BMX Negatively Regulates BAK Function, Thereby Increasing Apoptotic Resistance to Chemotherapeutic Drugs

Joanna L. Fox and Alan Storey

Abstract

The ability of chemotherapeutic agents to induce apoptosis, predominantly via the mitochondrial (intrinsic) apoptotic pathway, is thought to be a major determinant of the sensitivity of a given cancer to treatment. Intrinsic apoptosis, regulated by the BCL2 family, integrates diverse apoptotic signals to determine cell death commitment and then activates the nodal effector protein BAK to initiate the apoptotic cascade. In this study, we identified the tyrosine kinase BMX as a direct negative regulator of BAK function. BMX associates with BAK in viable cells and is the first kinase to phosphorylate the key tyrosine residue needed to maintain BAK in an inactive conformation. Importantly, elevated BMX expression prevents BAK activation in tumor cells treated with chemotherapeutic agents and is associated with increased resistance to apoptosis and decreased patient survival. Accordingly, BMX expression was elevated in prostate, breast, and colon cancers compared with normal tissue, including in aggressive triple-negative breast cancers where BMX overexpression may be a novel biomarker. Furthermore, BMX silencing potentiated BAK activation, rendering tumor cells hypersensitive to otherwise sublethal doses of clinically relevant chemotherapeutic agents. Our finding that BMX directly inhibits a core component of the intrinsic apoptosis machinery opens opportunities to improve the efficacy of existing chemotherapy by potentiating BAK-driven cell death in cancer cells. Cancer Res; 75(7): 1–11. ©2015 AACR.
there is often little response to chemotherapy, and there are only a few treatment options in this setting. Thus, there is an urgent clinical need to identify new therapeutic targets to improve the outlook for these patients. Recent genetic profiling of these tumors reveals that there are diverse changes that occur during their development that makes identifying therapeutically targetable proteins complex (19). We hypothesize then that if a BAK kinase was overexpressed or upregulated in these tumor types, this would effectively enable the cell to block initiation of BAK-dependent apoptosis, thereby increasing the apoptotic threshold of the tumor.

In this article, we identify BMX as the first tyrosine kinase that is able to modulate activation of BAK by direct phosphorylation of a key tyrosine residue, Y108. We find BMX expression to be upregulated in numerous tumor types of major importance, including TNBC, and that BMX overexpression in tumor cells increased the apoptotic threshold of these cells. Furthermore, we demonstrate that modulation of BMX expression levels by RNAi can resensitize tumor cells to existing chemotherapeutic agents by decreasing BAK phosphorylation, resulting in increased BAK activation potential and enhanced levels of apoptotic cell death.
Materials and Methods

Cell culture

HT1080, DU145, BT549, and MCF7 cells were obtained from and characterized by ATCC. HCT116\(^{bax-/-bax-/-}\)/C0 cells were a gift from R. Youle. All cells were mycoplasma-free when tested with MycoAlert (Lonza) and used for less than 6 months of continuous passage.

Flow cytometric analysis of BAK conformation was determined using Ab-1 (AM03; Calbiochem) as previously described (20). Cytochrome c release was determined using clone 6H2.B4 (BD Biosciences; ref. 21). Standard methods were used to determine Annexin V positivity.

RNA interference

Standard methods were used for siRNA and shRNA transfections with details in Supplementary Materials and Methods. Cells positive for shRNA constructs against BMX, PTPN21, and a
control shRNA construct (pRS; Origene) were selected with 0.4 μg/mL puromycin.

**Immunoprecipitation**

Protein A/G agarose beads (Santa Cruz Biotechnology) and 1 μg antibody as follows: pY108 BAK antibody previously characterized (16), PTPN21 (Abgent), BAK (BH3; Cell Signaling Technologies), BMX (C-17; Santa Cruz Biotechnology), pY100 (Cell Signaling) were used. Resultant samples were analyzed by immunoblotting.

Immunoblot analyses were carried out as previously described (16). Antibodies used were either previously published or listed (Supplementary Materials and Methods).

**Proximity ligation in situ assays (PLISA)** were performed using a Duolink II Kit (Olink Bioscience) with anti-BMX (C-17; Santa Cruz Biotechnology) and anti-BAK (BH3; Cell Signaling Technologies) antibodies.

For *in vitro* kinase assay, BMX was isolated by immunoprecipitation and incubated with GST, GST-WT BAK, or GST-Y108A BAK protein substrates produced in *Escherichia coli* and purified using standard techniques. Reactions were performed in kinase buffer containing 1.5 μCi [γ-32P]ATP and phosphorylated proteins detected by phosphoimage analysis (Typhoon analyzer). Immunoprecipitation kinase assay was performed using untagged wild-type (WT) or Y108A BAK as a substrate and phosphorylated protein...
immunoprecipitation with pY108BAK antibody and magnetic protein G beads.

**Tissue microarray and immunohistochemical analysis**

Tissue microarrays were purchased from OCHRe and staining performed with ethical approval (Reference Number C02.216).Slides were immunostained, evaluated by an independent histopathologist, and graded using a 2-score system based on intensity score and proportion score as described previously (22), see Supplementary Material for further details.

**Statistical analysis**

All data are presented as mean ± SEM of three independent biologic experiments. t tests were used to compare two experimental groups, differences were considered statistically significant where P < 0.05.

**Results**

**Identification of BMX as a potent inhibitor of BAK function**

A conformational change at the N-terminal of BAK can be used to measure levels of BAK activation, but this can only occur if the phosphate at residue Y108 of BAK is first removed and BAK is rendered “activation competent” (15, 16). We therefore wanted to identify kinases able to phosphorylate BAK at Y108, thus maintaining BAK in the inactive conformation. siRNA screening of the TEC tyrosine kinase family revealed that knockdown of BMX, but not the other family members, was able to significantly increase levels of BAK activation in response to camptothecin treatment (Fig. 1A). To determine whether BMX was acting directly on BAK or via upstream signalling pathways, we tested whether BMX could bind directly to BAK using coimmunoprecipitation/Western blotting. The BMX–BAK complex could be immunoprecipitated using either a BAK or BMX antibody (Fig. 1B), indicating that the two proteins were found in a stable complex in healthy cells. The specificity of these coimmunoprecipitations was confirmed using BAK-null and BMX-knockdown cells as controls (Supplementary Fig. S1). In contrast, BAX was not found to coimmunoprecipitate with BMX (Supplementary Fig. S2). To further examine the BMX–BAK complex in cells, we used a PLISA (23), which enables individual protein–protein complexes in cells to be studied. PLISA confirmed that in undamaged cells, BMX was in close proximity to endogenous BAK (Fig. 1C) whereas treatment of cells for 6 hours with camptothecin caused a detectable decrease in the number of PLISA foci present in a significant number of the cells studied (Fig. 1C and Supplementary Fig. S3A and S3B). Single-color controls confirmed that both BAK and BMX levels were unaltered following treatment, as both proteins could be detected at similar levels in both treated and untreated cells (Supplementary Fig. S3C and S3D). These data suggest that BMX associates with BAK in undamaged cells, but following an apoptotic stimulus, the BMX–BAK complex dissociates, which correlates with a decrease in pY108 BAK in cells following camptothecin treatment. The decrease in PLISA foci occurred before other morphologic changes associated with apoptosis revealing that dissociation of the BMX–BAK complex is an early step in the BAK activation process, in line with the fast kinetics of BAK activation. To assess whether BMX was able to phosphorylate BAK at residue Y108 and was not just present in a complex with inactive BAK, we performed an in vitro kinase assay. BMX protein was immunoprecipitated from HT1080 cells and incubated in the presence of [γ-32P]ATP was able to phosphorylate purified GST-tagged WT, but not the Y108A mutant BAK recombinant protein (Fig. 1D). Furthermore, when the protein from the kinase assay was
Figure 5.
BMX knockdown promotes BAK activation and cell killing. A, FACS analysis of BAK activation in HT1080 cells expressing BMX shRNA or empty vector (pRS) ± 4-hour camptothecin (CPT) or etoposide (ET) treatment. * P < 0.05. B, FACS analysis of BAK activation in DU145 prostate cancer cells expressing BMX shRNA or empty vector (pRS) ± 4-hour camptothecin or docetaxel (DOC) treatment. * P < 0.05. (Continued on the following page)
immunoprecipitated with an antiserum that specifically recognizes BAK that is phosphorylated at Y108, WT but not Y108A mutant BAK was pulled down (Fig. 1E). Together these assays show that BMX was able to phosphorylate BAK at this key regulatory residue. It is possible that there are other tyrosine kinases that are able to phosphorylate BAK that have yet to be identified, but we conclude that BMX is the first BAK tyrosine kinase identified to date.

BMX is upregulated in prostate, breast, and colorectal cancer

BMX is a member of the TEC family of non–receptor tyrosine kinases, which has been reported to have roles in modulating multiple cellular processes including proliferation, differentiation, and apoptosis. Interestingly and supporting a role in pathogenesis elevated BMX levels in bladder cancer were reported to be associated with poorer prognosis (24), and BMX is important in the migration and invasion of breast cancer cells (25). Increased BMX expression has also been observed in neoplastic skin (26), renal cell carcinoma (27), and in prostate cancer (22, 28). To extend and explore these findings in greater depth, we performed IHC on tissue microarrays of cancer biopsies to determine BMX levels in a range of human tumor types. This analysis revealed that BMX levels were significantly elevated in prostate, breast and colon cancers when compared to normal tissue (Fig. 2A and Supplementary Fig. S5). In conjunction with the IHC data, Western blotting of cell extracts from prostate cell lines revealed higher levels of BMX protein in PC3 and DU145 lines than in LNCaP cells (Fig. 2B). In a panel of colon cancer cell lines, we found that BMX overexpression was readily detectable in 5 of 20 cell lines investigated. BMX responds to receptor tyrosine kinase signaling that can be transduced through BRAF to MAP kinases, but we found no correlation between BMX levels and V600E BRAF mutation status (Fig. 2C). In a drive to identify individuals most at risk of developing the disease, genome-wide analysis of mutations and SNPs in breast cancers have revealed a complex tumor landscape and identified many new cancer-associated genes and loci; however, no activating or other mutations in BMX were reported (29–32). So-called TNBC are characterized by a lack of expression of 3 important receptors: estrogen, progesterone, and HER2 and display heterogeneity in mutation patterns. TNBC accounts for about 15% of all breast cancers at diagnosis, but the aggressive nature of the tumors and poor prognosis likely account for a disproportionate share in mortality rates (33). Importantly, we found that 6 of 6 TNBC lines investigated had much higher levels of BMX than cell lines in which at least one receptor was expressed and lacked any detectable BMX (Fig. 2D). Thus, while the acquisition of multiple mutations that in combination can alter the activity of the many diverse signaling pathways upstream of BMX activity, in some tumors, overexpression of BMX may promote evasion of apoptosis and help confer a survival advantage to cancer cells. However, the mechanism by which BMX is able to achieve this by modulating cellular apoptosis to promote cancer cell survival has not been identified to date. Importantly, our data suggest that the antiapoptotic role of BMX is largely through its regulation of BMX phosphorylation at Y108.

BMX requires PTPN21 for BAK phosphorylation

Activation of BMX requires both transphosphorylation and autophosphorylation. Once these events have occurred, a substantial increase in kinase activity is observed (34). In addition, non–receptor protein tyrosine phosphatase 21 (PTPN21) has been reported to bind to and regulate the activity of BMX (35), indeed in HT1080 cells, BMX can be immunoprecipitated with PTPN21 (Supplementary Fig. S6). Structure function studies of PTPN21 performed using the phosphatase domain failed to identify any substrate motifs (36), but the modulation of Src and EGF signaling pathways suggests that the full-length protein may be active (37). Previous siRNA screening of the non–receptor protein tyrosine phosphatase family to identify phosphatases involved in BAK activation revealed that knockdown of some phosphatases led to a modest increase in BAK activation (16). However, further investigations indicated that knockdown of PTPN21 both by siRNA and shRNA (knockdown determined by qRT-PCR; Supplementary Fig. S7) also increased the basal levels of BAK in a conformationally active state even in the absence of any damage signal, presumably due to an increase in the number of underphosphorylated BAK molecules. However, a dramatic potentiation of BAK activation occurred when an apoptotic signal was induced using a chemotherapeutic DNA-damaging agent such as camptothecin (Fig. 3A and Supplementary Fig. S8) or UV radiation (Supplementary Fig. S8A). Concomitant with the increase in levels of active BAK, we also found an increase in the number of cells showing cytochrome c release early after camptothecin treatment (Fig. 3B) and actively undergoing apoptosis as judged by Annexin V–positive staining (Fig. 3C and Supplementary Fig. S9) when PTPN21 was silenced—as compared with PTPN7, which we reported previously to have no effect on BAK activation (16). These results at first appear counterintuitive because such an observed increase in BAK activation would be predicted to be due to the relief from inhibition of BMX kinase activity that suppressed the initial step of BAK activation through phosphorylation at Y108. Measurement of the level of BMX autophosphorylation, a surrogate marker of BMX activity, by phospho-tyrosine immunoprecipitation–Western blotting showed that PTPN21 knockdown decreased the activity of BMX (Fig. 3D). This finding suggested that BMX acts in concert with and may require PTPN21 to direct its activity to BAK. It is therefore the role of PTPN21 in regulating BMX activity, rather than any direct activity on BAK itself that is important, implying that the PTPN21–BMX axis is key in determining the level of BMX phosphorylation at Y108. To test this, we performed immunoprecipitation–Western blots with an antisera that specifically recognizes BAK that is phosphorylated at Y108. While pY108–BAK was readily detectable in undamaged cells, we found that siRNA knockdown of either PTPN21 or BMX
each resulted in markedly decreased levels of pY108–BAK regardless of the presence of DNA damage (Fig. 3E and Supplementary Fig. S10). We conclude that BAK is a novel target of BMX signaling and that BMX is primarily responsible for maintaining pY108–BAK phosphorylation in these cells; however, BMX requires PTPN21 for this activity as knockdown of either PTPN21 or BMX leads to BAK underphosphorylation, enabling a potentiation of BAK activation and cell killing.

**BMX overexpression raises the apoptotic threshold of cancer cell lines**

To define further the link between BMX and BAK and simulate the effects of BMX overexpression in cancers, we developed a BMX overexpression cell line model in which we reasoned that the increased BMX levels would suppress BAK activation following camptothecin treatment. Transfection of HT1080 cells with plasmids expressing BMX showed that while expression of the wild-type protein was capable of suppressing BAK activation compared with cells transfected with the pEFires vector (38), expression of a previously characterized kinase-dead BMX mutant (K444Q; ref. 34) had no effect on BAK activation following camptothecin treatment (Fig. 4A). In line with this finding, overexpression of wild-type BMX also inhibited the release of cytochrome c from cells treated with camptothecin. Expression of the BMX K444Q mutant not only failed to suppress cytochrome c release following camptothecin treatment but instead resulted in a significant increase in cytochrome c release, suggesting either that this mutant acted in a dominant-negative manner or that the activity of other cellular targets that also impinge upon apoptotic signaling might have been altered (Fig. 4B). Increasing levels of active BMX in HT1080 cells also increased the cellular IC_{50} value about 4-fold as determined by MTT assay in response to camptothecin from 0.004 to 0.0165 μmol/L, whereas in comparison, cells expressing the K444Q BMX mutant had an IC_{50} of 0.005 μmol/L (that was not significantly different from control cells transfected with the pEFires vector). Consistent with these findings, HT1080 cells overexpressing BMX showed only a small decrease in phosphorylation of Y108 following camptothecin treatment (Fig. 4C and Supplementary Fig. S11). Together these findings indicate that cells with increased levels of BMX maintain BAK phosphorylation at Y108 and cannot initiate apoptosis, thereby conferring increased resistance to cytotoxic agents. To further confirm that the BMX activity on BAK is the key determinant for the observed increase in resistance to camptothecin, we similarly overexpressed BMX in HCT116{K444Q/BMX-/}/- cells that have been reconstituted to express either WT or Y108A-mutant BAK. Overexpression of BMX was able to block BAK activation in response to camptothecin treatment, as measured by N-terminal BAK conformational change, but only in cells expressing WT but not the Y108A BAK mutant (Fig. 4D).

**Knockdown of BMX lowers the apoptotic threshold by increasing BAK activation**

Following on from the reported roles of BMX in the pathogenesis of numerous cancers types, there has been increasing interest in developing BMX inhibitors (reviewed in ref. 39). Therefore, to evaluate the effect of BMX inhibition on BAK activation, we developed cell lines in which BMX was stably knocked down by shRNA. As predicted, knockdown of BMX alone modulated pY108 BAK levels (Fig. 3E) but did not cause BAK activation (Fig. 5A). However, knockdown of BMX in HT1080 cells significantly increased the amount of BAK that underwent activation, as measured by BAK N-terminal conformational change, in response to treatment with camptothecin or etoposide compared with empty vector controls (Fig. 5A). Levels of BAX conformational change, however, were not effect by BMX knockdown (Supplementary Fig. S12). Similar potentiation of BAK activation by BMX knockdown was seen in DU145 prostate cancer cells (Supplementary Fig. S13B) treated with docetaxel, an important therapeutic agent currently in widespread clinical use (Fig. 5B and Supplementary Fig. S13A), and in the breast cancer cell lines, BT549 and MCF7, which have high and low/undetectable levels of BMX, respectively (Supplementary Fig. S14). Overall, the magnitude of the sensitization observed did correlate with the levels of BMX in the cells, suggesting that BMX may be an excellent biomarker of cells that are using BMX overexpression as a mechanism to evade apoptosis. The increase in BAK activation in HT1080 cells correlated with a decrease in cellular IC_{50} values as determined by MTT assay (Supplementary Table S1), most strikingly in response to etoposide treatment where an approximate 10-fold increase in sensitivity was observed in cells with BMX levels knocked down compared with control cells (Fig. 5C). This suggested that a dose of etoposide that was nontoxic to the control cells would elicit cell death in BMX-knockdown cells, highlighting the huge potential that the use of a BMX inhibitor could have in combination with existing chemotherapeutic agents. To test this, BMX-silenced cells were treated with 0.5 μmol/L etoposide, a dose that caused minimal cell killing in control cells but caused significant levels of death in cells with silenced BMX. Examination of the effects this dose of etoposide had on BAK activation revealed that 0.5 μmol/L etoposide was insufficient to trigger BAK dephosphorylation (Fig. 5D) or N-terminal conformational change in the control cells (Fig. 5E). Whereas in the BMX-knockdown cells, where pY108 BMX levels were already significantly decreased (Fig. 5D and E), BAK activation was readily detectable as early as 4 hours following etoposide treatment and continued to increase with time (Fig. 5E), which correlated with the increase in apoptotic cells observed by Annexin V staining at the same time points (Fig. 5F). At later time points, a small increase in Annexin V–positive cells was apparent in the BMX-knockdown cells that may reflect the loss of other prosurvival activities of BMX unrelated to BAK. To confirm that the difference in apoptosis observed in the BMX-knockdown cells was BMX-dependent, transient knockdown of BAK expression by siRNA in BMX-knockdown cells partially reversed the sensitization to low-dose etoposide, indicating that the increase in sensitivity observed in this cell system was predominantly caused by the reduced activity of BMX on BAK (Fig. 5G).

**Discussion**

In this study, we investigated the molecular mechanism through which the activity of the apoptotic machinery can be suppressed in cancer cells. Chemotherapeutic drugs remain a mainstay of front-line clinical cancer therapy. Improving the efficacy of treatments or overcoming acquired resistance are priorities as they can have a major impact on disease progression and overall survival rates. We demonstrate for the first time here that BMX, acting in concert with PTPN21, phosphorylates the apoptotic regulator BAK, thereby suppressing BAK activation to favor survival of cells exposed to cytotoxic agents. These findings
define a novel mechanism that helps to explain how cancer cells can manifest increased resistance to multiple chemotherapeutic drugs that damage cells in different ways. The initial conversion of BAK from a Y108-phosphorylated to Y108-dephosphorylated form is the key switch that enables BAK activation to proceed and cause MOMP (16). We now propose that the size of the pool of Y108-dephosphorylated BAK will determine a given cell's sensitivity to undergo apoptosis, therefore setting the apoptotic threshold within that cell (Fig. 6). Upregulation of BMX expression, as observed in many cancer types and cancer cell lines (Fig. 2), maintains BAK in the inactive Y108 phosphorylated form, which in turn enables cancer cells to suppress BAK-driven cell death in response to diverse agents by modulating a single nodal point that integrates survival versus death signaling in the apoptotic cascade.

BMX has been implicated in the development of prostate cancer (22, 40), where elevated levels may compensate for androgen deprivation (28, 41). Furthermore, a requirement of BMX for glioblastoma stem cell survival has been identified (42), as well as a role for BMX in the pathogenesis of bladder cancer (24). At a molecular level, BMX has been shown to be involved in controlling a number of important pathways downstream of PI3K signaling (43, 44), including cell proliferation involving FAK and PAK1 to promote the migration and invasion of breast cancer cells (25). Furthermore, there are numerous reports that modulation of BMX expression levels is able to influence cellular apoptosis (45, 46); however, to date, no BMX substrates directly involved in the process have been identified. BMX is known to be activated by signaling pathways known to promote cell proliferation and survival (47); therefore, its role in phosphorylating Y108-BAK that we now propose maintains BAK in an inactive conformation, thereby providing another level of regulation that helps to push the life death cellular balance in the direction of survival. Our findings, therefore, reveal a mechanism through which BMX plays a major role in conferring apoptotic resistance to cancer cells, a function that underpins other pathogenic activities associated with BMX overexpression.

A clearer understanding of the mutational processes that drive cancer cells and how they impact on complex signaling networks that confer survival and growth advantage is ultimately needed. Genome sequencing has emerged as a powerful tool holding the exciting prospect of identifying genes and pathways altered in cancer cells that may provide new targets for intervention. However, BMX and BAK have been found to be mutated in only 0.51% and 0.095%, respectively, of the total samples analyzed (COSMIC database). Our findings raise the important prediction that malignancies may still be driven by tyrosine kinase signaling even though dominant mutations have not been identified. We propose a paradigm in which signaling pathways subverted through genetic and epigenetic alterations can impact directly on and suppress the activity of the otherwise intact apoptotic machinery. Modulation of a single nodal point in the apoptotic machinery, as exemplified by the phospho-regulation of BAK, enables diverse signaling pathways to converge and produce a common phenotypic outcome that favors survival of cancer cells.

With increasing interest in developing specific BMX inhibitors (reviewed in ref. 39) and the expansion of clinical trials of existing agents shown to have activity against BMX, for example, EGFR inhibitors (48), it would be interesting to investigate the effects of these agents on the BAK activation pathway. Our study suggests that, together with any stand-alone activity, these agents may have, they also have the potential to lower the apoptotic threshold of tumor cells. We present evidence that BMX inhibition presents an attractive novel approach to potentiate BAK-driven cell killing in appropriate combination with cytotoxic drugs. Such combination strategies could be used to both reverse the apoptotic resistance associated with cancers and render tumor cells susceptible to killing by otherwise sublethal doses of drug, by increasing the size of the Y108-dephosphorylated BAK pool within the cell. A further significant clinical implication of our results is that we can identify specific cancer cell types for which BMX may be an important biomarker for survival dependency.

In summary, we establish that BMX is the first kinase identified to phosphorylate the apoptotic regulator BAK. Consistent with published data, we find BMX expression to be upregulated in numerous cancer types and demonstrate that upregulation of BMX maintains BAK phosphorylation, thereby enabling the cancer cells to suppress BAK-driven apoptosis in response to diverse stimuli. Furthermore, reduction of BMX by RNAi significantly lowered the apoptotic threshold of tumor cells rendering them much more sensitive to chemotherapeutic agents. Finally, we propose that BMX expression could be used as a biomarker to...
identify patients in which inhibition of BMX could be used as a mechanism to lower the apoptotic threshold of cells to increase the efficacy of existing cytotoxic agents.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: J.L. Fox, A. Storey

Development of methodology: J.L. Fox, A. Storey

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.L. Fox

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L. Fox, A. Storey

Writing, review, and/or revision of the manuscript: J.L. Fox, A. Storey

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.L. Fox, A. Storey

**References**


**Acknowledgments**

The authors thank Peter McHugh and Marion MacFarlane for reading drafts of this article; Dr Richard Youle for kind gift of HCT116−/−/bax−/− double knockout cells; Leticia Campo for help with staining; and Drs. David Delaney and Francesco Pezzella for scoring the TMA, Sir Walter Bodmer for the colon cancer cell lines and Carolin Schmidke for assistance with Western blot analyses; members of the WIMM FACS core facility; MRC Toxicology Unit for reagents and support.

**Grant Support**

This work was funded by a CRUK program grant.

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Received May 2, 2014; revised January 19, 2015; accepted January 20, 2015; published OnlineFirst February 3, 2015.


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Cancer Res  Published OnlineFirst February 3, 2015.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-1340

Supplementary Material  Access the most recent supplemental material at:
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