Supporting Information for

Optical control of MKK6 reveals divergent roles in pro-apoptotic and anti-proliferative signaling

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This PDF file includes:

Figure S1

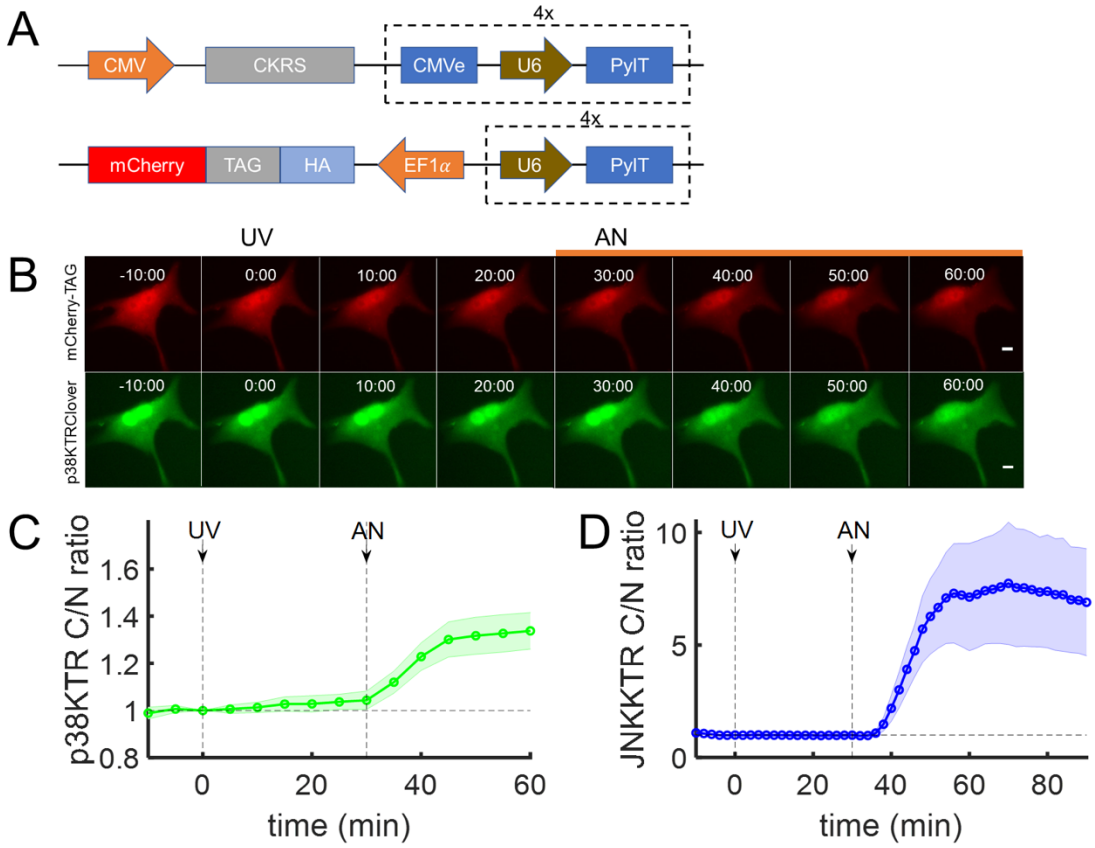

Fig. S1: No non-specific activation of p38 and JNK signaling following brief UV-light exposure. A) Plasmids used to express mCherry-tagged with TAG-codon which incorporate the caged lysine using CKRS and pyltRNA pair. **B)** Representative montage showing the expression of mCherry (first row) in a cell transfected with the plasmids in A and p38KTRClover. The cell was first (t = 0 min) irradiated with UV (365 nm, ~2 s), then (t = 30 min) treated with Anisomycin (AN, 50 ng/mL). Scale bar = 10 μm, and times are in minutes. **C)** Quantification of p38-kinase activity in cells prepared and treated as in B (mean ± 95% confidence interval, n = 14). **D)** Cells stably expressing JNK-activity reporter JNKKTRClover were first irradiated with UV-light (365 nm, ~2 s), then treated with Anisomycin (1 μg/mL). Quantification of normalized JNK-kinase activity is expressed as mean ± 95% confidence interval (n = 15).

Figure S2

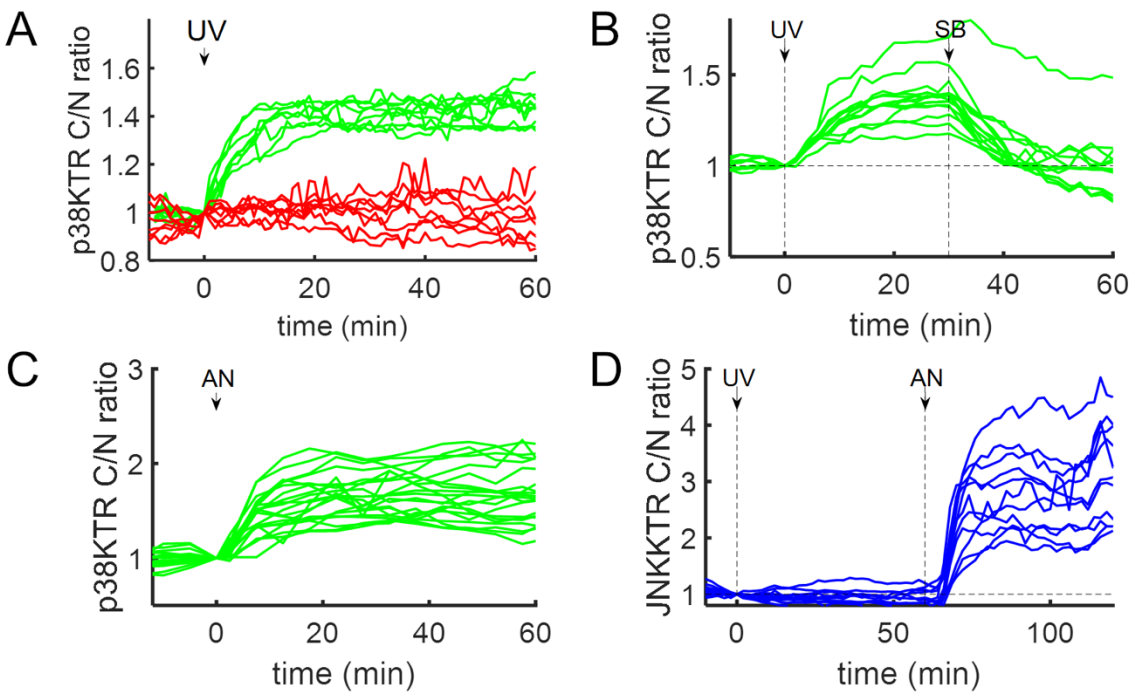

Fig. S2: Time courses of individual cell traces to assess variability in Fig. 2C-F. Panels A-D correspond to panels C-F in Fig. 2. **A)** Cells (n = 10 each) were expressing the p38 activity reporter along with caged MKK6 and growth media was supplemented with (green) or without (red) CK (2 mM). **B)** Cells (n = 12) were briefly exposed to UV light, then treated with the p38α/β-specific inhibitor SB239063 (10 μM). **C)** Analysis of NIH 3T3 cells (n = 27) transiently expressing p38KTRClover were treated with anisomycin (50 ng/ml). **D)** NIH 3T3 cells (n = 11) co-expressing caged MKK6 and the JNK activity reporter JNKKTRClover were briefly exposed to UV light, then treated with anisomycin (50 ng/ml).

Figure S3

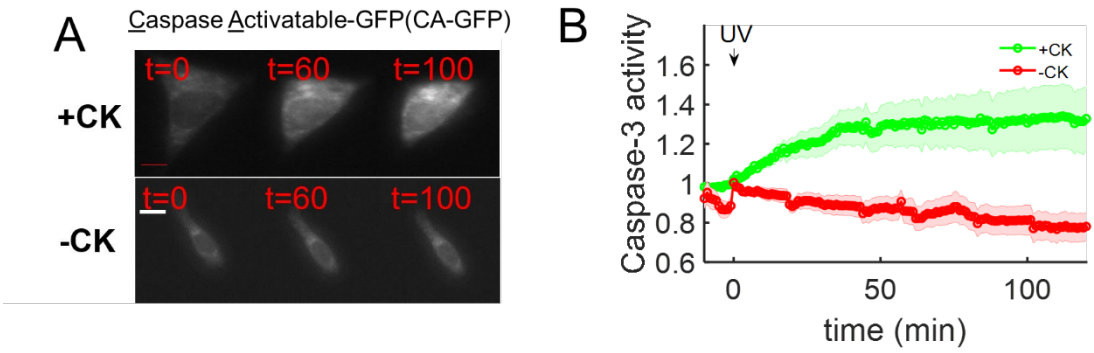

Fig. S3: Photo-activation of MKK6 elicits caspase-3/-7 activity, with no detectable lag. A) Representative montages of NIH 3T3 cells transiently transfected with plasmids to express caged MKK6 and caspase-3/-7 activity reporter CA-GFP and then cultured in the presence or absence of 2 mM CK. At t = 0, the cells were exposed to UV (365 nm, ~2 s). Scale bar = 10 μm, and times shown on the montages are in minutes. **B)** Quantification of the experiments represented in A (mean ± 95% confidence interval, n = 10 each). Representative of two independent experiments.

Figure S4

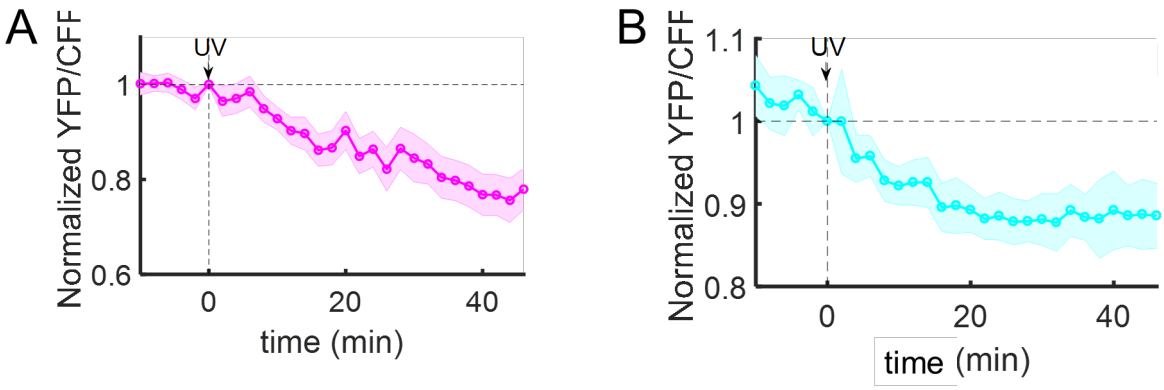

Fig. S4: Light-activated MKK6 negatively regulates ERK activity in both cytosol and nucleus. A) NIH 3T3 cells (n = 20) were transiently transfected with plasmids to express caged MKK6 along with nuclear ERK-kinase activity reporter Nuclear-EKAR. **B)** NIH 3T3 cells (n = 12) were transiently transfected with plasmids to express caged MKK6 and cytosolic ERK-kinase activity reporter Cyto-EKAR. Growth media was supplemented with 2 mM CK. The ERK-kinase activity was monitored before and after UV-irradiation (365nm, ~2s). The solid-line in each curve represents the average kinase activity and shaded regions represent 95% confidence intervals. Representative of two independent experiments.

Figure S5

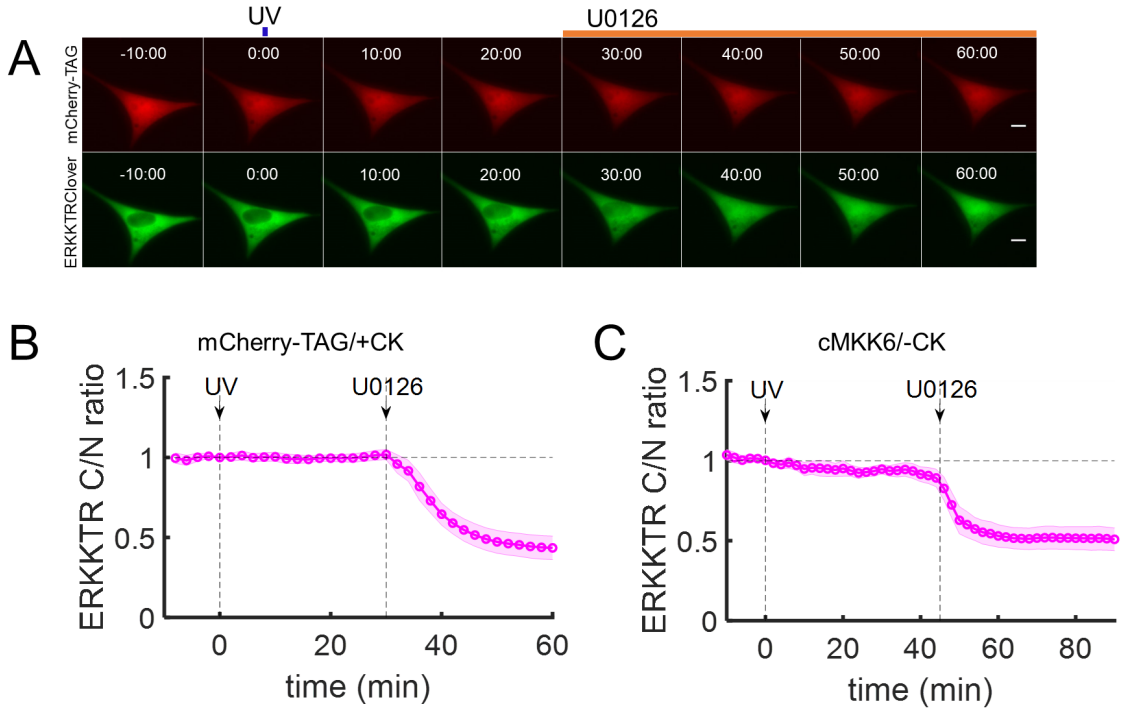

Fig. S5: No non-specific perturbation of ERK signaling due to decaging lysine incorporated in the endogenous proteome. A) Representative montage showing the expression of mCherry (first row) in a NIH 3T3 cell transfected with the plasmids in Fig. S1A and ERKKTRClover and incubated with CK. The cell was first (t = 0 min) irradiated with UV (365 nm, ~2 s), then (t = 30 min) treated with the MEK inhibitor, U0126 (10 μM). Scale bar = 10 μm, and times are in minutes. **B)** Quantification of ERK-kinase activity in cells prepared and treated as in B (mean ± 95% confidence interval, (n = 16). **C)** NIH 3T3 cells co-transfected with cMKK6 plasmids and ERKKTRClover, but not incubated with CK, was (t = 0 min) irradiated with UV (365 nm, ~2 s), then (t = 45 min) treated with the MEK inhibitor, U0126 (10 μM) (mean ± 95% confidence interval, (n = 15).

Figure S6

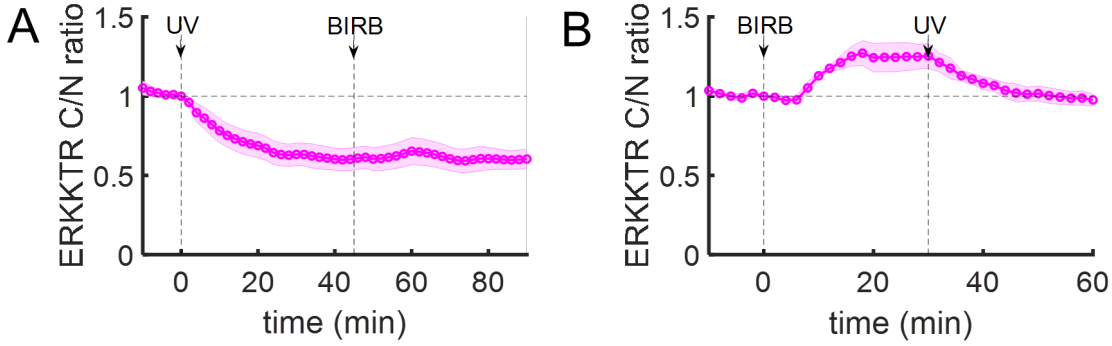

Fig. S6: Replication of the experiment shown in Fig. 5 B&C using the pan-p38 inhibitor, BIRB 796. A) NIH 3T3 cells (n = 6) expressing caged MKK6 and ERKKTRClover were irradiated with UV (365 nm, ~2 s), then treated with pan-p38 inhibitor BIRB 796 (10 μM) as indicated. **B)** NIH 3T3 cells (n = 13) expressing caged MKK6 and ERKKTRClover were pretreated with BIRB 796, then irradiated with UV (365 nm, ~2 s) as indicated. Both plots show mean ± 95% confidence interval.

Figure S7

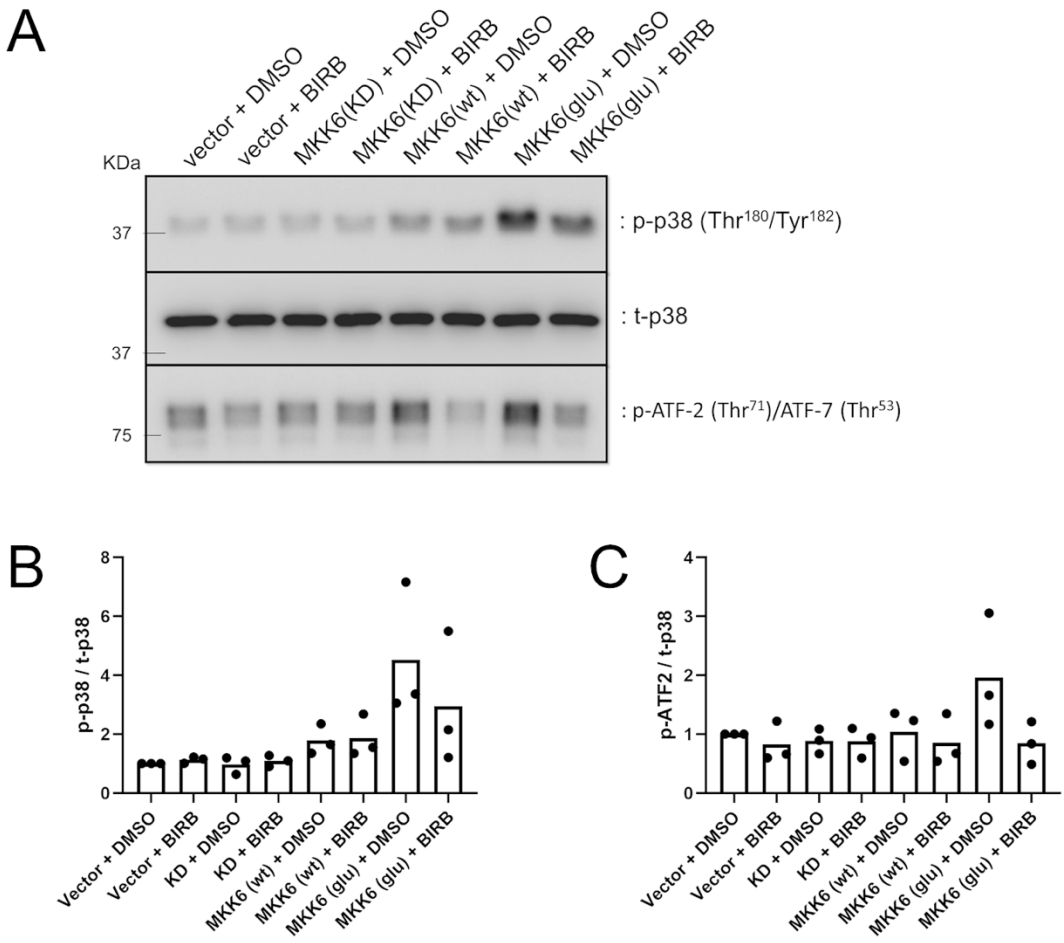

Fig. S7: Expression of kinase-competent MKK6 elicits p38 activation. NIH 3T3 cells were transfected with the indicated MKK6 construct (as in Fig. 6) and incubated in 3% FBS and either DMSO vehicle control or BIRB 796 pan-p38 inhibitor (10 μM). Lysates were probed with antibodies against phosphorylated p38 (p-p38), total p38 (t-p38), and phosphorylated ATF-2/7 (p-ATF-2/ATF-7). **A)** Immunoblot, representative of three independent experiments. **B & C)** Quantification of p38 **(B)** and ATF-2/7 **(C)** phosphorylation, first normalized by total p38 loading control for each sample, then by the value for the vector + DMSO control. The bars indicate the means of three independent experiments.

Figure S8

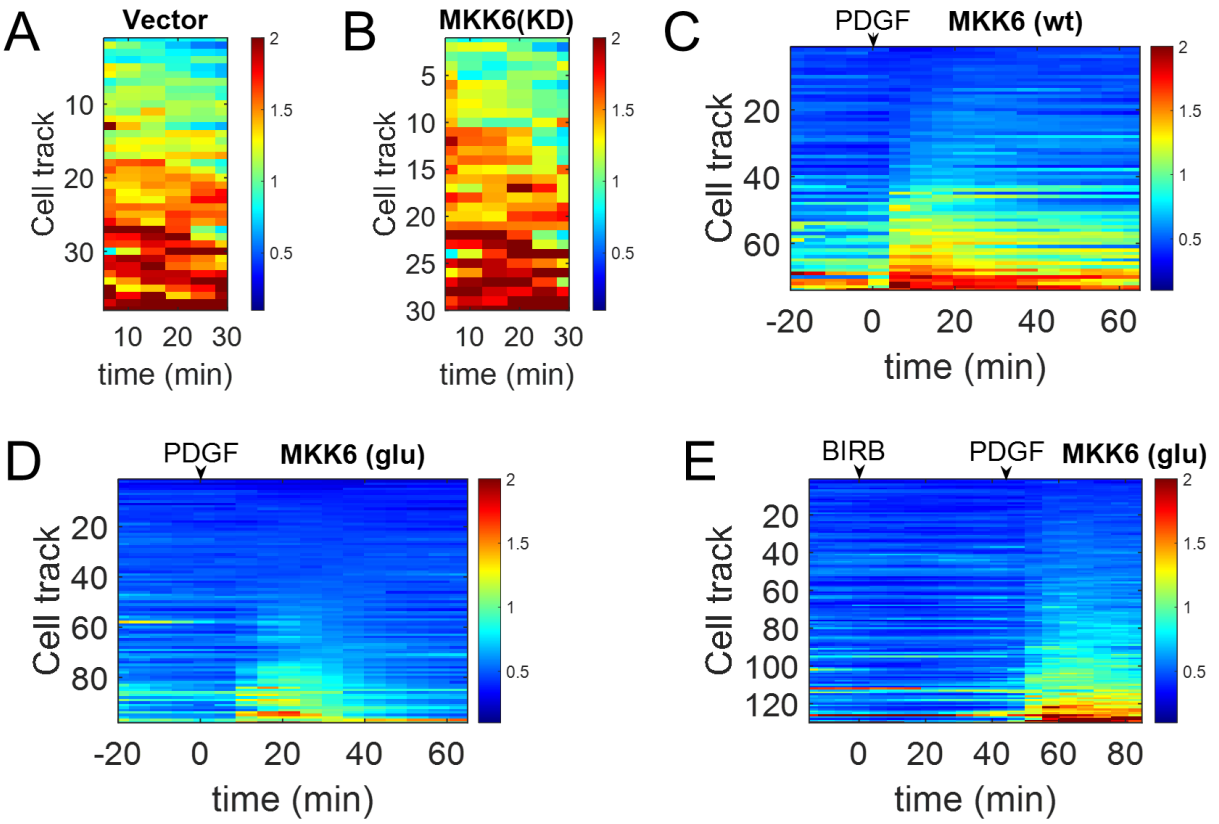

Fig. S8: Kinase-competent MKK6 suppresses ERK activity. NIH 3T3 cells were co-transfected with ERKKTR biosensor and the indicated vector control or MKK6 variant. The cells were maintained in 3% FBS to foster ERK activation. Time courses of ERK activity (Cytosol/nucleus ratio of ERKKTR, not normalized) are shown as heat maps, with other treatments are noted above. Co-transfection with matched empty vector (**A**) or kinase-dead (KD) MKK6 (K82A) (**B**) yields normal ERK activity levels. Co-transfection with wild-type MKK6 (**C**) or constitutively active (glu) MKK6 (S207E/T211E) (**D**) results in depressed ERK activity; subsequent stimulation with PDGF fails to restore ERK signaling in most of the cells. along with ERKKTRClover were imaged in presence of 3% FBS were stimulated with PDGF (1nM). **E)** In cells co-transfected with MKK6 (glu), inhibition of ERK signaling is not affected by inhibition of p38 (BIRB 796, 10 μM).

Figure S9

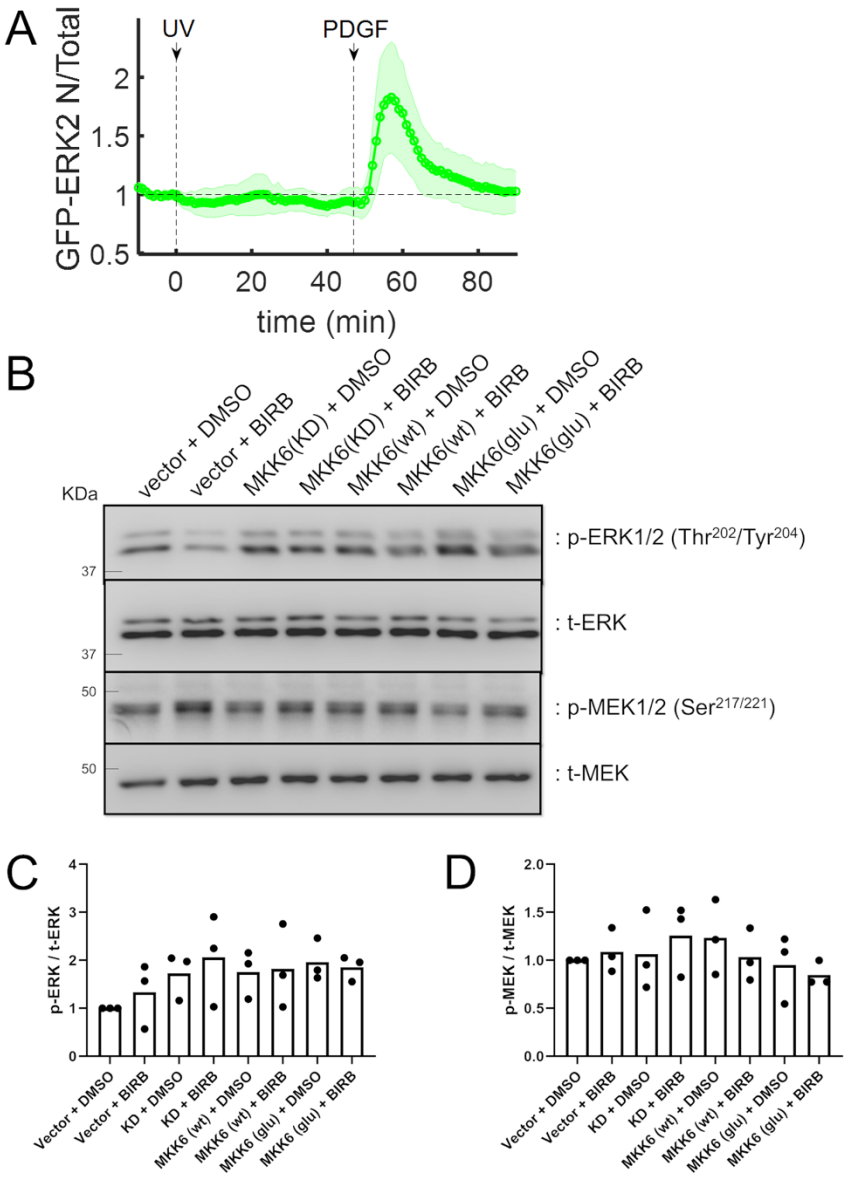

Fig. S9: No apparent perturbation of Raf/MEK/ERK phosphorylation cascade by active MKK6. A) NIH 3T3 cells expressing caged MKK6 and GFP-ERK2 were treated and imaged as in Fig. 4C. For each image, the ratio of nuclear/total GFP-ERK2 was determined and normalized by the value at time zero. Values are expressed as mean ± 95% confidence interval (n = 7). This is one of two independent experiments performed. **B-D**) The same lysates from Fig. S7 were probed for ERK1/2 and MEK1/2 phosphorylation, with loading controls. **B)** Immunoblot, representative of three independent experiments. **C & D)** Quantification of ERK1/2 **(C)** and MEK1/2 **(D)** phosphorylation, as in Fig. S7 B & C.

Figure S10

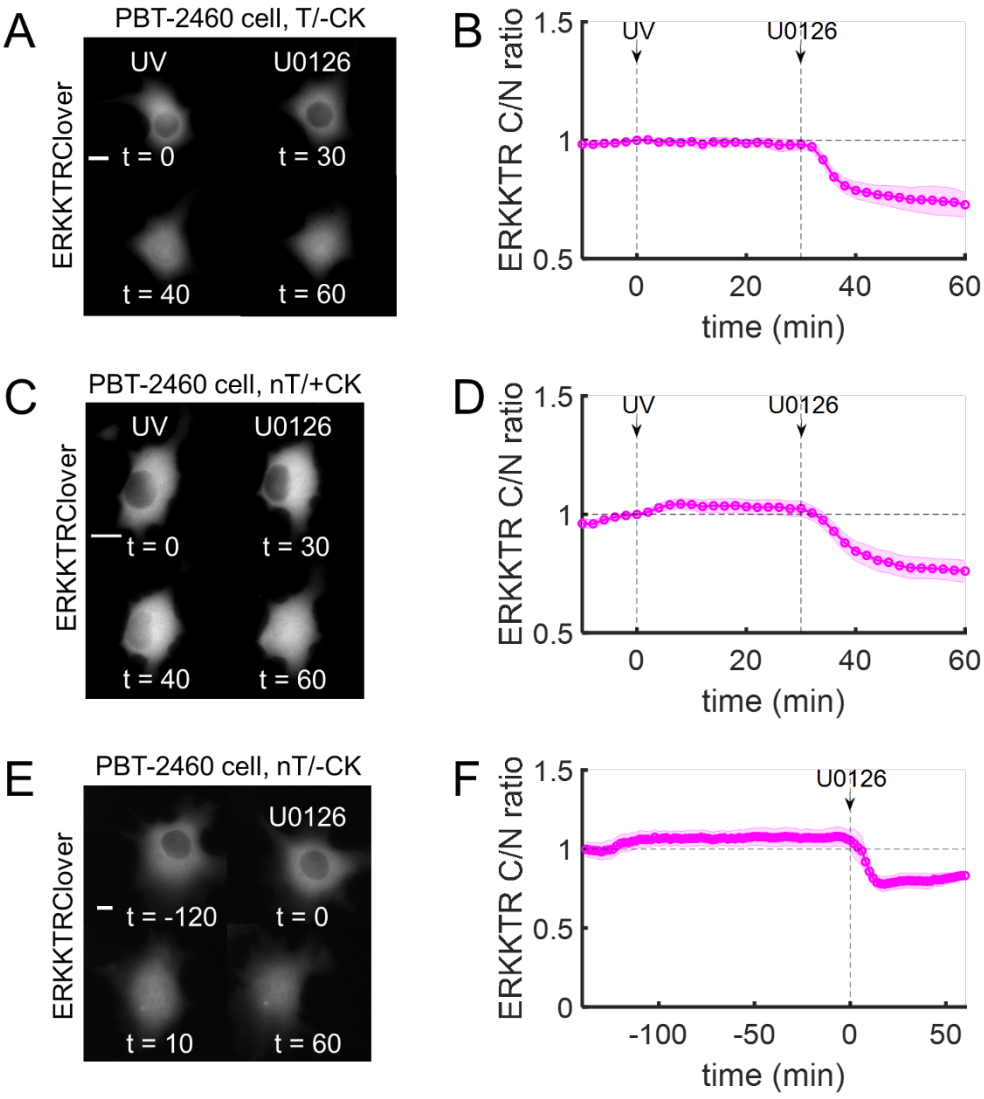

Fig. S10: Control experiments for the PBT-2460 melanoma line. A&B) In serum-starved PBT-2460 cells co-expressing the plasmids for caged MKK6 and ERKKTRClover, but with CK withheld (n = 14), there is no response to UV exposure (365 nm, ~2s). Subsequent addition of MEK inhibitor U0126 (10 μM) confirms that ERK activity was readily reversible. **C&D)** Same as A&B, respectively, without plasmids for caged MKK6 (nT) and with CK in the medium prior to the experiment (n = 15). **E&F)** Same as above, but with neither caged MKK6 plasmids nor CK (n = 13). In the representative montages shown in A, C, & E, the scale bars = 10 μm and all times are in minutes. The plots in B, D, and F show mean ± 95% confidence interval.

Figure S11

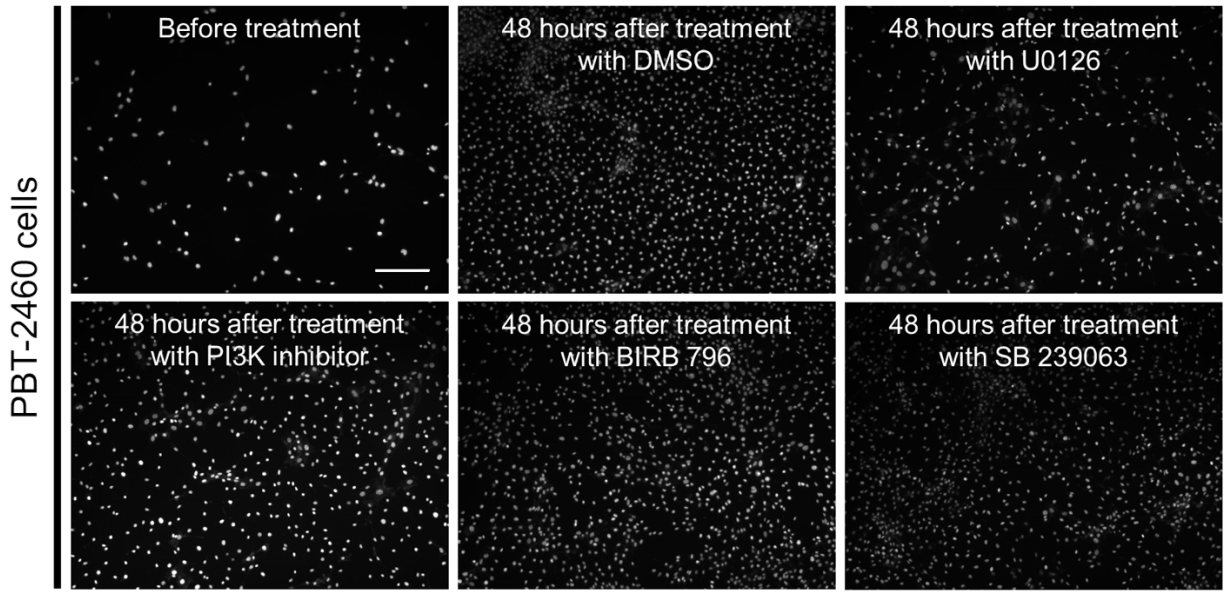

Fig. S11: Inhibition of MEK/ERK signaling suppresses PBT-2460 cell proliferation. Representative images of PBT-2460 cells stained with Hoechst 33342 (1 µg/ml) before and 48 hours after treatment with DMSO control, MEK1/2 inhibitor U0126 (10 µM), class 1A PI3K inhibitor IV (3 µM), BIRB-796 (10 µM) or SB239063 (10 µM). Scale bar = 10 µm. These images correspond to the quantification shown in Fig. 7C.

Figure S12

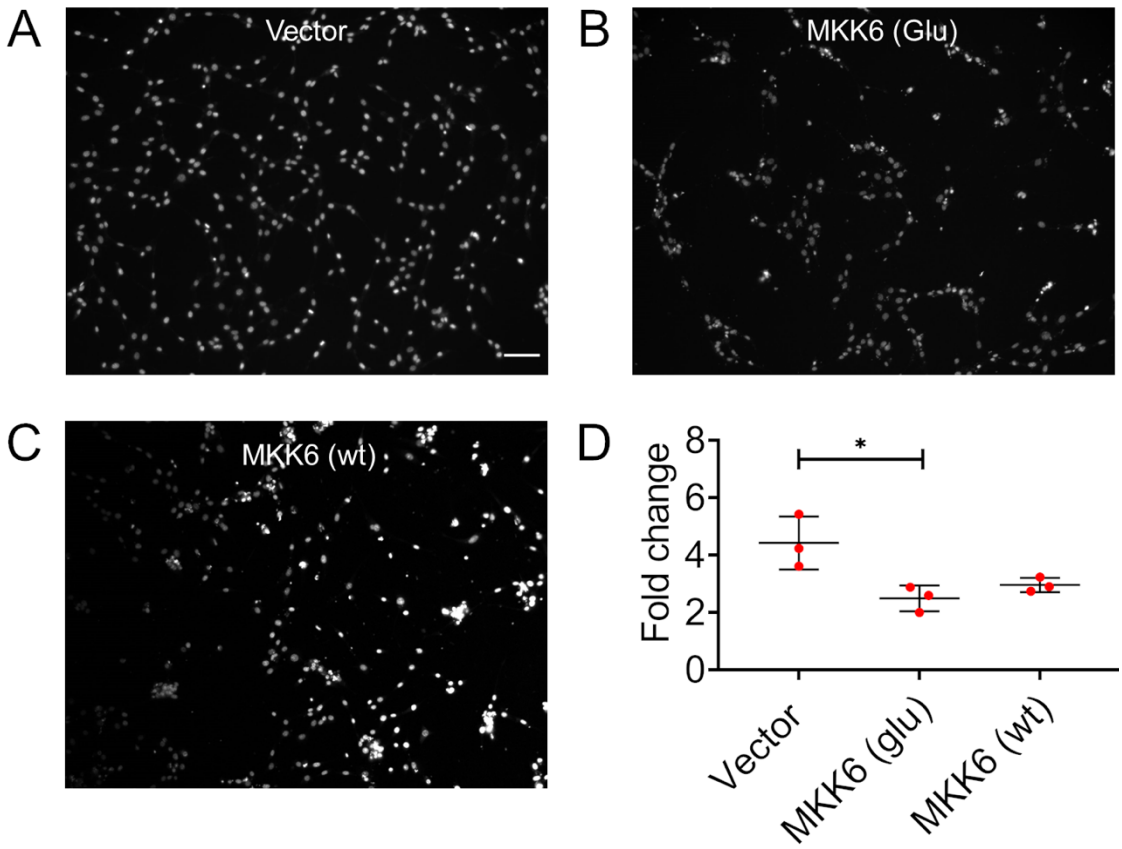

Fig. S12: Expression of kinase-active MKK6 elicits cell death. Representative images showing nuclei of NIH 3T3 cells transiently transfected with either empty vector (**A**), constitutively active MKK6 (glu, S207E/T211E) (**B**), or wild-type MKK6 (wt). The cells were maintained in normal growth media; 48 hours after transfection, the cells were stained with Hoechst 33342 (1 μg/ml). Scale bar = 100 μm). **D)** Quantification of the density of nuclei, relative to a reference plate that was stained at the time the other plates of cells were transfected (mean ± S.D., n = 3 independent experiments). The density of cells expressing MKK6 (glu) was reduced significantly relative to the vector control (*: $p < 0.05$, unpaired *t*-test); the density of MKK6 wt-expressing cells was also reduced relative to the control, but its significance did not meet the prescribed level.

Movie Captions

Movie S1. Decaging MKK6 induces p38 signaling and triggers apoptosis. In NIH 3T3 cells expressing caged MKK6 and p38KTRClover, brief UV exposure induces reporter translocation and, ultimately, morphological changes characteristic of apoptosis.

Movie S2. Decaging MKK6 triggers cytochrome c release from mitochondria. In NIH 3T3 cells expressing caged MKK6 and cytochrome c-GFP, brief UV exposure triggers, after a variable delay, the release of cytochrome c from mitochondria. Soon after, cells exhibit morphological changes characteristic of apoptosis.

Movie S3. PDGF-stimulated ERK activation measured by ERKKTR biosensor. Serum-starved NIH 3T3 cells expressing ERKKTRClover stimulated with 1 nM PDGF-BB, followed by addition of the MEK inhibitor, U0126 (10 μM).

Movie S4. Decaging MKK6 negatively regulates ERK signaling. In NIH 3T3 cells expressing caged MKK6 and ERKKTRClover, with 3% FBS in the imaging buffer, brief UV exposure induces reporter translocation to the nucleus (indicating loss of ERK activity). Subsequent addition of PDGF does not reverse the localization of ERKKTRClover.

Movie S5. MKK6-mediated regulation of ERK with p38 inhibited. In NIH 3T3 cells expressing caged MKK6 and ERKKTRClover, with 3% FBS in the imaging buffer, brief UV exposure induces reporter translocation to the nucleus (indicating loss of ERK activity). Subsequent addition of the p38 inhibitor, SB239063 (10 μM), had no discernible effect.

Movie S6. MKK6-mediated regulation of ERK in PBT-2460 cells. In serum-starved PBT-2460 cells expressing caged MKK6 and ERKKTRClover, brief UV exposure induces reporter translocation to the nucleus (indicating loss of ERK activity). Subsequent addition of the p38 inhibitor, SB239063 (10 μM), had no discernible effect.

Movie S7. Decaging MKK6 suppresses proliferation of PBT-2460 cells. In a PBT-2460 cell line with stable expression of ERKKTRClover and transient expression of caged MKK6, and with 3% FBS and amino acids added to the imaging buffer, brief UV exposure induces reporter translocation to the nucleus (indicating loss of ERK activity) in a subset of the cells. None of those cells divided during the 16 h period of observation.