

Expanded View Figures

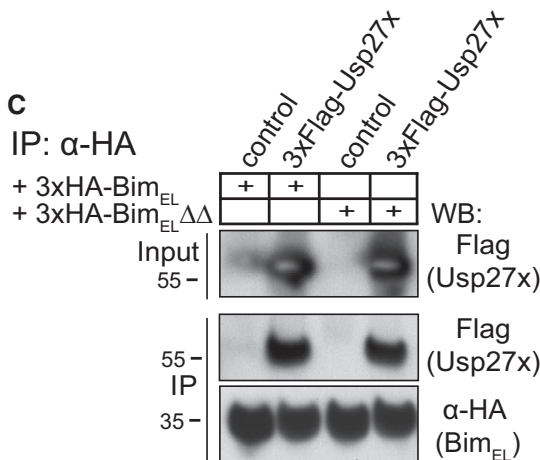
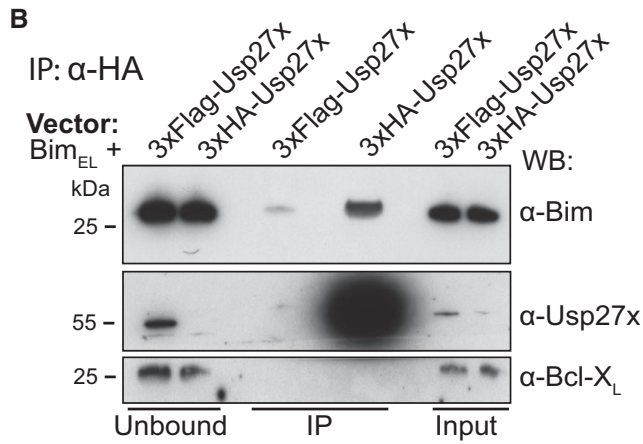
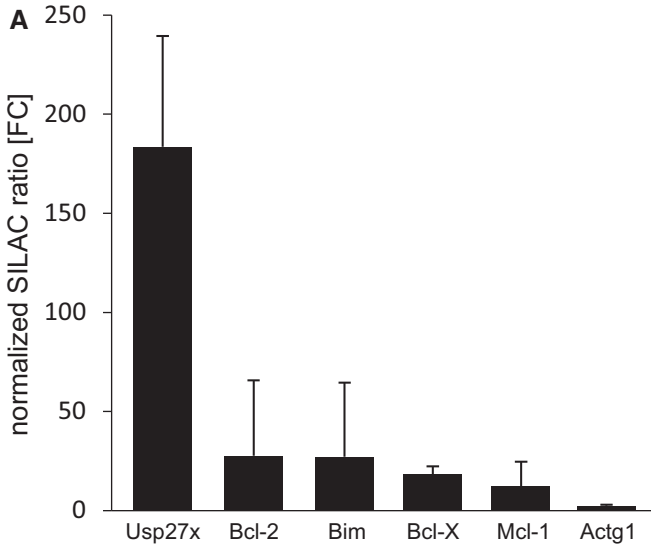


Figure EV1. Interaction of the Usp27x with Bim_{EL}.

A Profile of enriched proteins in anti-3xHA-Bim_{EL} immunoprecipitations of purified mitochondria from mouse embryonic fibroblasts (MEF Bax/Bak-double knockout cells). Cells expressing 3xHA-Bim_{EL} were labelled with light amino acids, whereas control cells (expressing only untagged Bim_{EL}) were labelled with heavy amino acids. Gamma actin (Actg1) served as a specificity control. Protein ratios are calculated from the respective peptide ratios. A minimum ratio count of two is needed to calculate a protein ratio. Error bars: protein quantification variability. Error bars indicate the coefficient of variability over all redundant quantifiable peptide signals. It is calculated as standard deviation of the fold enrichment of all peptide ratios (see also Table EV1). Identification of Usp27x as an interacting protein of Bim_{EL} was identified alongside the SILAC experiment published earlier. Enrichment of Bim and anti-apoptotic proteins already appear in the earlier report [25].

B 293FT cells were transfected with expression constructs for untagged Bim_{EL} together with 3xFlag-Usp27x or 3xHA-Usp27x (all pMIG-vector backbone). After cell lysis (1% Triton X-100) and subsequent anti-HA immunoprecipitation, the unbound fraction, input (50 μg each) and the IP-eluate (IP, ~60%) were run on SDS-PAGE. Flag- or HA-tagged Usp27x were detected using an antibody directed against Usp27x (Abgent). See also Fig 1A. Similar results were seen in *n* = 3 experiments.

C Usp27x binds a mutant of Bim incapable of binding to anti-apoptotic Bcl-2 proteins. 293FT cells transfected with constructs encoding 3xFLAG-Usp27x and 3xHA-tagged Bim_{EL} (Fig 1B) or 3xHA-tagged Bim_{EL}ΔΔ (a mutant with two mutations in the BH3 domain, incapable of binding anti-apoptotic Bcl-2 proteins [50]) were immunoprecipitated from whole-cell extracts using anti-HA resin. Bim and Usp27x were detected with anti-HA and anti-FLAG antibodies as indicated. Control, pEGFP-N1 vector. The caspase inhibitor Q-VD-OPH (QVD) was added to the cultures to inhibit Bim-induced apoptosis (see also Fig 1). Similar results were seen in *n* = 3 experiments.

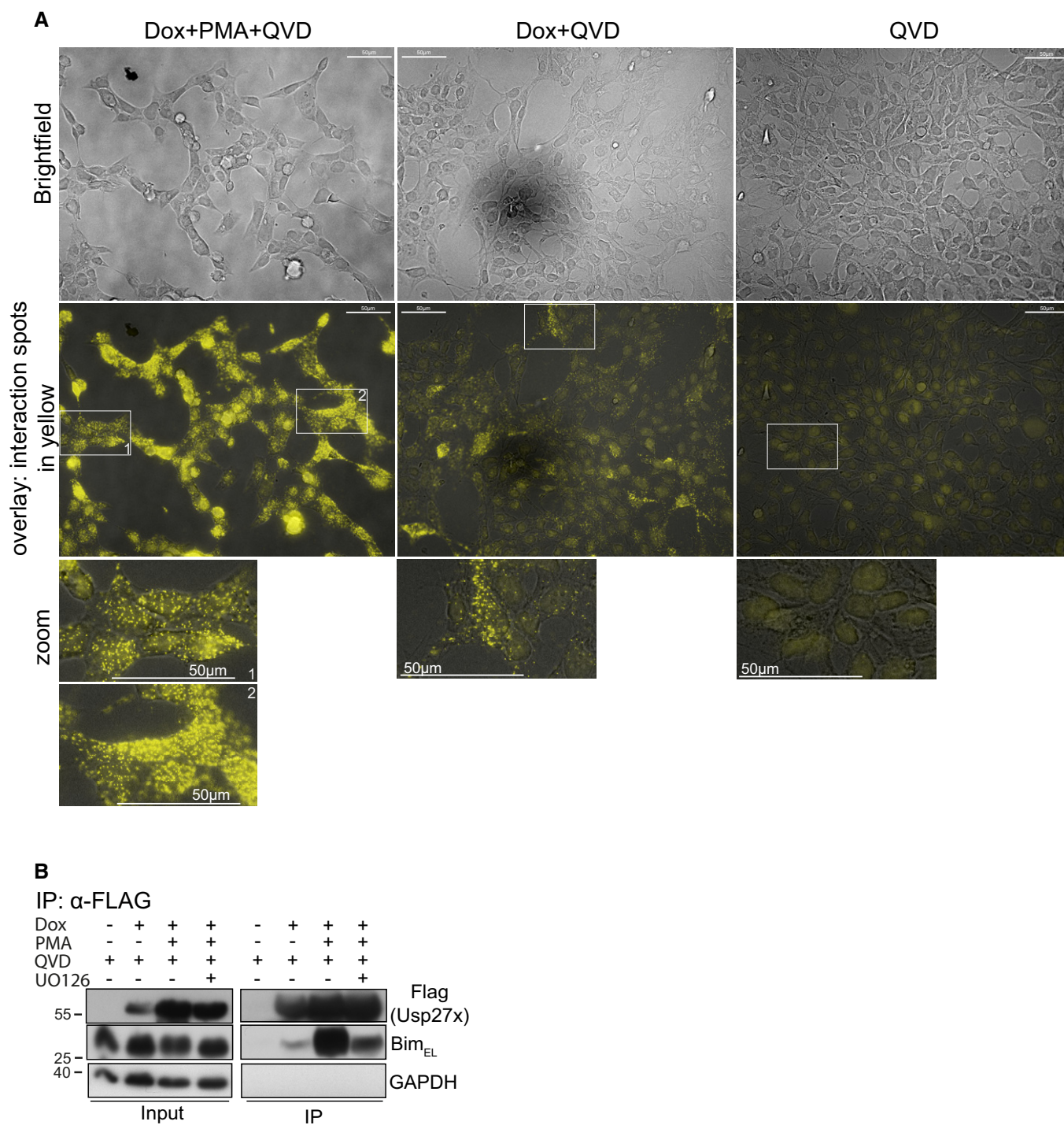


Figure EV2. Usp27x is in close proximity to Bim.

A *In situ* identification of complexes between Usp27x and Bim_{EL} molecules by proximity-ligation assay (PLA). 293FT cells carrying dox-inducible 3xFlag-Usp27x (293FT-3xFlag-Usp27x) were stimulated as indicated for 24 h, fixed and permeabilized. Yellow dots show interaction spots between endogenous Bim and inducible 3xFlag-Usp27x. For dox+PMA+QVD, two different zoom regions (1, 2) are shown from the overlay. Data are representative of $n = 2$ independent experiments. Scale bar, 50 μ m.

B Immunoprecipitation of 3xFlagUsp27x. The same cell line and conditions as in (A) were used. In one sample, UO126 was added 30 min before stimulation. Twenty-four hours after stimulation, cells were lysed and 3xFlag-Usp27x was immunoprecipitated using anti-Flag-resin. Detection of Usp27x was done using anti-Flag antibody, and Bim was detected using a Bim-specific antibody. GAPDH served as a loading control. Data are representative of $n = 4$ independent experiments.

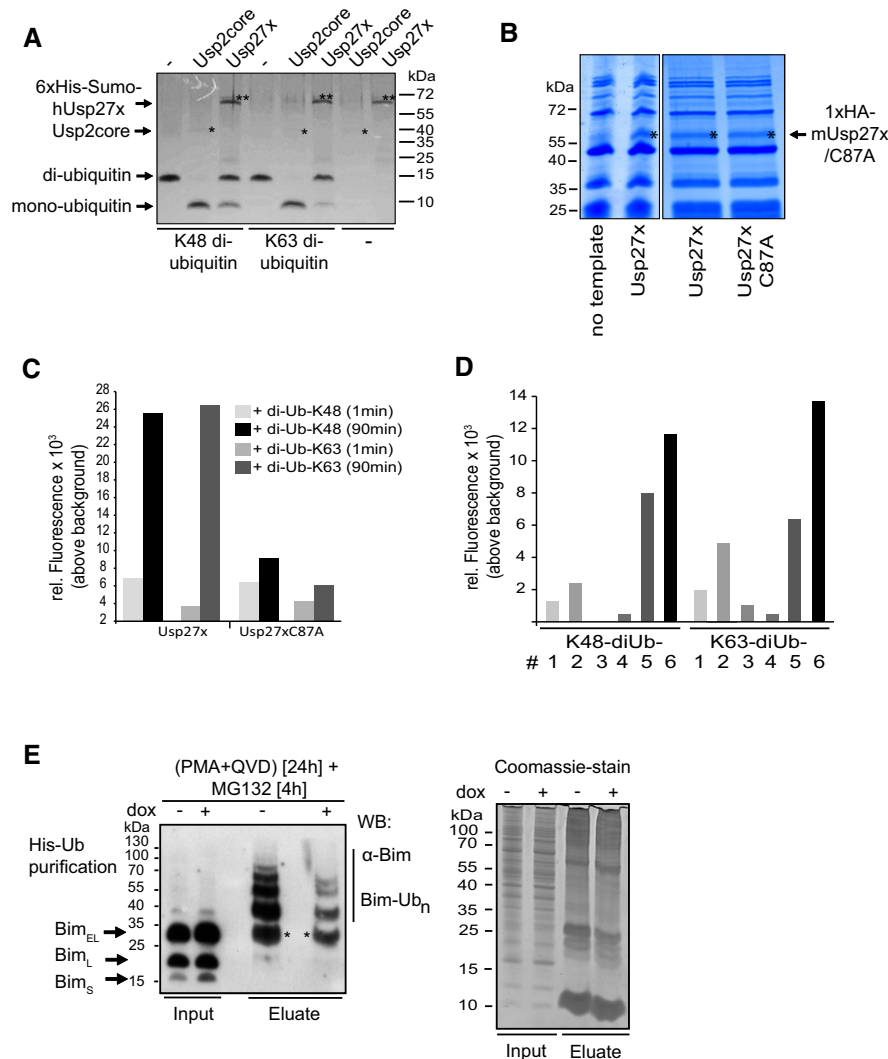


Figure EV3. Usp27x cleaves di-ubiquitin molecules.

- A** Human 6xHis-Sumo-Usp27x was purified from *E. coli*. Differently linked di-ubiquitin molecules (K48-, K63-linked di-ubiquitin; 1 μ g each) were incubated for 1 h at 37°C with 0.1 μ M Usp2core (* positive control, ~41 kDa) or 2.9 μ M of the affinity-purified 6xHis-Sumo-hUsp27x (**, ~63 kDa). Usp27x was able to cleave K48- and K63-linked di-ubiquitin molecules into mono-ubiquitin as visualized by Coomassie Blue staining. Similar results were observed in $n = 2$ separate experiments.
- B** Coomassie-stained gels of soluble fractions (4 μ l) from a PureExpress preparation without a template DNA (showing only proteins of the *in vitro* PureExpress translational *E. coli* system) or of *in vitro*-translated 1xHA-Usp27x (left gel) or the products of translation of 1xHA-Usp27x and its catalytically inactive mutant 1xHA-Usp27xC87A (right gel; Usp27x bands are marked with an asterisk). Reactions were used for (C). $n = 2$.
- C** Usp27x or Usp27xC87A were *in vitro*-translated with the PureExpress system (see B). Four microlitres of soluble fractions of the reactions were directly used for assays of proteolytic activity. As substrates, K48-linked IQF-di-UbK48-6 or K63-linked IQF-di-UbK63-6 (200 nM each) (the 6 in K48-6 and K63-6 refers to the substrate variant where the quencher and TAMRA dye is linked at specific positions; these substrates were selected based on the preliminary experiment with the label at various positions (see Fig EV3D)) were added, and reactions were incubated for 1–90 min at 23°C. As readout for cleavage of K48/K63-linked di-ubiquitin, the relative fluorescence generated by dequenching was measured. Bars indicate mean values of duplicate measurements in one experiment.
- D** Four microlitres of soluble fractions (from the reactions shown in A) was directly used for the activity assay. Six different K48-linked IQF-diUb substrates and six K63-linked IQF-diUb substrates (substrates #1–6; 200 nM each) were incubated with 1xHA-Usp27x for 60 min at 23°C. As readout for cleavage of K48/K63-linked di-ubiquitin, the relative fluorescence was measured using a Tecan M200 fluorescence reader. IQF-di-UbK48-6 and IQF-di-UbK63-6 substrates showed the best performance and were further used for activity assays (see Fig EV3C). Bars indicate mean values of an experiment measured in duplicate.
- E** Usp27x expression reduces ubiquitination of Bim. 293FT-TetR-3xFlagUsp27x cells were transfected with a vector coding for 6His-ubiquitin. After 24 h, cells were treated with PMA and QVD, and 3xFlag-Usp27x was induced by dox at the same time. After additional 20 h, cells were treated for 4 h with MG132 (40 μ M) to block proteasomal degradation of ubiquitinated proteins. Cells were lysed under denaturing conditions, and His-ubiquitin-labelled proteins were purified by Ni²⁺-NTA affinity chromatography. Ubiquitinated Bim (Bim-Ub_n) was detected by Western blot using anti-Bim antibodies (left). Asterisk (*) indicates non-His-ubiquitinated Bim that binds unspecifically to the Ni²⁺-agarose beads (see Fig 2E). Equal loading was verified by a Coomassie-stained SDS-PAGE from same protein extracts (right). Similar results were obtained in $n = 3$ separate experiments.

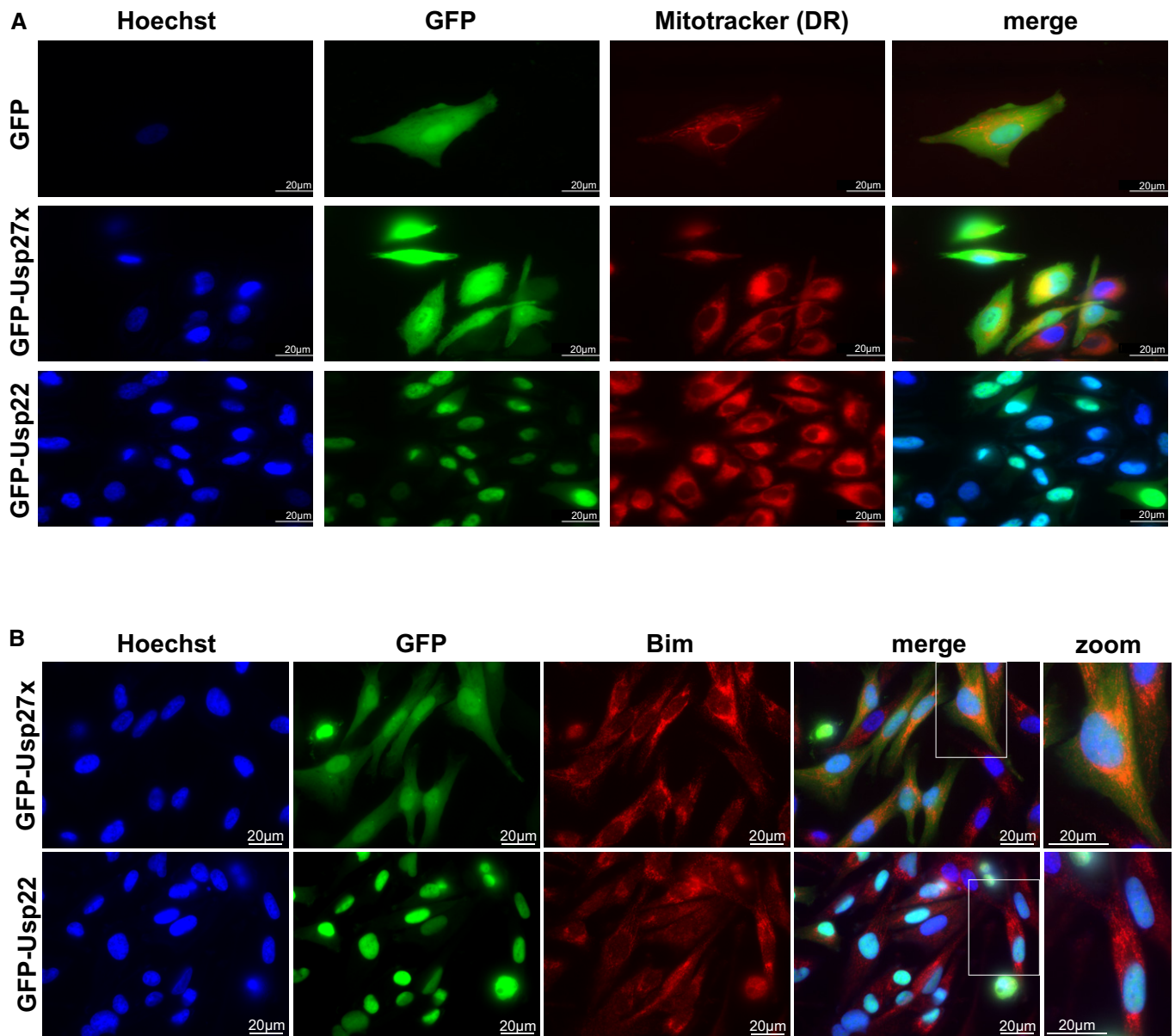


Figure EV4. Subcellular localization of Usp27x and Usp22 as analysed by fluorescence microscopy.

A 1205Lu melanoma cells carrying dox-inducible GFP, GFP-Usp27x or GFP-Usp22 were treated for 24 h with dox. Subcellular localization of GFP or GFP-fusion proteins (green) was visualized by microscopy. Nuclei were stained with Hoechst (blue), and mitochondria were identified by Mitotracker DeepRed FM staining (red). Scale bar, 20 μ m. $n = 2$. In the merged image for GFP-Usp22, the Mitotracker signal is not shown. GFP-Usp27x showed very similar localization in HCC827-GFP-Usp27x cells (not shown).

B 1205Lu-GFP-Usp27x and 1205Lu-GFP-Usp22 were treated for 48 h with dox. Subcellular localization of GFP-fusion proteins (green) was visualized by microscopy. Nuclei were stained with Hoechst (blue), and Bim was detected by intracellular staining for endogenous Bim (red). A higher magnification of merged figures is shown to the right (zoom). Scale bar, 20 μ m. $n = 2$. Similar results were seen in every visual field analysed.

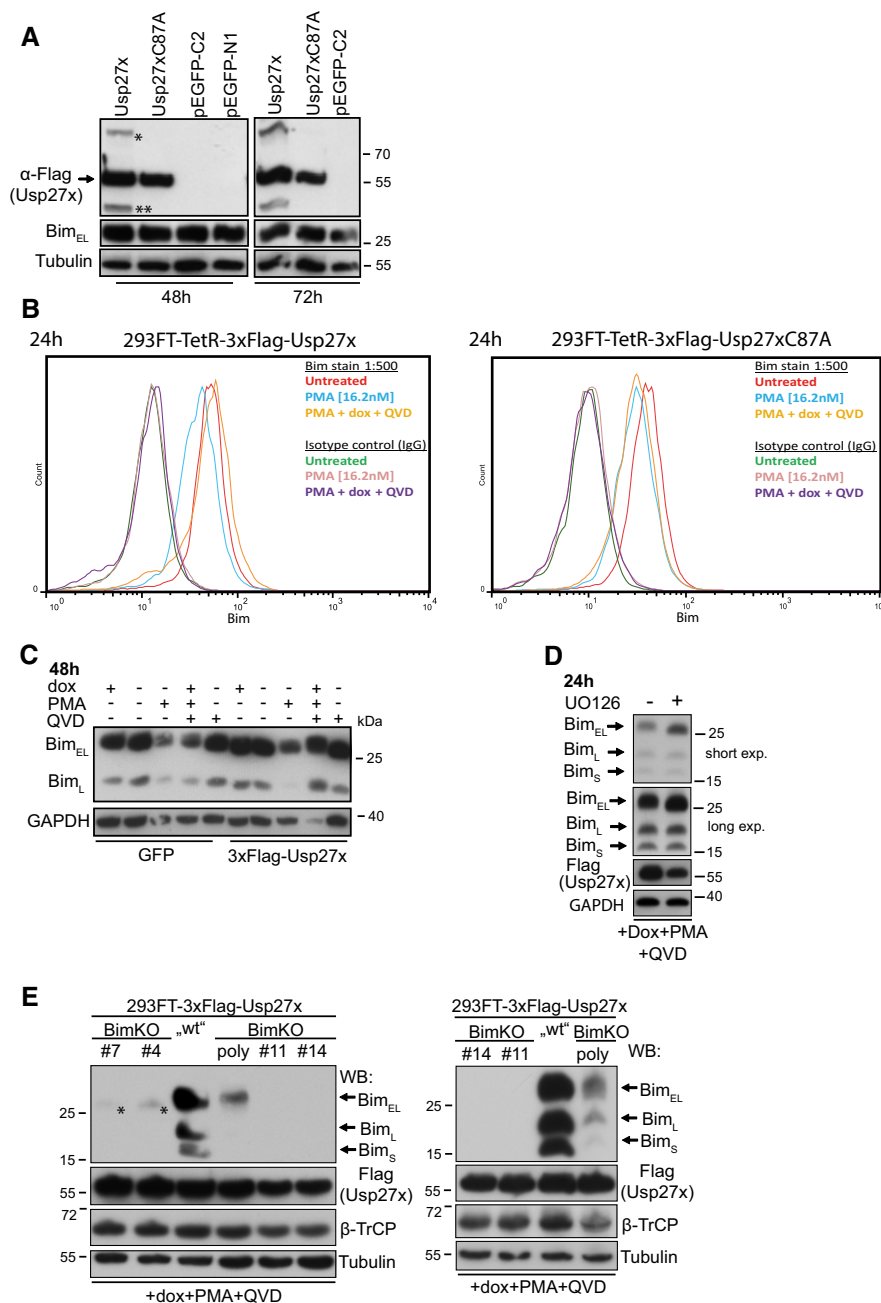


Figure EV5. Usp27x inhibits PMA-induced degradation of Bim.

- A** Expression of Usp27x does not increase Bim levels in the absence of PMA. 293FT cells were transfected with pCMV-7.1-3xFlag-Usp27x, pCMV-7.1-3xFlag-Usp27xC87A or with two different EGFP vectors (pEGFP-C2, pEGFP-N1). Forty-eight (left) or 72 h (right) later, cells were collected and lysed and the protein level of Bim_{EL} was analysed by immunoblotting. Asterisk (*) and double asterisk (**) represent Usp27x bands of unknown origin (compare Fig 2A). Similar results were obtained in $n = 2$ separate experiments.
- B** 293FT cells carrying dox-inducible 3xFlag-Usp27x (left) or 3xFlag-Usp27xC87A (right) were untreated or treated for 24 h as indicated. Cells were fixed, stained with anti-Bim antibodies or isotype control and secondary antibodies and analysed by flow cytometry. See also Fig 2A and B. $n = 2$.
- C** 293FT cells with dox-inducible GFP or 3xFlag-Usp27x were treated as indicated for 48 h prior to cell lysis. Bim was detected by Western blotting. Similar results were obtained in $n = 3$ separate experiments.
- D** The MEK inhibitor UO126 blocks phosphorylation of Bim_{EL}. 293FT-TetR-3xFlag-Usp27x were treated for 24 h with dox in the presence of PMA and QVD. As control, the same cells were additionally pre-treated with UO126 (10 μ M) for 30 min before stimulation. Shown are the immunoblots of Bim, Flag-Usp27x and as a loading control GAPDH. Similar results were obtained in $n = 3$ separate experiments (see also Fig 2B and D).
- E** Expression analysis of Bim and Flag-Usp27x in 293FT-3xFlag-Usp27x cells versus Bim-knockout cell lines. 293FT-TetR-3xFlag-Usp27x, one polyclonal Bim-knockout (Bim2KO) cell line established from 293FT-3xFlag-Usp27x cells using CRISPR/Cas9 and four additional single clones (#4, #7, #11, #14) obtained by serial dilution from the polyclonal Bim2KO cell line were treated for 24 h with dox plus PMA (16.2 nM) and QVD (10 μ M). Expression of Bim, Usp27x (Flag) and β -TrCP was analysed by immunoblotting. For apoptosis measurements, see Fig 3. Asterisk (*) indicates a signal obtained with the Bim antibody (recognizes the N-terminus of Bim (around proline 25)) only present in clones #4 and #7. This band runs slightly lower than full-length Bim_{EL}. Right immunoblot shows a second experiment ($n = 2$). Of note, the relative expression levels for Bim between the cell lines were the same in non-stimulated cells (data not shown).

Figure EV6. Usp27x stabilizes Bim.

- A Overexpression of Usp27x but not Usp22 stabilized endogenous Bim_{EL} in 1205Lu melanoma cells. 1205Lu cells carrying inducible constructs were treated with dox to induce GFP-Usp27x, GFP-Usp27xC87A or 3xFlag-tagged Usp22 for 48 h. Bim was detected by Western blotting. Similar results were obtained in *n* = 2 separate experiments.
- B 1205Lu cells with inducible constructs were treated as indicated to induce GFP or GFP-Usp27x. The MEK inhibitor UO126 was used to block ERK activity. Of note, Bim_{EL} protein shifted to a lower molecular size when cells were treated with UO126, presumably as an effect of dephosphorylation. Similar results were obtained in *n* = 2 separate experiments.
- C Caco2 colon carcinoma cells carrying dox-inducible HA-BRAF-V600E were treated as indicated. ERK phosphorylation was measured by Western blotting. Note the loss of Bim protein upon induction of HA-BRAF-V600E (*n* = 2).
- D 3xFlagUsp27x inhibits Bim loss upon BRAF-V600E induction. Maternal Caco2-HA-BRAF V600E cells or the same cells stably expressing constitutive 3xFlag-Usp27x were treated with dox for 3 (left) or 96 h (right) to induce BRAF-V600E (two separate experiments). Bim levels were measured by Western blotting. For each condition, Bim_{EL} levels were quantified from the shown immunoblot and normalized to the tubulin signal. Per cent Bim_{EL} gives the expression relative to the starting point set to 100%. Similar results were obtained in *n* = 3 separate experiments with varying induction times. See also Fig 4D.
- E Validation of Usp27x-specific siRNAs. 293FT cells were transfected with siRNAs targeting human Usp27x or with control siRNA. Knockdown of Usp27x was either tested by using one single siRNA against Usp27x (#861) or by using a combination of three siRNAs targeting Usp27x (#495, #498, #861). After 24 h, cells were transfected with a construct driving expression of human Usp27x and expression of GFP off an IRES. Usp27x and GFP were detected 24 h later by Western blotting (*n* = 2). The combination of three siRNAs was used for the later knockdown experiments (see Fig 5A).
- F Comparison of Bim expression levels between 293FT and 1205Lu. Western blot of whole-cell lysates obtained from 293FT cells and from 1205Lu melanoma cells. For both cell lines, 50 μg protein was run by SDS-PAGE, and Bim was identified using antibody against Bim. Tubulin served as a loading control. Similar results were obtained in *n* = 2 separate experiments.
- G Validation of 293FT-Usp27xKO (clone 2/10). Sequencing results (*n* = 1) indicate a thymidine (T) insertion a nucleotide (nt) 391 (arrow). This insertion leads to the addition of 14 different amino acids after aa 130 followed by a stop codon. Therefore, this clone expresses a truncated version of Usp27x (wt has 438 aa) deleting part of the catalytic activity/triad (involves aa C87A (nucleophile), and the predicted sites H380 (proton acceptor) and aa D396/D397 in Usp27x [28]).

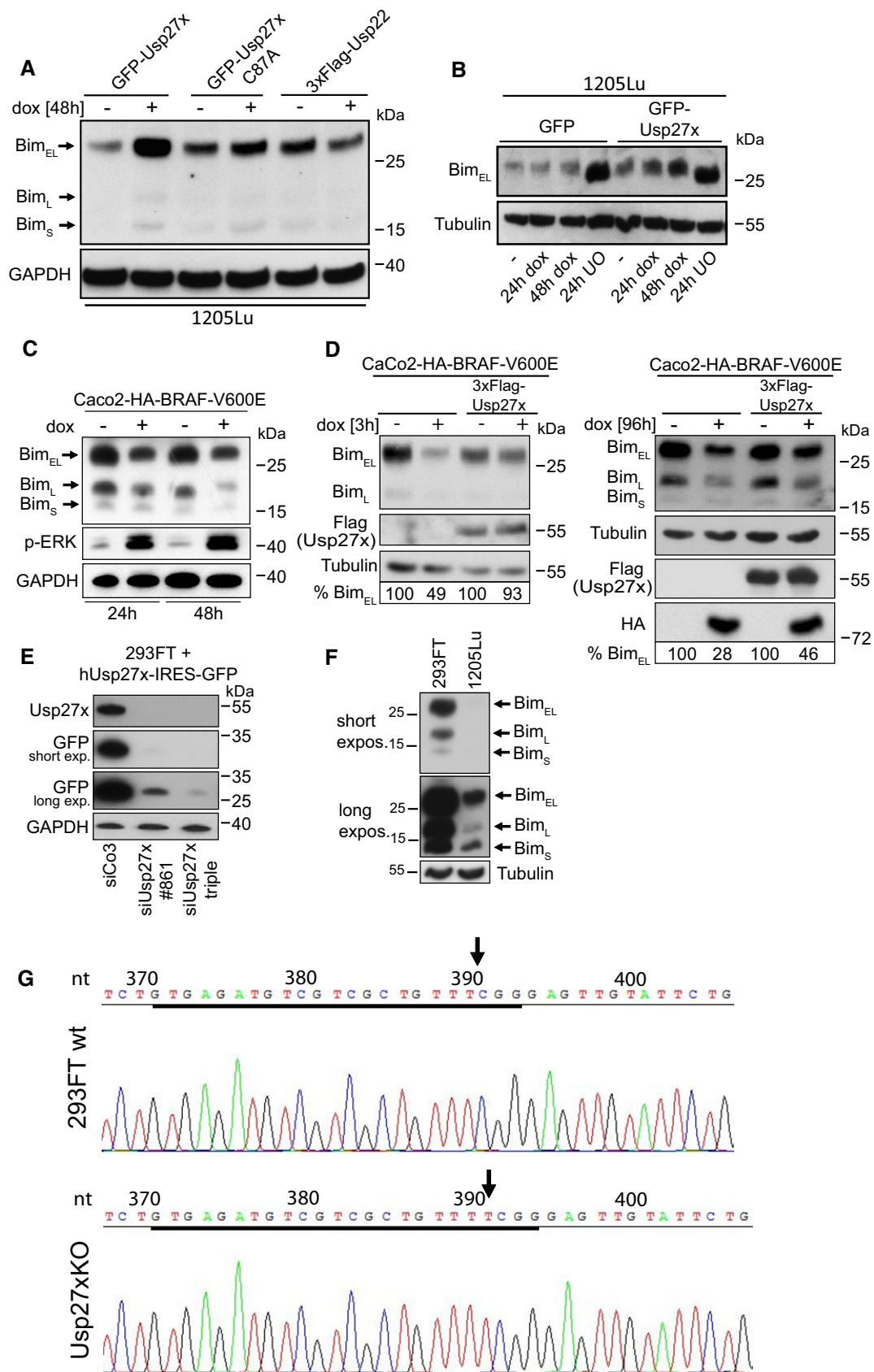


Figure EV6.

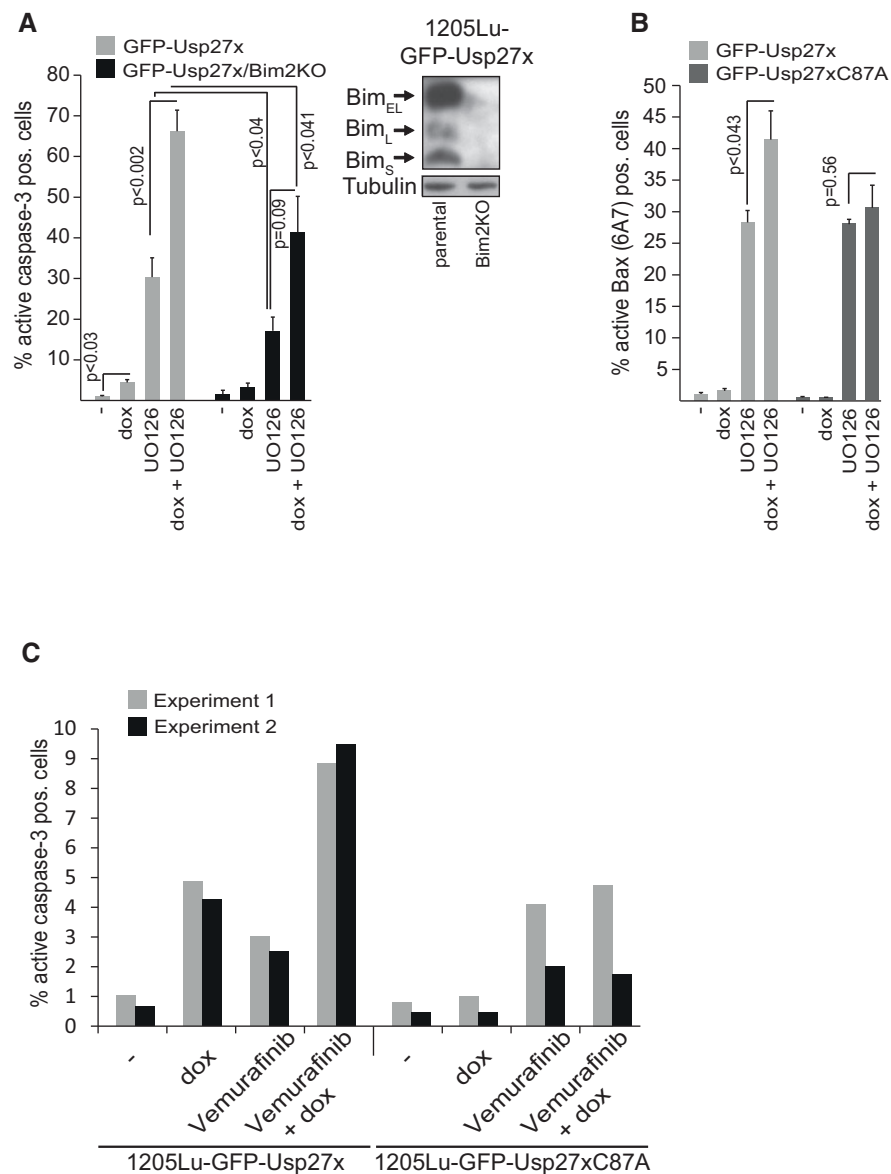


Figure EV7. Usp27x sensitizes 1205Lu melanoma cells to apoptosis induction by inhibition of the Raf-ERK pathway.

A 1205Lu melanoma cells carrying dox-inducible GFP-Usp27x and the same cells where Bim had been targeted using CRISPR/Cas9 (1205Lu-GFP-Usp27x/Bim2KO) were treated with dox as indicated. After 48 h, UO126 (10 μ M) was added for another 48 h. Apoptosis was measured by staining for active caspase-3, followed by flow cytometric analysis. All data (means \pm SEM) are from $n = 5$ separate experiments. P -values (t-test) for statistically significant differences are shown. The addition of QVD blocked the appearance of active caspase-3 staining (not shown). Inset shows knockout efficiency of Bim in 1205Lu-GFP-Usp27x melanoma cells ($n = 2$).

B GFP-Usp27x- or GFP-Usp27xC87A-expressing 1205Lu melanoma cells were treated as in (A) and percentage of cells with active Bax was measured by FACS using a conformation-specific antibody (6A7 clone) that recognizes only the activated form of Bax. All data (means \pm SEM) are from $n = 5$ (GFP-Usp27x) or $n = 3$ (GFP-Usp27xC87A) separate experiments. P -values (t-test) for statistically significant differences are shown.

C Dox-inducible GFP-Usp27x or GFP-Usp27xC87A were induced in 1205Lu melanoma cells as indicated. After 48 h, cells were treated with vemurafenib as indicated for additional 24 h. Apoptosis was measured by staining for active caspase-3 and flow cytometry. Bars show percentage of active caspase-3-positive cells from $n = 2$ independently performed experiments.