Bim short (Bim_S), Bim long (Bim_L) and Bim extra long (Bim_{EL}), generated by alternative splicing.[4,5] More recently, additional isoforms have been reported[6–8] so that recent estimates suggest that there may be up to 18 different Bim splice variants.[8] However, the physiological relevance of these unique splice variants has not yet been addressed and while the protein products of endogenous Bim_S, Bim_L and Bim_{EL} are readily detected, few, if any, studies have identified the naturally occurring protein products of these novel splice variants. For this reason, we have focused largely on the three, canonical forms of Bim, since even this diversity gives rise to proteins that exhibit different apoptotic potencies.

Bim_S is the most effective killer and is encoded by exon 2, exon 5 (which includes the BH3 domain) and exon 6 (which includes the hydrophobic tail, required for insertion into the outer mitochondrial membrane) (Figure 1). Bim_L further includes exon 4, which encodes a binding site for dynein light chain 1 (DLC1),[9] and is less effective at killing cells (Figures 1 and 2b). Bim_{EL} includes exons 2, 4, 5 and 6, but additionally includes exon 3 (Figures 1 and 2c), and is thought to be the least effective at killing cells;[4] presumably exon 3 must encode determinants which account for this reduced apoptotic activity. In addition, while all forms of Bim can bind to prosurvival Bcl-2 proteins, some forms may bind directly to Bax.[7] However, this is an area of considerable uncertainty; for example, while it is clear that the Bim BH3 domain can bind to all prosurvival Bcl-2 proteins with a physiologically relevant nM affinity in Biacore real-time binding assays,[10] no such evidence is available for Bim binding to Bax or Bak. Consequently, many in the field still believe that Bim and most other BH3-only proteins trigger apoptosis largely by binding to their prosurvival relatives and this remains an area of considerable debate. Bim is widely expressed in haematopoietic, neuronal, epithelial, fibroblast and germ cells,[11] and Bim_{EL} appears to be the most abundant isoform, whereas Bim_S is less frequently detected, possibly because it is the most effective killer.

Bim as a Sensor of Survival Factors and Trophic Support

Depending on the mouse strain, nearly half of Bim−/− mice die during development, while survivors exhibit elevated numbers of B cells, T cells, granulocytes and monocytes and may succumb to autoimmune disease.[12] In the immune system Bim is required for deletion of autoreactive B and T cells[13,14] and cessation of acute T-cell responses *in vivo*.[15,16] Bim-deficient B and T cells die normally in response to FasL but Bim−/− T cells exhibit reduced sensitivity to dexamethasone, ionomycin and γ-irradiation. However, the most striking phenotype is the resistance of both B and T cells to cytokine deprivation.[12] Indeed, Bim is expressed *de novo* following withdrawal of survival factors from haematopoietic cell lines,[12,17] primary sympathetic neurons,[18,19] osteoclasts[20] and fibroblasts[21] (Figure 2a–c). This is consistent with the observation that cell death following withdrawal of cytokines requires new gene expression in many cell types. Consequently, Bim is thought to play a major role in promoting cell death following withdrawal of survival factors.

Two major cytokine-activated cell survival pathways regulate Bim mRNA levels. Withdrawal of cytokines or pharmacological inhibition of the PI3K pathway causes inactivation of PKB, leading to de-phosphorylation and nuclear entry of the forkhead transcription factor FOXO-3A, which is sufficient to induce *Bim* mRNA expression.[17] The *Bim* gene is a direct target of FOXO-3A[17] and the 5′-UTR of the rat *Bim* gene contains two forkhead-binding sites which are required for regulated expression of Bim following withdrawal of NGF from

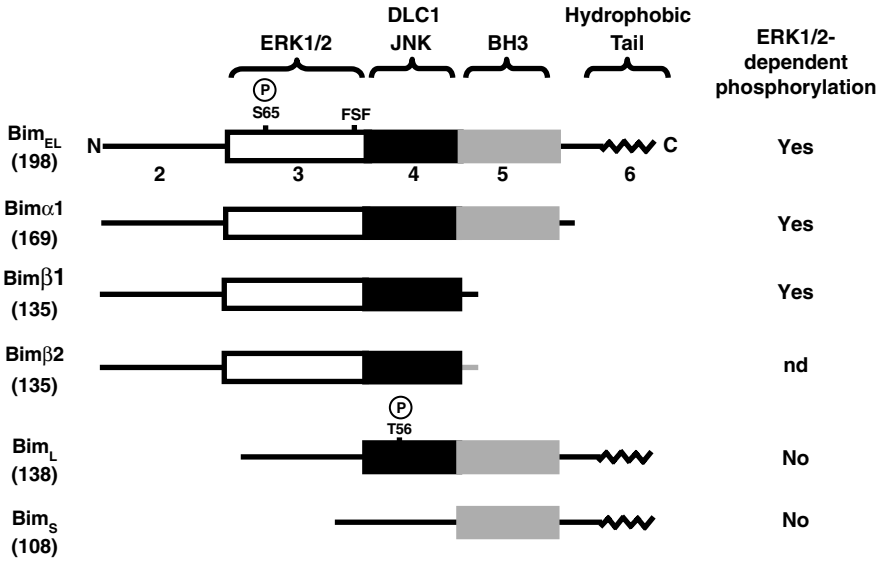

Figure 1 Bim splice variants that are subject to post-translational regulation. The domain structure of some of the proteins arising from alternative splicing of *Bim* is shown, with annotations to indicate functional domains such as the BH3-only domain and the hydrophobic C-terminal tail. Bim_S is encoded by exons 2, 5 and 6 and, to date, is not subject to post-translational regulation. Bim_L is encoded by exons 2, 4, 5 and 6. Exon 4 includes the sequence DKSTQTP, a binding site for DLC1,[9] and JNK phosphorylation sites, including Thr56 in Bim_L.[43] Bim_{EL} is encoded by exons 2, 3, 4, 5 and 6. Exon 3 includes an ERK1/2 docking domain (FSF) and ERK1/2 phosphorylation sites, including Ser69.[29–31,33] The presence of exon 3 in Bimα1, Bimβ1 and Bimβ2 also correlates with their ERK1/2-dependent phosphorylation *in vivo*.[33] ERK1/2-dependent phosphorylation of Bim_{EL} targets it for proteasomal degradation and may prevent binding to Bax. The numbers beneath each Bim protein represent the number of constituent amino acids (according to the human sequence), while the numbers below the Bim_{EL} figure represent the different exons; for details of the splicing of Bim, readers are encouraged to refer to the original studies[4,6–8]

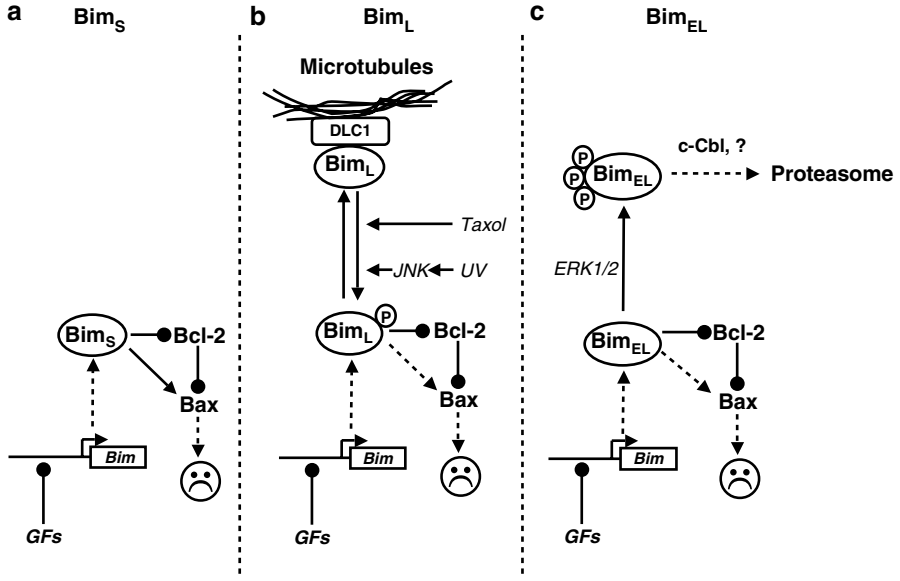

Figure 2 Regulation of Bim_L and Bim_{EL} by MAP kinase pathways. Expression from the *Bim* gene is normally repressed by the action of cytokines, survival factors and growth factors (GFs). Once the individual splice variants are expressed, they have unique properties based on individual patterns of post-translational regulation. (**a**) Bim_S can induce apoptosis by interacting with survival proteins such as Bcl-2, or maybe by binding directly to Bax. To date there have been no reports of post-translational regulation of Bim_S. (**b**) Bim_L can associate with microtubules through its interaction with DLC1;[9] this sequesters it away from Bcl-2 and Bax. Stresses such as Taxol, which disrupt microtubules, can cause re-location of Bim_L–DLC1 from microtubules so that Bim_L can now promote cell death. In addition, JNK can phosphorylate Bim_L at the DLC1-binding site, causing Bim_L to separate from DLC1.[43] This may be an important mechanism for cell death in response to stresses that activate JNK. Note that Bim_{EL} can also associate with DLC1, but to date there have been no reports that the Bim_{EL}–DLC1 interaction is regulated by JNK. (**c**) Upon activation by growth factors, ERK1/2 can physically associate with Bim_{EL} and phosphorylate it at Ser69 and probably other proline-directed sites. This phosphorylation targets the Bim_{EL} protein for ubiquitylation and proteasome-dependent degradation and may also impair interactions between Bim_{EL} and Bax[29–31,33]

sympathetic neurons.[22] The PI3K–PKB–FOXO pathway may operate to repress Bim in many other cell types including lymphocytes[17] and fibroblasts.[21] In addition, pharmacological inhibitors of the ERK1/2 MAP kinase pathway also induce *Bim* mRNA and protein levels in fibroblasts[21] and breast epithelial cells,[23,24] though the precise mechanism is not presently known. The ERK1/2 pathway does not act via the PI3K pathway to repress Bim,[21] but whether it acts independently of the FOXO transcription factors or decreases mRNA stability remains to be seen.

Expression of Bim following withdrawal of NGF from primary sympathetic neurons[18,19] may require AP-1 since an interfering mutant of c-Jun reduced the expression of Bim mRNA and protein.[18] Subsequently, it has been shown that CEP-1347, a mixed-lineage kinase-3 inhibitor, prevents activation of JNK, the induction of Bim and reduces the apoptotic response to NGF withdrawal.[25,26] This mode of regulation may be confined to neuronal cell types since c-Jun is not required for Bim expression in fibroblasts[21] and JNK activation is not a ubiquitous response to withdrawal of survival factors.

Phosphorylation of Bim_{EL} by ERK1/2

Bim_{EL} was first demonstrated to be a phospho-protein in IL-3-stimulated BaF3 cells[27] and subsequently in NGF-stimulated PC12 cells.[26] The use of U0126, an inhibitor of MEK1/2 and MEK5, implicated either the ERK1/2 or ERK5 pathways[26] (both activated by NGF), but the identity of the kinase responsible, the phosphorylation sites or the effect on Bim_{EL} were not resolved. Subsequent studies in fibroblasts, using the conditional kinase ΔRaf-1:ER*, confirmed that selective activation of the ERK1/2 pathway was sufficient to promote the phosphorylation of Bim_{EL} at multiple sites but did not apparently influence the phosphorylation of Bim_S or Bim_L.[21] However, the real complexity of Bim_{EL} phosphorylation first became apparent after metabolic labelling and 2-D gel analysis in the IL-2-dependent Bal17 cell line.[28] This revealed that Bim_{EL} was phosphorylated on four serine residues in viable cells and that withdrawal of IL-2 caused the loss of at least two of these phosphorylation sites prior to the onset of apoptosis, suggesting that there might be a causal relationship between de-phosphorylation of Bim_{EL} and apoptosis following withdrawal of IL-2.

The ability of ΔRaf-1:ER* or growth factors to promote the ERK1/2-dependent phosphorylation of Bim_{EL} could be explained by ERK1/2 phosphorylating Bim_{EL} directly, but were equally consistent with phosphorylation by ERK1/2-dependent downstream kinases such as RSK, MNK or MSK. However, the observation that activation of the ERK1/2 pathway was sufficient to promote the binding of Bim_{EL} to the Pin1 peptidyl–prolyl isomerase[29] strongly suggested that ERK1/2 was in fact the kinase responsible, since ERK1/2 phosphorylates proteins at S–P or T–P motifs and the Pin1 WW domain only binds proteins phosphorylated at such motifs (i.e. pS–P or pT–P). Bim_{EL} contains six S–P or T–P motifs and ERK1/2 can indeed phosphorylate recombinant Bim_{EL} at Ser69 *in vitro*[29–31] (Ser65 in rat and mouse Bim_{EL}). Mutation of this site also disrupts growth factor-stimulated, ERK1/2-dependent phosphorylation of Bim_{EL} in cells,[29–31] indicating that ERK1/2 can phosphorylate this site *in vivo.* Indeed, ERK1/2 exhibit a secondary preference for a proline residue at the −2 position relative to the phospho-acceptor site and Ser69 lies within the motif PPASP.

It is now apparent that ERK1/2-dependent phosphorylation *in vivo* is more complex than this. For example, 2-D gel analysis suggests that up to three of the four Bim_{EL} phosphorylation sites previously identified in Bal17 cells[28] are lost when the ERK1/2 pathway is inhibited.[29] In addition, mutation of just Ser69 completely abolishes ERK1/2-catalysed phosphorylation *in vitro*,[29–31] but causes the loss of two of the four phosphorylation sites *in vivo*.[29] One possible explanation for this is that Bim_{EL} may be phosphorylated in a hierarchical fashion in cells, with phosphorylation at Ser69 directing phosphorylation at an additional site. This also suggests that the third ERK1/2-dependent site may be phosphorylated independently of Ser69. Certainly a Ser69Ala or Ser69Gly mutant of Bim_{EL} still exhibits ERK1/2-dependent phosphorylation *in vivo.*[29–31] In summary, ERK1/2 can phosphorylate Bim_{EL} directly at Ser69 and can promote the phosphorylation of two other sites *in vivo;* phosphorylation of one of these additional sites may require prior Ser69 phosphorylation. These additional sites may be phosphorylated by ERK1/2 or by other ERK-dependent kinases; the identity of these sites is not known, but the proline-directed sites at Ser59 and Ser104 (Ser55 and Ser100 in rat and mouse) are good candidates.[31,32]

Efficient phosphorylation by ERK1/2 *in vivo* requires an appropriate phospho-acceptor site in the substrate (such as PPASP), but also requires a distinct docking domain for the kinase. Using GST–Bim fusion proteins as bait, it has been shown that Bim_{EL}, but not Bim_L or Bim_S, can bind to activated ERK1/2 in cell lysates.[29] ERK1/2 binding was specific (inactive ERK1/2 failed to bind) and was independent of the phospho-acceptor site. Subsequent analysis has identified a DEF/FXF-type ERK1/2 docking domain centred around the FSF motif (amino acids 97–99 in Bim_{EL}).[33] In common with other DEF domains, the phospho-acceptor site is situated N-terminal to the docking site. Mutation of this motif inhibits both ERK1/2–Bim_{EL} interactions *in vitro* and ERK1/2-dependent phosphorylation *in vivo*, while peptides spanning this motif inhibit the interaction between Bim_{EL} and ERK1/2 but do not affect the interaction between JNK and c-Jun. One attractive model is that active ERK1/2 binds to Bim_{EL} via the DEF domain, phosphorylates Ser69, which in turn induces a conformational change allowing phosphorylation at a second site. Interestingly, mutation of the DEF domain, like Ser69, blocks phosphorylation of two of the three ERK1/2-dependent sites. This may suggest that Bim_{EL} contains an additional ERK1/2 docking domain that directs phosphorylation at the third site.

ERK1/2-dependent Phosphorylation Antagonizes Bim_{EL}

The phosphorylation of Bim_{EL} by ERK1/2 has two main effects and both are consistent with the ability of the ERK1/2 pathway to protect cells against loss of trophic support. The best

characterized effect of phosphorylation is to promote the proteasomal degradation of Bim_{EL} (Figure 2c). Initial studies in fibroblasts showed that activation of the ERK1/2 pathway promoted a reduction in Bim_{EL} expression that was coincident with ERK1/2-dependent phosphorylation.[21] Subsequently, it was shown that activation of the ERK1/2 pathway was necessary and sufficient to accelerate the turnover of Bim_{EL} and ERK1/2-dependent Bim_{EL} degradation was shown to proceed via the proteasome.[34] Proteasome-dependent turnover has subsequently been confirmed in other cell types.[20,30,35,36] Phosphorylation at Ser69 seems to be an important signal for turnover of Bim_{EL} since Ser69Ala or Ser69Gly mutants are defective for turnover, accumulate to higher levels in cells and so exhibit enhanced toxicity.[29,30] Ubiquitylation of Bim_{EL} appears to take place at Lys3 or Lys112 (Lys 108 in rat and mouse), the only two lysines in the molecule, since a mutant form in which they are mutated to arginine fails to be ubiquitilated and exhibits enhanced cytotoxicity.[20] c-Cbl may function as an E3 ubiquitin ligase for Bim_{EL}, but is unlikely to be the only such molecule since MCSF-induced degradation of Bim is only partially inhibited in c-Cbl$-/-$ osteoclasts.[20]

In addition to promoting the turnover of Bim_{EL}, ERK1/2-dependent phosphorylation may also prevent Bim_{EL} from interacting with Bax. Stimulation of FL5.12 pro-B cells with IL-3 promotes the ERK1/2-dependent phosphorylation of Bim_{EL}.[32] In common with other studies,[27] this has no effect on the interaction of Bim_{EL} with Bcl-2 or Mcl-1, but rather prevented Bim_{EL} from interacting with Bax;[32] this would presumably have the effect of preventing the oligomerization of Bax and cell death. Mutation of three phosphorylation sites in Bim_{EL} (Ser59Ala, Ser69Ala and Ser104Ala) abolished phosphorylation *in vivo,* enhanced Bim_{EL}–Bax interactions and enhanced cell death following withdrawal of IL-3. The only question regarding this model is that others have argued that Bim_{EL} fails to interact directly with Bax and this whole issue remains uncertain at the present time.[7,8] Finally, ERK1/2-dependent phosphorylation of Bim_{EL} has been shown to occur at mitosis, although the functional relevance of this remains unclear.[37]

Taken together, these results suggest that the presence of exon 3 in Bim_{EL} provides an ERK1/2 docking domain and at least two ERK1/2 phosphorylation site(s), including Ser69. ERK1/2-dependent phosphorylation of Bim_{EL} can serve to target Bim_{EL} for ubiquitylation and proteasomal degradation, and may also disrupt Bim_{EL}–Bax interactions. Both mechanisms should act to inhibit Bim_{EL} function and may therefore provide an explanation for the reduced potency of Bim_{EL} in cell death assays.[2]

Phosphorylation of Other Bim Isoforms by ERK1/2: A Question of Attribution?

Two studies have argued that Bim_L is also subject to ERK1/2-dependent phosphorylation. NGF stimulation of PC12 cells was reported to promote the ERK1/2-dependent phosphorylation of Bim_L as well as Bim_{EL},[26] while mutation of Ser44 (Ser104 in Bim_{EL}) abolished the ERK1/2-dependent phosphorylation of Bim_L in FL5.12 cells.[32] Since Ser44 is a proline-directed site, this suggested that ERK1/2 might directly phosphorylate Bim_L. At first this seems difficult to reconcile with the fact that Bim_L lacks an ERK1/2 docking domain and is not phosphorylated by ERK1/2 *in vitro.*[29] However, one possibility is that *in vivo,* Bim_L is actually phosphorylated by ERK1/2 that is bound to a Bim_L partner protein. For example, ERK1/2 associate with microtubules (and were originally named microtubule-associated protein kinases) and so may associate in a ternary complex with Bim_L bound to the DLC1 motor protein. Indeed, since DLC1 is a dimer,[38,39] could one envisage ERK1/2 binding to Bim_{EL} on one DLC1 molecule and phosphorylating Bim_L on the other? Alternatively, ERK1/2 may bind to a prosurvival Bcl-2 protein and then phosphorylate Bim_L. In all such scenarios ERK1/2 would bind to a partner protein and phosphorylate Bim_L *in trans* rather than *in cis.* There is some precedent for this in the ability of c-Jun to provide a docking site for JNK, which then phosphorylates the c-Jun dimer partner, JunD, which otherwise fails to bind JNK directly.[40]

Despite these reports,[26,32] other studies suggest that Bim_L is not phosphorylated following activation of ERK1/2.[29–31,33] Aside from caveats regarding different cell types or stimulation conditions, an alternative explanation for this confusion is that another Bim splice variant is actually being studied. Of the six novel splice variants of Bim recently reported,[6,7] all contain the same amino-terminal sequences encoded by exon 2 and so should be recognized by many of the commercially available anti-Bim antibodies. Given that some of the novel splice variants are predicted to encode proteins with similar molecular weights to Bim_L, it is conceivable that immunereactive bands attributed to Bim_L may actually be other Bim splice variants. For example, Bimβ1 (135 aa) and Bimβ2 (135 aa), migrate very close to Bim_L (138 aa) on SDS-PAGE gels and, in contrast to Bim_L, at least one of these (Bimβ1) is subject to ERK1/2-dependent phosphorylation *in vivo.*[33] Interestingly, Bimα1, Bimβ1, Bimβ2 and Bim_{EL} all include Ser69 and the ERK1/2 docking domain encoded by exon 3 (Figure 1). Thus, when the individual splice variants are expressed from their own unique cDNAs, the presence or absence of exon 3 correlates well with their ability to be phosphorylated by ERK1/2 *in vivo* (Bim_{EL}, Bimα1 and Bimβ1) or not (Bim_L, Bim_S)[33] (Figure 1). Without clear and careful resolution of which isoform is which, for example by running 'standards' of each on the same gel, it can be difficult to unambiguously identify some of the smaller splice variants. With this caveat in mind, it seems likely that Bim_{EL}, Bimα1 and Bimβ1 (and conceivably Bimβ2) are targets of ERK1/2, but reports that Bim_L is phosphorylated by ERK1/2 *in vivo* should perhaps be treated with some caution at present.

Phosphorylation of Bim_L by JNK Regulates its Interaction with Dynein Light Chain-1

In viable cells Bim_L and Bim_{EL} can be sequestered at the microtubule-associated dynein motor complex, away from Bcl-2 and Bax proteins, by virtue of their binding to DLC1.[9] However, the degree of this binding varies substantially in different cells,[27,41] suggesting that this may not be a relevant

survival mechanism in all cell types. For example, most of the Bim is associated with Bcl-2 and Bcl-x_L at the mitochondria rather than at the microtubules in both healthy and apoptotic T cells.[42] The core DLC1-binding region maps to the sequence DKSTQTP (51–57 aa in Bim_L and 111–117 aa in Bim_{EL}) found in exon 4 of Bim_L and Bim_{EL}. Certain cellular stresses promote the release of Bim_L and Bim_{EL} from microtubules,[9] raising the possibility that this is a dynamic process regulated by stress-induced signalling pathways. Indeed, the stress kinase JNK has been shown to phosphorylate Bim_L on Thr56 within the DLC1-binding motif (and at either Ser44 or Ser58), reducing the Bim_L–DLC1 in whole cells and so unleashing Bim_L to kill the cell[43] (Figure 2b).

One attraction of this 'stress-induced redistribution' model is that it fits with evidence that JNK-induced apoptosis can proceed through the modulation of pre-existing components rather than via c-Jun-dependent expression of pro-death genes.[44,45] It could also explain the observation that Bim-null cells are resistant to taxol-induced death[12] even though taxol does not increase Bim expression. However, there is a major anomaly in the published studies to date that remains to be resolved. In the original studies on the Bim_L–DLC1 interaction it was shown that following a stress such as UV, which activates JNK, Bim_L migrated from the microtubules still bound to DLC1.[9] In contrast, the study of Lei and Davis clearly showed that UV-induced, JNK-dependent phosphorylation promoted the separation of Bim_L and DLC1.[43] Does this reflect differences in the cell types used or the degree of JNK activation? For example, stresses that strongly activate JNK (e.g. UV) may cause separation of Bim_L from DLC1, whereas others might disrupt microtubules but cause only a modest activation of JNK, thereby releasing Bim_L still bound to DLC1.

Other issues also remain to be resolved. For example, unlike the ERK1/2-dependent phosphorylation of Bim_{EL}, no JNK docking domain has been identified in Bim_L to date; again, does JNK phosphorylate Bim_L after binding to a Bim_L partner protein? Furthermore, while JNK has been shown to phosphorylate Bim_L *in vitro* and overexpression of activated JNK1 can promote phosphorylation of Bim_L *in vivo*, it remains to be seen if JNK is actually required for stress-induced phosphorylation of Bim_L or re-distribution of Bim_L from microtubules *in vivo.* The availability of JNK1/JNK2 double-knockout fibroblasts devoid of JNK activity[44] should allow this issue to be resolved. Finally, Bim_{EL} can also associate with DLC1,[9] but there are no reports that the Bim_{EL}–DLC1 interaction is regulated by JNK, despite Bim_{EL} having the same potential phosphorylation sites (Thr116 and Ser104 or Ser118). Is this mode of regulation unique to Bim_L?

JNK-dependent Phosphorylation of Bim_{EL}

The expression of Bim following withdrawal of NGF from primary sympathetic neurons requires JNK and is inhibited by an interfering mutant of c-Jun.[18,19,25,26] More recently, it has been proposed that JNK may also be responsible for phosphorylation of Bim_{EL} at Ser69 following NGF withdrawal, thereby enhancing its proapoptotic activity;[46] similar results were described in cerebellar granule neurons.[47] In light of reports that ERK1/2 is *necessary* and *sufficient* to induce phosphorylation of Bim_{EL} at Ser69, these studies in neurons raise several interesting issues. Evidence that the JNK pathway was *sufficient* to induce phosphorylation of Bim_{EL} stemmed from the use of constitutively active protein kinases. For example, phosphorylation of Bim_{EL} was induced by transient overexpression of ΔMEKK1;[47] however, it is well known that ΔMEKK1 activates ERK1/2 as well as JNK and p38 when overexpressed.[48,49] Similarly, the expression of activated MKK3 promoted Bim_{EL} phosphorylation,[47] but promoted activation of JNK as well as p38. Since MKK3 is established as a selective activator of p38,[50] this suggests that the assay conditions may not have been stringent. These examples highlight some of the concerns that arise when using transient overexpression systems to address issues of sufficiency.

Much of the evidence that JNK is *necessary* for Bim_{EL} phosphorylation *in vivo* stems from the use of inhibitors; for example, both SP600125 and CEP-1347 inhibit phosphorylation of Ser69, suggesting that JNK is the kinase responsible.[46] However, it is interesting that both drugs also reduce Bim_{EL} expression to a greater or lesser extent, making it unclear how much the reduction in phosphorylation of Bim_{EL} represents a reduction in stoichiometry of phosphorylation or more simply a reduction in total Bim_{EL} levels. The use of SP600125 *in vivo* is fraught with concerns since it can inhibit at least 12 other protein kinases;[51] however, the efficacy of CEP-1347 in these assays is more encouraging. Interestingly, in a separate study CEP-1347 reduced Bim_{EL} expression, but had no apparent impact on Bim_{EL} phosphorylation.[26] Indeed, Biswas and Greene,[26] who were the first to demonstrate phosphorylation of Bim_{EL} in neuronal cells, showed that NGF-dependent Bim_{EL} phosphorylation was inhibited by the pan-ERK pathway inhibitor U0126, a drug which does not inhibit the JNK pathway. However, the sites of ERK1/2-dependent phosphorylation of Bim_{EL} in neurons have not been defined.

Based on the evidence to date, it seems clear that Bim_{EL} may be phosphorylated by ERK1/2 or JNK in neurons;[26,46,47] certainly, both ERK1/2 and JNK1 can phosphorylate GST-Bim_{EL} *in vitro*[29,46,47] (though ERK1/2 is more effective[29]). So what determines which kinase acts *in vivo* and under what conditions? Interaction of JNK with Bim_{EL}, presumably mediated by a docking domain or as part of a larger complex, is likely to be a major determinant. In extracts from fibroblasts, active JNK1 binds only very weakly to fragments of Bim_{EL} that bind ERK1/2 strongly.[33] Furthermore, this weak binding 'maps' to a site adjacent to, but independent of, the ERK1/2-specific DEF-type docking domain. So Bim_{EL} may actually contain distinct docking domains that are either specific for ERK1/2 (DEF-type) or that can bind JNK and ERK1/2 (such as a D domain). Access of one kinase over another may be determined by occupancy of the adjacent docking domain, differences in affinity or differences in the activation state of ERK1/2 or JNK. For example, in the presence of NGF, active ERK1/2 will be abundant, will bind strongly and phosphorylate Bim_{EL}. Following NGF withdrawal, inactive ERK1/2 may dissociate from Bim_{EL}, perhaps allowing the otherwise weak JNK binding to predominate; so inactivation of ERK1/2 may be a prerequisite for JNK-dependent phosphorylation. In addition, the ability of JNK to phosphorylate Bim_{EL} may be determined by the expression of components unique to

neurons. JNK3 is only really found in nervous tissue but is actually the least effective JNK isoform at phosphorylating Bim_{EL} *in vitro*, whereas JNK1 is the most effective.[46] Alternatively, neurons may express a unique adaptor protein that facilitates JNK binding to Bim_{EL}.

The potential role of both JNK and ERK1/2 in Bim_{EL} phosphorylation in neurons is certainly very interesting. Quite apart from the details of how JNK phosphorylates Bim_{EL}, it is not presently known how phosphorylation at Ser69 enhances the proapoptotic activity of Bim_{EL}. Perhaps more importantly, it is not clear how phosphorylation of Ser69 can inhibit the proapoptotic activity of Bim_{EL} in some cells types,[29,30,32–35] but apparently have entirely the opposite biological effect in neurons.[46,47] Perhaps ERK1/2 and JNK induce different patterns of phosphorylation at other sites, which, together with phosphorylation at Ser69, provide different signals to regulate Bim_{EL}. Resolution of these issues should provide fascinating new insights into Bim regulation and Bim function.

A Role for Bim in Tumor Suppression?

Bim has emerged as a sensor of trophic support in a variety of cell types; so one can envisage that defects in its expression may relate to certain pathologies such as auto-immunity[12] or neurodegeneration. In addition, Bim expression is regulated by at least two signal pathways, Raf-MEK-ERK1/2 and PI3K-PDK-PKB, which are de-regulated in cancer.[52] Since cancer cells are characterized in part by their reduced dependency on exogenous growth and survival factors,[52] this begs the question – is Bim a tumor suppressor? Certainly, loss of even a single Bim allele accelerates Myc-induced tumorigenesis in mouse models[53] and more recently deletions of the Bim locus have been reported in 17% of mantle cell lymphomas, resulting in loss of all Bim expression.[54] In addition, Bim expression may indeed be deregulated indirectly in tumors by oncogene-dependent signalling events described in this review. For example, expression of Bim is uniformly low in blast cells from mouse models of chronic myelogenous leukaemia (CML) and from patients with CML. The Bcr–Abl oncoprotein provides cytokine-independent survival signals and appears to be important in repressing Bim expression since treatment with the Abl kinase inhibitor imatinib mesylate (STI571, Gleevec) induced Bim expression and Bim-dependent apoptosis.[55] Inhibition of Bcr–Abl resulted in inhibition of both the ERK1/2 and PI3K pathways and the dephosphorylation of Bim_{EL}. Such observations are not confined to CML. It has recently been shown that Bim−/− transformed baby mouse kidney epithelial (BMK) cells form tumors in mice, whereas their wild-type counterparts do not.[56] Bim expression correlates with sensitivity to paclitazel-induced apoptosis *in vitro* and paclitaxel causes regression of wild-type but not Bim−/−BMK tumors *in vivo.* Ras- and Raf-transformed BMK cells are resistant to paclitaxel-induced apoptosis *in vitro* due to their ability to reduce Bim expression by ERK1/2-dependent phosphorylation. Finally, proteasome inhibition reduced the Ras-imposed downregulation of Bim and restored paclitaxel sensitivity in wild-type but not Bim−/− cells. So, it appears that in at least two cases the ERK1/2-dependent phosphorylation and turnover of Bim_{EL} may be mechanisms of oncogene-induced cell survival that regulate tumorigenesis *in vivo.*

Summary – So Much Regulation for Such a Little Protein

The major theme that emerges from these various studies is the complex modes of post-translational regulation provided for by the alternative splicing of Bim. In a sense, Bim_S can be considered the simplest, 'stripped-down' version of Bim and exhibits the greatest proapoptotic activity, whereas Bim_L and Bim_{EL} come with 'bells and whistles' attached in the form of discrete functional domains. In both cases, these domains serve a dual purpose: impairing the proapoptotic activity of the basic Bim_S backbone, but also providing additional opportunities for post-translational regulation. For example, the presence of exon 4 may allow DLC1-dependent sequestration of Bim_L to microtubules as a protective mechanism, but also includes JNK phosphorylation sites that may allow dynamic redistribution of Bim_L in response to certain stress stimuli. In the case of Bim_{EL}, exon 3 provides an ERK1/2 docking domain and ERK1/2 phosphorylation sites; these serve to target Bim_{EL} for proteasomal degradation or impair its binding to Bax, thereby inhibiting its proapoptotic activity. Conversely, these same motifs also ensure the rapid stabilization and/or activation of Bim_{EL} following inactivation of the ERK1/2 pathway. This may explain why the increase in expression of Bim_{EL} is both quicker and greater than Bim_S or Bim_L following withdrawal of survival factors. The phosphorylation of Bim_{EL} by JNK in neurons may be a further variation on this theme, albeit with different biological effects. Looking forward, the key issue is to define the biological significance of the individual splice variants and their unique modes of regulation. In the cellular context, this may be achievable with splice form-selective RNAi reagents.[57] However, *in vivo,* the real challenge will be to derive knock-in mice in which splice donor/acceptor sites or individual phosphorylation sites are mutated.

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