Cell Reports
Report

Phosphorylation of the Proapoptotic BH3-Only Protein Bid Primes Mitochondria for Apoptosis during Mitotic Arrest

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http://dx.doi.org/10.1016/j.celrep.2014.03.050

SUMMARY

Mitosis is a moment of exquisite vulnerability for a metazoan cell. Failure to complete mitosis accurately can lead to aneuploidy and cancer initiation. Therefore, if the exit from mitosis is delayed, normal cells are usually removed by apoptosis. However, how failure to complete mitosis activates apoptosis is still unclear. Here, we demonstrate that a phosphorylated form of the BH3-only protein Bid regulates apoptosis if mitotic exit is delayed. Bid is phosphorylated on serine 66 as cells enter mitosis, and this phosphorylation is lost during the metaphase-to-anaphase transition. Cells expressing a nonphosphorylatable version of Bid or a BH3-domain mutant were resistant to mitotic-arrest-induced apoptosis. Thus, we show that Bid phosphorylation primes cells to undergo mitochondrial apoptosis if mitotic exit is delayed. Avoidance of this mechanism may explain the selective pressure for cancer cells to undergo mitotic slippage.

INTRODUCTION

During mitosis, the spindle assembly checkpoint (SAC) normally prevents cells progressing to anaphase until all chromosomes are correctly attached to spindle microtubules (Musacchio and Salmon, 2007). However, if normal cells persist in mitosis for too long, they die by apoptosis. Antimitotic drugs such as paclitaxel keep the SAC active in order to selectively induce apoptosis in rapidly dividing cancer cells (Sudo et al., 2004). However, cancer cells can develop resistance to paclitaxel by either exiting mitosis before apoptosis is initiated (termed mitotic slippage) or by blocking the apoptotic response to delayed mitotic exit (Rieder and Maiato, 2004). Mitotic slippage occurs due to the degradation of cyclin B1 before apoptosis can be activated (Gascoigne and Taylor, 2008). On the other hand, how delayed mitotic exit activates apoptosis is poorly understood, despite the possibility that activating this mechanism could sensitize cancer cells to antimitotic drugs.

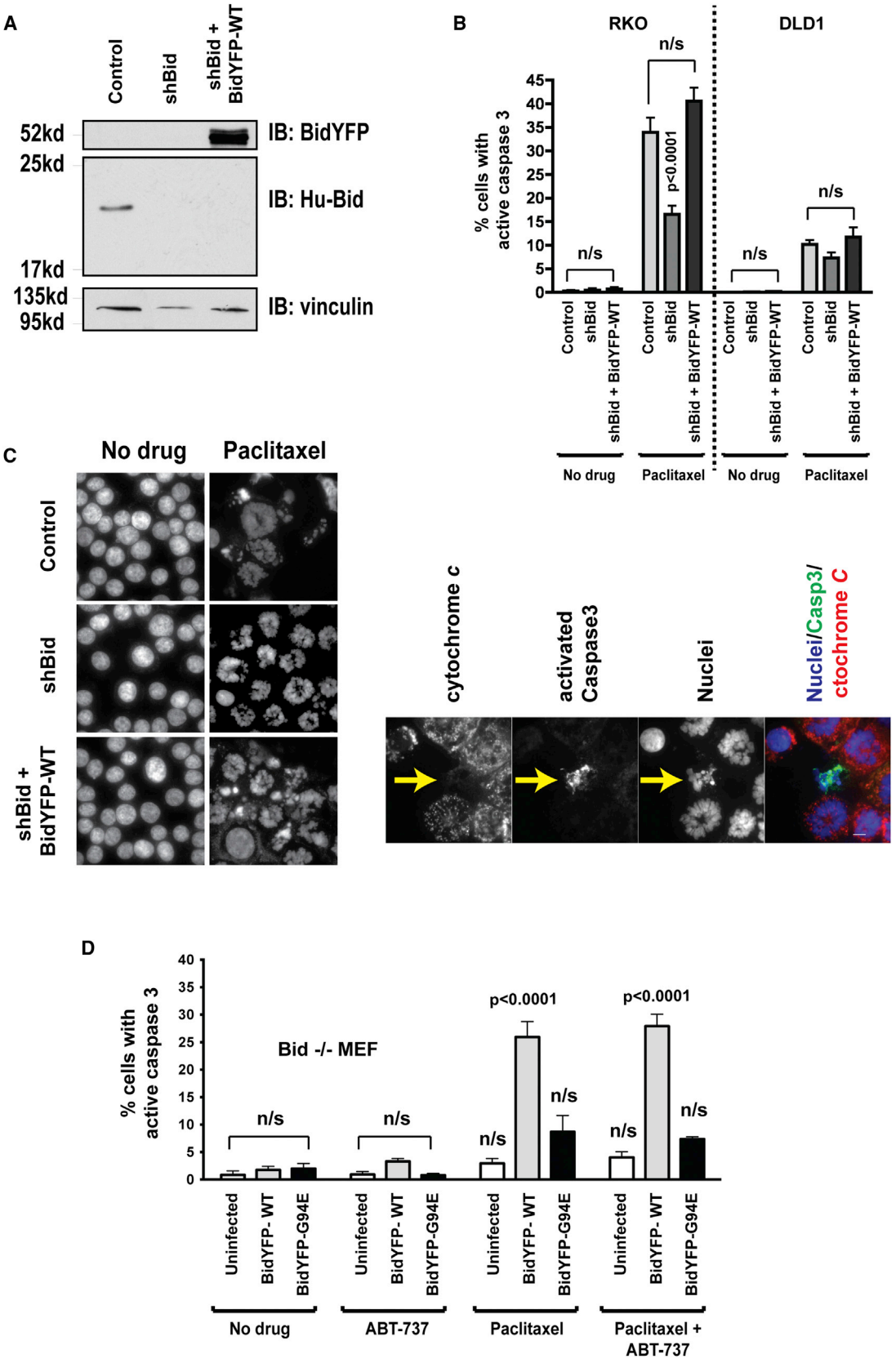
The Bcl-2 family of proteins regulates apoptosis. Activation of the Bcl-2 proteins, Bax and Bak, leads to mitochondrial outer membrane permeabilization (MOMP) (Youle and Strasser, 2008). The BH3-only members of the Bcl-2 family either activate Bax and Bak or inhibit antiapoptotic proteins such as Bcl-XL and Mcl-1. Different BH3-only proteins respond to distinct apoptotic signals and are regulated both transcriptionally and by posttranslational modification. For example, PUMA is transcriptionally upregulated by p53 (Nakano and Vousden, 2001), whereas Bad is phosphorylated via growth factor signaling (Gilmore et al., 2002). Another BH3-only protein, Bid, is regulated by proteolytic cleavage by caspase-8 downstream of death receptor signaling (Gross et al., 1999; Korsmeyer et al., 2000). Cleaved Bid then translocates to mitochondria where it activates MOMP. However, several studies have shown that Bid can be proapoptotic without being proteolytically cleaved (Sarig et al., 2003; Valentijn and Gilmore, 2004).

Here, we show that Bid is phosphorylated during mitosis within its regulatory loop. This phosphorylation sensitizes mitochondria for MOMP if mitotic exit is delayed. Our data suggest that BH3 mimetics may represent a viable strategy for targeting paclitaxel-resistant cancer cells.

RESULTS

Bid Is Required for Apoptosis following Delayed Mitotic Exit

As mitotic cells are transcriptionally inactive, we hypothesized a role for the posttranslationally regulated BH3-only protein, Bid, in mitotic-arrest-induced apoptosis. To examine this, we used two human colon carcinoma cell lines with different responses to mitotic arrest; RKO cells undergo apoptosis, whereas DLD1 cells are prone to mitotic slippage (Figure S1A; Gascoigne and Taylor, 2008). We knocked down endogenous human Bid (hBid) with lentiviral small hairpin RNA (shRNA) and re-expressed mouse Bid tagged with yellow fluorescent protein (YFP) (mBidYFP) or YFP (Figure 1A). Bid knockdown in the RKO cells significantly reduced the apoptotic response following arrest in paclitaxel (Figure 1B). The response of DLD1 cells to paclitaxel was unaffected by Bid knockdown. Furthermore, RKO cells lacking hBid remained in mitosis following paclitaxel treatment, indicating that the reduction in apoptosis was not due to mitotic



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slippage (Figures 1C and S1A). Death during mitotic arrest showed the hallmarks of classical mitochondrial apoptosis (Figure 1C). Furthermore, $Bax^{-/-}/Bak^{-/-}$ cells were completely resistant to paclitaxel-induced apoptosis (Figure S1B). Bid knockdown had no effect on RKO cell proliferation (Figure S1C).

To confirm a role for Bid in apoptosis during mitotic arrest, we generated $Bid^{-/-}$ mouse embryonic fibroblasts ($Bid^{-/-}$MEF) stably expressing mBidYFP-wild-type (WT) or mBidYFP-G94E, a substitution within the BH3 domain preventing it interacting with multidomain Bcl-2 proteins. Again, there was no effect of Bid expression on proliferation (Figure S1D). Paclitaxel did not induce apoptosis in $Bid^{-/-}$MEFs (Figure 1D). In contrast, expressing mBidYFP in $Bid^{-/-}$MEFs led to paclitaxel sensitivity, but this required a functional BH3 domain. The resistance of $Bid^{-/-}$MEFs or those expressing mBidYFP-G94E to paclitaxel was not reverted by the BH3 mimetic, ABT-737.

These results demonstrate that, in both human carcinoma cells and mouse fibroblasts, apoptosis caused by a paclitaxel-induced delay in mitotic exit requires the BH3-only protein Bid.

A Unique Form of Bid Phosphorylation Occurs during Mitosis

Bid is subject to posttranslational modifications within the loop between α helix 2 and α helix 3 (amino acids 39–81 in the mouse protein; Figure 2A). Following etoposide treatment, a fraction of Bid migrated more slowly than its predominant 22 kDa form, corresponding to phosphorylation on S61/S78 by ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) (Figure 2B; Kamer et al., 2005; Zinkel et al., 2005). We noted a slower-migrating form of Bid (termed pBid) in both untreated and etoposide-treated WT-MEFs. Both pBid and Bid-pS61/S78 were sensitive to alkaline phosphatase treatment, indicating that both were due to phosphorylation. To determine whether pBid was related to cell cycle, we arrested WT-MEFs in G1 (double thymidine block) or M (nocodazole). pBid was significantly enriched in mitosis (Figure 2C). After nocodazole washout, pBid disappeared synchronously with phosphorylated histone H3 (pSer10-H3), i.e., as the cell progressed through the metaphase-anaphase transition (Figure 2D). However, if mitotic exit following nocodazole washout was blocked with the proteasome inhibitor MG132 (confirmed by persistent pSer10-H3), pBid was not lost (Figure 2D).

To confirm that pBid accumulated as cells enter mitosis, WT-MEFs were arrested in G1 and then released, with or without a CDK1 inhibitor (RO-3306) to prevent entry into M or nocodazole to arrest cells before mitotic exit (Figure 2E). Both pBid and H3-pS10 failed to accumulate in RO-3306-treated cells. Furthermore, the pBid that accumulated over 8 hr in cells arrested in M by nocodazole was lost following brief treatment with RO-3306, indicating its maintenance in mitosis required Cdk1 activity. However, as RO-3306 will cause mitotic slippage in nocodazole-treated cells, we repeated the experiment but also included MG-132 to maintain cyclin B levels (Figure 2F). Again, pBid was lost upon inhibition of Cdk1, even if cyclin B degradation was inhibited. Bid phosphorylation also occurred in epithelial cells and in MEFs arrested with paclitaxel or monastrol, an Eg5 inhibitor, indicating that it was a general phenomenon associated with mitosis (Figures S2A and S2B). Interestingly, compromising the SAC with an aurora kinase inhibitor did not inhibit pBid accumulation in cells arrested in M (Figure S2C). However, Bid is probably not a direct Cdk1 target. Mouse Bid has no consensus Cdk1 sites, although human Bid has one possible phosphorylation site (Figure S2D). However, active Cdk1 did not phosphorylate recombinant Bid in vitro.

These data reveal that Bid is phosphorylated during mitosis and that pBid is lost concomitant with the metaphase to anaphase transition.

Mouse Bid Is Phosphorylated on S66 during Mitosis

To identify the mitotic phosphorylation sites in Bid, mBidYFP was isolated from nocodazole-treated human embryonic kidney 293T (HEK293T) cells and separated by SDS-PAGE. mBidYFP showed the same mobility shift as seen with endogenous mBid (cf. Figure 3A with 2C). The upper- and lower-molecular-weight bands of mBidYFP were excised, digested with AspN, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). A peptide corresponding to mBid amino acids 59–74 from the upper band had a single phosphate group, whereas the equivalent peptide from the lower band was unmodified. The fragmentation spectra of this peptide indicated the phosphate was on S66 (Figure 3B). Identical MS/MS data were obtained with a synthetic phosphopeptide corresponding to mBid residues 59–74 with phosphate on S66 (Figure 3C). We did not detect any other modifications in mBidYFP from mitotic cells. Furthermore, mBidYFP isolated from untreated cells was not phosphorylated.

To confirm the MS data, we generated a phosphospecific antibody to Bid S66, which detected the slower migrating form of mBidYFP-WT (Figure 3D). We also substituted all potential phosphorylation sites within the regulatory loop of mBidYFP to alanine. MEFs transiently expressing these mutated proteins were arrested by double-thymidine block or nocodazole and

Figure 1. Bid Is Required for Apoptosis following Delayed Mitotic Exit

(A) Knockdown and re-expression of Bid in human carcinoma cells. RKO cells stably expressing control pVenus, pVenus-shBid, or pVenus-shBid-mBidYFP were immunoblotted for human Bid (hBid) and BidYFP. Vinculin was immunoblotted as a loading control. IB, immunoblot.

(B) RKO and DLD1 cells expressing pVenus, pVenus-shBid, or pVenus-shBid-mBidYFP were left untreated or treated with paclitaxel for 18 hr. Cells were collected and apoptosis quantified by immunostaining for active caspase 3. The error bars represent SEM. Data represent the mean of three independent experiments. Data were analyzed by ANOVA. n/s, not significant.

(C) In the left panel, RKO cells stained with Hoechst. RKO cells remained in mitosis when knockdown of Bid prevented them undergoing apoptosis following 18 hr in paclitaxel. In the right panel, RKO cells treated with paclitaxel immunostained for cytochrome c and active caspase 3, as well as Hoechst. The cell indicated by the arrow shows that active caspase 3 corresponds with loss of mitochondrial cytochrome c and pyknotic nuclei.

(D) $Bid^{-/-}$ mouse embryonic fibroblasts (MEF) were stably infected with lentivirus expressing either BidYFP-WT or BidYFP-G94E, before being treated with combinations of paclitaxel and ABT-737 for 18 hr. Apoptosis was quantified as in (B). The error bars represent SEM. Data represent the mean of three independent experiments.

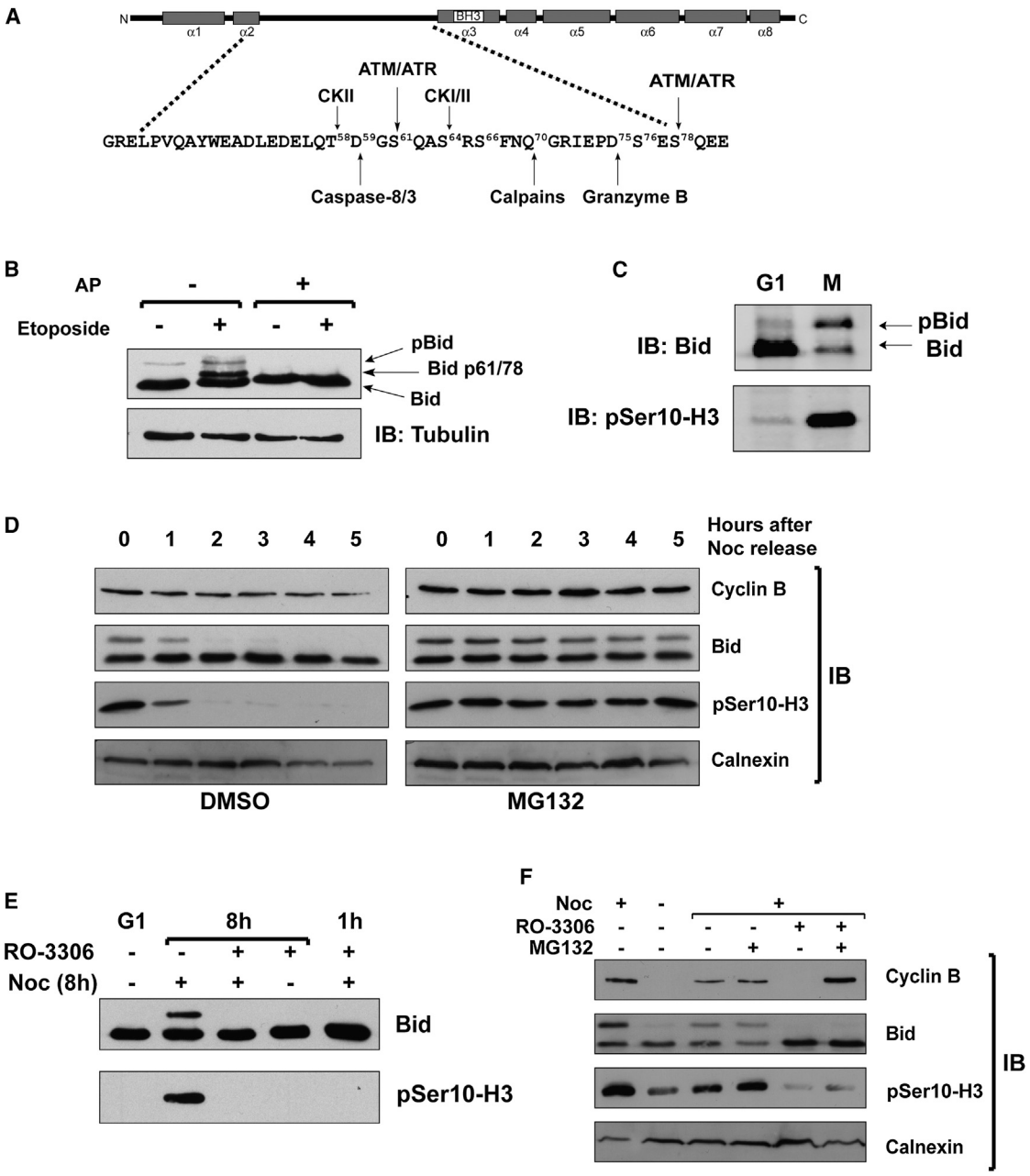


Figure 2. Endogenous Bid Undergoes Reversible Posttranslational Modification during Mitosis

(A) Schematic representation of mouse Bid, showing known sites of posttranslational modification within the loop between α helices 2 and 3.

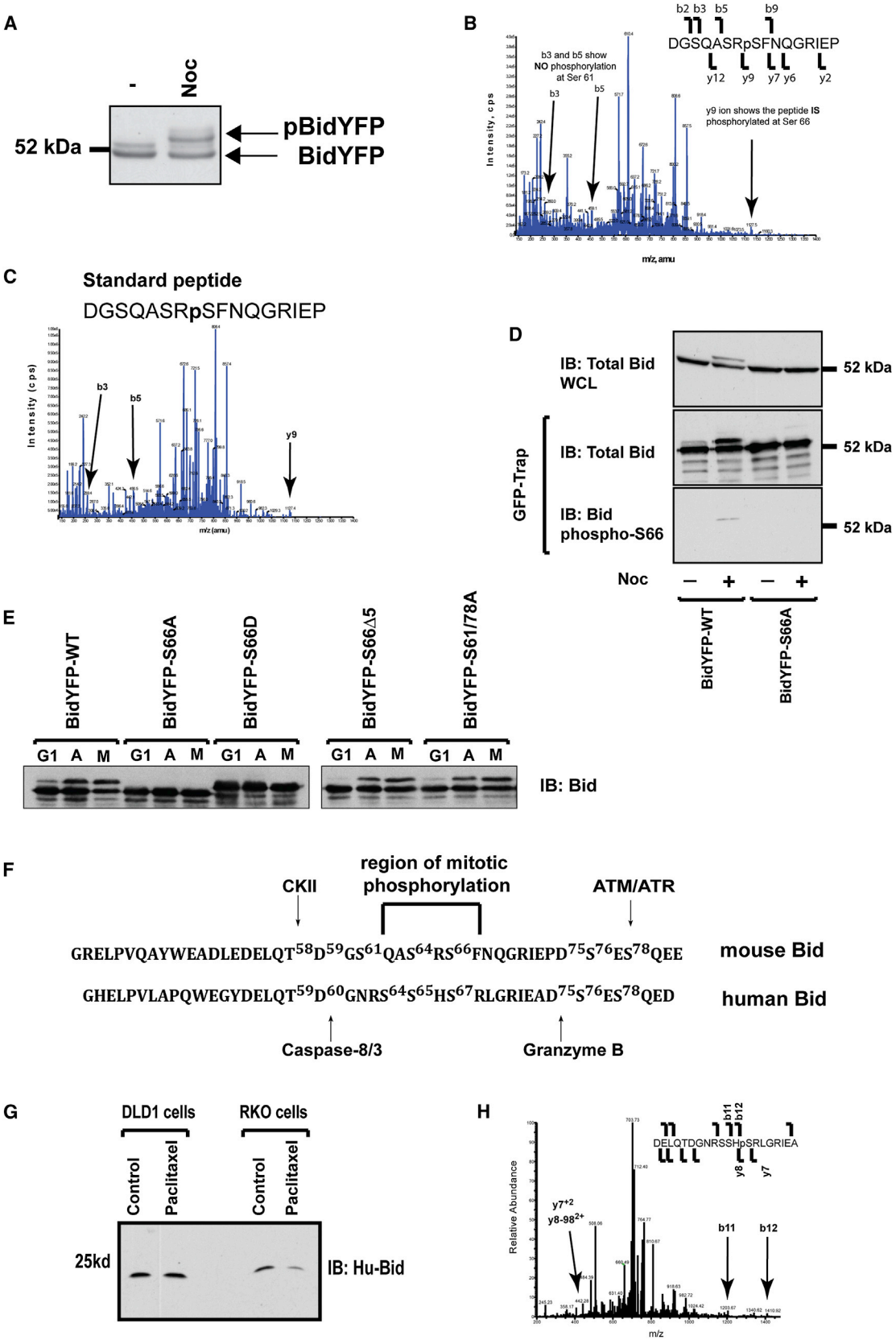
(B) Endogenous Bid in MEFs migrates as multiple forms. MEFs were treated with etoposide for 1 hr and lysates analyzed by immunoblotting for Bid, with or without alkaline phosphatase (AP) treatment. The higher-migrating form of Bid is indicated (pBid). The slower-migrating form of Bid phosphorylated in response to etoposide is also indicated (Bid p61/p78).

(C) Lysates from MEFs arrested in either G1 or mitosis were analyzed by immunoblotting for Bid or phosphorylated histone H3 (pSer10-H3).

(D) MEFs arrested in mitosis were collected by overnight treatment with nocodazole and shaking from the dish. These cells were replated into media with or without MG132. Lysates were collected at the indicated times and immunoblotted for Bid, cyclin B, phospho-histone H3, and calnexin.

(E) MEFs enriched in G1 were released for 8 hr into the indicated combinations of nocodazole and RO-3306. In the right lane, cells were released into nocodazole for 8 hr and RO-3306 added for 1 hr prior to lysis. Lysates were immunoblotted for Bid and pSer10-H3.

(F) Mitotic MEFs were collected by overnight treatment in nocodazole, followed by shake off, and replated in the indicated combinations of nocodazole, RO-3306, and MG132 for 2 hr. Lysates were immunoblotted for Bid, cyclin B, phospho-histone H3, and calnexin.



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the latter divided into those that could be detached by shaking (M) and those that remained attached to the culture dish (A) (Figure 3E). Substitution of all the potential phosphorylation sites other than S66 (BidYFP-S66Δ5) did not prevent the mobility shift in nocodazole-treated cells. Conversely, Bid containing a substitution at S66 alone (BidYFP-S66A) showed no mobility shift in mitosis. Furthermore, substituting S66 to aspartic acid (BidYFP-S66D) resulted in a similar size shift as seen for phosphorylated Bid, even in cells in G1. Phosphorylation of Bid on S66 was independent of the DNA-damage-induced phosphorylation on S61/S78 following etoposide-induced DNA damage (Figures 3E and S2E).

The sequences of human and mouse Bid diverge within the regulatory loop, with potential phosphorylation sites at S64, S65, and S67 in humans (Figure 3F). Furthermore, endogenous human Bid did not show a mobility shift when RKO or DLD1 cells were arrested in mitosis (Figure 3G). To ask if human Bid was phosphorylated in mitosis, hBidYFP was isolated from HEK293T cells and analyzed by LC-MS/MS. A peptide from hBidYFP isolated from mitotic cells corresponding to amino acids 55–74 was phosphorylated uniquely on S67 (Figure 3H). No modifications were found in hBidYFP isolated from untreated cells. No phosphorylation was detected by LC-MS/MS on the putative Cdk-1 consensus site at T163 (Figure S2D), in either untreated or nocodazole-treated cells.

These results demonstrate that Bid is phosphorylated on a unique serine residue specifically in mitosis.

Bid-pS66 Sensitizes Cells to Apoptosis following Delayed Mitotic Exit

To test if Bid-pS66 regulates apoptosis during mitotic arrest, we generated stable RKO lines where endogenous hBid was knocked down and substituted by mouse BidYFP-WT, BidYFP-S66A, BidYFP-S66D, or BidYFP-G94E. As expression of mBidYFP was significantly higher than endogenous hBid using the original pVenus vector with an EF1α promoter (Figure S3A), we replaced it with an ubiquitin (Ub) promoter. This led to expression of mBidYFP at levels comparable to endogenous hBid (Figures 4A and S3B). When the RKO lines were treated with paclitaxel for 18 hr, although BidYFP-WT rescued apoptosis following endogenous Bid knockdown, neither BidYFP-S66A nor BidYFP-G94E BH3 mutant were able to restore the response (Figures 4B–4D). Notably, BidYFP-S66D was not a functional phospho-mimetic and was also unable to restore the response. Similar results were obtained in $Bid^{-/-}$MEFs stably expressing Ub-promoter-driven BidYFP-WT, BidYFP-66A, and BidYFP-G94E (Figure 4E). To ask if phosphorylation of human Bid on S67 had the same role, we generated RKO cells where endogenous hBid was knocked down and hBidYFP-WT or hBidYFP-S67A expressed (Figure 4A). hBidYFP-WT rescued paclitaxel-induced apoptosis in Bid knockdown RKO cells, but hBidYFP-S67A did not (Figures 4F and S4A).

To determine whether the proapoptotic role of Bid during mitosis was seen when cells were treated with other antimitotic drugs, we treated RKO cells with monastrol. These cells also displayed Bid-S66-phosphorylation-dependent apoptosis (Figure 4G), although the level of cell death was much lower than with paclitaxel. However, RKO cells were more prone to slippage in monastrol than in paclitaxel (compare Figures S4A and S4B). Lastly, to determine whether knockdown of Bid altered the general sensitivity of cells to apoptosis, we treated RKO cells with etoposide. There was no effect of Bid knockdown, or expression of mBidYFP-WT or mBidYFP-S66A, on etoposide-induced apoptosis (Figure S4C).

These data reveal that apoptotic sensitivity of both MEFs and human colon carcinoma cells to mitotic arrest requires Bid to be phosphorylated on S66.

Bid-pS66 Controls Mitotic Apoptosis by Sensitizing the Mitochondrial Pathway

To understand how Bid S66 phosphorylation promotes apoptosis during delayed mitosis, we initially examined whether it influences the subcellular localization of Bid. However, both untreated and paclitaxel-treated RKO cells showed a mitochondrial distribution of stably expressed BidYFP-WT, BidYFP-S66A, and BidYFP-G94E, indicating that neither phosphorylation, nor a functional BH3 domain, were required for localization (Figure S5A). Consequently, we hypothesized that Bid-pS66 altered the interactions of Bid with antiapoptotic Bcl-2 proteins to increase mitochondrial priming during mitosis. To test this,

Figure 3. Bid Is Phosphorylated on Serine 66 during Mitosis

(A) BidYFP was transiently expressed in HEK293T cells, which were untreated, or treated with nocodazole. BidYFP was isolated on GFP-trap agarose, separated by SDS-PAGE, and stained with Coomassie blue. pBid is indicated as the sample isolated from nocodazole-treated cells.

(B) Fragmentation spectrum of the AspN-generated Bid peptide corresponding to amino acids 59–74. The data include diagnostic ions that identify the peptide and also a fragment ion that demonstrates that the peptide is phosphorylated at Ser66 (y_9). No fragment ions were observed supporting phosphorylation at any other serine residues present in the peptide. Fragment ions b_3 and b_5 discount S61 phosphorylation.

(C) Fragmentation spectrum of synthetic peptide representing mouse Bid amino acids 59–74, phosphorylated on S66. This peptide shows the same diagnostic fragment ions identified in (B).

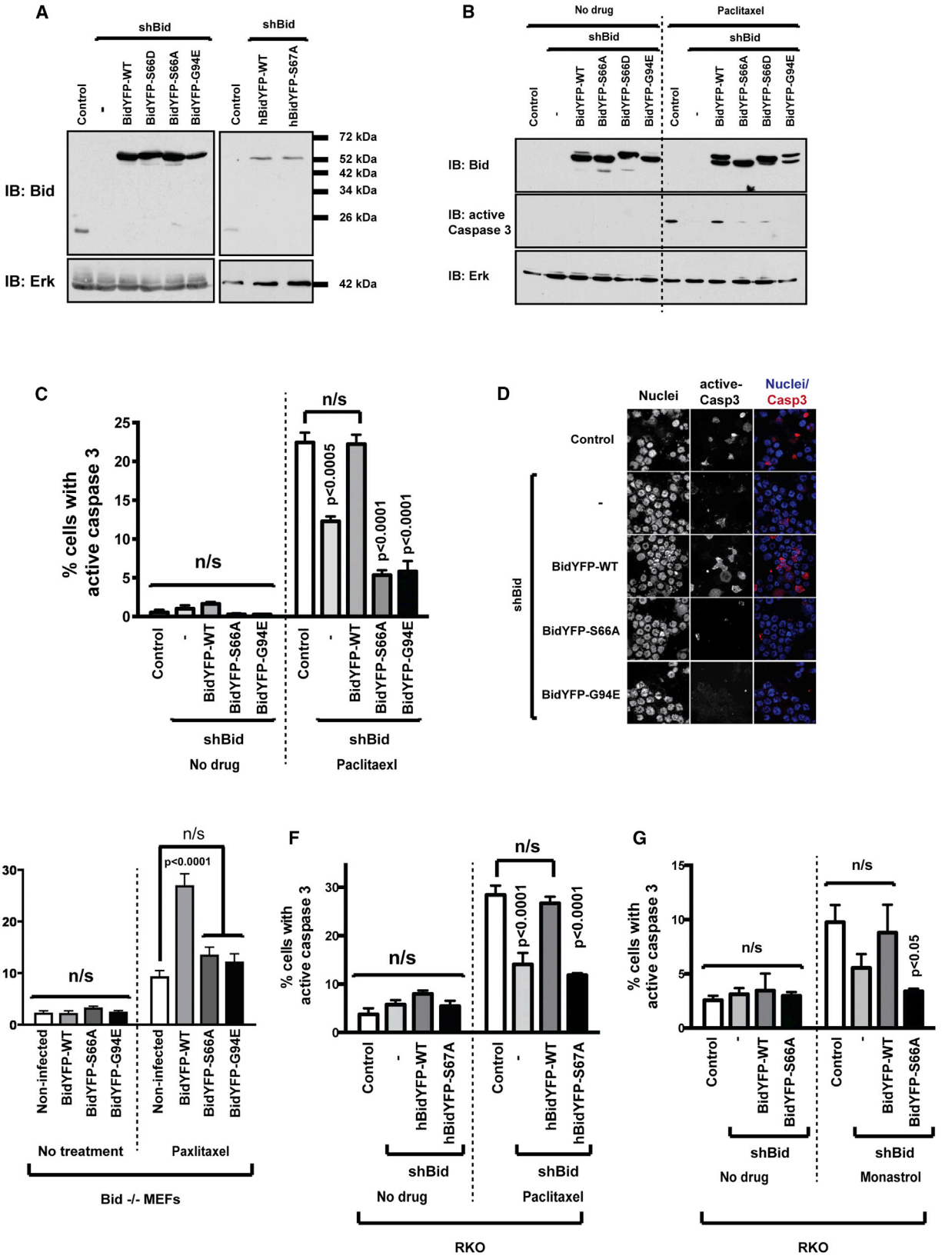
(D) $Bid^{-/-}$MEFs expressing either BidYFP-WT or BidYFP-S66A were untreated or treated with nocodazole and precipitated on GFP-Trap agarose. Precipitates and WCLs were separated by SDS-PAGE and immunoblotted for total Bid or Bid phospho-S66. A phospho-S66-positive band was only detected in the BidYFP-WT cells treated with nocodazole.

(E) MEFs transiently expressing the indicated BidYFP constructs were arrested in either G1 or mitosis. Mitotic cells were collected by shaking (M) and the remaining cells attached to the dish were also collected (A). Lysates were analyzed by immunoblotting for Bid.

(F) Comparison of the amino acids sequences from mouse (top) and human (bottom) Bid in the regulatory loop. The regions of divergence around mouse S66 are indicated.

(G) Human Bid does not show a shift in mobility by SDS-PAGE. DLD1 and RKO cells were treated with paclitaxel for 18 hr and lysates analyzed by immunoblotting for endogenous Bid.

(H) Fragmentation spectrum of the AspN-generated peptide corresponding to amino acids 55–74 from hBid-YFP, isolated on GFP-Trap agarose from nocodazole-treated HEK293T cells. The data include diagnostic ions showing hBid-YFP is phosphorylated uniquely on S67.



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we inhibited antiapoptotic Bcl-2 protein function with the BH3 mimetic, ABT-737. ABT-737 alone did not induce apoptosis in either WT- or Bid$^{-/-}$MEFs. However, whereas ABT-737 sensitized WT-MEFs to apoptosis following treatment with paclitaxel, it had no effect on the resistance of Bid$^{-/-}$MEFs, suggesting that apoptotic priming in mitosis requires Bid (Figure 5A). We then asked if the RKO lines in which hBid had been replaced with BidYFP-WT or BidYFP-S66A could be sensitized to paclitaxel by ABT-737. Again, ABT-737 alone did not increase apoptosis in any of the RKO lines. However, ABT-737 sensitized both the parental RKO cells and cells expressing BidYFP-WT to paclitaxel, but not those in which only endogenous hBid was knocked down (Figure 5B). Interestingly, ABT-737 did sensitize the BidYFP-S66A cells to paclitaxel, and apoptosis in these cells was rescued to almost the same level as parental and BidYFP-WT cells. This suggests that full-length Bid has a basal proapoptotic activity that is increased by phosphorylation on S66.

Lastly, we examined the paclitaxel-resistant DLD1 cells. Paclitaxel resistance was not due to defective Bid phosphorylation, as mBidYFP-WT was phosphorylated on S66 in mitotic DLD1 cells (Figure 5C). Furthermore, when mitotic RKO and DLD1 cells were compared, similar proportions of phosphorylated mBidYFP were seen (Figure 5D). Death during arrested mitosis has been proposed to be initiated by the degradation of Mcl-1 (Harley et al., 2010; Wertz et al., 2011). Surprisingly, both DLD1 and Bid$^{-/-}$MEFs expressed less Mcl-1 than the paclitaxel-sensitive RKO cells (Figure S5B). However, all the lines showed loss of Mcl-1 during mitotic arrest. Consequently, we asked if paclitaxel sensitivity might be achieved in DLD1 cells with BH3 mimetics. DLD1 cells were not sensitive to ABT-737 alone, similar to the RKO and MEFs, and DLD1 cells in paclitaxel tend to exit mitosis by slippage (Figures S1A and 5F). However, ABT-737 dramatically sensitized DLD1 cells to apoptosis in paclitaxel, quantified by active caspase 3 (Figures 5E and 5F). We generated DLD1 cells where endogenous Bid was knocked down and either hBidYFP-WT or hBidYFP-S67A were expressed (Figure S5C). When these were treated with paclitaxel and ABT-737, the same sensitization was seen as with RKO cells (compare Figures 5B and S5D). These data suggest that paclitaxel-resistant DLD1 cells become primed by Bid phosphorylation just like RKO cells but tend to undergo slippage before MOMP can be initiated.

Together, these results show that mitochondrial priming for apoptosis via Bid phosphorylation is a general event in mitosis, even in cells that are otherwise resistant to mitotic-arrest-induced death.

DISCUSSION

As cells progress through mitosis, they initiate two timers, each driven by the proteolysis of a regulator (Topham and Taylor, 2013). Thus, loss of cyclin B leads to mitotic slippage (Brito and Rieder, 2006), whereas degradation of Mcl-1 results in MOMP (Harley et al., 2010; Wertz et al., 2011). Normally, inactivation of the SAC following spindle attachment leads to rapid cyclin B degradation and mitotic exit before Mcl-1 loss reaches the point where death occurs. However, although mitotic cells are sensitive to loss or inhibition of antiapoptotic Bcl-2 proteins, outside of mitosis they are not. The relative dependence of cells on antiapoptotic Bcl-2 proteins is termed mitochondrial priming and changes according to the milieu of Bcl-2 proteins on the outer mitochondrial membrane (OMM) (Del Gaizo Moore and Letai, 2013; Schellenberg et al., 2013). Increased priming results in a cell becoming more dependent upon antiapoptotic Bcl-2 proteins to suppress MOMP, seen by their sensitivity to BH3 mimetics (Certo et al., 2006; Deng et al., 2007; Llambi et al., 2011). Our data presented here indicate that Bid S66 phosphorylation sensitizes cells to apoptosis, making them dependent upon antiapoptotic Bcl-2 proteins as they enter mitosis.

Bid can be phosphorylated on several residues with the regulatory loop between helices 2 and 3 (Degli Esposti et al., 2003; Desagher et al., 2001; Kamer et al., 2005; Zinkel et al., 2005). How phosphorylation on S66 alters Bid function is unclear at present, but we found no evidence that it alters its susceptibility to cleavage by caspase 8 (P.W., J.L., and A.P.G., unpublished data). Indeed, we found that the noncleavable BidD59E mutant was both phosphorylated in mitosis and restored paclitaxel sensitivity to RKO cells following endogenous hBid knockdown.

Loss of endogenous Bid did not completely desensitize RKO cells to apoptosis during mitotic arrest (Figures 1B and 4). Whereas this may be due to incomplete knockdown, it was notable that re-expression of the nonphosphorylatable S66A

Figure 4. Bid Phosphorylation on Serine 66 Sensitizes Cells to Apoptosis during Mitotic Arrest

(A) RKO cells stably infected with pVenus, pVenus-shBid, or pVenus-shBid coexpressing the indicated mouse (left panel) and human (right panel) BidYFP variants under the ubiquitin promoter were analyzed by immunoblotting with an antibody that recognizes both human and mouse Bid. Immunoblotting for Erk was used as a loading control. Endogenous human Bid is only present in the control cells.

(B) The control RKO lines and those expressing mouse BidYFP variants were untreated or treated with 1 μM paclitaxel for 18 hr. Lysates were analyzed by immunoblotting for Bid and active caspase 3. Erk was a loading control. Note the shift in mobility of BidYFP-WT and BidYFP-G94E in paclitaxel-treated RKO cells.

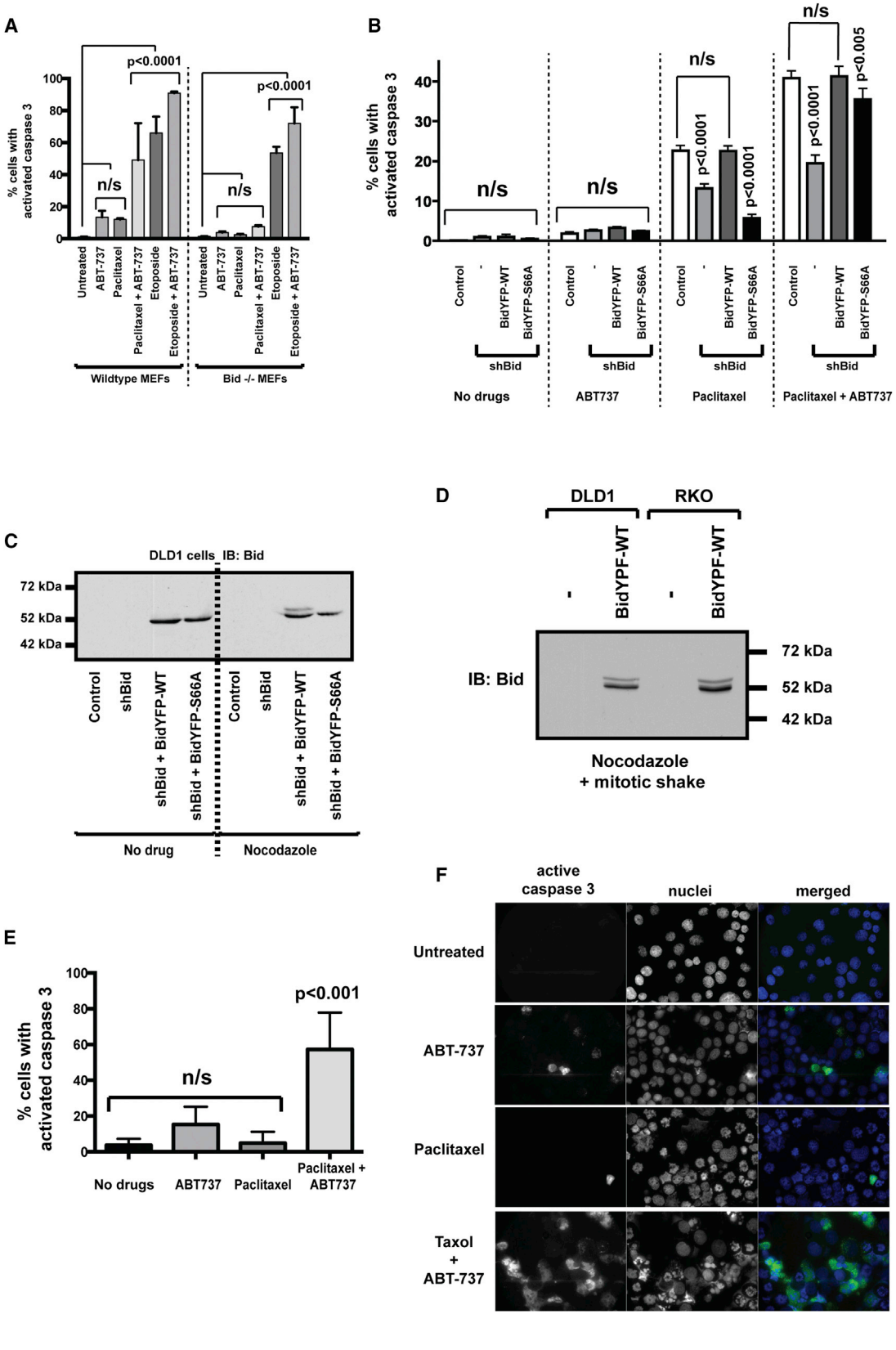
(C) The RKO lines from (A), untreated or treated with 1 μM paclitaxel for 18 hr, were immunostained for active caspase 3 and apoptosis quantified. The data represent the mean of three independent experiments. The error bars represent SEM. Data were analyzed by ANOVA.

(D) Images of the paclitaxel-treated RKO cell lines from (C), immunostained for active caspase 3. Nuclei were stained with Hoechst.

(E) Bid$^{-/-}$ MEFs, infected with the indicated pVenus lentiviruses, were left untreated or treated with 1 μM paclitaxel. Apoptosis was quantified as above. The data represent the mean of three independent experiments. The error bars represent SEM. Data were analyzed by ANOVA.

(F) RKO cells infected with the indicated lentiviruses expressing human Bid or human BidS67A were treated with paclitaxel as in (C). Cells showed similar responses to those expressing the mouse BidYFP. The data represent the mean of three independent experiments. The error bars represent SEM. Data were analyzed by ANOVA.

(G) The indicated RKO lines, untreated or treated with monastrol for 18 hr, were immunostained for active caspase 3 and apoptosis quantified. The data represent the mean of three independent experiments. The error bars represent SEM. Data were analyzed by ANOVA.



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or BH3 domain mutants resulted in further suppression of apoptosis. Consequently, we think that phosphorylation on S66 might bring about a conformational change to alter BH3 domain availability, altering how Bid interacts with multidomain Bcl-2 proteins, and might explain a dominant-negative effect of nonfunctional Bid at the mitochondria. Furthermore, the observation that BidYFP-S66A cells could be sensitized to apoptosis with ABT-737, whereas the Bid deficient or BidYFP-G94E cells could not, suggests that phosphorylation increases a basal proapoptotic activity inherent to full-length Bid. Possible mechanisms are currently under investigation.

Resistance to antimitotics in cancer can occur by either resistance to MOMP or increased mitotic slippage. Maintaining mitotic arrest for longer allows resistant cells to reach the threshold for MOMP (Huang et al., 2009). Similarly, BH3 mimetics such as navitoclax (ABT-263), the orally active variant of ABT-737, accelerate apoptosis during mitotic arrest (Shi et al., 2011). As the paclitaxel-resistant DLD1 cells still underwent apoptotic priming by Bid phosphorylation, they could be sensitized to mitotic-arrest-induced apoptosis by ABT-737, without directly targeting the SAC. Thus, reducing the threshold for MOMP using BH3 mimetics achieves the same goal as prolonging arrest in mitosis.

In summary, we have found that phosphorylation of Bid primes mitochondria for apoptosis and makes a cell dependent upon antiapoptotic Bcl-2 proteins. At anaphase, as soon as the cell has satisfied the requirements to exit mitosis, Bid phosphorylation is lost and mitochondrial priming restored to interphase levels. It is also interesting to note that Bid-deficient mice spontaneously develop myeloid tumors with multiple chromosomal abnormalities, which is expected if loss of Bid function allows cells to survive aberrant mitosis (Zinkel et al., 2003). Furthermore, ATM/ATR phosphorylation of Bid is required for an S phase checkpoint (Kamer et al., 2005; Zinkel et al., 2005) and is involved in the DNA damage response in vivo (Biswas et al., 2013; Maryanovich et al., 2012). Together with those studies, our results support a role for Bid as a sentinel of genomic integrity during the cell cycle.

EXPERIMENTAL PROCEDURES

Detailed descriptions of reagents, cell culture, immunofluorescent imaging, cell proliferation and apoptosis assays, and immunoblotting are provided in the Supplemental Experimental Procedures.

Expression Constructs

BidYFP expression and endogenous Bid knockdown were achieved using the pVenus lentiviral transfer vector, a modified version of pLVTHM in which a multiple cloning site was introduced downstream of the EF1α promoter (a gift from Didier Trono). The hBid shRNA hairpin was introduced downstream of the H1 promoter (target sequence AAGAAGACATCATCCGGAATA). BidYFP was amplified by PCR and inserted in the multiple cloning site regulated by the EF1α promoter. Amino acid substitutions were introduced into the Bid sequence by oligonucleotide-directed mutagenesis. To reduce BidYFP expression, the ubiquitin promoter was PCR amplified from p199-UbTAzeo and cloned in place of the EF1α promoter. To re-express hBid in the shBid knockdown cells, the target sequence for the shRNA was mutated in hBid to AAGAGGATATAATACGGAATA (substitutions are underlined). The amino acid sequence of the expressed protein was unaltered.

Cell Cycle Arrest and Drug Treatments

Cells were arrested in G1 by double thymidine block. Cells were incubated overnight with 2.5 mM thymidine and released from the block in medium without thymidine for 8 hr followed by another overnight treatment with 2.5 mM thymidine. To arrest cells in mitosis, G1-arrested cells were rinsed and incubated in the presence of 200 ng/ml nocodazole for 8 hr or unsynchronized cells were treated with nocodazole overnight. Mitotic cells were collected by shake off. In mitotic release experiments, cells were arrested in mitosis by an overnight incubation in nocodazole (200 ng/ml) and then incubated in the normal growth medium lacking nocodazole for various times. The cdk1 inhibitors RO-3306 (20 μM) and RO-31-8220 (10 μM) were used to arrest cells at G2/M prior to entry into mitosis. The aurora A inhibitor, ZM447439, was used at 2 μM. Mitotic exit was blocked with 10 μM MG132 to prevent cyclin B degradation.

Mass Spectrometry Analysis of Bid Phosphorylation

BidYFP was isolated from transfected HEK293T cells lysed in Radio-Immunoprecipitation Assay buffer (50 mM Tris Cl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, protease inhibitors) by precipitation on GFP-Trap agarose (ChromoTek). BidYFP was separated by SDS-PAGE, stained with Coomasie blue, and cut out from the gel. Excised gel bands were reduced, alkylated with iodoacetamide, and digested overnight with Asp-N (Sigma-Aldrich). Mouse sample digests were analyzed by multiple reaction monitoring (MRM)-LC-MS/MS using a nanoAcquity (Waters) coupled to a 4000 Q-Trap (Applied Biosystems). The mass spectrometer was employed to detect (by MRM) a neutral loss from the predicted *m/z* values for the phosphorylated Bid peptides of interest. Human Bid samples were analyzed by LC-MS/MS using an UltiMate 3000 Rapid Separation LC (RSLC; Dionex) coupled to an Orbitrap Elite (Thermo Fisher Scientific) mass spectrometer. Peptide mixtures were separated by liquid chromatography and selected for fragmentation by MRM initiation (4000 Q-Trap) or automatically via data-dependent analysis (Orbitrap Elite). Data produced were searched using Mascot (Matrix Science UK), against the Uniprot database. Phosphorylation was included in the search parameters as a variable modification. The Mascot

Figure 5. Bid Phosphorylation on Serine 66 Increases Mitochondrial Priming in Mitotic Cells

(A) Wild-type and $Bid^{-/-}$MEFs were treated for 18 hr with the indicated combinations of ABT-737, paclitaxel, and etoposide. Apoptosis was quantified by immunostaining for active caspase 3. Data represent the mean of three independent experiments. The error bars represent SEM.

(B) RKO cells infected with control pVenus, pVenus-shBid, pVenus-shBid-BidYFP-WT, or pVenus-shBid-BidYFP-S66A were untreated or treated for 18 hr with 5 μM ABT-737, 1 μM paclitaxel, or both ABT-737 and paclitaxel. Apoptosis was quantified by immunostaining for active caspase 3. Data represent the mean of three independent experiments. The error bars represent SEM.

(C) DLD1 cells infected with pVenus, pVenus-shBid, pVenus-shBid-BidYFP-WT, or pVenus-shBid-BidYFP-S66A were left untreated or treated with nocodazole overnight. Lysates were immunoblotted for Bid.

(D) DLD1 and RKO cells, control or expressing mouse BidYFP-WT, were arrested overnight in nocodazole and mitotic cells collected by shaking. Lysates were immunoblotted for Bid. Quantification of the pBid/Bid ratios using Odyssey-based imaging showed no difference between the cell types.

(E) DLD1 cells expressing endogenous Bid were treated with the indicated combinations of drugs for 18 hr. Cells were immunostained for active caspase 3 and apoptosis quantified. Data represent the mean of three independent experiments and analyzed by ANOVA. The error bars represent SEM.

(F). Representative images from (E).

output was subsequently validated using Scaffold (Proteome Software). The product ion spectra were also assessed manually.

Statistical Analysis

Quantitative data were analyzed by either one-way or two-way ANOVA using Bonferroni's multiple comparison test where indicated (GraphPad Prism).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.050.

ACKNOWLEDGMENTS

We thank Dr. David Knight for discussions about the mass spectrometry, Prof. Stephen Taylor for advice on mitotic arrest, and members of the Wellcome Trust Centre for Cell Matrix Research for discussions. This work was supported by grants from Breast Cancer Campaign, Cancer Research UK, and The Wellcome Trust. The Wellcome Trust Centre for Cell-Matrix Research is supported by core funding from the Wellcome Trust (grant 088785/Z/09/Z).

Received: October 15, 2013
Revised: February 12, 2014
Accepted: March 20, 2014
Published: April 24, 2014

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