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JNK mediates UVB-induced apoptosis upstream lysosomal membrane permeabilization and Bcl-2 family proteins

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Abbreviated title: JNK mediates UVB-induced apoptosis

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Abstract

UVB irradiation induced phosphorylation of JNK and subsequent apoptosis in human melanocytes. Depletion of both JNK1 and JNK2 expression using siRNA transfection, protected against apoptosis, as detected by decreased nuclear fragmentation and caspase-3 activity, as well as reduced translocation of Bax to mitochondria. Moreover, release of cathepsin B and D from lysosomes to the cytosol was reduced when JNK expression was suppressed by siRNA, demonstrating a JNK dependent regulation of lysosomal membrane permeabilization. In unirradiated control melanocytes, coimmunoprecipitation showed that Bim was sequestered by Mcl-1, which had a pro-survival function. After UVB irradiation, a significant decrease in Mcl-1 protein level was found, which was prevented by addition of a proteasome inhibitor. The interaction between Bim and Mcl-1 was reduced in response to UVB irradiation and Bim was phosphorylated in a JNK dependent manner. In conclusion, these findings suggest JNK to have an important pro-apoptotic function following UVB irradiation in human melanocytes, by acting upstream of lysosomal membrane permeabilization and Bim phosphorylation.

Introduction

A critical step in the intrinsic pathway of apoptosis is the permeabilization of the mitochondrial outer membrane, which results in release of several pro-apoptotic proteins, including cytochrome c and Smac/DIABLO to the cytosol. Such release promotes activation of caspases, which are designed to induce the biochemical and morphological alterations characterizing apoptosis. The mitochondrial membrane permeabilization is regulated mainly by pro- and anti-apoptotic proteins belonging to the Bcl-2 family [1]. The anti-apoptotic proteins, including Bcl-2, Bcl-X_L, Mcl-1, Bcl-w, and A1 counteract apoptosis by interacting with pro-apoptotic Bcl-2 members in the mitochondrial membrane, which are either BH3-only proteins (e.g. Bid, Bim, Bad, Bmf, Puma, and Noxa) or proteins that contain multiple BH domains (e.g. Bax, and Bak). Following a death stimulus, some BH3-only proteins are transcriptionally induced, while others, constitutively expressed in their inactive form, are activated by post-translational modifications, such as proteolytic processing, phosphorylation, or dephosphorylation [2,3]. After activation, the BH3-only proteins translocate to mitochondria and stimulate apoptosis either by forming heterodimers with anti-apoptotic members to unleash pro-apoptotic proteins, such as Bax and Bak, or by direct interaction and activation of Bax and Bak proteins.

c-Jun NH_2-terminal kinases (JNKs), belonging to the group of stress-activated protein kinases (SAPKs), are encoded by three genes, namely *Jnk1* and *Jnk2* that are ubiquitously expressed, and *Jnk3*, whose expression is restricted to the brain, heart and testis [4]. Ten different JNK isoforms exist due to alternative splicing. The role of JNKs in apoptosis is unclear as these proteins have been assigned both pro- and anti-apoptotic properties. However, there are also studies demonstrating JNK to be insignificant in regulation of apoptosis. JNK becomes activated through phosphorylation and some tumor cell lines contain constitutively active JNK [4]. Anti-sense JNK oligonucleotides have been

reported to inhibit the growth of such tumor cells and to induce apoptosis [5]. In contrast, activated JNK is required for UV-induced apoptosis and is suggested to be essential for cytochrome c release from mitochondria in mouse fibroblasts [6]. Moreover *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ cells were shown to be almost completely resistant to apoptosis induced by UV irradiation. The exact consequence of JNK activation in UV-irradiated cells has, however, not been fully investigated.

In a previous study, using human epidermal melanocytes, we have demonstrated lysosomal membrane permeabilization (LMP) to be essential in UV-induced apoptosis signaling [7]. UV exposure caused release of cathepsins from lysosomes to the cytosol, where they acted as pro-apoptotic mediators upstream translocation of Bax to mitochondria and mitochondrial membrane permeabilization. The aim of the present study was to investigate the significance of JNK in regulation of Bcl-2 family proteins and LMP during UV-induced apoptosis in human epidermal melanocytes.

Materials and Methods

Cell culture

The use of residual skin after surgery for cell culture studies of UV effects on melanocytes has been approved by the Ethical Committee at Linköping University, Linköping, Sweden. Normal human melanocytes were isolated from foreskins obtained from Caucasian donors (0-2 years of age). Melanocyte cultures were established as described previously [8] and cultured in Medium 199 (Invitrogen, Paisley, Scotland, UK) with 2% fetal bovine serum and incubated at 37°C in a humidified 5% CO_2 in air, according to Gilchrest *et al*. [9]. Melanocytes in passage 2-5 were used in all experiments and no cells were cultured for more than three weeks in total. Untreated controls from the same donor were analyzed in parallel. Protease activity was inhibited by pretreatment with the proteasome inhibitor MG-132 (10 μM for 4 h; Calbiochem, Darmstadt, Germany), the caspase inhibitor zVAD (10 μM for 1 h), and the aspartic protease inhibitor Pepstatin A (100 μM for 16 h; stock in DMSO, Sigma-Aldrich, St. Louis, MO, USA) before UV exposure. Controls for DMSO effects were also analyzed and no interference with the experiments was noted.

UVB exposure

The UVB source was two Philips TL20W/12 tubes (Philips, Eindhoven, The Netherlands), emitting in the spectral range 280-370 nm with a main output of 305-320 nm, equipped with a Schott WG 305 cut off filter (50% absorption below 305 nm, Mainz, Germany). The output was 1.44 mW/cm^2, measured with a PUVA Combi Light dosimeter (Leuven, Belgium), using adequate adjustments for UVB. The cultures were irradiated with 500 mJ/cm^2 in phosphate buffered saline (PBS). This procedure did not cause increase in temperature of the PBS during irradiation. Directly after irradiation, the PBS was replaced with fresh pre-warmed (37ºC)

medium. Control cells were treated identical but not irradiated. The irradiation dose was selected to achieve a frequency of apoptosis of 30-40 % with a minimum number of necrotic cells [7].

Nuclear morphology and caspase activity

Six hours following UVB exposure, the cultures were fixed in 4% neutral buffered formaldehyde and mounted in Vectashield® Mounting Media supplemented with 4',6-diamidino-2-phenylindole (DAPI, 5 μg/ml, Vector Laboratories, Burlingame, CA, USA). 200 randomly selected nuclei were evaluated using a fluorescence microscope (λ_{ex} 350 nm, Nikon, Tokyo, Japan). In the control cells, most nuclei were round in shape and glowed homogenously, while apoptotic cells were identified by either fragmented nuclei or by a condensed chromatin pattern gathered at the periphery of the nuclear membrane.

In order to analyze caspase-3 activity, the cells were collected in lysis buffer (10 mM Tris-HCl pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM $NaH_2PO_4/NaHPO_4$) and incubated with the substrate Ac-DEVD-AMC according to the manufacturer's recommendations (BD Pharmingen, San Diego, CA, USA). The fluorescence of proteolytically released AMC (7-amino-4-methylcoumarin) was analyzed in a Shimadzu RF-540 spectrofluorometer ($\lambda_{ex}380/\lambda_{em}435$, Shimadzu Kyoto, Japan). Protein concentrations were analyzed with Bio-Rad D_C Protein Assay System (Bio-Rad Laboratories, Hercules, CA, USA) and caspase activity was expressed as arbitrary units/μg protein/h.

siRNA transfection

Human melanocytes were seeded one day prior to transfection at 25×10^3 cells/cm^2. The cells were transfected with 1 μg (12-well plate) of either MAPK8 (JNK1;

AAGCCCAGTAATATAGTAGTA), MAPK9 (JNK2; CATGATGTTATCATATCTTAT), a combination of MAPK8 and MAPK9 (0.5 µg of each), Mcl-1 (CGGGACTGGCTAGTTAAACAA) or Bim (CGGAGACGAGTTTAACGCTTA) siRNA, together with 6 µl RNAiFect Transfection Reagent according to the manufacturer's instructions (Qiagen, Germantown, MD, USA). Optimal transfection conditions were determined by transfection with Alexa Fluor 555 labeled non-silencing siRNA, consisting of a scrambled sequence with no homology to mammalian genes (AATTCTCCGAACGTGTCACGT). This siRNA sequence was used in the experiments as negative control and siRNA targeting Lamin A/C (AACTGGACTTCCAGAAGAACA) was positive control as recommended by the manufacturer. The cells were incubated at standard culture conditions (37ºC, 5% CO_2) and after 8 h, the siRNA transfection medium was replaced with fresh medium. A significant decrease in protein level was observed 48 h following siRNA addition. Silencing of JNK and Mcl-1 siRNA was confirmed by Western blot analysis. All siRNA and reagent used were obtained from Qiagen.

Isolation of cytosol

Cytosol was extracted by adding digitonin (Sigma-Aldrich) in a buffer consisting of 250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM Pefabloc, 8 mM dithiotriol, pH 7.5 to the melanocyte cultures for 12 min on ice. This procedure permeabilizes the cholesterol-rich plasma membrane, but leaves membranes of intracellular organelles intact, as determined by analysis of lactate dehydrogenase (LDH) and the lysosomal enzyme β-N-acetylglucoseaminidase (NAG) activities, respectively [10,11]. The digitonin concentration (10-15 µg/ml) was individually titrated for each melanocyte donor. Proteins of the extracted cytosol were precipitated in trichloric acid (50%), incubated on ice for 10 min, and subsequently pelleted by centrifugation. For Western blot analysis, the

pellet was resuspended in urea-lysis buffer (6 M urea, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM Tris, pH 8.0, 5 mM EDTA), sample buffer (5% β-mercaptoethanol in Laemmli sample buffer, Bio-Rad Laboratories) and 1 M NaOH.

Western blot analysis

Gel electrophoresis and Western blotting were performed as described previously [7]. Primary monoclonal Lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Mcl-1 (Calbiochem) antibodies or polyclonal JNK (Santa Cruz Biotechnology), JNK-phos (Santa Cruz Biotechnology), Bim-phos (Bim_{EL} pS65, Millipore, Billerica, MA), Bim (23 kDa; Sigma-Aldrich), cathepsin D (Upstate Biotechnology, Lake Placid, NY,USA), and Mcl-1 (Santa Cruz Biotechnology) antibodies were used, followed by horse radish peroxidase (HRP)-conjugated sheep anti-mouse or donkey anti-rabbit secondary antibodies (Amersham Biosciences, Buckinghamshire, UK). The bands were visualized using enhanced ECL-Plus Western blotting detection system (Amersham Biosciences) and the protein concentrations were determined with Bio-Rad protein assay. The membranes were reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Biogenesis, Poole, UK) as internal control. Densitometric quantification was performed of the immunoblots and normalized to the amount of GAPDH using Gel-Pro Analyzer 3.1 (MediaCybernetics, Silver Spring, MD, USA). Representative blots are presented in the figures.

Immunocytochemistry

Melanocytes were fixed in 4% paraformaldehyde for 20 min at 4°C and processed for immunocytochemistry [12]. After permeabilization with 0.1% saponin, the cultures were incubated overnight at 4°C with the monoclonal antibodies anti-Mcl-1 (Calbiochem), or anti-

LAMP-2 (lysosomal associated membrane protein-2; SouthernBiotech, Birmingham, AL, USA), or the polyclonal antibodies towards Bax (Upstate Biotechnology), Cathepsin B (Calbiochem), Mcl-1 (Santa Cruz Biotechnology), or Bim (Sigma-Aldrich) followed by incubation with a secondary donkey anti-mouse Alexa Fluor® 594 (Molecular Probes, Eugene, OR, USA) or goat anti-rabbit Alexa Fluor®488 conjugate (Molecular Probes) for 1 h at room temperature. Vital staining of mitochondria was accomplished by incubation of cells with 200 nM Mitotracker® Red (Molecular Probes) for 30 min at 37°C before fixation. The specimens were mounted in Vectashield® Hardset Mounting Media and inspected in a Nikon Eclipse E600W fluorescence confocal microscope. In each culture dish, 200 cells were randomly selected and the localization of the proteins was analyzed. Negative controls, incubated without primary antibody, showed no staining.

Immunoprecipitation

Six hours after UVB exposure, the melanocytes were incubated in Chaps buffer (10 mg/ml Chaps (Sigma-Aldrich), 10 µl/ml protease inhibitor cocktail (Sigma-Aldrich), 150 mM NaCl, 10 mM Hepes, pH 7.4) on ice for 30 min. The lysate was pre-cleared by incubation with protein G-agarose beads (Santa Cruz Biotechnology) at 4°C for 1 h on an orbital shaker. This procedure reduces non-specific binding of proteins to the agarose. The protein G-agarose beads were then removed by centrifugation (2700 × g for 5 min at 4°C). Protein concentration was analyzed by Bio-Rad D_C Protein Assay System and 25 µg total protein was diluted to a total volume of 200 µl in Chaps buffer. The proteins were subsequently incubated with polyclonal Bim antibody (2 µg, Sigma-Aldrich) or monoclonal Mcl-1 antibody (1 µg, Calbiochem) over night followed by incubation with protein G-agarose beads for 1 h. Both incubations were performed at 4°C on an orbital shaker. The beads were then collected by centrifugation (2700 × g for 5 min at 4°C) and washed four times in Chaps buffer. The beads

were boiled in sample buffer (5% β-mercaptoethanol in Laemmli sample buffer, Bio-Rad Laboratories) for 5 min and proteins that copreciptitated were visualized by a Western blot. A negative control sample, without antibody addition, was handled in parallel.

Statistics

Statistical comparisons were performed with Kruskal Wallis test as pre-test, followed by Mann-Whitney U tests. P-values below 0.05 were considered significant.

Results

UVB exposure of human melanocytes resulted in phosphorylation of the JNK protein, while the total JNK level remained unaltered (Fig. 1A). To investigate the impact of JNK activation in the regulation of apoptosis, melanocytes were transfected with siRNA to silence the expression of both JNK1 and JNK2. The protein expression was reduced by two thirds 48 h after transfection (Fig. 1B). When melanocytes were exposed to UVB irradiation, 37 % of the cells showed fragmented or condensed nuclei after 6 h (Fig. 1C). In cells transfected with JNK siRNA before UVB irradiation, a significant reduction in apoptotic frequency was detected compared to non-transfected cells and to cells transfected with a negative control siRNA sequence. In accordance, cells with silenced JNK expression demonstrated significantly lower caspase-3 activity (Fig. 1D).

To investigate if JNK had any impact on lysosomal membrane integrity, we studied the release of the lysosomal enzyme cathepsin D into the cytosol 6 h following UVB irradiation. A marked loss of lysosomal membrane integrity (2,5-fold increase compared to non-exposed controls) was observed after UVB irradiation in non-transfected cells. In JNK-depleted cells a minor lysosomal release of cathepsin D was detected (1.6-fold increase compared to non-exposed controls) (Fig. 2A). Total protein level of cathepsin D was unaltered after UVB (Fig. 2A). Lysosomal membrane permeabilization (LMP) was further studied by immunostaining of cathepsin B. As presented in Figure 2B, cathepsin B staining showed a punctate perinuclear pattern in unexposed cells, but after UVB irradiation, the intracellular staining pattern became more diffuse, representing release of cathepsin B into the cytosol. In addition, punctate staining pattern of cathepsin B correlated with normal shaped nucleus, while diffuse staining pattern characterized cells having a fragmented apoptotic nucleus (not shown). Quantification of immunostained cells displaying LMP showed that JNK siRNA transfection significantly reduced the release of cathepsin B to the cytosol,

indicating that JNK operates upstream of lysosomal permeabilization (Fig. 2C). The experiments described in Figure 1 and 2 were also performed in melanocytes selectively depleted in JNK1 or JNK2, and gave similar results (not shown). However, the depletion of JNK1 and JNK2 simultaneously resulted in a more pronounced protective effect.

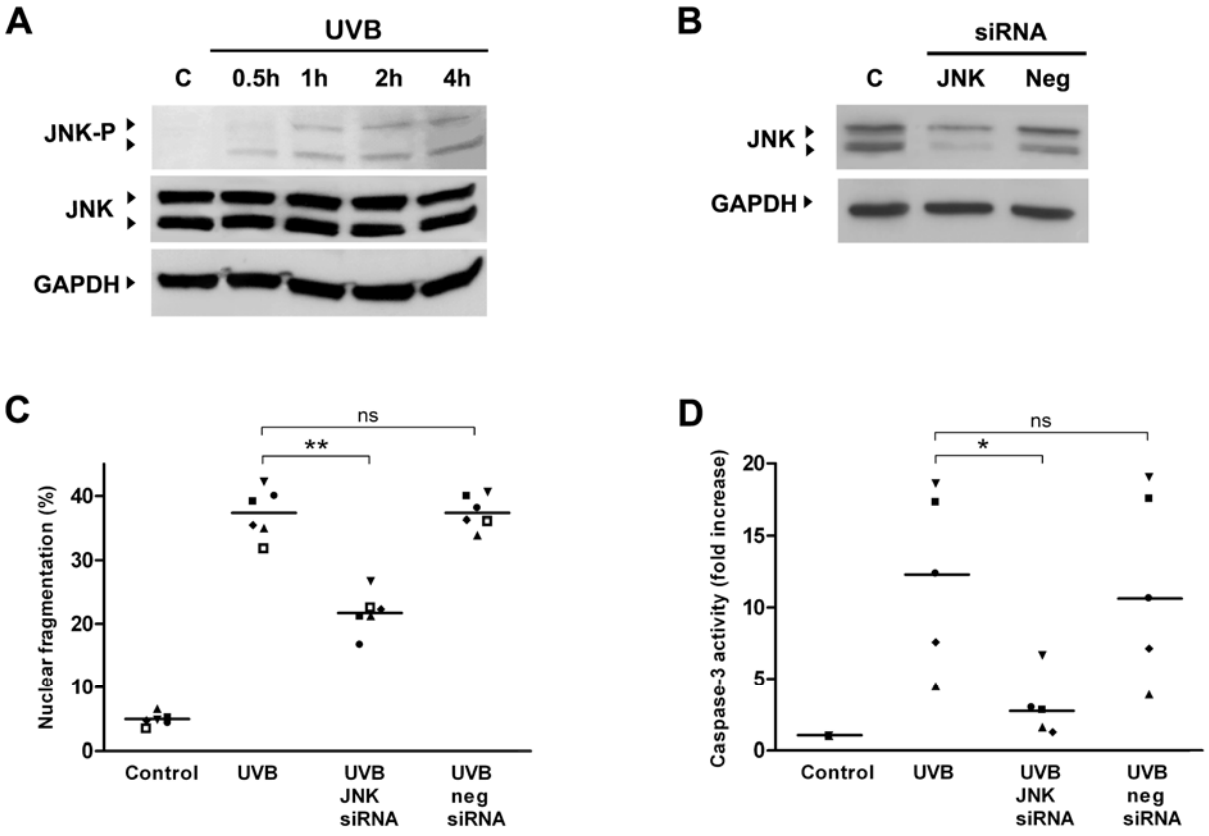

Fig. 1 Bivik & Öllinger

Figure. 1. JNK has pro-apoptotic effect in UVB-irradiated (500 mJ/cm^2) melanocytes. (A) Western blot analysis of phosphorylated JNK and total JNK protein levels after UVB irradiation. One representative blot out of 3 is presented and GAPDH is used as internal control. (B) Western blot analysis of JNK1 and JNK2 after siRNA transfection. One representative blot out of 4 is shown and GAPDH is used as internal control. (C) Frequency of apoptosis 6 h after UVB exposure in JNK siRNA transfected melanocytes, determined by microscopic inspection of nuclear morphology in DAPI stained cells (n=6). A scrambled siRNA sequence was used as negative control for siRNA transfection. (D) Caspase-3 activity following UVB irradiation in JNK siRNA transfected melanocytes was detected as cleavage of the substrate Ac-DEVD-AMC and presented as fold increase of control samples (n=5). The symbols in C and D represent individual melanocyte donors and median values are marked with a horizontal line. * $p<0.05$, ** $p<0.01$, ns; non-significant.

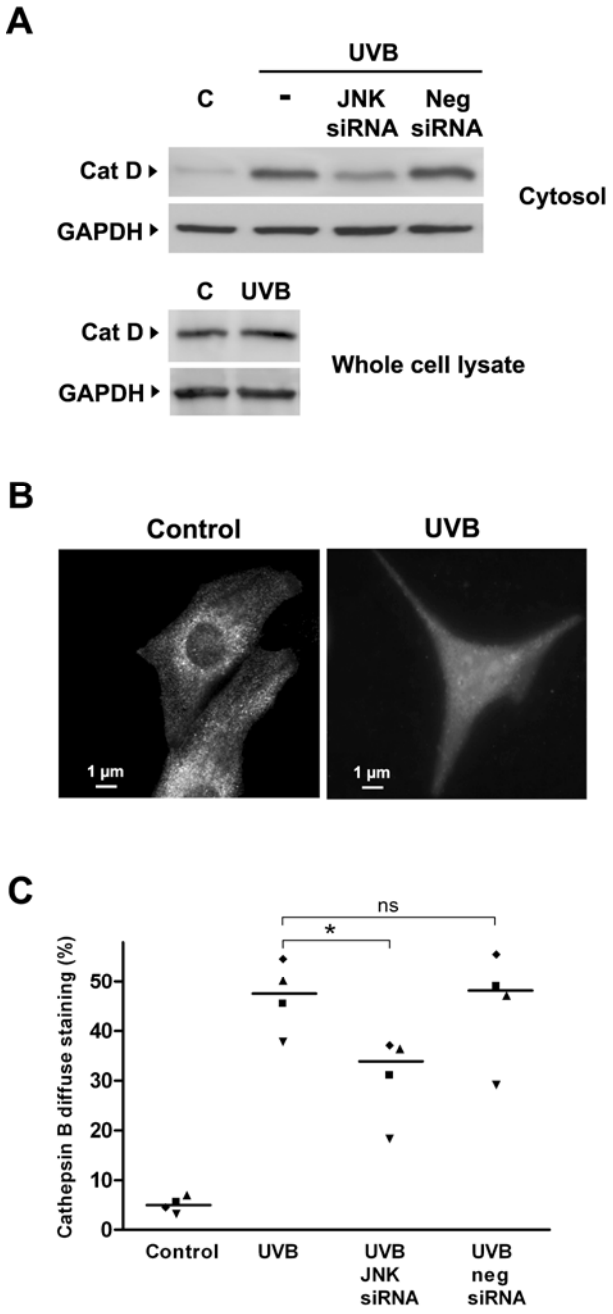

Fig. 2 Bivik & Öllinger

Figure 2. JNK siRNA reduces lysosomal permeabilization after UVB irradiation (500 mJ/cm^2). (A) Melanocytes transfected with JNK siRNA were exposed to UVB and the cytosolic fraction was isolated by digitonin extraction after 6 h. Release of cathepsin D was analyzed by Western blot. A scrambled siRNA sequence was used as negative control for siRNA transfection. Cathepsin D protein expression in whole cell lysate from unexposed and UV exposed cells is also shown. One representative blot out of 3 is presented and GAPDH is used as internal control. (B) Representative immunocytochemistry images of cathepsin B in unexposed and UVB exposed melanocytes. Note the change in cathepsin B staining pattern from punctate in control cells to diffuse following UVB exposure. (C) Quantification of JNK siRNA transfected melanocytes with diffuse staining of cathepsin B 6 h after UVB irradiation (n=4, * $p<0.05$, ns; non-significant). A scrambled siRNA sequence was used as negative control for siRNA transfection. The symbols represent individual melanocyte donors and median values are marked with a horizontal line.

JNK is known to phosphorylate, and thereby activate, the pro-apoptotic protein Bim [13]. Indeed, in our cell system Bim was phosphorylated at serine 65 in the Bim_{EL} isoform in response to UVB irradiation (Fig. 3A). When the protein level of JNK was reduced by siRNA transfection, the amount of phosphorylated Bim was decreased, indicating that Bim activation is under the control of JNK. No change in total Bim protein expression was observed following UVB exposure (Fig. 3B). The pro-apoptotic action of Bim was confirmed by Bim protein suppression with siRNA, showing decreased frequency of apoptosis following UV exposure (Fig. 3C,D). Moreover, immunoprecipitation followed by Western blot analysis revealed that Bim interacts with the anti-apoptotic protein Mcl-1 in unirradiated control cells. However, following UVB exposure the interaction between the two proteins was reduced (Fig. 3E). The same results were achieved regardless if Mcl-1 or Bim was used as capturing antibody (Mcl-1 antibodies were used in experiments presented in Fig. 3E). In addition, double immunostaining of Bim and Mcl-1 in control melanocytes revealed similar staining pattern and analysis of merged confocal images indicated colocalization of the proteins (Fig. 3F). Following UVB irradiation, the Mcl-1 staining intensity decreased and colocalization of Bim and Mcl-1 was reduced. Both Bim and Mcl-1 protein levels were unaffected by JNK downregulation (not shown).

A marked depletion of the Mcl-1 protein was observed 2 h after UVB irradiation (Fig. 4A). In order to investigate the mechanism of the Mcl-1 decrease, we inhibited the proteasome activity with MG-132, caspases by using the broad-spectrum caspase inhibitor z-VAD, and aspartic cathepsins with the inhibitor Pepstatin A. We found that the Mcl-1 level was retained after UVB exposure in melanocytes treated with the proteasome inhibitor (Fig. 4B), and accordingly MG-132 treatment reduced the number of cells with fragmented nuclei following UV (Fig. 4C). Inhibition of caspases or cathepsins had no significant effect on Mcl-1 degradation (Fig. 4D). To explore the role of Mcl-1 in UVB-induced apoptosis,

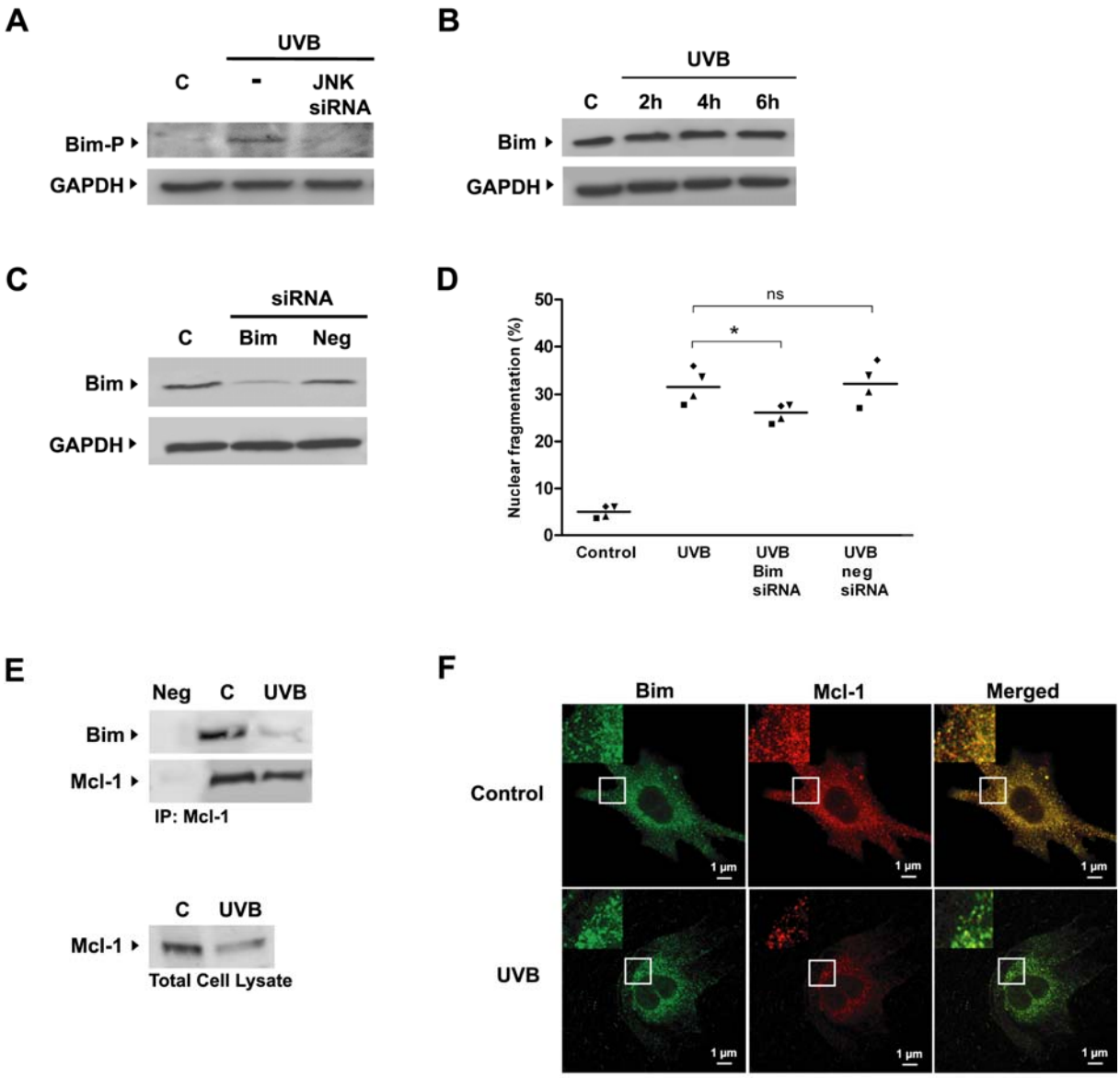

Fig. 3 Bivik & Öllinger

Figure 3. Bim interacts with Mcl-1 and becomes phosphorylated in a JNK-dependent manner. (A) Western blot analysis of phosphorylated Bim in the in response to UVB irradiation (500 mJ/cm^2) in JNK siRNA silenced melanocytes. One representative blot out of 3 is shown and GAPDH is used as internal control. (B) Western blot analysis of Bim expression 2-6 h after UVB irradiation. One representative blot out of 3 is shown and GAPDH is used as internal control. (C) Western blot analysis of Bim after siRNA transfection. GAPDH is used as internal control. (D) Frequency of apoptosis 6 h after UVB exposure in Bim siRNA transfected melanocytes, determined by microscopic inspection of nuclear morphology in DAPI stained cells (n=4). A scrambled siRNA sequence was used as negative control for siRNA transfection. (E) Immunoblot of coimmunoprecipitation experiments (6 h after UVB exposure) with Mcl-1 as capturing antibody demonstrating interaction with Bim. Negative control represents precipitation without antibodies. Mcl-1 expression is also shown in total cell lysate (F) Control and UVB exposed melanocytes were immunostained and examined by fluorescence confocal microscopy. Colocalization of Mcl-1 (red) and Bim (green) is presented in yellow in merged images.

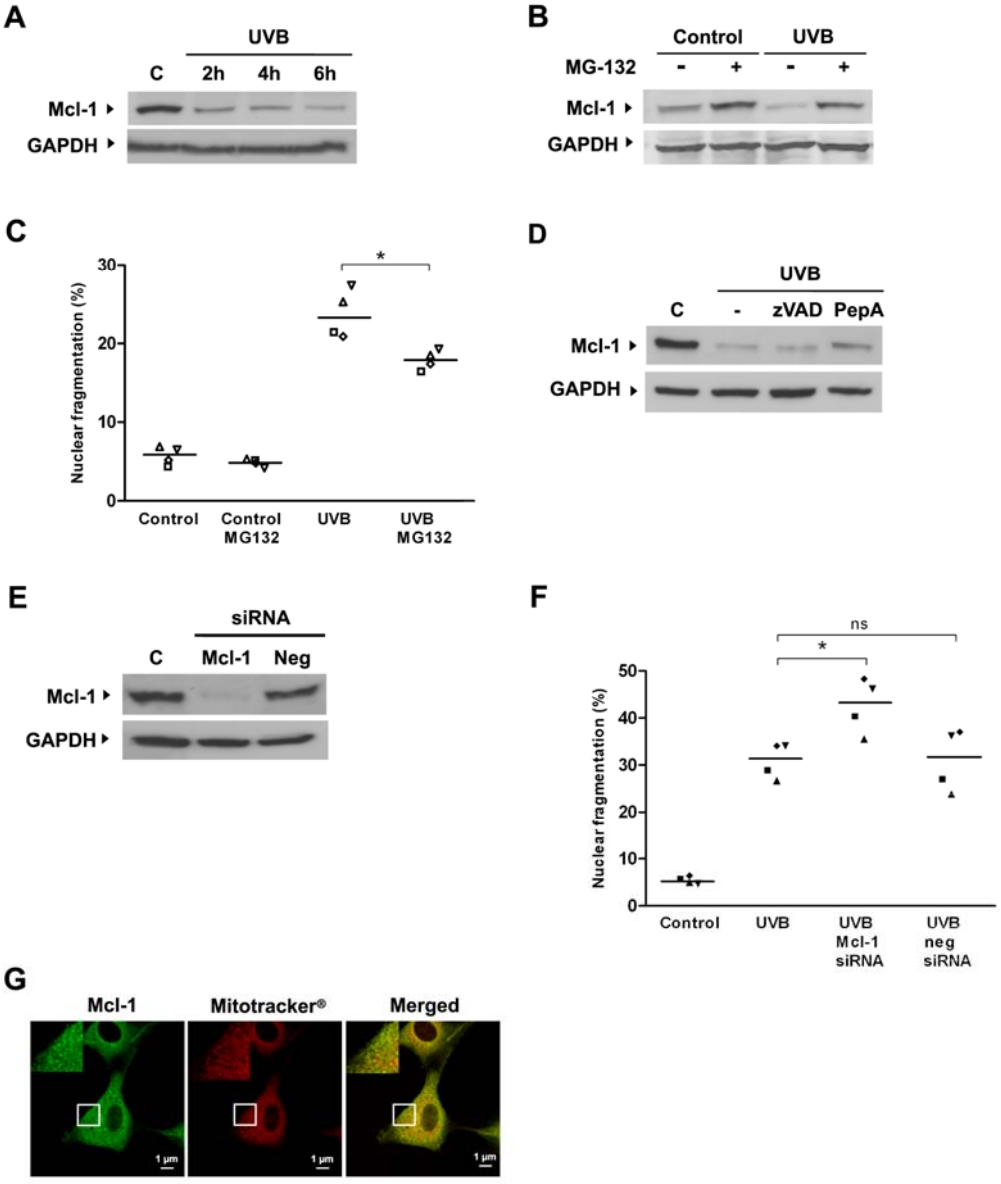

Fig. 4 Bivik & Öllinger

Figure 4. Mcl-1 has a pro-survival function and is depleted by proteasome degradation in UVB irradiated (500 mJ/cm^2) melanocytes. (A) Western blot analysis of Mcl-1 protein level after UVB irradiation. One representative blot out of 6 is presented and GAPDH is used as internal control. (B) Western blot analysis of Mcl-1 6 h upon UVB irradiation in melanocytes pretreated with or without the proteasome inhibitor MG-132. One representative blot out of 4 is presented and GAPDH is used as internal control. (C) Frequency of apoptosis, detected as fragmented nuclei, in UVB-exposed melanocytes pretreated with or without MG-132 (n=4 *, $p<0.05$). The symbols represent individual melanocyte donors and median values are marked with a horizontal line. (D) Western blot analysis of Mcl-1 expression 6 h after UVB exposure in melanocytes treated with or without the caspase inhibitor zVAD or the aspartic cathepsin inhibitor Pepstatin A. One representative blot out of 3 is shown and GAPDH is used as internal control. (E) Western blot analysis of Mcl-1 48 h after siRNA transfection. One representative blot out of 3 is shown and GAPDH is used as internal control. (F) Frequency of apoptosis 6 h after UVB exposure in Mcl-1 siRNA transfected melanocytes determined by microscopic inspection of nuclear morphology in DAPI stained cells (n=4 *, $p<0.05$, ns; non-significant). A scrambled siRNA sequence was used as a negative control for siRNA transfection. The symbols represent individual melanocyte donors and median values are marked with a horizontal line. (G) Mcl-1 localization in unexposed melanocytes determined by immunocytochemistry. Intracellular colocalization of Mcl-1 (green) and Mitotracker® (red) is shown in yellow in merged images.

melanocytes were transfected with Mcl-1 siRNA, which almost completely silenced the expression (Fig. 4E). Nuclear inspection of Mcl-1 siRNA transfected cells 6 h after UVB exposure showed that Mcl-1 has an anti-apoptotic function, since the number of cells with fragmented nuclei increased in these cultures as compared to non-transfected cells and cells transfected with negative control siRNA before irradiation (Fig. 4F). Downregulation of Mcl-1 alone did not induce any increase in apoptosis frequency (not shown). To further investigate the intracellular localization of Mcl-1, cells were double stained for Mcl-1 and either the lysosomal membrane specific protein LAMP-2, or the mitochondria by vital staining using Mitotracker® Red before Mcl-1 immunostaining. Confocal microscopy showed Mcl-1 staining in both cytosol and mitochondria in untreated control cells (Fig. 4G). No colocalization was detected between Mcl-1 and lysosomes (not shown). Parallel experiments were performed to determine the intracellular localization of Bim. We found similar distribution as for Mcl-1 i.e. co-staining of mitochondria but not lysosomes, and no change after UVB irradiation (not shown).

In a previous study, we have shown that Bax translocation from the cytosol to mitochondria is a prerequisite for apoptosis induction following UVB exposure [7]. Immunocytochemistry of Bax showed a diffuse cytosolic staining pattern and normal shaped nuclei in control cells, while in UVB irradiated cells, the Bax staining pattern changed into a punctate organelle-restricted pattern, which paralleled detection of fragmented nuclei. Figure 5A shows representative images of the appearance of Bax staining in an apoptotic and a non-apoptotic melanocyte. Mcl-1-silenced melanocytes displayed an increased Bax translocation in response to UVB irradiation (Fig. 5B). The subcellular localization of Bax was also explored in cells transfected with JNK siRNA, and as presented in Figure 5C, JNK silencing resulted in a

decreased number of cells exhibiting Bax translocation in response to UVB exposure. Thus, these results suggest that Bax translocation is stimulated by JNK and counteracted by Mcl-1.

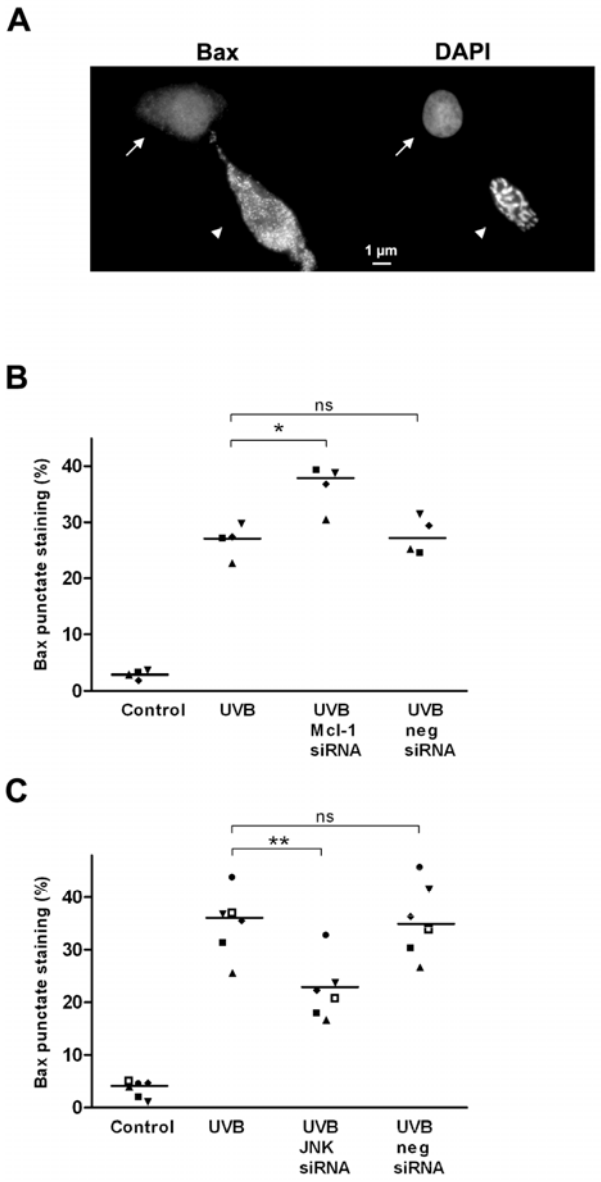

Fig. 5 Bivik & Öllinger

Figure 5. Effect of Mcl-1 and JNK on Bax translocation 6 h following UVB exposure (500 mJ/cm^2). (A) Bax localization was analyzed by immunocytochemistry and representative images of Bax and the corresponding DAPI-stained nuclei, in healthy (arrow) and apoptotic (arrowhead) cells are presented. Note redistribution of Bax from a diffuse cytosolic location in healthy cells to a punctate mitochondrial-like pattern in apoptotic cells with fragmented nuclei. (B) Quantification of Mcl-1 siRNA transfected melanocytes with punctate staining of Bax 6 h after UVB exposure (n=4). A scrambled siRNA sequence was used as a negative control for siRNA transfection. (C) Quantification of JNK siRNA transfected melanocytes with punctate staining of Bax 6 h after UVB exposure (n=6). The symbols represent individual melanocyte donors and median values are marked with a horizontal line. * $p<0.05$, ** $p<0.01$, ns; non-significant.

Discussion

The present study identifies JNK as an important pro-apoptotic factor during UVB-induced apoptosis in human melanocytes. UVB irradiation resulted in JNK activation through phosphorylation. In order to explore the function of JNK, melanocytes were transfected with JNK siRNA, suppressing both JNK1 and JNK2 expression. Such treatment revealed JNK to be essential for apoptosis signaling, upstream phosphorylation of Bim, induction of LMP, and translocation of Bax to mitochondria. These data are in agreement with previous studies, in which JNK was found to be required for cytochrome c release in mouse embryonic fibroblasts (MEFs) exposed to TNF and UV irradiation [6,14].

We have recently shown that cathepsins are released from lysosomes to the cytosol during UV-induced apoptosis in human melanocytes and that these proteases are involved in the intrinsic pathway by mediating pro-apoptotic effects upstream translocation of Bax to mitochondria [7]. Here, we present consistent data from Western blot analyses on cytosolic fractions (cathepsin D) and immunocytochemistry experiments (cathepsin B), demonstrating that reduced JNK expression prevents LMP. JNK-mediated LMP has also been shown to be involved in the extrinsic pathway. In a recent report Werneburg *et al.* showed that chemical inhibition of JNK significantly reduced cathepsin B release from lysosomes and prevented death receptor mediated cell death following TRAIL exposure in MZ-CHA-1 cells [15]. By comparing JNK1 and JNK2 deficient MEFs, Dietrich *et al*. suggested that TNF-α-induced LMP was regulated by JNK2 only [14]. In our model, selective siRNA knock-down of JNK1 or JNK2 reduced UVB-induced LMP independently, even though depletion of both proteins showed higher efficiency. This suggests that the role of different JNK isoforms is stimulus- and/or cell type-specific. However, the phosphorylation target for JNK-dependent regulation of LMP remains unidentified. Interestingly, a recent report demonstrates that Bim is translocated to lysosomes in a JNK-dependent manner during TRAIL-induced apoptosis

[15]. However, we found no colocalization of Bim and lysosomes in human melanocytes after UVB irradiation.

Bim is normally sequestered in the cytoskeleton, and is detached and activated upon phosphorylation [13,16]. By immunocytochemistry and immunoprecipitation we identified colocalization of Bim and the anti-apoptotic protein Mcl-1 in the melanocytes, and the interaction decreased following UVB exposure. Furthermore, the experiments suggest JNK to trigger phosphorylation of Bim, since JNK-depleted melanocytes showed reduced Bim phosphorylation. Previously, Bim phosphorylation by Erk 1/2 was found to promote proteasome-dependent degradation of the Bim protein [17,18]. On the other hand, JNK has been reported to be involved in transcriptional upregulation of Bim in neurons [19]. However, in melanocytes, Bim protein level was unchanged up to 6 h after UV exposure. Instead, a prominent reduction of the Mcl-1 protein level was detected in UV-irradiated cells. Previous studies have shown that Mcl-1 depletion is due to degradation by caspase-3, granzyme B, or the proteasome [20-22]. We found that the Mcl-1 level was retained by inhibition of the proteasome before UV exposure. In addition, proteasome inhibition resulted in a decreased apoptotic frequency after UV exposure compared to UV-only treatment.

Mcl-1 has been reported to counteract apoptosis by binding pro-apoptotic Bcl-2 members, including Bim, Bid, and Bak [20,23,24]. We here confirm interaction between Mcl-1 and Bim in melanocytes. We propose that Bim is phosphorylated by a JNK-mediated mechanism, which activates and releases it from Mcl-1, allowing its pro-apoptotic effect. It has earlier been reported that some BH3-only proteins, such as Bid and Bim, directly activate Bax [25]. Here, we present data showing that Bax translocation is also caused by JNK-dependent induction of LMP. Cathepsins released to the cytosol have been shown to cleave and activate Bid, which in turn activates Bax [26-28]. Thus, pro-apoptotic signaling by cathepsin release and Bim liberation converge by Bax translocation, resulting in

mitochondrial membrane permeabilization. Interestingly, a recent study, performed in human embryonic kidney cells, proposes another model of interactions between Bcl-2 family proteins, in which BH3-only proteins, once activated, would bind and inactivate the pro-survival proteins Mcl-1 and Bcl-X_L, resulting in indirect activation of Bax/Bak [29]. Thus, the action of BH3-only proteins is still unclear and the direct and indirect models for Bax/Bak activation might reflect differences between cell types and/or stress stimuli.

Melanocytes are generally considered relatively resistant to apoptosis [30]. Previously, we have reported that melanocytes have a high basal level of the Bcl-2 protein, which is unaltered by UVB exposure [31]. Here we found that Mcl-1 was degraded following UVB, which might provide an opportunity for pro-apoptotic proteins to exert their action. Although Bim had a minor but significant pro-apoptotic effect in melanocytes, additional apoptosis signaling by BH3-only proteins must occur for induction of Bax translocation and melanocyte death. The present and previous [7] results obtained after UVB irradiation suggest the redistribution of Bax to mitochondria to be the apoptosis determinant event.

Our results, summarized in Figure 6, suggest that UVB activates JNK, which mediates LMP and phosphorylation of Bim. These events might be followed by cathepsin-mediated activation of Bid [26,27] and proteasome degradation of Mcl-1 that both facilitate translocation of Bax to mitochondria and release of cytochrome c.

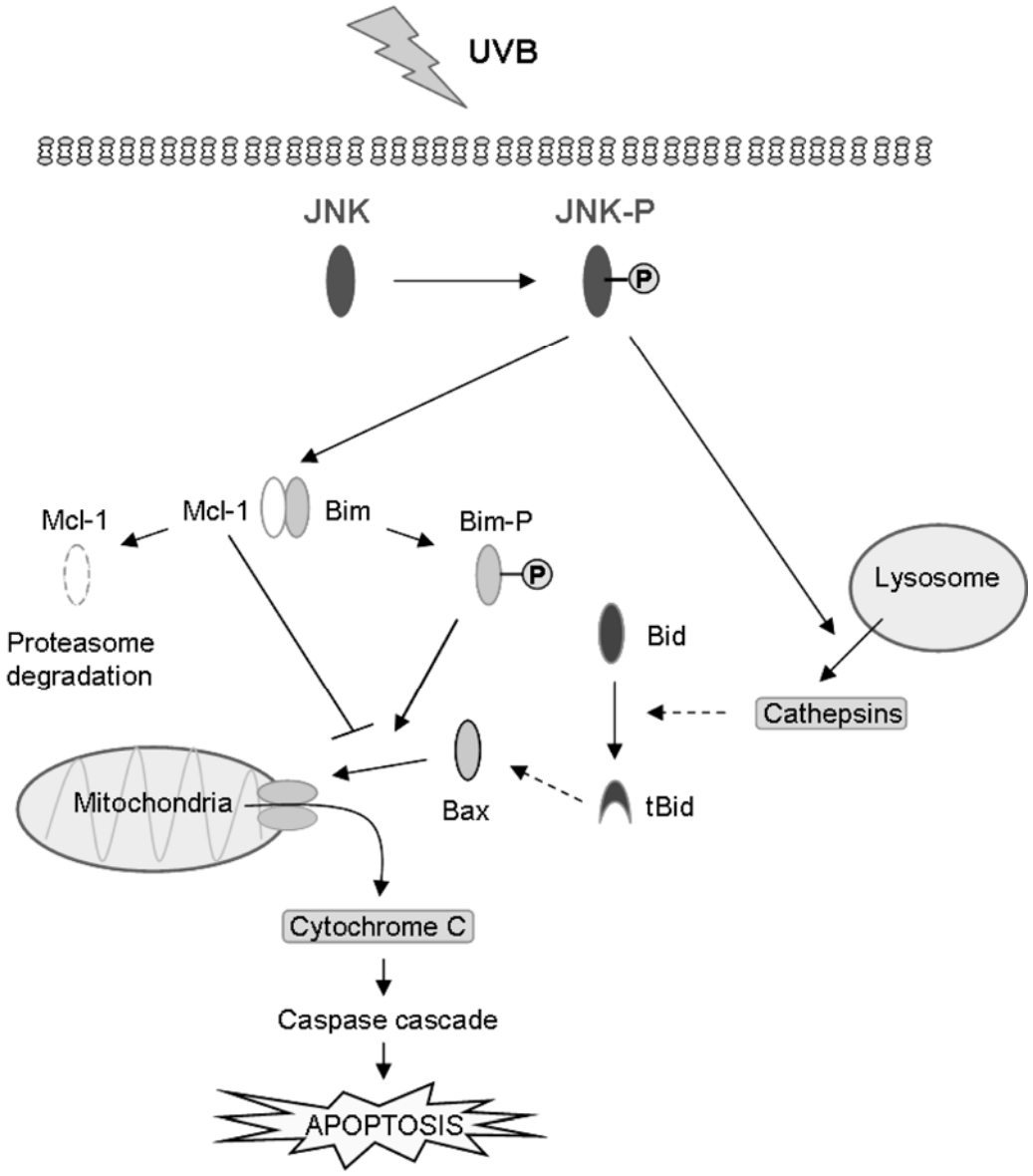

Fig. 6 Bivik & Öllinger

Figure 6. Proposed model for JNK regulation of UVB-induced apoptosis. Our results suggest that JNK triggers apoptosis by induction of lysosomal membrane permeabilization with release of cathepsins to the cytosol, and by phosphorylation of the BH3-only protein Bim. Cathepsins have been shown to cleave and activate Bid [26,27], which in turn activates Bax. Furthermore, JNK mediates phosphorylation of Bim, which normally is sequestered and kept inactive by Mcl-1. UV irradiation causes proteasome degradation of Mcl-1, and active Bim is then free to promote apoptosis. Thus, both cathepsin release and activation of Bim converge by triggering of Bax translocation, resulting in mitochondrial membrane permeabilization [7] and apoptosis.

References

1. Lucken-Ardjomande S, Martinou JC. Regulation of Bcl-2 proteins and of the permeability of the outer mitochondrial membrane. C R Biol 2005;328:616-631.
2. Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ 2002;9:505-512.
3. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol 2005;17:617-625.
4. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000;103:239-252.
5. Potapova O, Gorospe M, Bost F, Dean NM, Gaarde WA, Mercola D, Holbrook NJ. c-Jun N-terminal kinase is essential for growth of human T98G glioblastoma cells. J Biol Chem 2000;275:24767-24775.
6. Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science 2000;288:870-874.
7. Bivik CA, Larsson PK, Kågedal KM, Rosdahl IK, Öllinger KM. UVA/B-induced apoptosis in human melanocytes involves translocation of cathepsins and Bcl-2 family members. J Invest Dermatol 2006;126:1119-1127.
8. Andersson E, Vahlquist A, Rosdahl I. Beta-carotene uptake and bioconversion to retinol differ between human melanocytes and keratinocytes. Nutr Cancer 2001;39:300-306.
9. Gilchrest BA, Vrabel MA, Flynn E, Szabo G. Selective cultivation of human melanocytes from newborn and adult epidermis. J Invest Dermatol 1984;83:370-376.
10. Leaback DH, Walker PG. Studies on glucosaminidase. 4. The fluorimetric assay of N-acetyl-beta-glucosaminidase. Biochem J 1961;78:151-156.

11. Vanderlinde RE. Measurement of total lactate dehydrogenase activity. Ann Clin Lab Sci 1985;15:13-31.
12. Brunk UT, Dalen H, Roberg K, Hellquist HB. Photo-oxidative disruption of lysosomal membranes causes apoptosis of cultured human fibroblasts. Free Radic Biol Med 1997;23:616-626.
13. Lei K, Davis RJ. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. Proc Natl Acad Sci U S A 2003;100:2432-2437.
14. Dietrich N, Thastrup J, Holmberg C, Gyrd-Hansen M, Fehrenbacher N, Lademann U, Lerdrup M, Herdegen T, Jäättelä M, Kallunki T. JNK2 mediates TNF-induced cell death in mouse embryonic fibroblasts via regulation of both caspase and cathepsin protease pathways. Cell Death Differ 2004;11:301-313.
15. Werneburg NW, Guicciardi ME, Bronk SF, Kaufmann SH, Gores GJ. Tumor necrosis factor-related apoptosis-inducing ligand activates a lysosomal pathway of apoptosis that is regulated by Bcl-2 proteins. J Biol Chem 2007;282:28960-28970.
16. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell 1999;3:287-296.
17. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. J Biol Chem 2003;278:18811-18816.
18. Luciano F, Jacquel A, Colosetti P, Herrant M, Cagnol S, Pages G, Auberger P. Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. Oncogene 2003;22:6785-6793.

19. Harris CA, Johnson EM, Jr. BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. J Biol Chem 2001;276:37754-37760.
20. Han J, Goldstein LA, Gastman BR, Froelich CJ, Yin XM, Rabinowich H. Degradation of Mcl-1 by granzyme B: implications for Bim-mediated mitochondrial apoptotic events. J Biol Chem 2004;279:22020-22029.
21. Weng C, Li Y, Xu D, Shi Y, Tang H. Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells. J Biol Chem 2005;280:10491-10500.
22. Nijhawan D, Fang M, Traer E, Zhong Q, Gao W, Du F, Wang X. Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev 2003;17:1475-1486.
23. Clohessy JG, Zhuang J, de Boer J, Gil-Gomez G, Brady HJ. Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis. J Biol Chem 2006;281:5750-5759.
24. Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, Adams JM, Huang DC. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 2005;19:1294-1305.
25. Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, Newmeyer DD. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol Cell 2005;17:525-535.
26. Cirman T, Oresic K, Mazovec GD, Turk V, Reed JC, Myers RM, Salvesen GS, Turk B. Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. J Biol Chem 2004;279:3578-3587.

27. Heinrich M, Neumeyer J, Jakob M, Hallas C, Tchikov V, Winoto-Morbach S, Wickel M, Schneider-Brachert W, Trauzold A, Hethke A, Schutze S. Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. Cell Death Differ 2004;11:550-563.
28. Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. Cell Death Differ 2000;7:1166-1173.
29. Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE, Ierino H, Lee EF, Fairlie WD, Bouillet P, Strasser A, Kluck RM, Adams JM, Huang DC. Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science 2007;315:856-859.
30. Gilchrest BA, Eller MS, Geller AC, Yaar M. The pathogenesis of melanoma induced by ultraviolet radiation. N Engl J Med 1999;340:1341-1348.
31. Bivik CA, Andersson EB, Rosdahl IK. Wavelength-specific effects on UVB-induced apoptosis in melanocytes. A study of Bcl-2/Bax expression and keratinocyte rescue effects. Melanoma Res 2005;15:7-13.