Supplementary Information for Oberoi *et al*. (EMBOJ-2011-79557R)

Supplemental Materials and methods

Cell Culture and reagents

HeLa, A549 and HEp-2 were cultured in RPMI-1640 medium (Gibco BRL) supplemented with 10 % FCS (Gibco BRL), and 0.2 % penicillin (100 U/ml)/streptomycin (100 μg/ml) (Gibco BRL) and 2 mM L-glutamine at 37°C in 5 % CO_2. A second HeLa cell line, 293T cells, HCT-116, MDA-MB-231, HuH-7, $XIAP^{-/-}$, c-$IAP1^{-/-}$ MEFs and WT-MEFs cultured in DMEM supplemented with 10 % FCS and antibiotics were also employed. MCF-7 cells were cultured in RPMI supplemented with 10 % FCS, 1mM NEAA, 1mM sodium pyruvate, 10 μg/ml Insulin. ReNcell CX cells (Millipore) were grown in ReNcell CX maintenance medium (Chemicon) as per manufacturer's instructions. HMEC primary cells were cultured in MEBM as per manufacturer's instructions (Lonza). Sbcl2 cells (a kind gift from Prof. Richard Marais, ICR, UK) were cultured in MCDB153 (Sigma)/L15 medium (Invitrogen; v/v: 4/1) supplemented with $CaCl_2$ (2 mmol/L), insulin (5 μg/mL, Sigma) and 2 % FCS. The immortalized cerebellar granule neurons were a kind gift from Susan Cotman (Massachutes general hospital, USA). The cells were cultured as described in (Fossale et al, 2004). The cells were treated with U0126 (Calbiochem, #662005) at a final concentration of 10 μM when needed. For treatment with IAP inhibitor, various concentrations of IAC (BV6, Genentech) were added to cell culture media directly. The control cells were treated with DMSO alone. The following inhibitors were employed at

various concentrations; Rac1 inhibitor: NSC-23766 (Calbiochem, #553502) 50-100 μM, Bortezomib (#S1013, Selleck Chemicals).

Cloning of zebrafish expression constructs

Based on the pDon122 vector containing inverted repeats of the Tol1 transposon a pTol1-5xUAS-vector was generated containing 5 Gal4 binding sites (UAS) followed by the E1b basal promoter (Koga *et al.*, 2008).

#2630 pTol1-5xUAS:FynTagRFPTGIpA: an EcoRI/NotI fragment encoding membrane targeted FynTagRFP-T (Fyn:alkylation sequence of human Fyn Kinase, TagRFP-T: photostable variant of the fluorescent protein TagRFP) (Shaner *et al.*, 2008) followed by the rabbit β-globin intron and SV40 polyadenylation derived from pCSGI was cloned behind the 5xUASEIb-sequence (Distel *et al.*, 2009).

#2575 pTol1-5xUAS:FyntagRFP-T-T2A-Rac(T17N)GIpA: an EcoRI/NotI-cassette encoding FynTagRFP-T followed by the peptide backbone breaking T2A sequence (Provost *et al.*, 2007) and dominant-negative Rac1(T17N) variant was derived from a pCSGI vector and cloned behind the 5xUASE1b-sequence. #2691 pTol1-5xUAS:FyntagRFP-T-T2A-XIAPGIpA: an EcoRI/NotI-cassette encoding FynTagRFP-T followed by the peptide backbone breaking T2A sequence and zebrafish XIAP was derived from a pCSGI vector and cloned behind the 5xUASE1b-sequence. Gal4-mediated UAS:FyntagRFP-T2A-Rac1 expression construct was made by the replacement of T2A-Rac1 by T2A-XIAP, whereas the triple expression plasmid, UAS:FyntagRFP-T2A-XIAP-T2A-Rac1, was generated by insertion of T2A-XIAP DNA fragment behind

tagRFP in UAS:FyntagRFP-T2A-Rac1 construct. Further cloning details are available upon request.

Mass spectrometry

In gel trypsin digestion of the proteins was performed as described previously (Shevchenko *et al.*, 2007). Chloroacetamide was used for alkylation to minimize formation of lysine modifications isobaric to Gly-Gly (Nielsen *et al.*, 2008). After peptide desalting on C_{18} StageTips, LC-MS analyses were performed using an Easy-LC system (ProxeonBiosystems) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) as described previously (Borchert *et al.*, 2010). Peptides were eluted using a segmented gradient of 5–80% of solvent B (80% ACN in 0.5% acetic acid) with a constant flow of 200 nl min^{-1} over 60 min. Full scan MS spectra were acquired in a mass range from *m/z* 300 to 2000 with a resolution of 60,000 in the Orbitrap mass analyser using the lock mass option for internal calibration. The five most intense ions were sequentially isolated for CID fragmentation in the linear ion trap. Inclusion list containing 9 ions of special interest (ubiquitinated Rac1 peptides) was used and up to 500 precursor ion masses selected for MS/MS were dynamically excluded for 90 s. Mass spectra were processed using the MaxQuant software suite (Cox and Mann, 2008) (version 1.0.14.3) and the data were searched using Mascot search engine (Matrix Science) against a decoy human database (ipi.HUMAN.v3.64) containing 168,584 protein entries. Carbamidomethylation (Cys) was defined as fixed-, and protein N-terminal acetylation, oxidation (Met) and Gly-Gly (Lys) were defined as variable modifications. Initial mass tolerance was set to 7 p.p.m. for precursor ions, and to 0.5 Da for fragment ions. The Gly-Gly (Lys) modification sites were considered localized if the localization probability

(calculated by MaxQuant software) was higher than 0.75. Spectra of modified peptides were manually validated.

SDS-PAGE and Western Blot

For SDS-PAGE, cells were lysed in 4X Laemmli buffer and boiled at 100°C for 5 min before loading onto the polyacrylamide gels. After separation, the proteins were transferred to nitrocellulose membranes. For immunoblot analysis, membranes were blocked with 5 % low fat milk in Phosphate-Buffered Saline for 1 h and then incubated with various primary antibodies diluted in blocking buffer or in TBST + 3 % BSA. Antigen antibody complexes were detected by incubating with horseradish peroxidase coupled secondary antibodies followed by enhanced chemiluminescence (Amersham Biosciences). Quantification of Western Blots was performed by densitometry (ImageJ software, NIH). The following antibodies have been employed in this study: anti-C-RAF rabbit polyclonal C-12 antibody (Santa Cruz Biotechnology), anti-C-RAF-pSer338 rabbit monoclonal antibody, anti-beta-actin rabbit polyclonal antibody (Sigma), anti-phospho-ERK1/2 rabbit polyclonal antibody (Thr202/Tyr204) (Cell Signaling Technology), anti-total ERK1/2 (Cell Signaling Technology), anti-FLAG (Sigma), anti-RAS (SantaCruz), anti-GST (Santa Cruz), anti-c-IAP2 antibody (BD pharmingen), anti-XIAP mouse monoclonal antibody (BD Pharmingen), anti-N-terminal XIAP rabbit polyclonal antibody (ALEXIS), anti-c-IAP1 goat antibody (R&D Systems), anti-Ubiquitin mouse monoclonal antibody (Zytomed), anti-Rac1 mouse monoclonal antibody (BD Pharmingen), anti-Rac1 rabbit polyclonal (C11, Santa Cruz Biotechnology), anti-HA-probe (12CA5) mouse monoclonal antibody (Santa Cruz Biotechnology), anti-c-Myc (9E10) mouse monoclonal antibody (Santa Cruz Biotechnology, anti-RhoA (26C4) mouse monoclonal antibody

(Santa Cruz Biotechnology), normal rabbit IgG (Santa Cruz Biotechnology), normal mouse IgG (Santa Cruz Biotechnology).

Maintenance of Fish

Zebrafish strains were raised and maintained at 27°C in a manufactured fish facility (Aqua Schwarz GmbH, Göttingen) with circulating and constantly filtered water at 800-1000 µS salinity (Westerfield, 2000). In the *Tg(atoh1a:Gal4TA4)*^{hzm2} strain regulatory elements of the atonal1a gene drives expression of Gal4TA4 in cells of the upper and lower rhombic lip in the cerebellum and hindbrain (Distel *et al.*, 2010).

***In vitro* pull down experiments**

Recombinant full-length XIAP, c-IAP1, Rac1/2 and 3 were expressed as GST fusion proteins in BL-21 *Escherichia coli* strain (Stratagene, Cedar Creek, TX) according to manufacturer's instructions. The proteins were purified following standard procedures. Purified GST fusion proteins GST-Rac1, GST-Rac2, GST-Rac3 (2 µg) were incubated with 50 µl of Glutatione Sepharose Beads in 500µl of 1X binding buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1 % NP-40) for 1h at 4°C on a rotator. Beads were washed three times with 500 µl of binding buffer and pelleted at 2600 rpm for 2 min and unspecific binding was blocked by incubation with 5 % BSA containing binding buffer for 1-2 h. Beads were washed three times again, and then XIAP (self purified or R&D Sytems) or c-IAP1 purified from *E.coli* were incubated with GST-tagged proteins in a high-stringency binding buffer (200 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1 % NP-40) at 4°C on a rotator for 1 h. Again the beads were washed three times with high-stringency binding buffer and boiled at 100°C for 5 min in 50 µl of sample buffer. The interaction between proteins of interest was checked by SDS-PAGE as described above.

To check whether active as well as inactive Rac1 could also bind to IAPs, GST-tagged Rac1 proteins were incubated with GTPγS (0.1mM) or GDP (1mM) in presence of 0.5 M EDTA (pH 8.0) in 500 µL of binding buffer at 30°C for 15 min with constant agitation. Reaction is stopped with addition of 60 mM of $MgCl_2$. The beads were washed three times with binding buffer. Background protein binding on GST-beads was blocked by incubation with 5 % BSA containing binding buffer for 1-2 hours. After this step, the same procedure was followed as described above. The loading of the nucleotides was checked by EZ-Detect™ Rac1 Activation Kit (PIERCE) following manufacturer's instructions after cleaving off the GST tag.

RT-PCR

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN) from HeLa cells transfected with XIAP-siRNA or Control-siRNA for 48 hours. To synthesize cDNA, First Strand cDNA Synthesis Kit (Fermentas) was used according to the manufacturer's instructions. For PCR amplification, the following primers were employed:

GAPDH-for: 5'-TGCACCACCAACTGCTTAGC-3'

GAPDH-rev: 5'- GGCATGGACTGTGGTCATGAG-3'

Rac1-for: 5'-GACGGAGCTGTAGGTAAAACTTG-3'

Rac1-rev: 5'-GATAGGGGGCGTAATCTGTCA-3'

The PCR machine was programmed as follows: 1 cycle for 5 min at 94°C; 30 cycles for 30 s at 94°C, 30 s at 60°C, 45 s at 72°C, and 1 cycle 5 min at 72°C .

Time lapse microscopy and quantification of various parameters

HeLa cells seeded on collagen coated chambers were treated with IAC, 2.5 - 5 µM for 2-15 h and the morphologic changes were monitored under Leica time lapse imaging

system. Images were taken at frequent intervals (every 15 min) for 15 or 24 h. The images were processed using Leica application suite.

Cell tracking Analysis

The images were processed with standard routines ("plugins") on Image J (Scion Image, NIH, USA), and the cells were tracked with three different programs to ensure consistency of results: the imaging and analysis software on Meta Morph (Universal Imaging, USA), on Volocity (Improvision, USA) and with Cell Track (Sacan *et al.*, 2008). The cell speed and size were calculated for cells indicated by tracks in the supporting movies with acquisition as described in the previous section. For comparison and analysis of data presented in all figures we have considered all of the cells whether elongated or amoeboid (rounded) for all treated conditions. Elongation was calculated from each frame for each cell as axis length divided by the width.

Wound healing assay

$XIAP^{-/-}$ MEFs and strain matched WT-MEFs were seeded on to tissue culture plates and scratches (4-5) were made on confluent monolayers with a pipette tip. The extent of wound closure was monitored for 0 and 8h. The images were acquired using a Leica fluorescent microscope (DMIRE2) fitted with a digital camera with both high NA and low NA 10X objectives.

Immunoprecipitation

To immunoprecipitate endogenous proteins, HeLa cells were seeded on 10 cm dishes. Next day, 80 % confluent cells were lysed with a buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 % NP-40, 0.5 % Triton X-100, 1 mM $NaVO_3$, 10 mM Na-pyrophosphate, 1 mM NaF, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 µg/ml

Aprotinin, 0,5 μg/ml Leupeptin, 10 μM Pepstatin for 30 min on ice and sonicated twice for 10 sec. Lysates were cleared by centrifugation for 15 min at 14,000 rpm. Endogenous Rac1 was then immunoprecipitated with Rac1 antibody (BD Transduction Lab) for 15 hours. The antigen-antibody complexes were precipitated by sepharose coupled protein A/G beads (Roche). The beads were washed with the lysis buffer, and bound proteins were analyzed by SDS-PAGE and immunoblotting. For immunoprecipitation of co-expressed proteins in 293T cells, we have transfected 293T cells with various plasmids. The cells were lysed at 48 h post-transfection and proteins were immunoprecipitated as mentioned above. Whenever needed, the cells were treated with proteasome inhibitor MG132 at a final concentration of 10 μM for 6 hours before lysis. Controls IPs were performed with IgG isotype control antibodies (Santa Cruz).

Plasmids and constructs

The eukaryotic expression plasmids which are not mentioned elsewhere are listed here:

Rac:

pGEX-2p Rac1, pGEX4T3 Rac2, pGEX-2T Rac3

pRK5 myc-Rac1, pRK5 myc-Rac1 Q61L, pRK5 myc-Rac1 T17N, pRK5 myc-Rac1 K147R, pRK5 myc-Rac1 Q61LK147R

pCDNA3 HA-Rac1, pCDNA3 HA-Rac1 G12V, pCDNA3 HA- Rac2 T17N

Ubi:

pRK5 HA-Ubi

pCS2 His-Ubi (a kind gift from Prof. Rape)

c-IAP1:

CMV Flag-c-IAP1, CMV Flag-c-IAP1 H588A

pCDNA3 myc-c-IAP1, pCDNA3 myc-c-IAP1 H588A

pEGFP-C1-c-IAP1, pEGFP-C1-c-IAP1 H588A

XIAP:

pEGZ Flag-XIAP

pCDNA3 myc-XIAP, pCDNA3 myc-XIAP 1-240, pCDNA3 myc-XIAP 1-336

pCDNA3-zfXIAP-myc-His

Supplementary movies Legend

Movie S1. HeLa cells were seeded on gelatin coated plates and treated with DMSO. The cells were observed under a microscope and images were made for every 15 min for 22 h. The images from 14-18 h post treatment were considered for cell tracking analyses.

Movie S2. HeLa cells seeded on gelatin were treated with 5 μM of IAC and the morphological changes were observed under a microscope for 22 h. The images were captured for every 15 min. The images from 14-18 h post treatment were considered for cell tracking analyses.

Movie S3. HeLa cells seeded on gelatin were treated with 2.5 μM of IAC and the morphological changes were observed under a microscope for 22 h. The images were captured for every 15 min. The images from 14-18 h post treatment were considered for cell tracking analyses.

Movie S4. HeLa cells seeded on gelatin were treated with 2.5 μM of IAC and 50 mM of Rac1 inhibitor (NSC-23766) and the morphological changes were observed under a microscope for 22 h. The images were captured for every 15 min. The images from 14-18 h post treatment were considered for cell tracking analyses.

Supplementary References

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Supplementary Figure S1

Oberoi et al.

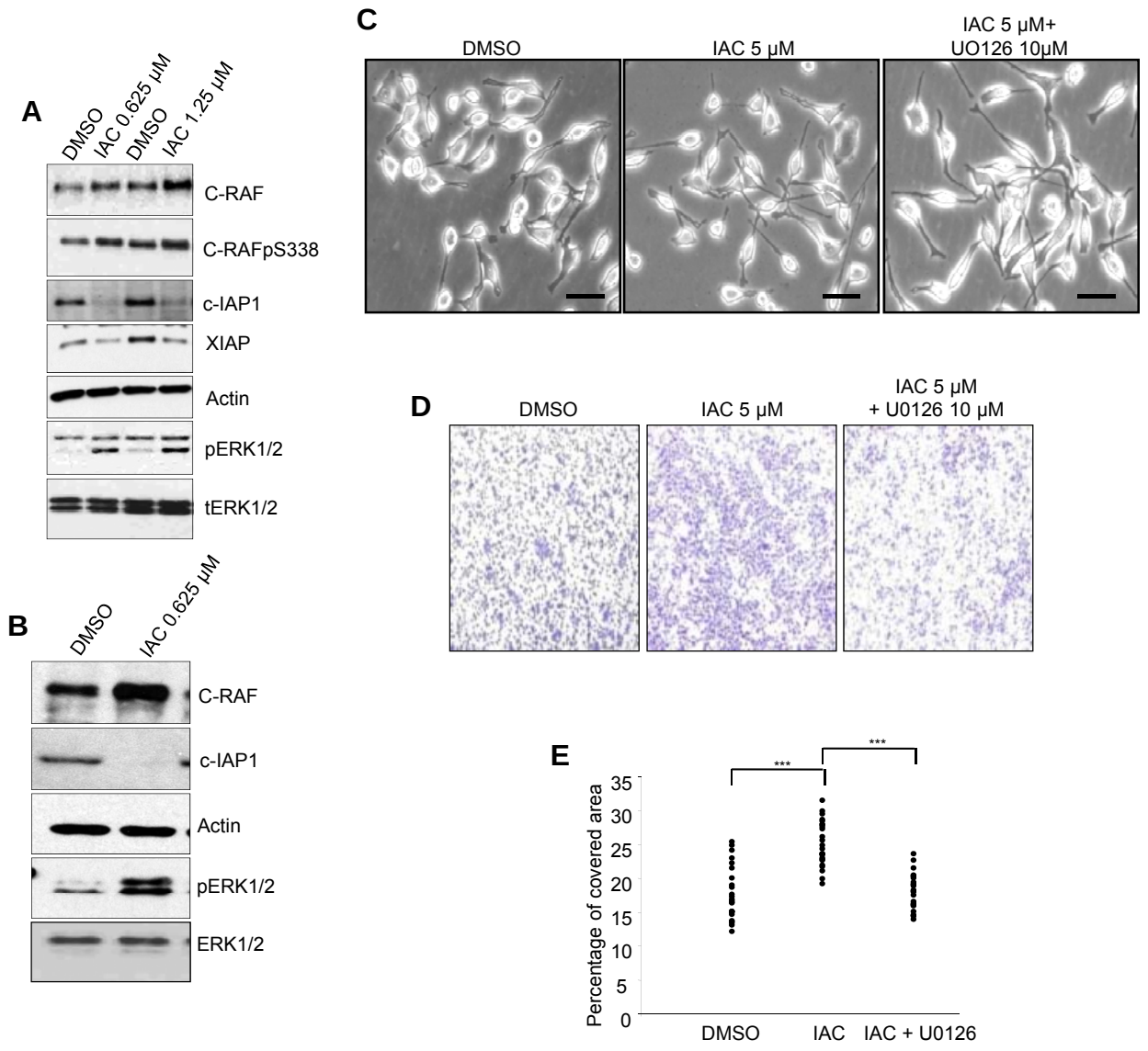

Figure S1. IAC depletes IAPs inducing ERK1/2 activation that further contributes to directional migration but not to elongated morphology. (**A**) Western blot analyses of serum starved HeLa cells treated with various concentrations of IAC for 15 h. Total cell lysates were western blotted with various antibodies as indicated. (**B**) Western blot analyses of Sbcl2 cells treated with IAC or DMSO showing C-RAF stability as well as activation of ERK1/2 upon depletion of c-IAP1 by IAC. (**C**) HeLa cells were treated with DMSO or IAC (5 µM) or IAC+U0126 and the morphological changes are monitored by phase contrast microscopy. (**D**) IAC mediated cell migration is MAPK dependent. HeLa cells were treated with IAC or in combination with U0126 and transwell migration experiments were performed. The migrated cells were fixed and stained with crystal violet staining. Shown in (**E**) are the quantifications of transwell migration experiments from three independent experiments (*** = P<0.005).

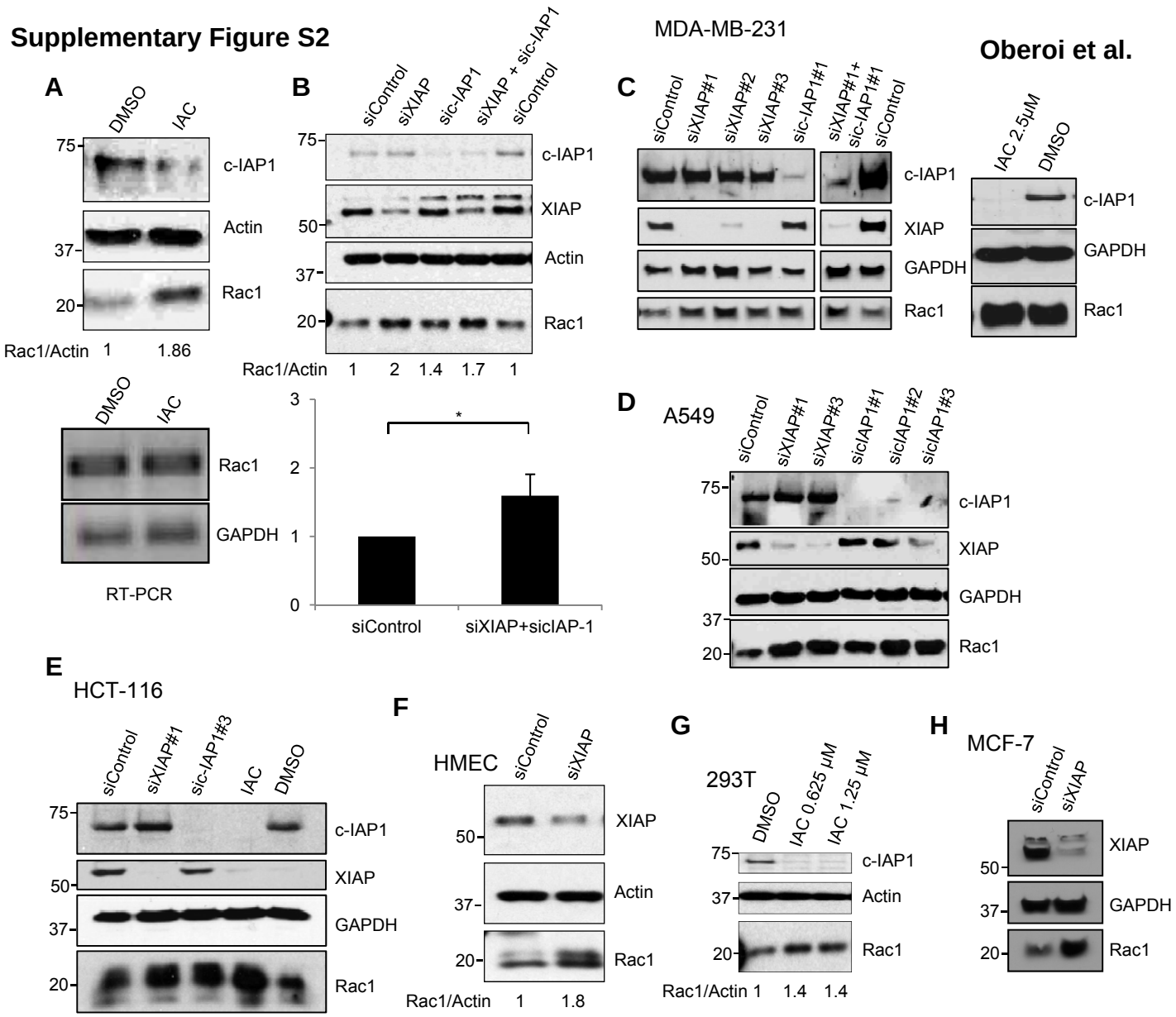

Figure S2. IAPs modulate Rac1 protein levels. (**A**) Validation of mRNA level of Rac1 in HeLa cells treated with IAC by RT-PCR. GAPDH was used as a control (lower panel). The increase in Rac1 protein levels was monitored by immunoblots (upper panel). (**B**) Western blot analyses and quantification of Rac1 levels from HeLa cells. Shown are the data from three independent experiments. Error bars represent ±SD of the mean. (*= P<0.05) (**C**) Western blot analyses of Rac1 levels in MDA-MB-231, A549 (**D**), HCT-116 (**E**), HMEC primary cells (**F**), 293T (**G**), MCF-7 (**H**), HuH-7 (**I**) and ReNcell CX (**J**) transfected with control, XIAP siRNAs, c-IAP1 siRNAs and/or treated with IAC and the total Rac1, c-IAP1, XIAP and actin levels are monitored by immunoblots.

Supplementary Figure S3

Oberoi et al.

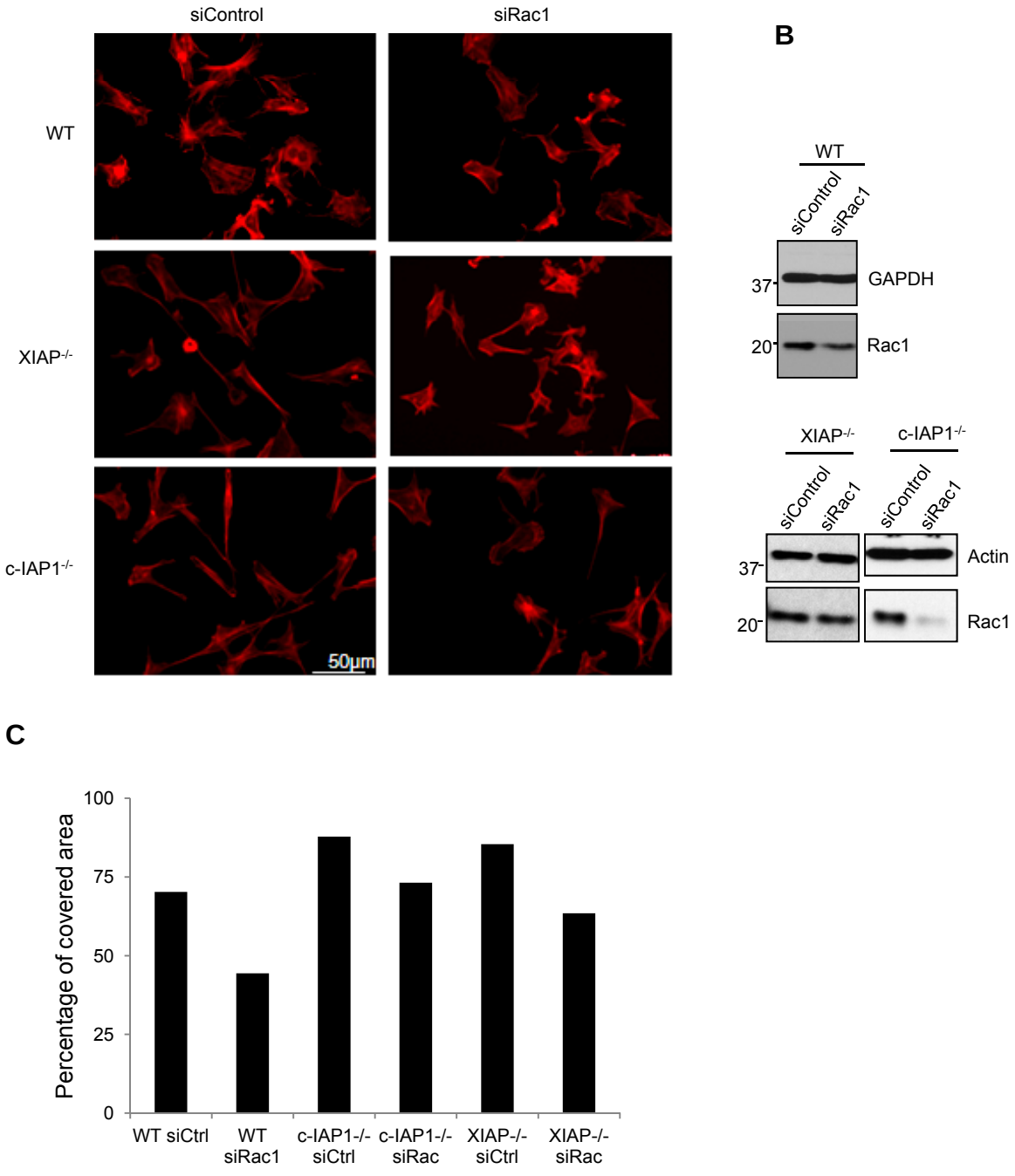

Figure S3. IAPs regulate cell morphology and migration in Rac1 dependent manner. (**A**) WT, XIAP$^{-/-}$ as well as c-IAP1$^{-/-}$ MEFs were trasfected with Rac1 siRNA, seeded on coverslips 24 hr post transfection and the morphology was checked after phalloidin staining 48 hr post-transfection. (**B**) The efficiency of Rac1 knockdown was checked by western blot analyses. (**C**) Quantification of transwell migration assay performed with WT, XIAP$^{-/-}$ and c-IAP1$^{-/-}$ MEFs transfected with control or Rac1 siRNA. Shown are data from one representative experiment.

Supplementary Figure S4

Oberoi et al.

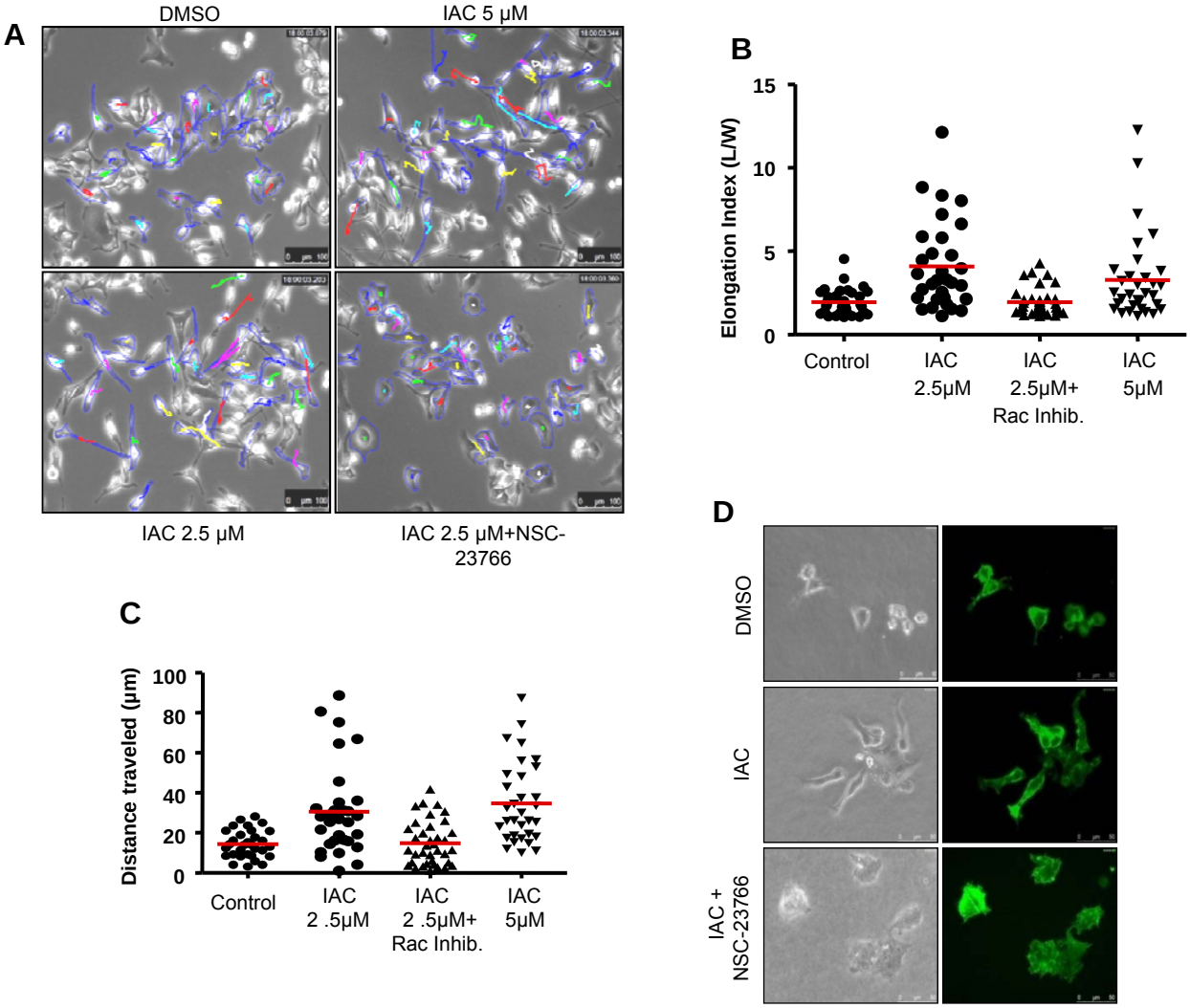

Figure S4. IAC-mediated elongated morphology is Rac1 dependent. (**A**) HeLa cells seeded on gelatin were treated with DMSO, IAC or IAC+NSC-23766 (50 µM) and observed under a microscope at various time intervals. Shown are snap shots from movies S1-S4. (**B**) Quantification of elongation index and distance travelled (**C**) of control and treated cells from supporting movies S1-S4. (**D**) HeLa cells were treated with IAC (2.5 µM) and NSC-23766 (100 µM) for 5 min before embedding in 3D collagen matrices. The cells were fixed and stained with Phalloidin-green after 24 h.

Supplementary Figure S5 **Oberoi et al.**

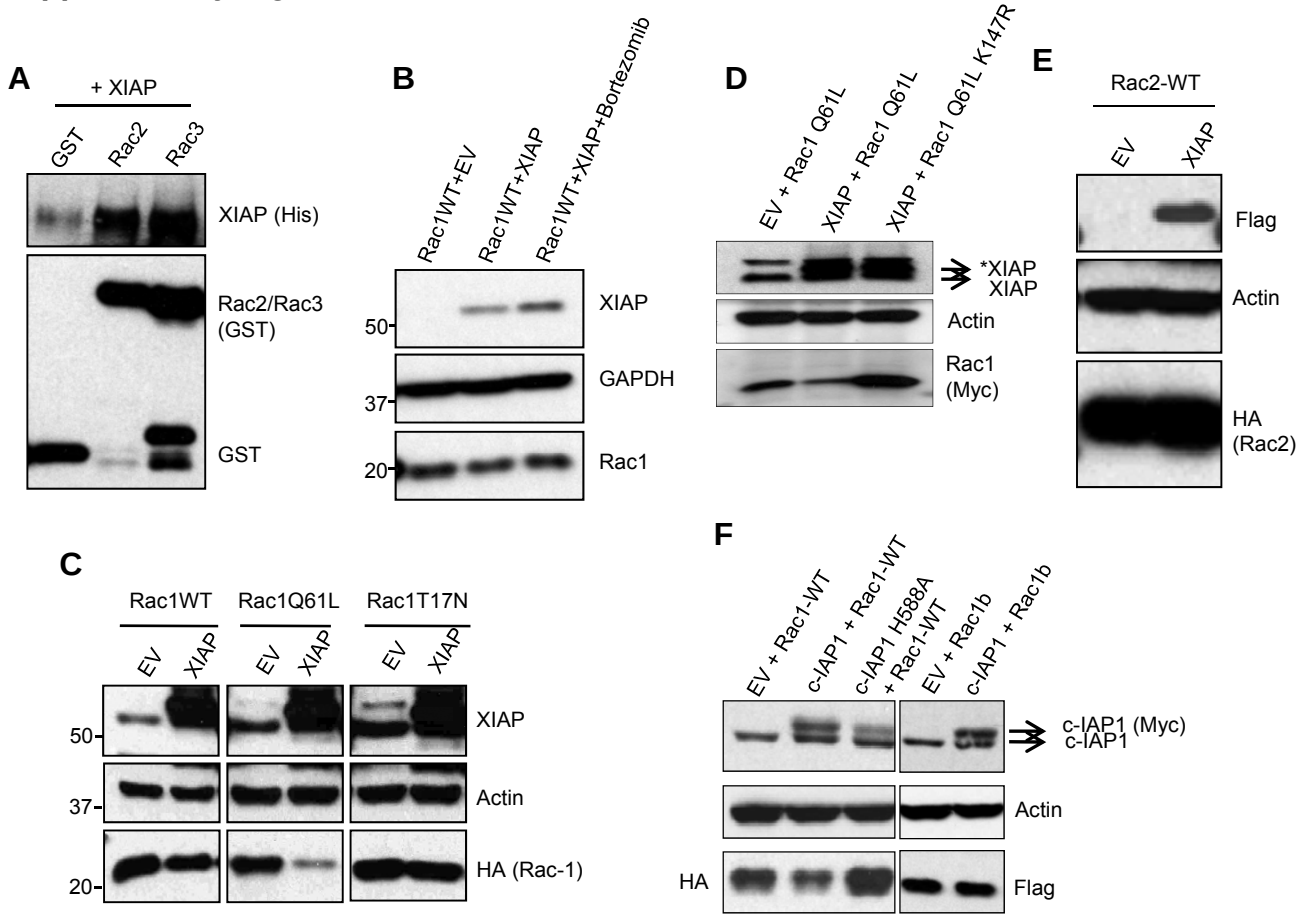

Figure S5. IAPs directly bind to and promote the ubiquitination and degradation of Rac1 but not Rac2. (**A**) XIAP binds directly to Rac2 and Rac3. *In vitro* binding of GST-Rac2 and GST-Rac3 to His-XIAP was detected by Western blotting using purified proteins. GST was used to detect non-specific binding. (**B**) The rescue in the levels of Rac1 under the influence of proteasomal inhibitors Bortezomib upon IAP overexpression is monitored by western blotting. (**C**) XIAP degrades Rac1. 293T cells were transfected with various constructs as indicated. The degradation of HA-tagged Rac1 was monitored by immunoblots. (**D**) Western blot analyses showing degradation of Rac1Q61L by XIAP in 293T cells is rescued with K147R mutant of Rac1Q61L. * represents overexpressed XIAP. (**E**) Rac2 is not degraded by XIAP overexpression. (**F**) Analyses of degradation of Rac1 as well as Flag-Rac1b by c-IAP1 or c-IAP1H588A mutant in 293T cells by Western blotting.

Supplementary Figure S6

Oberoi et al.

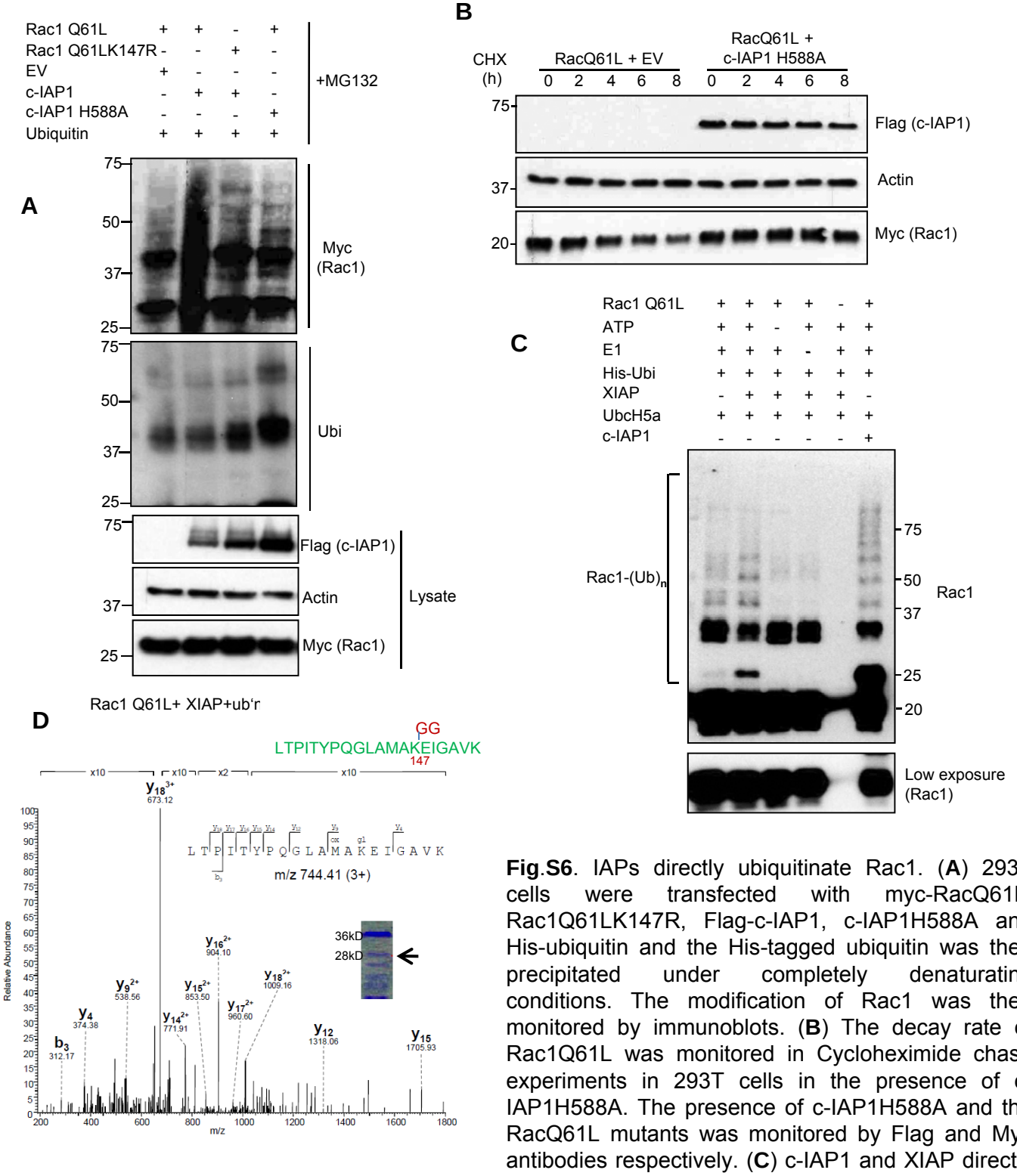

Fig.S6. IAPs directly ubiquitinate Rac1. (**A**) 293T cells were transfected with myc-RacQ61L, Rac1Q61LK147R, Flag-c-IAP1, c-IAP1H588A and His-ubiquitin and the His-tagged ubiquitin was then precipitated under completely denaturating conditions. The modification of Rac1 was then monitored by immunoblots. (**B**) The decay rate of Rac1Q61L was monitored in Cycloheximide chase experiments in 293T cells in the presence of c-IAP1H588A. The presence of c-IAP1H588A and the RacQ61L mutants was monitored by Flag and Myc antibodies respectively. (**C**) c-IAP1 and XIAP directly ubiquitinates Rac1 at lysine 147. Recombinant Rac1Q61L was subjected to an *in vitro* ubiquitination reaction as mentioned in the methods section with c-IAP1 or XIAP. (**D**) The reaction sample was subjected to mass spectrometry analysis of the Gly-Gly conjugated peptides.

Supplementary Figure S7

Oberoi et al.

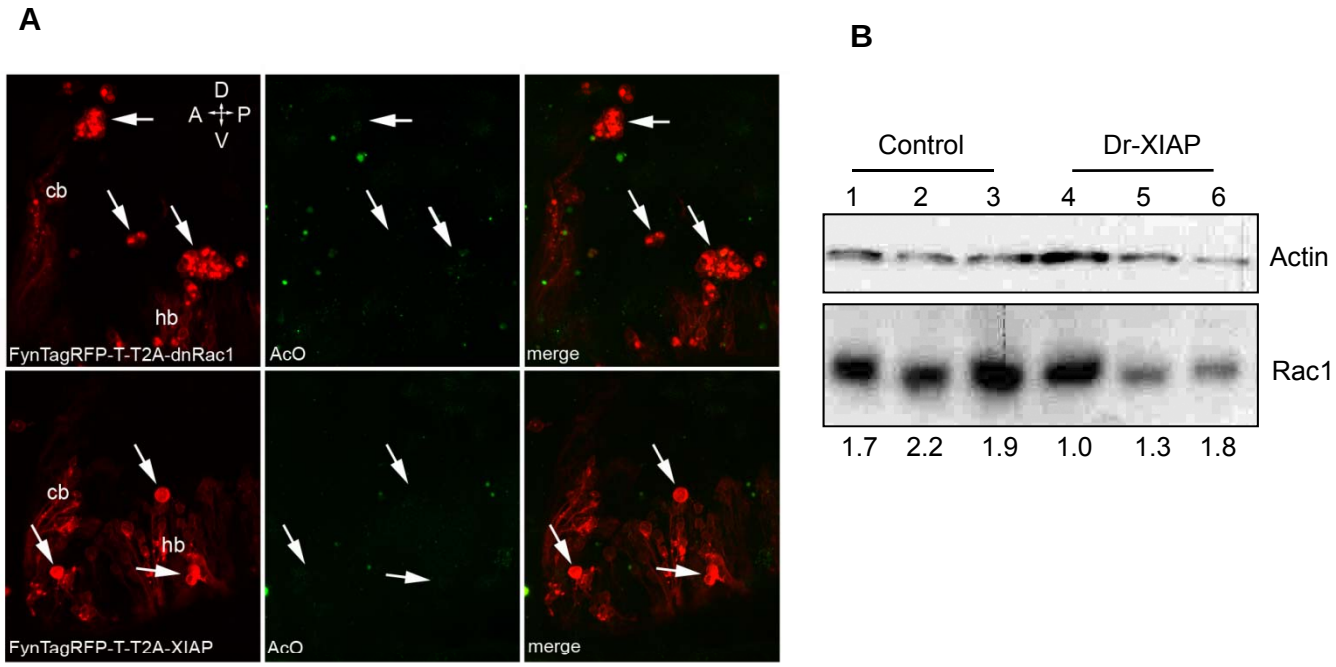

Fig.S7 dnRac1- and XIAP-expressing cells that have delaminated from the rhombic lip do not immediately undergo apoptosis. (**A**) Top panel shows lateral views of the cerebellum and anterior hindbrain of a 48 hpf embryo expressing FynTagRFP-T-T2A-dnRac1 (red), and labeled with acridine orange (AcO) dye (green). AcO-positive cells (green) are dying cells. Arrows point to ectopic clusters of dnRac1-expressing cells, none of which contain a cell positive for AcO staining. Only 3% of ectopic dnRac1-expressing cells were positive for AcO (n=233 cells, 3 fish). Lower panel shows lateral views of the cerebellum and anterior hindbrain of a 48 hpf embryo expressing FynTagRFP-T-T2A-XIAP (red) and labeled with acridine orange (green). Arrows point to the ectopic XIAP-expressing cells, none of which are AcO-positive. Only 6% of the XIAP-expressing cells with an abnormal rounded morphology were positive for AcO staining (n=17 cells, 5 fish). (**B**) Western blot of Zebrafish embryos (one-cell stage) lysates injected with mRNA encoding GFP alone or XIAP-GFP was analysed for the expression levels of endogenous Rac1. Each lane has lysates from 20 different embryos.