UV irradiation resistance-associated gene suppresses apoptosis by interfering with BAX activation

Xiaocheng Yin¹, Lizhi Cao²⁺, Rui Kang^{2,3}, Minghua Yang², Zhuo Wang², Yanhui Peng¹, Yanfang Tan¹, Liying Liu², Min Xie², Yiming Zhao², Kristen M. Livesey³ & Daolin Tang³⁺⁺

¹Department of Pediatrics, The First Affiliated Hospital of Nanhua University, Hengyang, Hunan, People's Republic of China,

²Department of Pediatrics, Xiangya Hospital, Central South University, Changsha, Hunan, People's Republic of China, and

³Hillman Cancer Center, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania, USA

Ultraviolet irradiation resistance-associated gene (UVRAG) is a well-known regulator of autophagy by promoting autophagosome formation and maturation. However, little is known about the non-autophagic functions of UVRAG. Here, we present evidence that UVRAG functions as an unusual BCL2-associated X protein (Bax) suppressor to regulate apoptosis. Chemotherapy and radiation induces UVRAG expression and subsequently upregulates autophagy and apoptosis in tumour cells. Depletion of UVRAG expression by RNA interference renders tumour cells more sensitive to chemotherapy- and radiation-induced apoptosis *in vitro* and *in vivo*. Moreover, UVRAG interacts with Bax, which inhibits apoptotic stimuli-induced mitochondrial translocation of Bax, reduction of mitochondrial membrane potential, cytochrome *c* release and activation of caspase-9 and -3. Our findings show that UVRAG has an essential role in the intrinsic mitochondrial pathway of apoptosis by regulating the localization of Bax. This pathway represents a target for clinical intervention against tumours.

Keywords: apoptosis; autophagy; BAX; mitochondria

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INTRODUCTION

Apoptosis is induced by two main routes involving either the mitochondria (the 'intrinsic' pathway) or the activation of death receptors (the 'extrinsic' pathway). Permeabilization of the mitochondrial outer membrane, which is mainly regulated by BCL2 family members (Rong & Distelhorst, 2008), is the first step

in the mitochondrial pathway of apoptosis. BCL2-associated X protein (BAX) is a proapoptotic member of the BCL2 family of proteins and has a crucial role in apoptosis (Zhang *et al*, 2000). Bax normally resides in the cytosol and translocates to the mitochondria in response to a variety of apoptotic stimuli. Bax performs its proapoptotic action by disrupting the mitochondrial membrane—which results in the release of cytochrome *c* from the organelle—and by forming heterodimers with anti-apoptotic BCL2 proteins to neutralize their actions (Rosse *et al*, 1998). Bax shows differential detergent-induced conformations (Hsu & Youle, 1998). It has been suggested that caspase activation, mitogen-activated protein kinase or association with BID, PUMA or BIM initiate changes in BAX conformation, which induces mitochondrial translocation (Ghibelli & Diederich, 2010; Ren *et al*, 2010). However, the mechanism by which the proapoptotic functions of Bax are suppressed has remained unclear.

Autophagy, a process of 'self-eating' induced by stress, functions as a survival mechanism, but might also contribute to cell death (Kroemer *et al*, 2010). The functional relationship between apoptosis and autophagy is complex; under certain circumstances, autophagy constitutes an adaptation to stress by which cells avoid death (and suppress apoptosis), whereas in other cellular settings it constitutes an alternative cell-death pathway (Maiuri *et al*, 2007b). The well-known autophagy regulator localized to the human chromosomal subregion 11q13, ultraviolet irradiation resistance-associated gene (UVRAG), was first cloned from the KCL22 myeloid leukaemia cell line in 1997 (Perelman *et al*, 1997). UVRAG shows tumour suppressor activity through the induction of autophagy in cancer cells (Liang *et al*, 2006). Remarkably, UVRAG promotes autophagosome formation by activation of the Beclin 1 complex (Liang *et al*, 2006), and autophagosome maturation by recruitment of the fusion machinery to the late endosome (Liang *et al*, 2008). However, accumulating evidence indicates that autophagy-related proteins might not only be specific for autophagy; they might have autophagy-independent functions, including a role in cell death, endocytosis and immunity-related GTPase transport (Mizushima *et al*, 2010). As apoptosis and autophagy share components and exert overlapping functions (Maiuri *et al*, 2007b), we reasoned that UVRAG might have a role in apoptosis.

¹Department of Pediatrics, The First Affiliated Hospital of Nanhua University, Hengyang, Hunan 421001, People's Republic of China

²Department of Pediatrics, Xiangya Hospital, Central South University, Changsha, Hunan 410008, People's Republic of China

³Hillman Cancer Center, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15219, USA

⁺Corresponding author. Tel: +86 731 84327208; Fax: +86 731 84327332; E-mail: lizhicao@gmail.com

⁺⁺Corresponding author. Tel: +01 412 623 1211; Fax: +01 412 623 1212; E-mail: tangd2@upmc.edu

Here, we demonstrate that UVRAG interacts with BAX, which inhibits apoptotic-stimuli-induced mitochondrial translocation of BAX and prevents BAX-mediated downstream events that are required for the induction of apoptosis. Moreover, depletion of UVRAG made tumour cells more sensitive to radiation- and chemotherapy-induced apoptosis *in vitro* and *in vivo*. These results indicate that in addition to its previously recognized proautophagy activity in response to starvation, UVRAG has a cytoprotective function in the cytosol that controls the localization of BAX when tumour cells are subjected to apoptotic stimuli.

RESULTS AND DISCUSSION

Genotoxic or metabolic stress induce UVRAG expression

Genetic and metabolic stressors that induce apoptosis have recently been shown to also induce autophagy. Autophagy is viewed as a 'doubled-edged sword' in the regulation of tumorigenesis and therapy (White & DiPaola, 2009). However, in response to various forms of genetic and metabolic stress (for example, doxorubicin, cisplatin and ultraviolet radiation), autophagy—as detected by microtubule-associated protein light chain 3 (LC3) spot formation and LC3-II expression—precedes apoptosis—detected by Annexin V or cleaved poly-ADP ribose polymerase—in HL60 and HCT116 tumour cells (Fig 1A–C). Interestingly, in human tumour cells exposed to genotoxic or metabolic stress, the level of UVRAG expression was upregulated at early time points (for example, 12 h) and restored to normal levels at later time points (for example, 36 h) by immunofluorescence and western blot analyses (Fig 1A–C). These data indicate that UVRAG expression is related to levels of autophagy.

To determine the influence of autophagy on the expression of UVRAG, we treated cells with ultraviolet radiation and doxorubicin in the presence or absence of an autophagy inhibitor, such as 3-methyladenine. 3-methyladenine decreased the protein and messenger RNA (mRNA) expression of UVRAG during apoptosis (Fig 1D). Moreover, autophagy-deficient ATG5^{−/−} murine embryonic fibroblasts had decreased protein and mRNA levels of UVRAG during apoptosis (Fig 1E). Consistent with previous studies (Ito *et al*, 2005; Apel *et al*, 2008), inhibition of autophagy increased anti-cancer-therapy-induced apoptosis (Fig 1D,E). These findings indicate that autophagy-mediated UVRAG overexpression might be a negative regulator of apoptosis.

UVRAG regulates sensitivity to cancer therapy

The primary cause of treatment failure in patients with cancer is multidrug resistance. To explore the potential role for UVRAG in therapy, a target-specific short-hairpin RNA (shRNA) against UVRAG was transfected into human HL60 leukaemia cells, HCT116-clone cancer cells and HeLa cervical cancer cells. Transfection of UVRAG-shRNA led to a decrease in UVRAG protein in these cell types (Fig 2A–C). Depletion of UVRAG expression in these cells made them more susceptible to doxorubicin, cisplatin, ultraviolet radiation or starvation-induced apoptotic cell death, which was associated with low levels of LC3 punctae formation (Fig 2A–C). Treatment with the lysosomal protease inhibitors pepstatin/E64D (Mizushima *et al*, 2010) induced a further increase in LC3 punctae after doxorubicin treatment in cells transfected with control shRNA, but not UVRAG shRNA (Fig 2D). Moreover, knockdown of UVRAG in ATG5^{−/−} murine embryonic fibroblasts increased doxorubicin-induced

apoptosis, which is inhibited by the pan caspase inhibitor Z-VAD-FMK (Fig 2E). These results indicate that the increase in apoptosis is a direct result of loss of UVRAG expression rather than a secondary result of autophagy inhibition causing a shift to the apoptosis pathway.

To determine whether targeted knockdown of UVRAG also increased sensitivity to chemotherapy *in vivo*, we inoculated nude mice with 4×10^6 HL60 tumour cells transfected with control or UVRAG-specific shRNA and treated with doxorubicin. *In vivo*, inhibition of UVRAG expression increased tumour cell sensitivity to doxorubicin and decreased tumour growth (Fig 2F), which were associated with increased apoptosis by terminal deoxynucleotidyl transferase uridine triphosphate nick-end labelling assay at day 21 (Fig 2G). Together, these results show that UVRAG has a crucial role in modulating apoptosis in stressed cancer cells *in vivo* and *in vivo*.

UVRAG inhibits Bax-induced apoptosis

Among the proapoptotic BCL2 family members, BAX and BAK are central to the induction of apoptosis, as cells derived from BAX/BAK knockout mice demonstrate resistance to several cell-death stimuli that are known to trigger mitochondria-dependent apoptosis (Wei *et al*, 2001). Knockdown of UVRAG increased BAX-induced apoptosis in tumour cells (Fig 3A). BAX activation leads to mitochondrial membrane depolarization and cytochrome c release from the organelle. These events result in caspase-3 and -9 activation and subsequent cell death. Knockdown of UVRAG by shRNA increased BAX induction of these downstream events resulting in apoptosis (Fig 3B). By contrast, overexpression of UVRAG by gene transfection inhibited BAX-induced apoptosis (Fig 3C), suggesting that UVRAG regulates BAX-mediated signalling in apoptosis. In addition, UVRAG does not suppress apoptosis induced by other cytoplasmic proapoptotic BCL2 proteins—such as BAD or BID or by 'extrinsic' cell death stimuli—such as FAS or TRAIL (TNF-related apoptosis-inducing ligand; Fig 3C). Furthermore, knockdown of UVRAG in BAX^{−/−} HCT116 cells restored sensitivity to ultraviolet treatment-induced apoptosis (Fig 3D), although the precise mechanism requires further investigation. One explanation would be an additional role of UVRAG involving BAK, another requisite gateway to mitochondrial dysfunction and death (Wei *et al*, 2001). These results support our hypothesis that UVRAG selectively inhibits BAX induction of intrinsic apoptotic signalling.

UVRAG interacts with Bax

Knockdown of UVRAG did not affect the expression level of endogenous BAX (Fig 2A–C), suggesting that UVRAG regulates BAX-mediated apoptotic signalling rather than cellular levels of BAX. To explore whether UVRAG binds to BAX, we performed co-immunoprecipitation analysis using UVRAG and BAX antibodies. We found that under normal conditions, endogenous UVRAG and BAX co-immunoprecipitate with each other in HL60 and HCT116 cells and this interaction decreased after ultraviolet treatment in radioimmunoprecipitation assay buffer or 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphate buffer (Fig 4A,B). Moreover, glutathione-S-transferase (GST) pull-down assays confirmed an interaction between UVRAG and BAX, but not between UVRAG and a control protein (cyclin D1) *in vitro* (Fig 4C). These results indicate that there is an interaction between UVRAG and BAX.

significant interaction between Beclin 1 and BAX in HL60 and HCT116 cells (Fig 4A), suggesting that UVRAG forms two complexes—UVRAG–Beclin 1 and UVRAG–BAX—during cell stress to regulate levels of apoptosis and autophagy. UVRAG

Fig 2 | Inhibition of UVRAG expression increases tumour-cell sensitivity to chemotherapy and radiation therapy *in vivo* and *in vitro*. (A–C) After transfection with UVRAG shRNA or control shRNA for 48 h, HL60 (A), HCT116 (B) and HeLa (C) cells were treated with doxorubicin (Doxo, 1 µg/ml), cisplatin (Cis, 10 µM) and UV irradiation (5 min after 50 mJ/cm²) for 12 h or starvation (HBSS) for 3 h, and then apoptosis and autophagy were assayed (*n* = 3, **P* < 0.05). (D) Analysis of LC3 processing by autophagy in the presence or absence of lysosomal protease inhibitors pepstatin A (10 µg/ml) and E64D (10 µg/ml) after doxorubicin (1 µg/ml) treatment for 12 h. **P* < 0.05 compared with control shRNA group. (E) After transfection with UVRAG shRNA or control shRNA for 48 h, ATG5^{+/+} and ATG5^{-/-} murine embryonic fibroblasts (MEFs) were treated with doxorubicin (1 µg/ml), with or without Z-VAD-FMK (20 µM) for 12 h, and then apoptosis was assayed (*n* = 3, **P* < 0.05). (F) Nude mice were inoculated with 4 × 10⁶ HL60 tumour cells following transfection of control (shControl) or UVRAG (shUVRAG)-specific shRNA and treated with doxorubicin (10 mg/kg) beginning at day 7. Tumours were measured twice weekly, and volumes were calculated for 21 days (*n* = 10, **P* < 0.05; shControl + Doxo compared with shUVRAG + Doxo). (G) On day 21 in the experiments described in (F), apoptosis and UVRAG expression in tumour samples were assayed by TUNEL or western blotting, respectively (*n* = 10, **P* < 0.05). BAX, BCL2-associated X protein; HBSS, Hank's Buffered Salt Solution; LC3, light chain 3; shRNA, short-hairpin RNA; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; UV, ultraviolet; UVRAG, ultraviolet irradiation resistance associated gene.

contains an amino-terminal proline-rich sequence followed by a potential calcium-dependent phospholipid binding C2 domain, a central Beclin 1-binding coiled-coil domain and a carboxy-terminal region. A previous study demonstrated that UVRAG binds to Beclin 1 through its coiled-coil domain (Liang *et al*, 2006). By contrast, we demonstrated that deletion of C2 (ΔC2) inhibits the interaction of UVRAG with BAX (Fig 4D), making it unable to inhibit chemotherapy and radiation-induced apoptosis (Fig 4E).

UVRAG inhibits mitochondrial translocation of BAX

BAX translocation to the mitochondria is an important event in inducing apoptotic cell death. To explore whether the interaction between UVRAG and BAX regulates BAX translocation, we isolated mitochondrial and cytosolic cellular fractions and then performed immunoblots. Suppression of UVRAG promoted doxorubicin and ultraviolet-radiation-induced mitochondrial translocation of BAX (Fig 5A). By contrast, overexpression of UVRAG inhibited mitochondrial translocation of BAX (Fig 5B).

Fig 3 | UVRAG inhibits BAX-induced apoptosis. (A) Control shRNA or UVRAG shRNA cells were transfected with BAX plasmid, and apoptosis was assayed ($n = 3$, $*P < 0.05$). (B) In parallel, the mitochondrial membrane potential, cytochrome *c* release from mitochondria, and caspase-3 and -9 activation in HL60 cells were analysed ($n = 3$, $*P < 0.05$). (C) After transfection with UVRAG vector or control vector for 48 h, cells were transfected or treated with BAX (1 µg), BAD (1 µg), BID (1 µg), FAS antibody (10 µg/ml) or TRAIL (100 ng/ml) for 24 h, then cell apoptosis was analysed ($n = 3$, $*P < 0.05$). (D) Knockdown of UVRAG in BAX^{-/-} HCT116 cells restored the sensitivity to UV treatment-induced apoptosis ($n = 3$, $*P < 0.05$). AU, arbitrary units; BAX, BCL2-associated X protein; shRNA, short-hairpin RNA; UV, ultraviolet; UVRAG, ultraviolet irradiation resistance associated gene.

Interestingly, control vector, but not control shRNA, induced more doxorubicin-induced mitochondrial translocation of BAX than non-transfected cells (Fig 5C). This suggests that there might be a non-specific effect of this control construct on BAX location.

Apoptotic signals can induce conformational changes in the BAX protein, leading to the exposure of the BAX N-terminus before mitochondrial translocation. This conformational change can be detected with the BAX monoclonal antibody clone 6A7 (Suzuki *et al*, 2000). We next evaluated whether loss of UVRAG can induce the exposure of the BAX N-terminus. We found that suppression of UVRAG did not induce exposure of the BAX N-terminus, but that loss of UVRAG enhanced this conformational change after doxorubicin and ultraviolet radiation (Fig 5D). We also found that overexpression of UVRAG suppressed binding of the 6A7 antibody to BAX in the cells treated by doxorubicin or ultraviolet radiation (Fig 5E). Similarly, there is a non-specific effect of control vector on the binding of the 6A7 antibody to BAX (Fig 5F). These results indicate that UVRAG can inhibit exposure of the BAX N-terminus during apoptosis.

In summary, we demonstrate the involvement of UVRAG in the regulation of BAX-mediated mitochondrial apoptotic events. Recent studies suggest that a cytoprotective mechanism regulates BAX translocation to mitochondria through BAX–Humanin (Guo *et al*, 2003) and BAX-apoptosis-associated speck-like protein (Ohtsuka *et al*, 2004). Thus, a more detailed understanding of Bax–UVRAG interactions and its partners might lead to the development of new drugs for the treatment of human tumours.

METHODS

Reagents. The antibodies to LC3 were obtained from MBL International (Woburn, MA, USA). The antibodies to BAX, Beclin 1, COX IV, tubulin, GST and actin were obtained from Cell Signaling Technology (Danvers, MA, USA). BAX monoclonal antibody (clone 6A7) and EEA1 antibody were from Abcam (Cambridge, MA, USA). The antibodies to UVRAG, cytochrome *c* and FAS were obtained from Santa Cruz Technology (Santa Cruz, CA, USA). The antibody to ATP synthase-β was obtained from Affinity BioReagents (Rockford, IL, USA). Other reagents were from Sigma (St Louis, MO, USA). The human HL60, HCT116 and HeLa cells were derived from the American Type Culture Collection (Manassas, VA, USA). ATG5^{+/+} and ATG5^{-/-} mouse embryonic fibroblasts were a kind gift from Dr Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). BAX^{-/-} HCT116 cells were a kind gift from Dr Lin Zhang (University of Pittsburgh, Pittsburgh, USA).

Immunoprecipitation analysis. Cells were lysed at 4 °C in ice-cold radioimmunoprecipitation assay lysis buffer (Millipore, Billerica, MA, USA) or 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate lysis buffer (Cell Signaling Technology) as indicated in figure legends. Before immunoprecipitation, samples containing equal amounts of proteins were precleared with protein A or protein G agarose/sepharose (Millipore) and subsequently incubated with various irrelevant IgG or specific antibodies in the presence of protein A or G agarose/sepharose

Fig 4 | UVRAG interacts with Bax. (A,B) HL60 and HCT116 cells were treated with UV irradiation for 24 h and then assayed for protein expression levels as indicated by co-immunoprecipitation (RIPA or CHAPS buffer) or western blotting. (C) Proteins in the pellet after GST pull-down assays in HCT116 cell lysate were analysed by SDS-PAGE and immunodetected with UVRAG or GST antibodies. (D) UVRAG C2 domain deletion terminates BAX binding. HCT116 cells were transfected with Flag-UVRAG or its mutants (Δ C2, Δ CCD), and then assayed for protein expression levels as indicated by IP or western blot. (E) Apoptosis was assayed in indicated HCT116 cells after treatment with doxorubicin (Doxo, 1 μ g/ml), UV irradiation (5 min after 50 mJ/cm²) or transfected with BAX (1 μ g) for 24 h. BAX, BCL2-associated X protein; CCD, coiled-coil domain; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate; GST, glutathione-S-transferase; IB, immunoblotting; IgG, immunoglobulin G; IP, immunoprecipitation; RIPA, radioimmunoprecipitation assay; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; UV, ultraviolet; UVRAG, ultraviolet irradiation resistance associated gene.

beads, as described previously (Hsu & Youle, 1998; Guo *et al*, 2003; Tang *et al*, 2007; Kang *et al*, 2010b). Following incubation, agarose/sepharose beads were washed extensively with phosphate-buffered saline and proteins eluted by boiling in 2 $\times$ SDS sample buffer before SDS-polyacrylamide gel electrophoresis.

Gene transfection and RNA interference. Transfection with UVRAG cDNA and mutants (Liang *et al*, 2006, 2008), pEGFP-BAX (Nechushtan *et al*, 1999), pEGFP-BAD (Kang *et al*, 2010b), pEGFP-BID (Kang *et al*, 2010b) vector and/or UVRAG-shRNA, Beclin 1-shRNA, BAX-shRNA and control shRNA (from Sigma, USA) were performed using the FuGENE HD Transfection Reagent (Roche Applied Science, Stockholm, Sweden) according to the manufacturer's instructions.

Apoptosis assays. Apoptosis in cells was assessed by using an Annexin V/PI Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA) by flow cytometric analysis. Apoptosis in tissue was assessed using a terminal deoxynucleotidyl transferase uridine triphosphate

nick-end labelling kit from Roche Applied Science. Caspase-3 and -9 activity assays were performed using Colorimetric Assay Kits from Calbiochem (Gibbstown, NJ, USA). Mitochondrial membrane potential depolarization was measured by flow cytometry using a fluorescent cationic dye, 1,1',3,3'-tetraethylbenzamidazolocarboxyanin iodide (JC-1, Molecular Probes, San Diego, CA, USA).

Autophagy assays. The formation of autophagic vesicles was monitored by endogenous LC3 aggregation in cell lines by immunofluorescence analysis. The average LC3 punctae per cell from at least 100 cells was determined using Image-Pro Plus 5.1 software (Media Cybernetics, Bethesda, MD, USA), as described previously (Tang *et al*, 2010a,b; Liu *et al*, 2011; Wang *et al*, 2011).

Animal experiments. To generate murine subcutaneous tumours, 4 $\times$ 10⁶ HL60 cells transfected with control or UVRAG-specific shRNA were injected subcutaneously to the right of the dorsal midline in nude mice, as described previously (Karajannis *et al*,

Fig 5 | UVRAG inhibits mitochondrial translocation of BAX. After transfection with indicated shRNA or cDNA for 48 h, HL60 cells were treated with doxorubicin (Doxo) and UV irradiation for 12 h, and then BAX in cytosol (Cyt) and mitochondria (Mit) was assayed by western blotting (A–C) and activation of BAX (D–F) was assayed by western blotting after IP using BAX monoclonal antibody (clone 6A7) **P* < 0.05. AU, arbitrary units; BAX, BCL2-associated X protein; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate buffer; Cyt c, cytochrome c; IP, immunoprecipitation; shRNA, short-hairpin RNA; UV, ultraviolet; UVRAG, ultraviolet irradiation resistance associated gene.

2006). One week after injection, when the subcutaneous tumour size had reached a diameter of approximately 3 mm, the mice received intraperitoneal injections of doxorubicin (10 mg/kg) twice a week. Tumours were measured twice weekly, and volumes were calculated using the formula: length $\times$ width² $\times$ $\pi/6$ (Kang et al, 2010a). The procedures for performing animal experiments were approved and in accordance with the guidelines of the institutional review board.

GST pull-down assay. GST pull-downs were performed using the Pierce GST Protein Interaction Pull-Down Kit (Pierce, Rockford, IL, USA), according the manufacturer's instructions.

Isolation and subcellular fractionation of mitochondria. Subcellular fractionation of tumour cells was carried out with a mitochondria isolation kit obtained from Pierce, according the manufacturer's instructions.

Statistical analysis. Data are expressed as means $\pm$ s.d. of three independent experiments. Significance of differences between

groups was determined by two-tailed Student's *t*-test or analysis of variance least significant difference test. A *P*-value < 0.05 was considered to be significant.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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