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Synchronised phosphorylation of BNIP3, Bcl-2 and Bcl-xL in response to microtubule-active drugs is JNK-independent and requires a mitotic kinase

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ABSTRACT

BNIP3 is a hypoxia-inducible BH3-only member of the Bcl-2 family of proteins that regulate apoptosis and autophagy. However the role of BNIP3 in the hypoxia response has proved difficult to define and remains controversial. In this study we show that in cancer cells, knockdown or forced expression of BNIP3 fails to modulate cell survival under hypoxic or normoxic conditions. However, we demonstrate that BNIP3 is regulated post-translationally, existing as multiple monomeric and dimeric phosphorylated forms. Upon treatment with microtubule inhibitors, but not other classes of chemotherapeutics, BNIP3 becomes hyperphosphorylated. We demonstrate that the phosphorylation of BNIP3 occurs in synchrony with phosphorylation of its binding partners Bcl-2 and Bcl-xL. Microtubule inhibitor-induced phosphorylation of these proteins occurs independently of the AKT/mTor and JNK kinase pathways and requires Mps1 mitotic checkpoint kinase activity. Inhibition of mitotic arrest in the presence of paclitaxel blocks the phosphorylation of BNIP3, Bcl-2 and Bcl-xL, demonstrating that these proteins are phosphorylated by a mitochondrially active mitotic kinase. We show that phosphorylation increases the stability of BNIP3 and that BNIP3 predominantly interacts with the phosphorylated form of Bcl-2. This study provides new insight into the post-translational functional control of these Bcl-2 family members.

1. Introduction

Hypoxia is a reduction in the normal level of tissue oxygen tension and is a key feature of solid tumours where it arises due to an inadequate blood supply. Hypoxia evokes a range of adaptive changes in cells that facilitate survival under low oxygen conditions [1]. The transcription factor hypoxia-inducible-factor-1α (HIF-1α) is stabilised in response to hypoxia where it combines with HIF-1β to form a transcriptionally active HIF-1 heterodimer. HIF-1 binds to hypoxia response elements (HRE) in a diverse range of target genes [1,2] including the Bcl-2 family members BNIP3 and, its homologue, BNIP3L (Nix) [3–7].

The Bcl-2 family of proteins play a critical role in the regulation of apoptosis (for review see [8–11]). Members of this family share up to four homologous regions, called Bcl-2 homology domains (BH1-4). Antiapoptotic members including Bcl-2, Bcl-xL, Bcl-w, A1 and Mcl-1 contain all four BH domains and predominantly reside at the outer mitochondrial membrane, where they integrate via a transmembrane (TM) domain. The antiapoptotic members bind to and inhibit two groups of pro-apoptotic Bcl-2 family proteins. The first group of pro-apoptotic members, termed the BH3-only proteins, include BAD, BID, BIK, BIM, BNIP3, BNIP3L (NIX), HRK, NOXA and PUMA. These are transcriptionally induced and/or post-translationally activated in response to specific stress stimuli. The second pro-apoptotic group, or effectors, including BAK and BAX (containing BH1-3), induce apoptosis via pore formation in the mitochondria leading to cytochrome *c* release, APAF1 initiation and caspase activation. Bcl-2 and Bcl-xL also regulate autophagy, the sequestration and degradation of cytoplasmic contents in vacuoles via the lysosome, by binding to and inhibiting the BH3-only autophagy activator Beclin-1. Some other BH3-only proteins have been shown to activate autophagy via binding to Bcl-2/Bcl-xL and releasing Beclin-1 [12].

Abbreviations: BAD, Bcl-2-associated death promoter; Bcl-2, B-cell CLL/lymphoma 2; Bcl-xL, B-cell leukemia XL; Bcl-w, B-cell leukemia w; BID, Bcl-2 interacting domain; BIK, BCL2-interacting killer (apoptosis-inducing); BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; BNIP3L, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 like; DMEM, Dulbecco's Modified Eagle Medium; DTT, dithiothreitol; ECL, enhanced chemoilluminescence; FBS, foetal bovine serum; HIF-1, hypoxia-inducible factor-1; HRK, harakiri; HRP, horse radish peroxidase; Mcl-1, myeloid cell leukemia-1; NOXA, Phorbol-12-myristate-13-acetate-induced protein 1; NP40, nonyl phenoxylpolyethoxylethanol; PUMA, p53-upregulated modulator of apoptosis; RIPA, radioimmunoprecipitation assay; RPMI, Roswell Park Memorial Institute Medium.

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The role of BNIP3 in the hypoxia response remains controversial. Many of the early studies implicated BNIP3 as an inducer of cell death [4,13–15] and the more recent work of Gibson and colleagues supports this [5,16,17]. However, other groups have found no pro-death effect of BNIP3 and most cultured cells can tolerate long periods of hypoxia without deleterious effects, despite expression of BNIP3 [6].

Several groups have implicated BNIP3 as having a direct or indirect role in hypoxia-induced autophagy or mitophagy [14,18–21]. However, this is also controversial, as another study demonstrated that hypoxia-induced autophagy occurs independently of BNIP3 and BNIP3L expression [22].

As the majority of Bcl-2 family members are regulated post-translationally, we hypothesised that BNIP3 may also be subject to this form of control. A clearer understanding of the mechanism of control of BNIP3 may explain the controversy surrounding the protein and help to clarify its role in the response to hypoxia.

2. Experimental procedures

2.1. Materials

LS174T, MDA-MB-231 cells, RPMI and DMEM media were from Clare Hall laboratories, UK. Fetal bovine serum (FBS) Gold was from PAA Laboratories. Tet System Approved FBS was from Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France. Protein A-agarose was from Santa Cruz Biotechnology Inc., Heidelberg, Germany. Oligofectamine™ and Optimem™ were from Invitrogen Ltd., Paisley, UK. Lambda Protein Phosphatase was from New England BioLabs, Hitchin, UK. Protease inhibitor cocktail tablets were from Roche Diagnostics Ltd., Burgess Hill, UK. RIPA buffer, Triton-X100 and phosphatase inhibitor cocktail 1 and 2 were from Sigma–Aldrich Company Ltd., Gillingham, UK. DRAQ5 was from Alexis Biochemicals and Immobilon-P PVDF membrane (0.45 μm) was from Millipore. The PhosphoProtein Purification kit was from Qiagen Ltd., Crawley, UK. Fluoromount was from Dako, Ely, UK.

2.2. Antibodies

Akt, Phospho-Akt (Ser473), Phospho-Bcl-2 (Thr56), Phospho-Bcl-2 (Ser70), Bcl-xL, JNK, Phospho-JNK (Thr183/Tyr185), PARP, Phospho-vimentin (Ser 56), p70 S6 kinase, phospho-p70 S6 kinase (Thr389) were from Cell Signalling Technology (via New England BioLabs, Hitchin, UK). BNIP3 and BNIP3L were from Sigma–Aldrich Ltd., Gillingham, UK. Cyclin B1 and HIF-1α were from BD Pharmingen, Oxford, UK. Bcl-2, Bax and Tom20 were from Santa Cruz Biotechnology Inc., Heidelberg, Germany and TrueBlot™ Ultra HRP anti-mouse IgG was from eBioscience Ltd., Hatfield, UK. Alexa-Fluor 488 goat-anti-mouse IgG_{2b} and Alexa-Fluor 546 goat-anti-mouse IgG_{2a} were from Invitrogen Ltd., Paisley, UK.

2.3. Chemicals

Doxorubicin hydrochloride, vinblastine sulfate, paclitaxel, doxycycline hyclate, vinorelbine ditartrate salt, colchicine, nocodazole and sulforhodamine B sodium salt were from Sigma–Aldrich Ltd., Gillingham, UK. Rapamycin and SP600125 were from Calbiochem (Merck Chemicals Ltd., Nottingham, UK). Bortezomib was a gift from Millenium Pharmaceuticals, Cambridge, MA, USA. Cisplatin was a gift from Dr. Richard Callaghan, University of Oxford.

2.4. BNIP3 silencing by RNAi

BNIP3 RNAi duplexes were designed using Dharmacon si*DESIGN*® centre (http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx), scrambled control (SCR) RNAi duplexes were designed using InvivoGen siRNA wizard™ and all RNAi duplexes were synthesised and annealed by Eurogentec, Seraing, Belgium. The oligonucleotide sense strand sequences were: BNIP3(1), 5′-GAACUGCACUUCAGCAAUAdTdT-3′; BNIP3(2), 5′-GCCUCGGUUUCUAUUUAUAdTdT-3′; BNIP3(3), 5′-ACACGAGCGUCAUGAAGAAdTdT-3′; SCR(1), 5′-GGAAAUAAUCACGGUACAUdTdT-3′; SCR(2), 5′-GAGCCAAAGCACGAUAGGUdTdT-3′; SCR(3), 5′-GGCUAAACGUAAGUUGACUdTdT-3′. A pool of the three respective duplexes was used to maximise target selectivity and transfected with Oligofectamine™, according to the manufacturer's instructions, and cultured in Optimem™ for 4 h prior to the addition of serum for overnight recovery.

2.5. Generation of HCT116-BNIP3 cells

Tetracycline inducible HCT116 cells were prepared using the flp-in T-Rex system according to the manufacturer's instructions (Invitrogen Ltd., Paisley, UK).

2.6. Hypoxic and anoxic incubations

Hypoxic incubations (0.1% O_2, 5% CO_2) were performed using an INVIVO$_2$ 400 hypoxic workstation (Pro-Lab Diagnostics, Neston, UK). To generate a combined anoxic/low pH environment, cells were placed in an anaerobic jar containing an Anaero*Gen*™ sachet (Oxoid Ltd., Basingstoke, UK) at 37 °C for the duration of the experiment.

2.7. Immunoblotting

Cells were washed with 4 °C PBS and homogenised on ice in lysis buffer containing 6.2 M urea, 10% glycerol, 5 mM DTT, 1% SDS, protease inhibitors and phosphatase inhibitors. Proteins were separated by SDS-PAGE, using 12% Tris–HCl gels. Immunoreactivity was detected using Amersham ECL™ western blotting detection reagent and Hyperfilm™ (GE Healthcare, Slough, UK).

2.8. Sulforhodamine B (SRB) cell viability assay

Cells were seeded in 96-well plates and exposed to normoxia or hypoxia for 72 h. SRB-staining was conducted to manufacturer's instructions and the absorbance was measured at 540 nM.

2.9. Growth curves for HCT116-BNIP3 cells

Cells were seeded in McCoys' medium supplemented with 10% tet-free FBS. Cells were left to attach overnight before doxycyline (1 μg/mL) was added to induce BNIP3 expression before cells were exposed to normoxia or hypoxia for various times. After the incubation period, cells were counted using a Coulter Z2 particle count and size analyser.

2.10. Phosphoprotein purification column

Cells were exposed to hypoxia for 24 h. Cell lysis and phosphorylated protein enrichment was performed using a PhosphoProtein purification kit according to the manufacturer's instructions. BNIP3 levels in the eluate fraction (enriched for phosphoproteins) were compared to the input lysate by loading equivalent amounts of total protein on a 12% gel and immunoblotting as described.

2.11. Lambda protein phosphatase digestion

Cells were seeded overnight and then treated with 1 μM paclitaxel prior to 24 h normoxic or hypoxic exposure. Post-incubation, cells were washed with 4 °C PBS and lysed in RIPA

buffer containing protease inhibitors. Lysates were centrifuged at 22,000 × g and the pellet was discarded. For each treatment, a volume of supernatant equivalent to 45 μg total protein was subjected to phosphatase digestion according to the manufacturer's instructions. Samples were analysed by SDS-PAGE, and then immunoblotted for BNIP3 as described.

2.12. Immunofluorescence and confocal microscopy

Cells were seeded on coverslips and exposed to normoxia or hypoxia for 24 h +/− vinblastine or paclitaxel. Cells were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton-X100 and non-specific binding was inhibited with 10% FBS in PBS. Primary antibodies used were BNIP3 (Ana40) mouse IGg_{2b} (1:100) and Tom20 (F-10) mouse IgG_{2a} (1:200) were applied in 10% FBS in PBS for 90 min. Alexa-Fluor 488 goat-anti-mouse IgG_{2b} and Alexa-Fluor 546 goat-anti-mouse IgG_{2a} secondary antibodies were then applied. For nuclear counterstaining, DRAQ5 used. Cells were visualized using a Carl Zeiss LSM510 confocal laser-scanning microscope.

2.13. BNIP3 immunoprecipitation

LS174T cells were incubated in hypoxia for 24 h. Then cells were washed with 4 °C PBS and lysed in a buffer containing 20 mM Tris–HCl pH 7.4, 137 mM CaCl, 2 mM EDTA pH 7.4, 1.5 mM $MgCl_2$, 0.2% NP-40, 10% glycerol, protease inhibitors and phosphatase inhibitors. Lysates were centrifuged and supernatants were incubated with Protein A-Agarose beads, coated with mouse anti-BNIP3. The beads were then washed and resuspended in sample buffer and analysed by SDS-PAGE and immunoblotting as described.

2.14. Data analysis

All data analyses were performed using GraphPad Prism® v4.0 software. Statistical significance was determined using unpaired, two-tailed, *t*-tests with confidence intervals set at 95%. For calculation of IC_{50} values from the paclitaxel-sensitivity data, nonlinear regression was applied; curves were fit to the data using the sigmoidal dose–response equation.

3. Results

3.1. BNIP3 exists as multiple monomeric and dimeric species

A timecourse experiment of LS174T cells exposed to hypoxia (0.1%) revealed stabilisation of HIF-1α by 2 h with expression reaching maximum by 6 h (Fig. 1A). Consistent with the established HIF-1 dependency of BNIP3 expression [3,4,7], anti-BNIP3 reactive bands appeared after 4 h of hypoxia and expression increased up to 12 h. Anti-BNIP3 reactive bands migrated at 21.5 kDa, consistent with the predicted molecular weight of the polypeptide, and also at both 26 and 30 kDa. Another group of anti-BNIP3 reactive bands migrated around 60 kDa (Fig. 1A). It has previously been reported that BNIP3 exists in both monomeric and dimeric forms and the dimer is stable even under reducing conditions [13]. We speculated that the 30 kDa and lower bands represented BNIP3 monomers and that the 60 kDa forms represented BNIP3 homodimers. To confirm that all of these

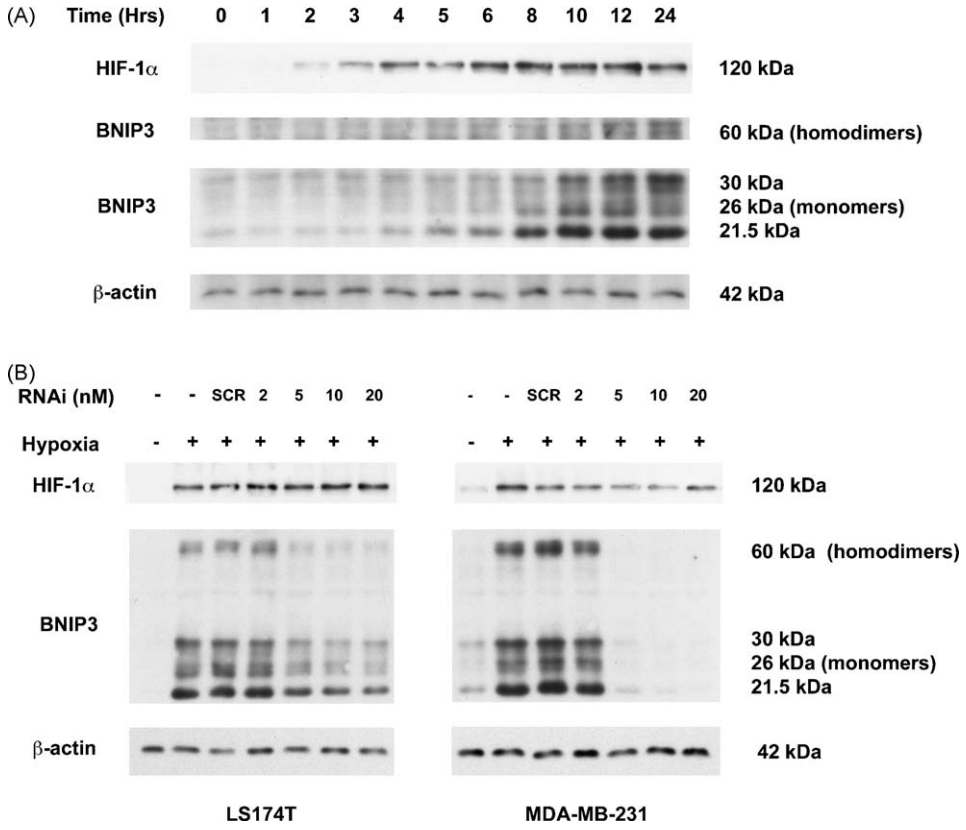

Fig. 1. Cancer cells express multiple forms of BNIP3 in hypoxia without an effect on cell viability. A, LS174T cells were seeded at 3×10^5 cells per well in 6-well plates and exposed to hypoxia for the indicated times. Lysates were prepared and immunoblotted for BNIP3, HIF-1α and β-actin as described. B, LS174T and MDA-MB-231 cells were mock transfected (oligofectamine only), transfected with a pool of three scrambled (SCR) RNAi duplexes at 20 nM or transfected with increasing concentrations of a BNIP3 RNAi pool. The following day, cells were exposed to normoxia or hypoxia for 24 h prior to lysates being prepared and immunoblotted for BNIP3 and HIF-1α.

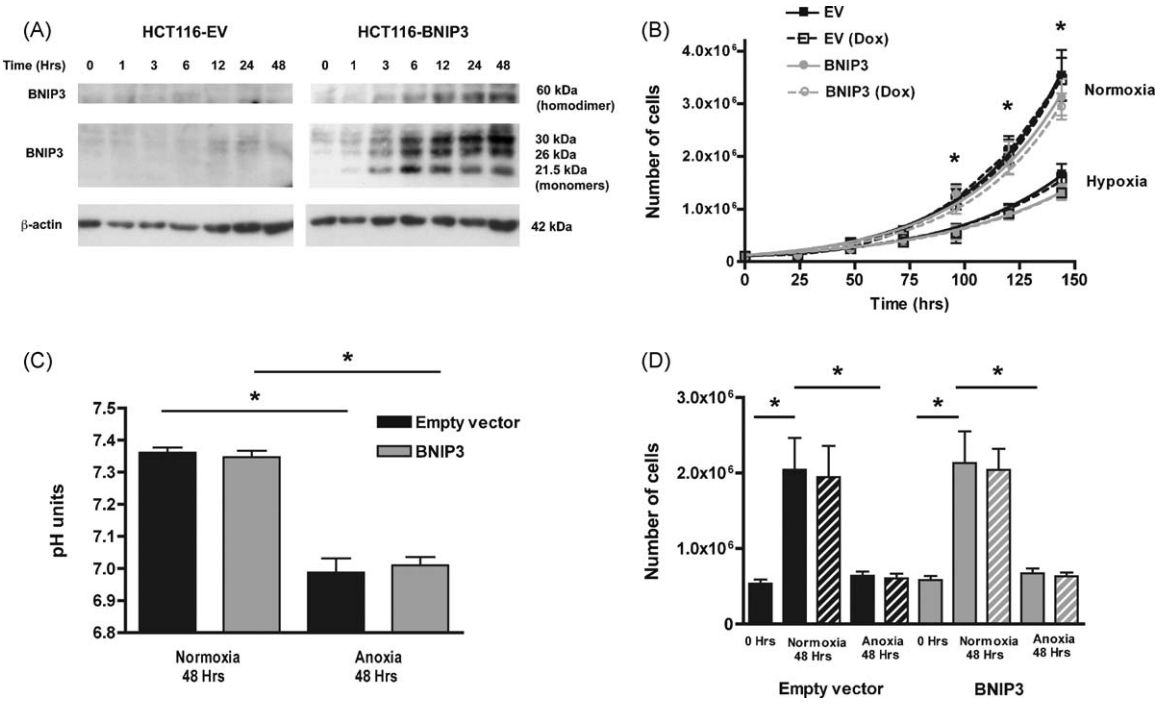

Fig. 2. Inducible-expression of BNIP3 in HCT116 cells does not influence normoxic or hypoxic growth. A, HCT116-EV (empty vector) or HCT116-BNIP3 cells were seeded in 6-well plates at 3×10^5 cells per well and left to adhere overnight. Doxycycline (1 μg/mL) was added to the culture medium to induce gene expression. Cells were harvested at the indicated timepoints and lysates were prepared and immunoblotted for BNIP3 and β-actin as described. B, HCT116-EV or HCT116-BNIP3 cells were seeded at 2×10^5 cells per 10 cm^2 dish and left to adhere overnight before being exposed to hypoxia or normoxia +/− doxycycline (1 μg/mL) for up to 6 days. At various timepoints, attached cells were counted as described and growth curves were plotted. Data represents the S.E.M. from three independent experiments, each including duplicate measurements. C, HCT116-EV or HCT116-BNIP3 cells were seeded at 10^6 per 10 cm^2 dish and left overnight to adhere. The next day doxycycline (1 μg/mL) was added (bars with diagonal white lines) and cells were exposed to normoxia or anoxia (severe hypoxia <0.01% O_2) for 48 h as described. After exposure, C, the pH of the culture medium was measured and D, the attached cells were counted. Data represents the S.E.M. from three independent experiments, each including duplicate measurements. $^*p < 0.05$ based on unpaired *t*-test.

species were in fact forms of BNIP3, we transfected LS174T and MDA-MB-231 cells with a pool of three BNIP3 RNAi duplexes. Reduced expression of all anti-BNIP3 reactive bands was observed in both cell lines (Fig. 1B), demonstrating that these all represent forms of BNIP3.

3.2. BNIP3 expression does not modulate cell proliferation or survival under hypoxia

Next, we examined the effect of BNIP3 knockdown on cell survival under hypoxia. The sulforhodamine B (SRB) assay was used, as enzyme-based viability assays can be influenced by hypoxia [23]. No difference in cell viability was observed between SCR or BNIP3 RNAi treated cells after 72 h of either normoxic or hypoxic exposure (Supp. Fig. 1).

We postulated that forced expression of BNIP3 in a cell line in which it is silenced [24] could reveal a potential function that had been circumvented in BNIP3 expressing lines such as LS174T or MDA-MB-231. Therefore we expressed BNIP3 under a doxycyline-inducible promoter in HCT116 cells. Addition of doxycycline to the culture medium resulted in the appearance of BNIP3 in the expressor from 3 h but not the empty vector (EV) (Fig. 2A). Importantly, all of the monomeric and dimeric forms of BNIP3 were present in normoxia, demonstrating that hypoxia is not required for the formation of the more slowly migrating species (Fig. 2A). Next, we examined the effect of BNIP3 expression on normoxic and hypoxic growth of HCT116 cells. Although hypoxia suppressed proliferation [25,26], BNIP3 expression did not influence normoxic or hypoxic growth over 6 days (Fig. 2B). These results are in agreement with the work of Papandreou et al. [6]. A previous report suggested that acidosis could act as a trigger to activate BNIP3 in cardiac myocytes under hypoxia [27]. Therefore we exposed HCT116 cells to a combination of severe hypoxia (anoxia <0.1% O_2) and low pH (by increasing atmospheric CO_2 and thus carbonic acid in the culture medium) (Fig. 2C). Although this combination greatly reduced viable cell number after 48 h compared to normoxia, the presence or absence of BNIP3 did not influence this in any way (Fig. 2D).

3.3. Post-translational modification of BNIP3 through microtubule-active drug treatment

We hypothesised that the slowly migrating BNIP3 species (26 and 30 kDa) represented post-translationally modified forms of the native protein (21.5 kDa). To test if this modification was influenced by cellular stress, we exposed hypoxic LS174T cells (Fig. 3A) and MDA-MB-231 cells (Supp. Fig. 2A) to various anti-cancer drugs. Treatment with the proteasome inhibitor bortezomib led to an accumulation of all BNIP3 forms including the dimer, consistent with the inhibition of proteasome-targeted BNIP3 degradation. Treatment with the anthracycline doxorubicin (1 μM) had a mildly suppressive effect on BNIP3 expression without effecting HIF-1α levels particularly in the MDA-MB-231 cells (Supp. Fig. 2A), most likely through its recently described inhibition of HIF-1 binding to DNA [28]. The DNA crosslinking agent cisplatin had a minimal effect on BNIP3 expression (Fig. 3A and Supp. Fig. 2A). However, treatment with either of two microtubule-active agents, paclitaxel and vinblastine, resulted in a marked upwards shift in migration of the monomeric BNIP3 species from the 21.5 and 26 kDa forms to the 30 kDa form. Paclitaxel and vinblastine also partially suppressed HIF-1α expression (Fig. 3A). All of the compounds tested had the same effect in MDA-MB-231 cells (Supp. Fig. 2A).

To examine if the effect on BNIP3 was unique to paclitaxel and vinblastine or was shared by other microtubule-active drugs, we

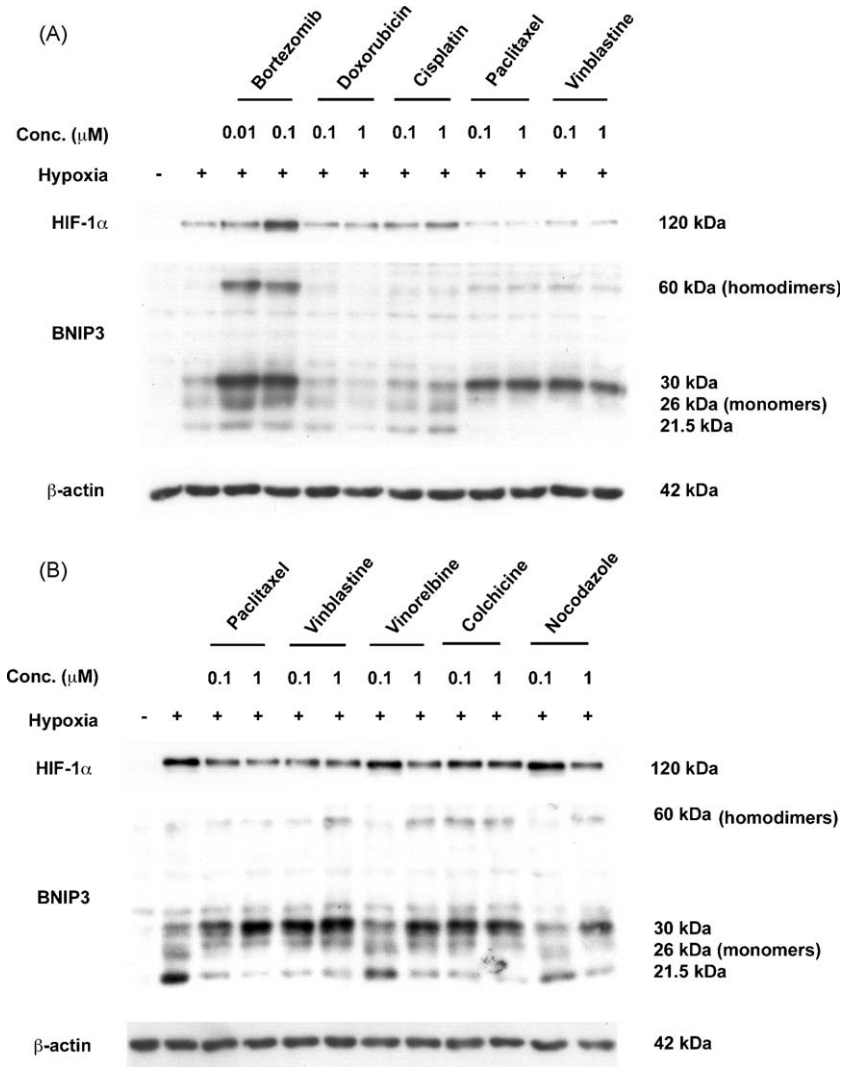

Fig. 3. Microtubule-active drugs modulate post-translational modification of BNIP3. A and B, LS174T cells were seeded in 6-well plates at 3×10^5 cells per well and left to adhere overnight. The medium was replaced with fresh medium containing drugs at the indicated concentrations and the cells were exposed to hypoxia or normoxia for 24 h. After the incubation period, cell lysates were prepared and immunoblotted for BNIP3, HIF-1α and β-actin as described.

repeated the experiment with vinorelbine, colchicine and nocodazole. Although the potency varied, all of the microtubule-active agents tested resulted in the same increase in the 30 kDa form of BNIP3 (Fig. 3B).

3.4. BNIP3 is phosphorylated

BNIP3 does not contain a signal-peptide sequence, so is unlikely to be *N*- or *O*-glycosylated. However, Phosphosite™ predicted a number of potential phosphorylation sites. To test the phosphorylation status of BNIP3, we took lysates from hypoxic LS174T or MDA-MB-231 cells and attempted to enrich BNIP3 using a PhosphoProtein purification column. Both monomeric and dimeric forms of BNIP3 were highly enriched in the phosphoprotein fraction, along with some other anti-BNIP3 reactive bands including one at 40 kDa (Fig. 4A). As controls, we also probed for phospho-AKT and phospho-p70 S6 kinase, both of which were highly enriched in the phosphoprotein fraction, as expected. Phospho-AKT in MDA-MB-231 cells was the exception to this, as only a slight enrichment was observed. This is likely to reflect low levels of AKT activation in this cell line under hypoxia compared to LS174T cells (Fig. 4A). As expected, β-actin, which is not phosphorylated, was present in the input, but was not present in the phosphoprotein fraction (Fig. 4A).

To further confirm that BNIP3 is phosphorylated, we incubated normoxic or hypoxic (+/− paclitaxel) LS174T or MDA-MB-231 cell protein extracts with Lambda phosphatase. This is an Mn^{2+}-dependent phosphatase active against phosphorylated serine, threonine and tyrosine residues. After phosphatase treatment, the 30 and 26 kDa BNIP3 monomers collapsed down to the faster migrating 21.5 kDa form. This is consistent with slower migration (26 and 30 kDa) representing increasing multi-site phosphorylation and with the 21.5 kDa species being the unmodified polypeptide. The shift was observed in normoxic, hypoxic and paclitaxel treated hypoxic extracts from both cell lines (Fig. 4B). Incubation of extracts at 30 °C for 1 h in the absence of phosphatase did not effect BNIP3 migration. The 60 kDa BNIP3 homodimer also migrated more rapidly after phosphatase treatment, consistent with it being a phospho-dimer of BNIP3 (Fig. 4B). This also demonstrates that phosphorylation of BNIP3 is not required for stabilisation of dimers.

To test if BNIP3 hyper-phosphorylation by microtubule inhibitors resulted in a change in the subcellular localization of the protein, we exposed LS174T cells to hypoxia in the presence or

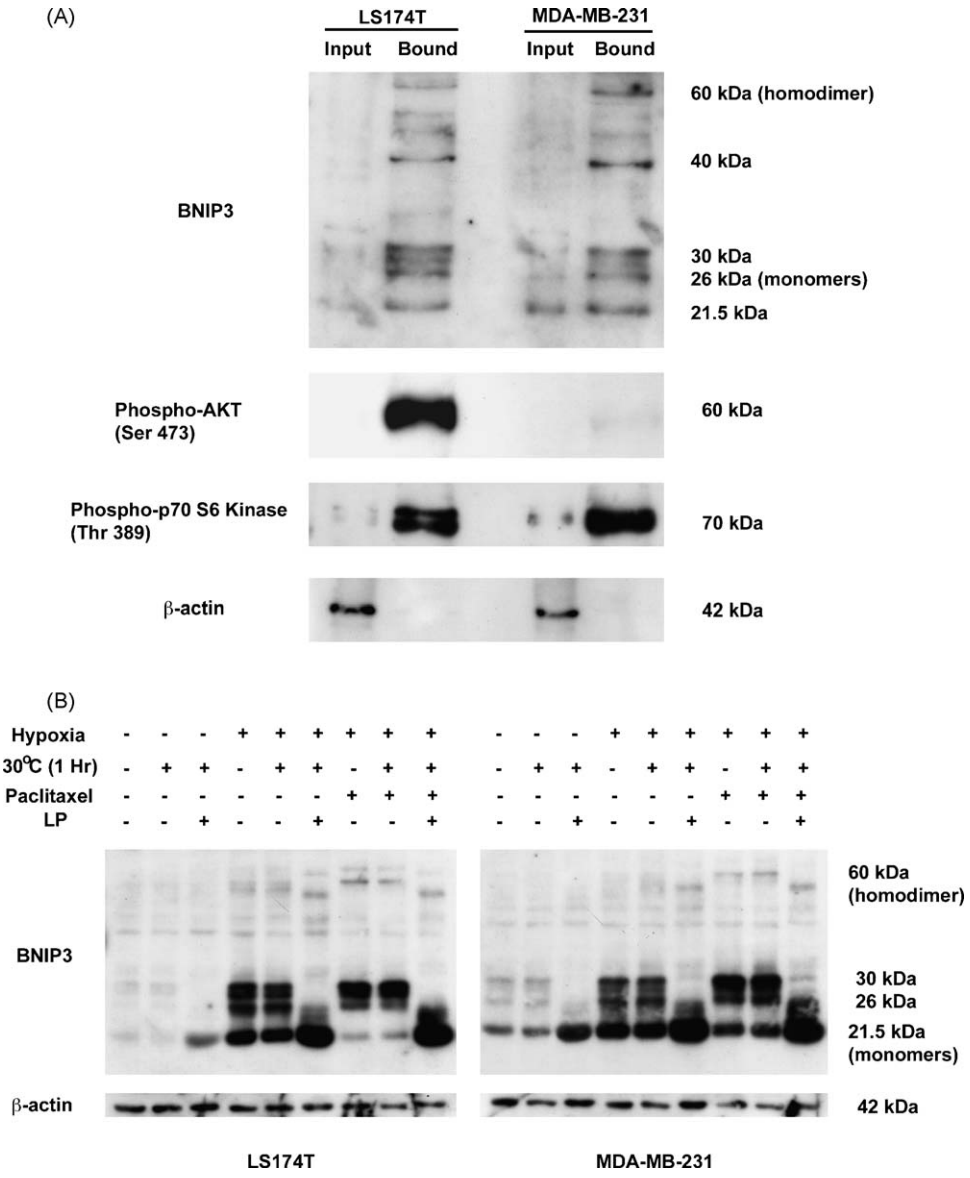

Fig. 4. BNIP3 exists in multiple phospho-monomeric and a phospho-dimeric forms. A, LS174T and MDA-MB-231 cells (10^7) were seeded in 15 cm^2 dishes and exposed to hypoxia for 24 h. The cells were lysed and phosphorylated proteins were enriched using a PhosphoProtein purification kit according to the manufacturer's instructions. BNIP3 levels in the eluate (bound) fraction (enriched for phosphoproteins) were compared to the input lysate by loading equivalent amounts of total protein on a 12% gel and immunoblotting for BNIP3, phospho-AKT (Ser 473), phospho-p70 S6 kinase and β-actin as described. B, LS174T and MDA-MB-231 cells were seeded at 5×10^5 in 6-well dishes and left overnight to adhere. The next day, the medium was replaced with fresh medium +/− 1 μM paclitaxel prior to 24 h normoxic or hypoxic exposure. Lambda phosphatase (LP) digestion was performed on cell lysates as described. Experimental controls were included where samples were exposed to the 1 h 30 °C incubation in the absence of LP. SDS-PAGE sample buffer (6×) was added to the digested lysates, which were then immunoblotted for BNIP3 and β-actin as described.

absence of paclitaxel or vinblastine. BNIP3 predominantly exhibits mitochondrial localization [13,29]. We found this to be independent of phosphorylation status (Fig. 5A) or oxygen tension, as BNIP3 localized to mitochondria in inducible HCT116 cells in both normoxia and hypoxia (data not shown).

3.5. Microtubule inhibitor treatment induces post-translational modification of multiple Bcl-2 family members

We noted previous reports that two antiapoptotic mitochondrial Bcl-2 family members (Bcl-2 and Bcl-xL) are also phosphorylated in response to microtubule inhibitor treatment [30–36]. In contrast to BNIP3, we found that the expression of Bcl-2 and Bcl-xL was unaltered by hypoxic exposure. However, like BNIP3, treatment with paclitaxel or vinblastine induced hyper-phosphorylation of both (Fig. 5B). For Bcl-2 we confirmed that two of the phosphorylation sites were Thr56 and Ser70 (Supp. Fig. 2B).

The hypoxia-inducible BNIP3 homologue BNIP3L (Nix) exhibited a small down-shift upon drug treatment, indicating a post-translational change, and the antiapoptotic family member Mcl-1 showed decreased expression, consistent with stress-induced degradation [37] (Fig. 5B). Bak levels were partially suppressed by microtubule inhibitor treatment in MDA-MB-231 but not in LS174T cells. LS174T cells did not express Bax, as shown previously [38]. Taken together, these results suggest that of the Bcl-2 family proteins studied, hyper-phosphorylation is common to BNIP3, Bcl-2 and Bcl-xL.

3.6. BNIP3, Bcl-2 and Bcl-xL undergo synchronised phosphorylation during paclitaxel-induced mitotic arrest

Next we examined the phosphorylation kinetics of BNIP3, Bcl-2 and Bcl-xL after paclitaxel treatment. LS174T cells were exposed to hypoxia for 24 h to transcriptionally upregulate BNIP3 prior to the

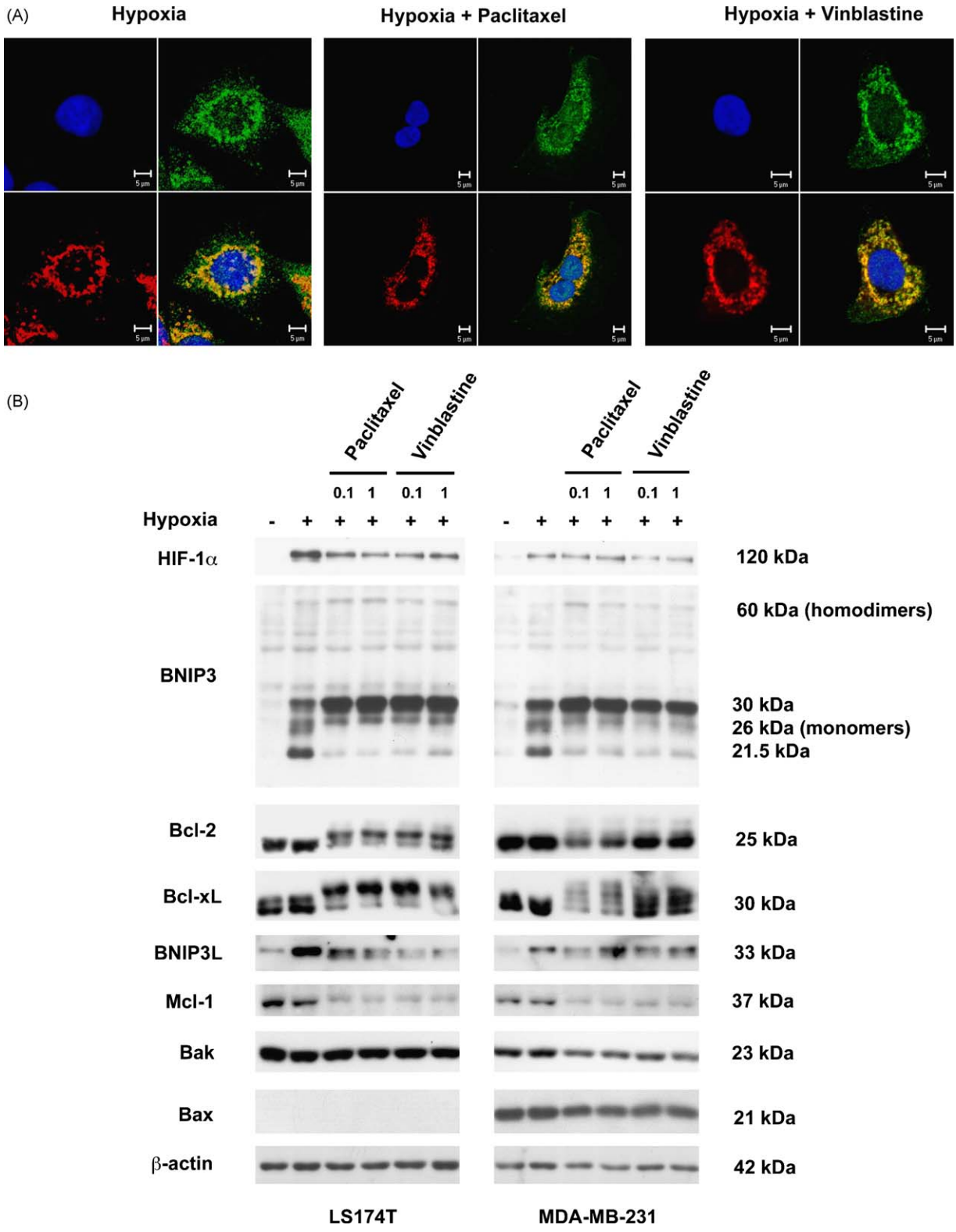

Fig. 5. Multiple Bcl-2 family members are phosphorylated in response to paclitaxel or vinblastine treatment. A, LS174T cells were seeded on coverslips in 6-well plates at 3×10^5/well and exposed hypoxia for 24 h +/− vinblastine or paclitaxel. Cells were fixed and immunofluorescent labelling of BNIP3 (green, upper right) and Tom20 (red, lower left) was performed as described. DRAQ5 (blue, upper left) was applied to observe nuclei. Cells were visualized using a Carl Zeiss LSM510 confocal laser-scanning microscope. Co-localization of BNIP3 and Tom20 is indicated by a yellow pattern in the merged panels (lower right). B, LS174T and MDA-MB-231 cells were seeded in 6-well plates at 3×10^5 cells per well and left to adhere overnight. The medium was replaced with fresh medium containing paclitaxel or vinblastine at the indicated concentrations and the cells were exposed to hypoxia or normoxia for 24 h. After the incubation period, cell lysates were prepared and immunoblotted for BNIP3, HIF-1α, Bcl-2, Bcl-xL, BNIP3L, Mcl-1, Bak, Bax and β-actin as described. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

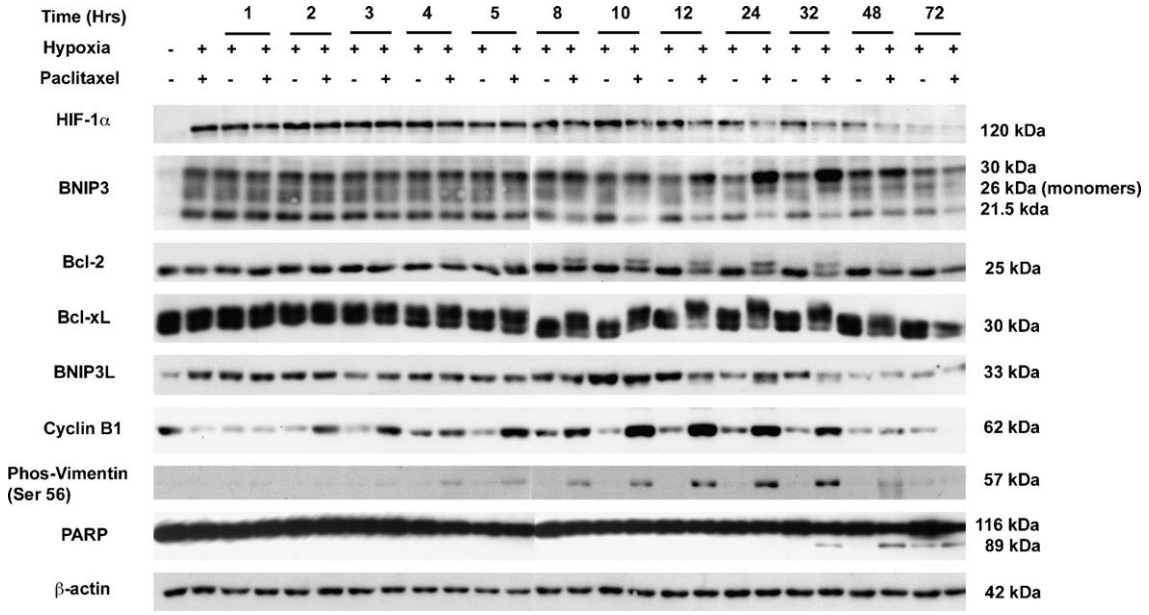

Fig. 6. Paclitaxel induces the synchronised phosphorylation/dephosphorylation of BNIP3, Bcl-2 and Bcl-xL. LS174T cells were seeded in 6-well plates at 3×10^5 cells per well and left to adhere overnight. Cells were exposed to hypoxia for 24 h, at which point paclitaxel (1 μM) was added (still under hypoxia) and the cells were maintained in hypoxia for various timepoints, up to a further 72 h, as indicated. After the paclitaxel exposure period, cell lysates were prepared and immunoblotted for BNIP3, HIF-1α, Bcl-2, Bcl-xL, BNIP3L, cyclin B1, Phos-vimentin, PARP and β-actin as described.

addition of paclitaxel. The upward phosphorylation shift was clearly visible for all three proteins after 8 h of drug treatment (Fig. 6). Phosphorylation of BNIP3, Bcl-2 and Bcl-xL continued to increase as the cells arrested in M-phase, as measured by cyclin B1 accumulation and phosphorylation of the CDK1 substrate vimentin [39] (Fig. 6). BNIP3, Bcl-2 and Bcl-xL phosphorylation peaked at 24 h before dropping through 48 and 72 h as the cells exited mitosis and underwent apoptosis, as measured by PARP cleavage (Fig. 6). These data suggested that the synchronised phosphorylation of BNIP3, Bcl-2 and Bcl-xL was tightly linked to the paclitaxel-induced mitotic arrest.

3.7. Paclitaxel-induced BNIP3, Bcl-2 and Bcl-xL phosphorylation requires mitotic checkpoint kinase activity

The AKT/mTOR pathway has previously been implicated in microtubule inhibitor-induced Bcl-2 phosphorylation [40]. However, we found that paclitaxel actually suppresses AKT and downstream mTOR activation, as measured by phospho-AKT and phospho-p70 S6 kinase levels, respectively (Supp. Fig. 3). In addition, treatment with the mTOR inhibitor rapamycin failed to block BNIP3 phosphorylation (Supp. Fig. 3).

We speculated that microtubule inhibitor-induced BNIP3, Bcl-2 and Bcl-xL phosphorylation was the result of prolonged exposure to a mitotic kinase as a consequence of mitotic arrest. To test this hypothesis, we treated cells with paclitaxel in the presence of SP600125, an inhibitor of the mitotic checkpoint kinase Mps1. Inhibition of Mps1 allows progression through mitosis even in the presence of microtubule inhibitors [41]. Treatment with SP600125 at 10 μM partially inhibited the paclitaxel-induced M-phase arrest (cyclin B1 accumulation) and the phosphorylation of BNIP3, Bcl-2 and Bcl-xL. At the higher concentration of 25 μM, SP600125 completely inhibited the M-phase arrest and phosphorylation of BNIP3, Bcl-2 and Bcl-xL. It also blocked the BNIP3L down-shift (Fig. 7).

SP600125 is also known to inhibit JNK kinase [42], however JNK kinase was not activated (phosphorylated) by paclitaxel in LS174T cells (Fig. 7). JNK could be activated by anisomycin [43] in LS174T

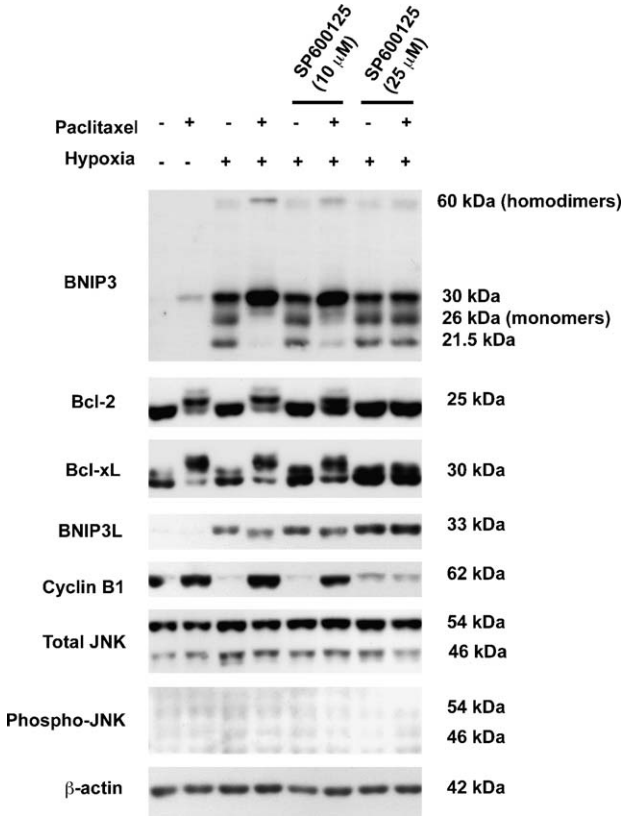

Fig. 7. Paclitaxel-induced BNIP3, Bcl-2 and Bcl-xL phosphorylation requires mitotic checkpoint kinase activity and M-phase arrest. LS174T cells were seeded in 6-well plates at 3×10^5 cells per well and left to adhere overnight. Cells were pre-treated with SP600125, at the indicated concentration, and then exposed to hypoxia (in the continued presence of the drug) +/− paclitaxel for 24 h. After the incubation period, cell lysates were prepared and immunoblotted for the proteins shown as described.

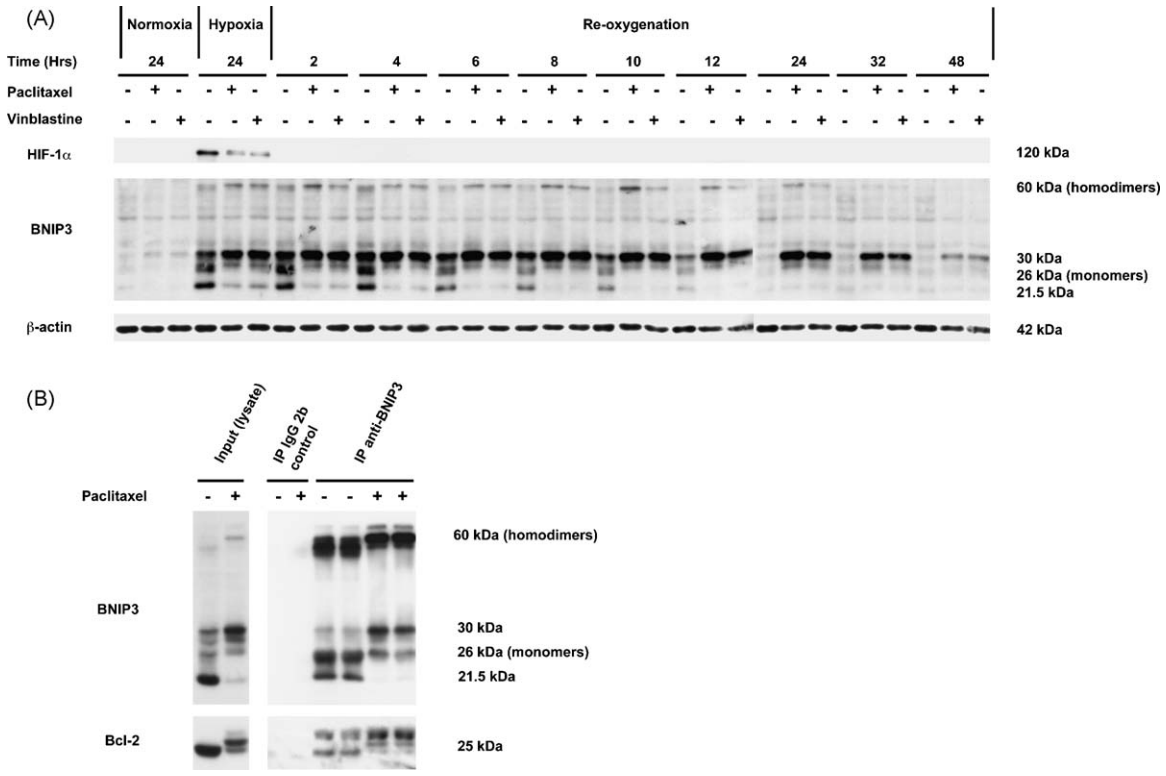

Fig. 8. Paclitaxel and vinblastine increase the half-life of BNIP3 and BNIP3 predominantly interacts with phospho-Bcl-2. A, LS174T cells were seeded in 6-well plates at 3×10^5 cells per well and left to adhere overnight. Cells were exposed to hypoxia for 24 h +/− paclitaxel (1 μM) or vinblastine (1 μM). After the hypoxic exposure period, cells were returned to normoxia (re-oxygenated) and lysed at timepoints up to 48 h. Lysates were immunoblotted for BNIP3, HIF-1 and β-actin as described. B, LS174T cells were seeded at 2×10^6 on 10 cm^2 dishes and left overnight to adhere. The next day, the medium was replaced with fresh (+/− 1 μM paclitaxel) and the cells were placed in hypoxia for 24 h. Immunoprecipitation of BNIP3 was performed as described in Section 2 and total cell lysates and immunoprecipitates were analysed for BNIP3 and Bcl-2 by SDS-PAGE and immunoblotting as described. A control immunoprecipitation was performed using an isotype control antibody (mouse IgG 2b) to account for non-specific protein binding.

cells, but this did not induce BNIP3 or Bcl-2 phosphorylation (Supp. Fig. 2C). Taken together, these results show that BNIP3, Bcl-2 and Bcl-xL are phosphorylated independently of the AKT/mTOR and JNK kinase pathways by a kinase active in M-phase of the cell cycle.

3.8. Microtubule inhibitors increase the stability of BNIP3

Phosphorylation has previously been shown to increase the stability of Bcl-2. To examine if this was also the case for BNIP3, we exposed cells to hypoxia for 24 h +/− paclitaxel and then re-oxygenated cells to examine the longevity of BNIP3 expression in the absence of HIF-1 transcriptional activity (Fig. 8A). In cells exposed to hypoxia only, BNIP3 expression had returned to basal levels 24 h post-re-oxygenation. However, in the paclitaxel-induced hyper-phosphorylated state, BNIP3 expression persisted even 48 h post-reoxygenation (Fig. 8A), suggesting that phosphorylation increases the stability of BNIP3.

3.9. BNIP3 does not influence the sensitivity of cells to paclitaxel

To test if BNIP3 modulated the cellular response to paclitaxel, we transfected LS174T and MDA-MB-231 with BNIP3 RNAi prior to performing a dose–response cell viability experiment. Examples of the survival curves obtained are shown in Supp. Fig. 4 and the IC_{50} values for paclitaxel are shown in Table 1. Hypoxia significantly reduced the paclitaxel sensitivity of LS174T cells relative to normoxia, but the mechanism was BNIP3-independent as SCR and BNIP3 RNAi cells were equally sensitive under both conditions (Table 1). Hypoxia did not alter paclitaxel sensitivity in MDA-MB-231 cells and knockdown of BNIP3 also had no effect (Table 1). We conclude that BNIP3 expression does not modulate paclitaxel sensitivity.

3.10. BNIP3 predominantly interacts with the phosphorylated form of Bcl-2

We investigated whether phosphorylation modulated the interaction between BNIP3 and Bcl-2. When we immunoprecipitated (IP) BNIP3 from hypoxic cells +/− paclitaxel, we enriched both monomeric and dimeric forms of the protein (Fig. 8B). However, it is interesting to note that the dimeric forms of BNIP3 more selectively immunoprecipitated under these conditions than the monomers (Fig. 8B). This may be due to dimers forming at the antibody-BNIP3 complex, where the local BNIP3 concentration is high. Alternatively, the dimeric conformation may form a more stable complex with the antibody. Upon probing the same IP for Bcl-2, we found that all forms of Bcl-2 IP with BNIP3, however the most highly phosphorylated form of Bcl-2 showed a preferential interaction (Fig. 8B). As would be expected, this form of Bcl-2 was enriched in the paclitaxel treated cells, but also formed a high proportion of the Bcl-2 to co-IP with BNIP3 from untreated cells (Fig. 8B). This demonstrates that BNIP3 preferentially interacts with phosphorylated Bcl-2.

4. Discussion

Several of the early studies on BNIP3 reported that it induced cell death [4,13–15]. However many of these studies involved the overexpression of non-physiological levels of the protein. The levels of BNIP3 in our HCT116-inducible cells were consistent with the hypoxia-induced level observed in another colorectal carcinoma

Table 1
The influence of BNIP3 expression on paclitaxel sensitivity (IC_{50}) in LS174T and MDA-MB-231 cells.

	MOCK		SCR		BNIP3	
Cell line	**Normoxia**	**Hypoxia**	**Normoxia**	**Hypoxia**	**Normoxia**	**Hypoxia**
		**		*		**
LS174T	**2.87 ± 0.08**	**4.50 ± 0.36**	**2.82 ± 0.22**	**4.17 ± 0.42**	**2.96 ± 0.33**	**4.10 ± 0.14**
MDA-MB-213	**0.91 ± 0.15**	**0.62 ± 0.27**	**0.82 ± 0.22**	**0.56 ± 0.35**	**0.97 ± 0.16**	**0.66 ± 0.18**

* p = < 0.05 Unpaired t-test, two-tailed, 95% confidence intervals
** p = < 0.01
*** p = < 0.001

$^{*}p < 0.05$ unpaired *t*-test, two-tailed, 95% confidence intervals.
$^{**}p < 0.01$.
$^{***}p < 0.001$.
To determine the role of BNIP3 in paclitaxel sensitivity in LS174T and MDA-MB-231 cells, cells were pre-transfected with BNIP3 or SCR RNAi or mock transfected (oligofectamine only) and 10^3 cells (per well of a 96-well dish) were exposed to a range of concentrations of paclitaxel (1 nM to 100 μM) under nomoxia or hypoxia for 72 h. After the exposure period, the SRB cell viability assay was performed as described. Sigmoidal dose–response curves were fitted to the data using GraphPad Prism® v4.0 software and IC_{50} values for paclitaxel were calculated. IC_{50} values represent the S.E.M. from three independent experiments, each including six replicate measurements. Statistical significance was determined using unpaired, two-tailed, *t*-tests with confidence intervals set at 95%.

line, LS174T (Fig. 1A) and the breast carcinoma line MDA-MB-231. However, modulation of BNIP3 expression failed to influence cell survival under hypoxia or normoxia in any of the three cell lines used (Figs. 1 and 2 and Supp. Fig. 1). These results are consistent with other recent reports showing that BNIP3 expression does not induce cell death. There is some controversy as to whether BNIP3 has a role in autophagy [14,18–20,22]. When we examined this, we found that hypoxia-induced autophagy occurred independently of BNIP3 induction (unpublished observation) consistent with a recent report [22].

The lack of a survival/death phenotype with respect to BNIP3 expression in hypoxia and the existence of multiple forms of the protein, led us to investigate the possibility that BNIP3 is regulated by post-translational modification. We found that treatment of cells with microtubule inhibitors, but not other chemotherapeutics, resulted in hyper-phosphorylation of BNIP3 (Figs. 3 and 4). Upon hyper-phosphorylation, after paclitaxel or vinblastine treatment, BNIP3 remained localized to the mitochondria (Fig. 5A), demonstrating that phosphorylation is not a localization signal. The mitochondrial localization and membrane insertion of Bcl-2 is also retained after phosphorylation in response to paclitaxel [31] or vinblastine [33]. Therefore, the kinase responsible must be active at the mitochondria and this is supported by the observation that the mitochondrial fraction extracted from vinblastine, but not control cells, was able to phosphorylate recombinant Bcl-xL [33].

There is conflicting evidence regarding a role for JNK kinase in the paclitaxel-induced phosphorylation of Bcl-2 [36,44,45]. However, here we have shown that in LS174T cells, paclitaxel induces hyper-phosphorylation of Bcl-2, Bcl-xL and BNIP3 in the absence of JNK activation, thus ruling it out as the kinase responsible. Phosphorylation of BNIP3, Bcl-2 and Bcl-xL was tightly associated with cyclin B1 expression and mitotic arrest (Fig. 6). Inhibition of Mps1, and thus blockade of M-phase arrest in the presence of microtubule inhibitors [41], inhibited the phosphorylation of BNIP3, Bcl-2 and Bcl-xL (Fig. 7). This demonstrates that a mitochondrially active mitotic kinase is responsible for the phosphorylation of the aforementioned proteins. After 48 h of paclitaxel treatment, BNIP3, Bcl-2 and Bcl-xL phosphorylation decreased and dropped to basal levels by 72 h. This event was concurrent with mitotic exit and cell death (Fig. 6) and is likely to be caused by a drop in the activity of the mitotic kinase responsible for phosphorylating these proteins. A loss of the kinase activity would render BNIP3, Bcl-2 and Bcl-xL susceptible to dephosphorylation by a phosphatase. Indeed, the phosphatase inhibitor okadaic acid has previously been shown to block this late dephosphorylation of Bcl-2 [34].

The activities of many BH3-only proteins are regulated by phosphorylation. In many, but not all, cases this is inhibitory, for example phosphorylation of BAD inhibits its apoptotic effect by preventing its interaction with Bcl-xL [46,47]. Similarly, phosphorylation of BID by casein kinase I (CKI) and CKII inhibits its cleavage (activation) by caspase 8 [48] and the ERK-dependent phosphorylation of BIM inhibits its interaction with BAX [49]. In contrast, phosphorylation of BIK, by a CKII-related enzyme, augments its pro-apoptotic activity through enhanced binding to Bcl-2 and Bcl-xL [50,51]. Phosphorylation of Ser70 of Bcl-2 has been associated with either an enhancement [52] or inhibition [36] of its antiapoptotic activity.

Microtubule inhibitors are an important class of chemotherapeutic used to treat a broad range of malignancies. Paclitaxel is frequently prescribed for breast cancer [53]. Despite inducing phosphorylation of BNIP3, the mechanism of paclitaxel-induced cell death operates independently of BNIP3 in hypoxia (Table 1, Supp. Fig. 4). However, one of the functional consequences of paclitaxel-induced BNIP3 phosphorylation is that it extended the half-life of the protein (Fig. 8A). Interestingly, the same phenomenon has been previously observed for mono- and multi-site phosphorylation of Bcl-2 [31,52]. The mechanism for this effect remains unclear, but phosphorylation may inhibit the proteasomal degradation of Bcl-2 and BNIP3. An interesting observation is that BNIP3 interacts with the phosphorylated form of Bcl-2 (Fig. 8B). This indicates that the BNIP3/Bcl-2 interaction is modulated during mitosis. Future studies will focus on understanding the functional consequences of these phosphorylation events, the phosphorylation sites in BNIP3 and the kinase pathway(s) involved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.01.019.

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