Supporting Information

Krieg et al. 10.1073/pnas.0907131106

SI Text

Reagents. Recombinant human TNF-α was from Peprotech. Monoclonal rat anti-RIP2 was purchased from Alexis, whereas monoclonal mouse anti-XIAP was obtained from BD Biosciences, mouse-monoclonal anti β-actin, mouse-monoclonal anti-GST-antibody and mouse-monoclonal anti-FLAG antibodies from Sigma Aldrich, mouse-monoclonal anti-GFP antibody from Santa Cruz Biotechnology, mouse-monoclonal anti-His-antibody from Qiagen and mouse-monoclonal anti-myc-antibody from Roche Applied Science. Anti-SMAC antiserum generated in rabbits has been described (1).

Expression Plasmids. To subclone the complete ORF of XIAP and the BIR2-coding region into pEGFP-C2 (Clontech), the corresponding inserts were excised from previously described pcDNA3 plasmids (2) by digestion with *Eco*RI/*Xho*I, purified, and ligated into pEGFP-C2 at the *Eco*RI/*Sal*I cloning sites. Various codons in the XIAP and BIR2 cDNAs were mutated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). RIP2 cloned into pCR3.V64-Met-FLAG was a gift of J. Tschopp (University of Lausanne). Plasmids encoding 6×-His-FLAG-tagged versions of *NOD1* and *NOD2* human cDNAs were generated by PCR and subcloned into HIV-based self-inactivating lentiviral vector pCSC-SP-PW.

IL-8 ELISA. Human IL-8 secreted into cell culture media was measured using an ELISA (eBioscience). Briefly, 10^5 cells per well were seeded into 24 well plates. The next day cells were stimulated with MDP (20 μg/mL) or γTri-DAP (20 μg/mL) for 24 h. Subsequently, the media was removed and centrifuged at 15,000 $\times$ *g* for 5 min. Cell-free supernatants containing the secreted IL-8 were stored at -80°C until performance of ELISA assays.

Real-Time RT-PCR. Total RNA was extracted from HCT-116 and MEF cell lines by TRIzol® solution (Gibco BRL, Life Technologies). After isolation, 1–5 μg total RNA were treated with DNase (DNA-free, Ambion) and reverse-transcribed, in the presence of oligo(dT) primer, according to the manufacturer (SuperScript First-Strand Synthesis, Invitrogen). First-strand cDNA was diluted and analyzed in triplicates with gene-specific primers by real-time PCR, using a Stratagene Mx3000p sequence detection system with SYBR Green PCR master mix (Applied Biosystems). The gene expression (fold induction) was normalized with the respective levels of 18S or cyclophilin (CPH) expression.

Protein Expression and Purification. Recombinant GST and His-6-tagged proteins were produced in bacteria and purified, essentially as described (2–5). Briefly, recombinant GST-tagged human full length XIAP, BIR1–3, BIR1+2, BIR2+3, BIR1+3, BIR3+RING, BIR1, BIR2, BIR3, and Survivin were produced from previously described pGEX4T-1 constructs into *E. coli* strain BL21(DE3). Cultures were induced with 50 μM IPTG at 22°C for 3 h and fusion proteins affinity purified by incubating cleared lysates with glutathione-Sepharose at 4°C for 1 h under mild rotation. Glutathione Sepharose beads were washed three times and GST-fusion proteins eluted with 10 mM reduced glutathione dissolved in 50 mM Tris-HCl (pH 8) and 1 mM DTT. Eluted proteins were concentrated and purified in exchange buffer (50 mM Tris-HCl pH 8 and 1 mM DTT) with Amicon Ultra Centrifugal Filters. Purification of His-Ssel protein has been previously described (4)}.

Expression plasmid for the active form of SMAC (56–239) containing nine Histidines at the C terminus was kindly supplied by Xiaodong Wang (5). Transformed cells (BL21 DE3) were grown at 37°C to an OD_{600} of 1.0 and induced with 1 mM IPTG and growth was continued at room temperature for 3 h. Cells were harvested by centrifugation at 6,000 rpm for 15 min. Cell pellets were frozen and stored at – 20°C. Pellets were resuspended in 60 mL of 50 mM Tris at pH 8.0/1 mM DTT/1 mM PMSF/100 μg/mL lysozyme. Cells were sonicated for 10 min and then centrifuged at 20,000 rpm for 30 min. The resulting supernatant was then added to 9 mL preequilibrated (50 mM Tris at pH 8.0/1 mM DTT) Ni-NTA resin (Qiagen) and rotated slowly for 1 h at 4°C. The slurry was poured into a column and the settled resin was washed over-night with 400 mL of 50 mM Tris at pH 8.0/1 mM DTT. SMAC protein was then eluted with a 200 mL linear gradient of 100 mL of 50 mM Tris at pH 8.0/1 mM DTT to 100 mL of 50 mM Tris at pH 8.0/1 mM DTT/250 mM imidazole. Fractions were scanned at OD_{280} to locate eluted protein, pooled, and frozen on dry ice/ethanol and stored at -80°C.

1. Krajewska M, et al. (2005) Tumor-associated alterations in caspase-14 expression in epithelial malignancies. *Clin* Cancer Res 11:5462–5471.
2. Takahashi R, et al. (1998) A single BIR domain of XIAP sufficient for inhibiting caspases. *J Biol Chem* 273:7787–7790.
3. Marusawa H, et al. (2003) HBXIP functions as a cofactor of survivin in apoptosis suppression. *EMBO J* 22:2729–2740.
4. Le Negrate G, et al. (2008) *Salmonella* secreted factor L deubiquitinase of *Salmonella typhimurium* inhibits NF-kappaB, suppresses IkappaBalpha ubiquitination and modulates innate immune responses. *J Immunol* 180:5045–5056.
5. Du C, Fang M, Li Y, Li L, Wang X (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102:33–42.

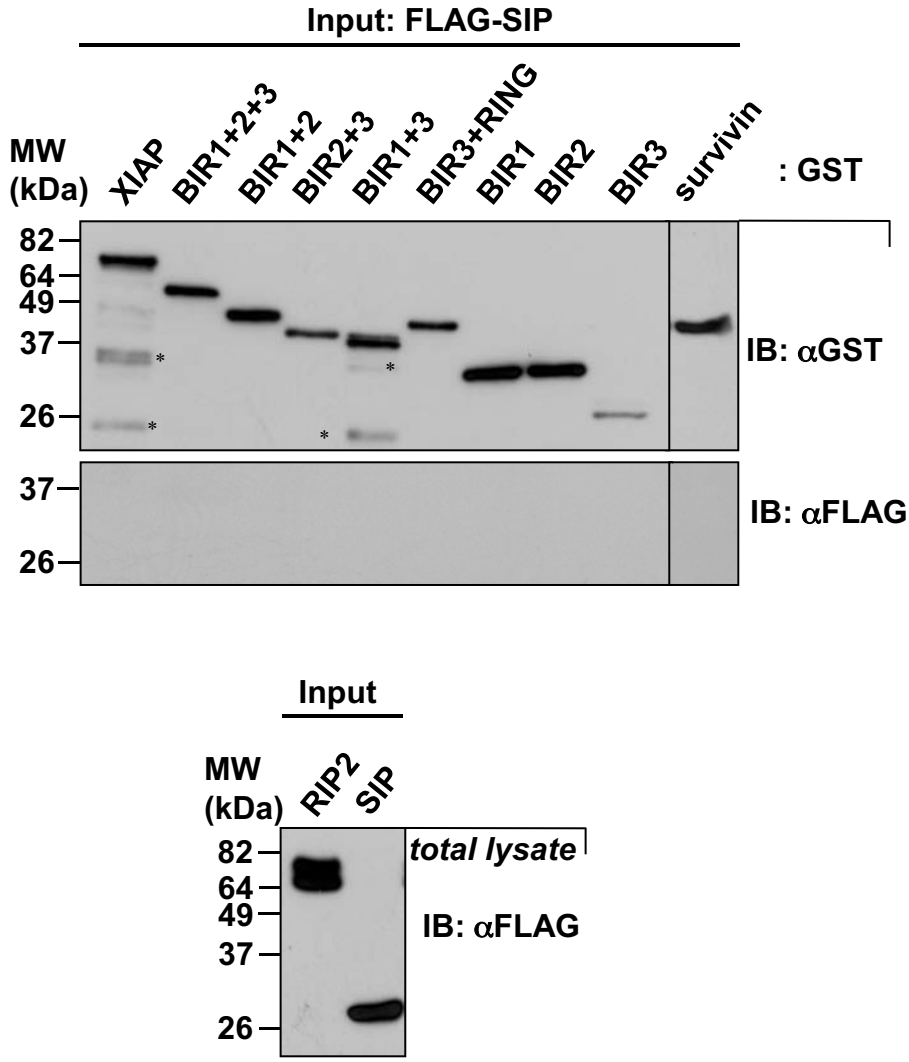

Fig. S1. XIAP does not bind to SIP. As a control for specificity of XIAP binding to RIP2, lysates of transfected HEK293T cells expressing FLAG-SIP were incubated overnight with recombinant GST-XIAP, GST-XIAP mutants, or GST-Survivin immobilized on glutathione-Sepharose. Beads were washed, boiled in Laemmli sample buffer, centrifuged and the supernatants were analyzed by SDS/PAGE/immunoblotting using mouse monoclonal anti-GST and peroxidase linked anti-FLAG antibodies. Asterisks denote nonspecific bands.

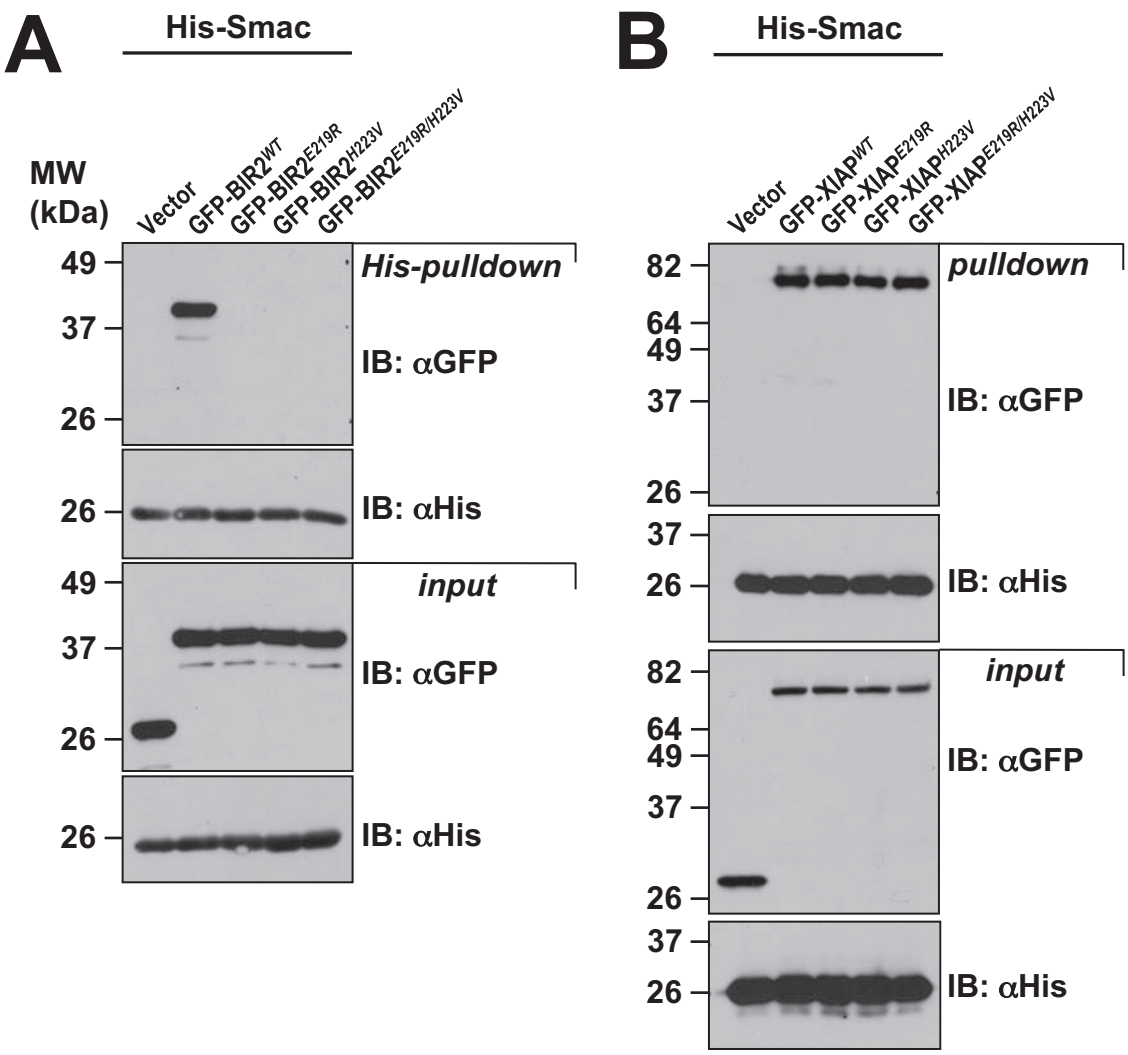

Fig. S2. Analysis of SMAC protein binding to XIAP mutants. (*A*) Lysates of transfected HEK293T cells expressing (*A*) GFP-BIR2^{WT}, GFP-BIR2^{E219R}, GFP-BIR2^{H223V}, GFP-BIR2$^{E219R/H223V}$ or (*B*) full-length GFP-XIAP^{WT}, GFP-XIAP^{E219R}, GFP-XIAP^{H223V}, GFP-XIAP$^{E219R/H223V}$ or empty GFP-plasmid were incubated with His-6-SMAC protein. Lysates were then incubated with Ni-NTA-Agarose with gentle rotation overnight at 4°C. Beads were washed, boiled in Laemmli sample buffer, centrifuged, and the supernatants were subjected to immunoblotting anti-GFP and anti-His-6-tag antibodies.

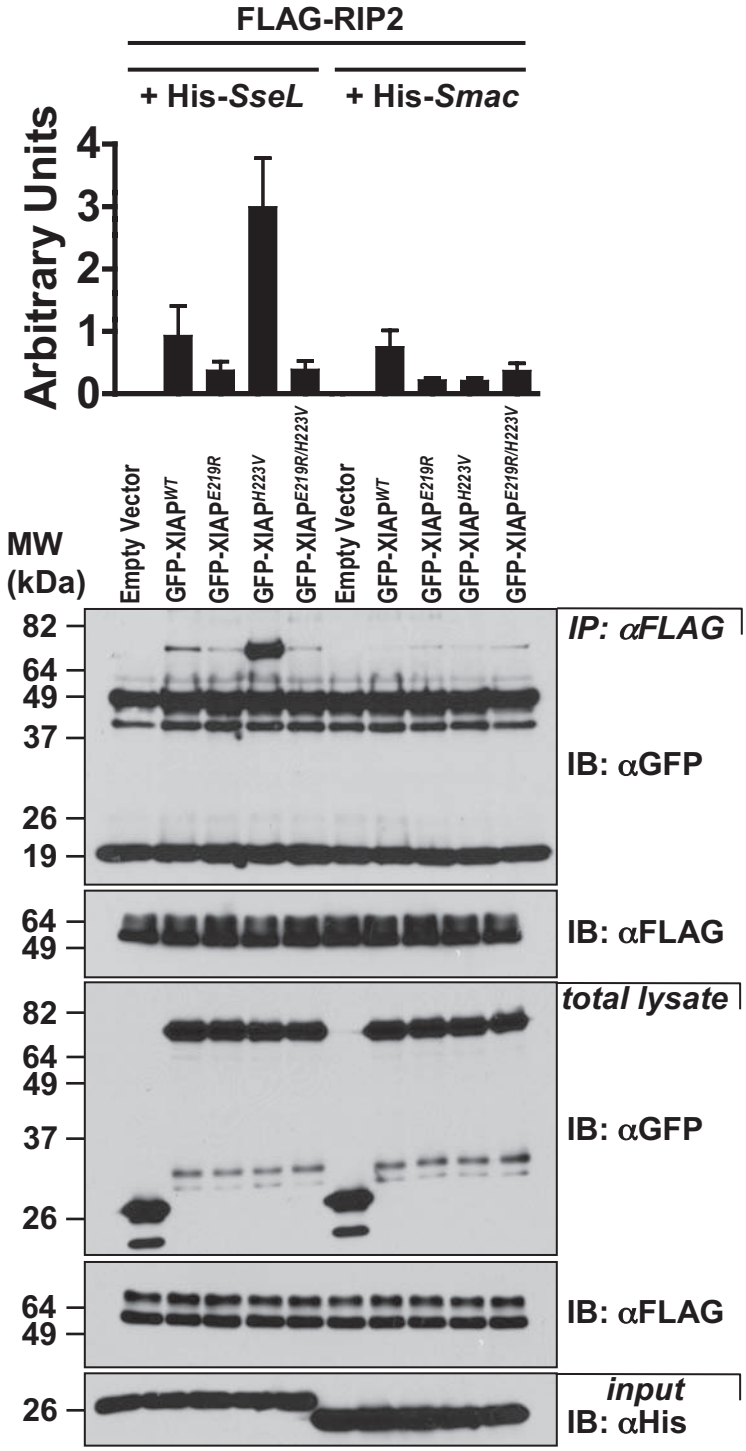

Fig. S3. SMAC protein blocks binding of XIAP to RIP2. HEK293T cells were transfected with plasmids encoding FLAG-RIP2 and GFP-XIAP, GFP-XIAP mutants or GFP. After 24 h, lysates were prepared (1 mg) and 2 μg recombinant His-6-SMAC or His-6-SseI was added along with anti-FLAG-antibody. Washed anti-FLAG immunoprecipitates were analyzed by immunoblotting and results were quantified as above (mean $\pm$ SD; $n = 3$).

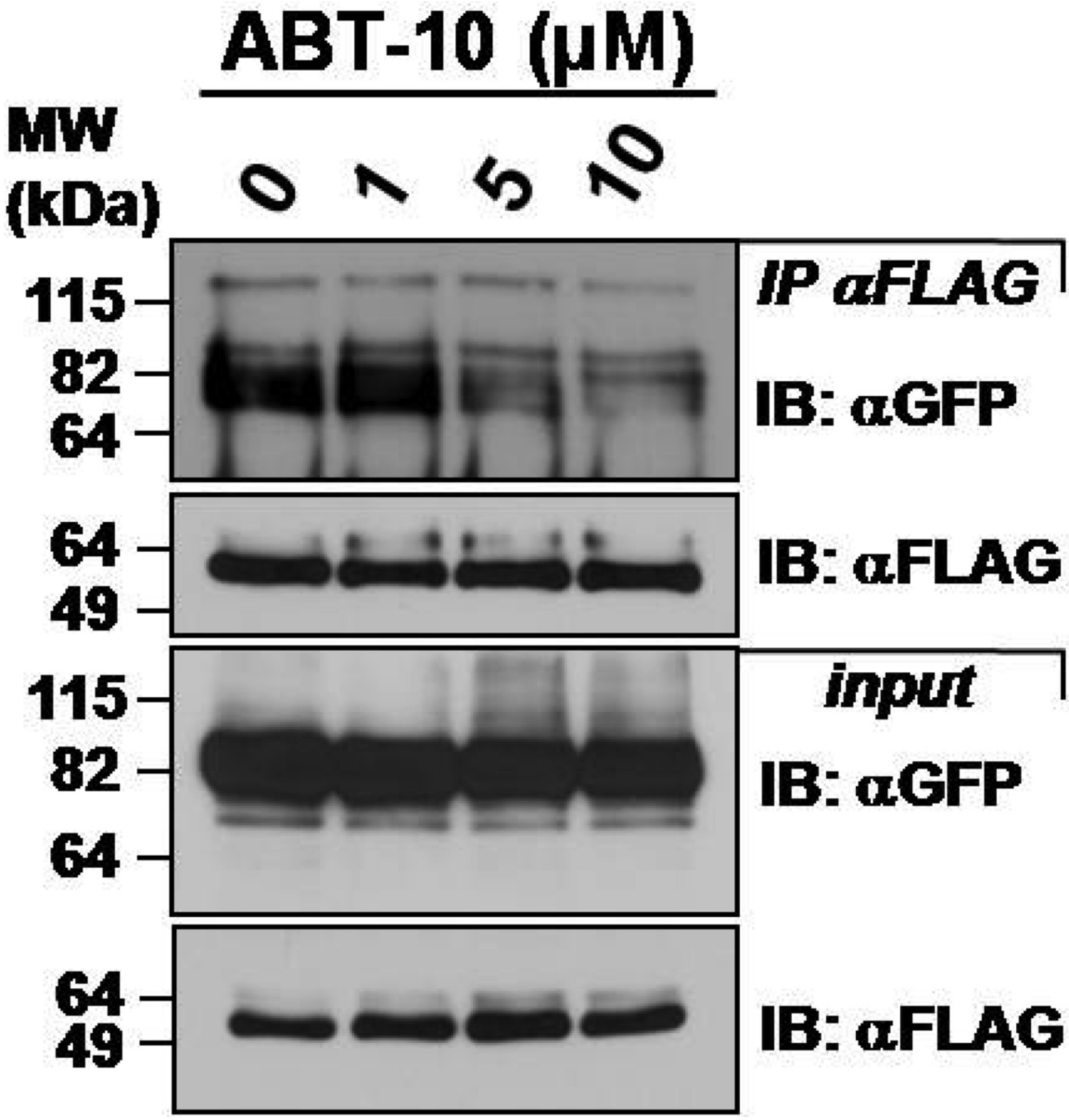

Fig. S4. *S*MAC-mimic compound inhibits RIP2/XIAP interaction in cells. HEK293T cells were co-transfected with plasmids encoding FLAG-XIAP and GFP-RIP2. SMAC-mimicking compound ABT-10 was added to cultures after 6 h. Lysates were prepared after 24 h and subjected to immunoprecipitation using monoclonal anti-FLAG antibody. Immunoprecipitates and lysates (50 μg) (''input'') were analyzed by immunoblotting as above. with indicated antibodies.

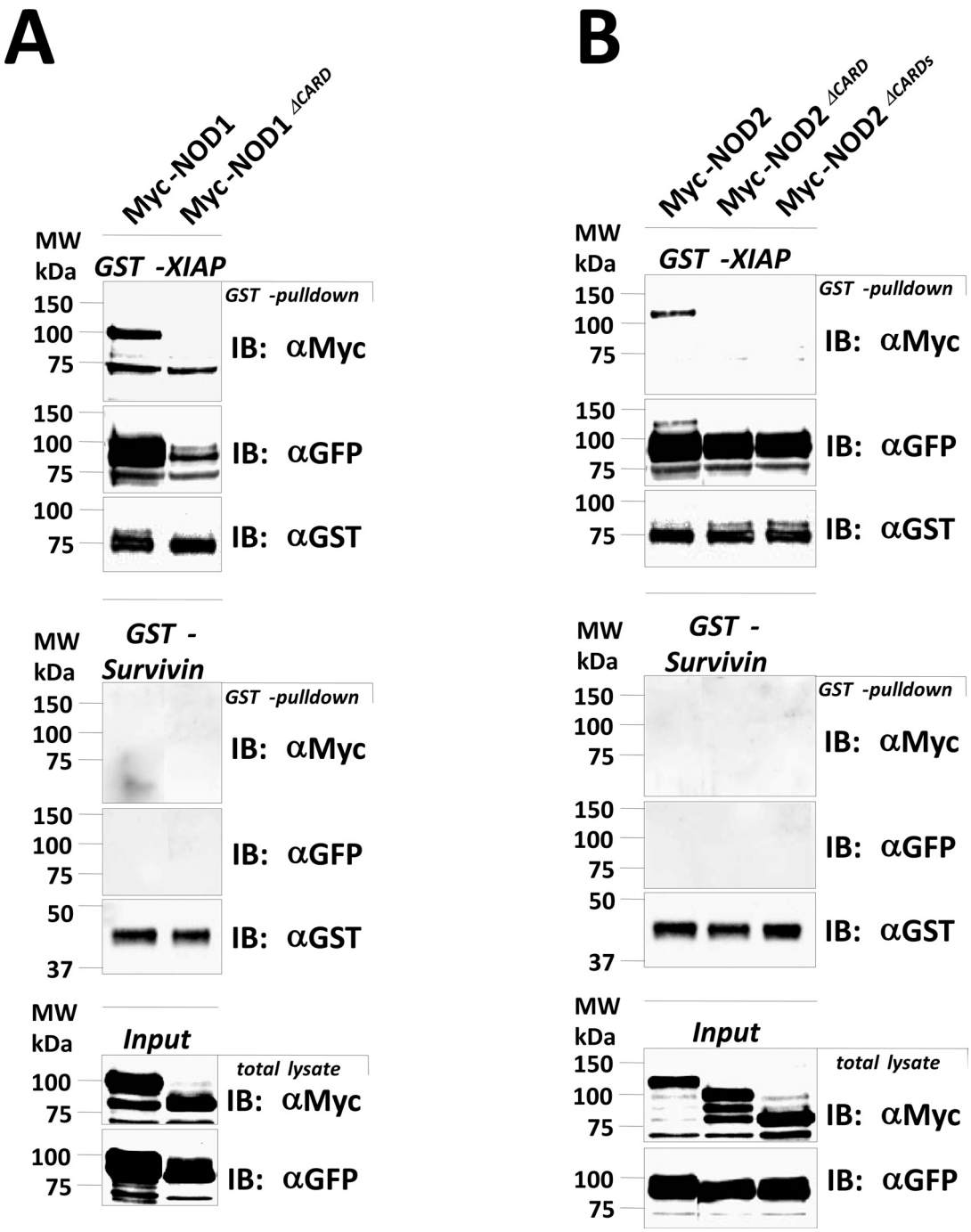

Fig. S5. CARD domains are required for association of XIAP with NOD1 and NOD2. HEK293T cells were transfected with plasmids encoding GFP-RIP2 along with (*A*) Myc-tagged NOD1 or NOD1ΔCARD or with (*B*) NOD2, NOD2ΔCARD, or NOD2ΔCARDs. Cell lysates were prepared after 24 h (1 mg) and GST pull-down assays were performed using either GST-XIAP *(Top)* or GST-Survivin (*Bottom*). Glutathione-Sepharose beads were analyzed by SDS/PAGE/immunoblotting as above. An aliquot of lysates (input) was analyzed directly by immunoblotting.