Supporting Information

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SI Text

Immunoblotting and Immunoprecipitations. Total cell lysis buffer (50 mM Tris-HCl; 150 mM NaCl; 1% Triton X-100; 1% SDS) was added directly to the culture wells, and the lysates were "sheared" using a 28-g insulin syringe. Protein content was determined using the Lowry assay (Bio-Rad Laboratories). Total cell lysates were prepared in SDS sample buffer, and 5–50 μg of total protein was separated by SDS-PAGE on 10% Bis-Tris gels and transferred to nitrocellulose membranes. Membranes were probed with primary antibodies diluted in 5% skim milk powder (SMP) overnight at 4°C, followed by HRP-conjugated secondary antibodies diluted in 5% SMP for 1 h at room temperature. Membranes were then treated with ECL reagent, exposed to x-ray film and developed (Kodak X-Omat 2000A). For endogenous immunoprecipitations, $\approx 1 \times 10^7$ cells were washed twice with PBS and lysed in buffer (20 mM Tris, pH 7.5; 0.2% Nonidet P-40; 10% glycerol; 150 mM NaCl) on ice for 15 min. TNF-R1 and TRAF2 were immunoprecipitated overnight at 4°C with 1 μg of antibody, and complexes were recovered with 50 μl of protein-G-agarose slurry. Protein complexes were separated and individual proteins denatured in SDS sample buffer.

Luciferase Activity Analyses. To measure luciferase activity, cells were lysed directly on plates in lysis buffer (25 mM Gly-Gly; 15 mM $MgSO_4$; 1% Triton X-100; 1 mM DTT). 10 μl of cell lysate was added to 90 μl of assay buffer (80 mM Gly-Gly; 12 mM $MgSO_4$; 16 mM KPO_4; 2 mM ATP; 2 mM DTT) and 100 μl of Luciferin Reagent (0.1 mM Luciferin; 90 mM Gly-Gly; 15 mM $MgSO_4$) and read, in triplicate, on a luminometer.

Viability Assay. Cell viability was assessed using the WST viability assay, as outlined in the manufacturer's instructions (Promega).

Primary Cell Extractions. For primary myoblast and fibroblast extraction, 4-week-old mice were killed by cervical dislocation, and lower limb muscles were carefully dissected away from the bone. Muscle samples were minced with scissors and digested with collagenase-Dispase solution (500 mg Collagenase B [Roche]; 50 ml Dispase II (Roche); 250 μl 0.5 M $CaCl_2$). Myoblasts were filtered through a 0.22-μM membrane, spun at $300 \times g$ for 5 min, resuspended in Hams Complete Media (HCM; Ham's F-10 Media supplemented with 20 FCS, 2.5 ng/ml bovine FGF, penicillin, and streptomycin) and enriched by "preplating" onto a standard 100-mm culture plate for 1 h. Nonadherent myoblasts were transferred to a 60-mm collagen-coated plate in HCM, whereas the adherent fibroblasts were grown in DMEM supplemented with 10% FCS. After approximately 48 h in culture, the myoblasts were preplated again for 20 min. It took approximately 10–14 days to generate enough myoblasts for experimentation.

For primary hepatocyte extraction, 3-month-old mice were anesthetized with Somnitol, and the liver was perfused first with EGTA and then with collagenase (type IV; Sigma). The liver was carefully dissected out, and hepatocytes were carefully released into a Petri dish containing Williams Media E (Invitrogen) supplemented with L-Glutamine (Gibco) by gentle agitation with fine forceps. The hepatocytes were then filtered through a nitex membrane and centrifuged at $250 \times g$ for 5 min. The cell pellet was resuspended in 30 ml of Williams Media E, carefully triturated, and centrifuged again at $250 \times g$ for 5 min. The cell pellet was then resuspended in 20 ml of Complete Media (Williams Media E supplemented with 10% FCS, penicillin, and streptomycin), counted on a hemocytometer, and 2.5×10^5 viable cells were seeded onto 35-mm plates coated with fibronectin. Two hours later the cells were washed, and 2 ml of fresh complete media was added. The cells were used for experiments the following day.

SiRNA-Mediated Knock-Down. The following sequences were used for all experiments: *non-targeting*: GGA UCC UUG ACA AUA CCA A[dT][dT] and UUG GUA UUG UCA AGG AUC C[dT][dT]; *cIAP1*: GCA AGU GCU GGA UUC UAU U[dT][dT] and AAU AGA AUC CAG CAC UUG C[dT][dT]; *cIAP2*: GCA CAA GUC CCU ACC ACU U[dT][dT] and AAG UGG UAG GGA CUU GUG C[dT][dT]; *XIAP*: GGA CAU CCU CAG UUA ACA A[dT][dT] and UUG UUA ACU GAG GAU GUC C[dT][dT].

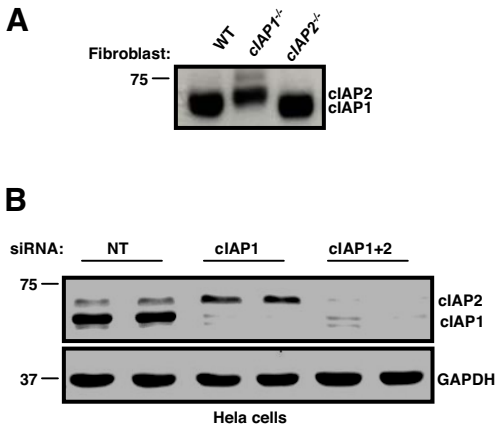

Fig. S1. Characterization of our rabbit anti-rat IAP1 antibody. (*A*) Primary fibroblasts were extracted from WT as well as cIAP1 and 2 null mice, cultured, and total cell lysates were separated on an 8% gel and immunoblotted for cIAP1 and 2 using our anti-RIAP1 antibody. (*B*) HeLa cells were cultured and treated with siRNA targeting either cIAP1 ± 2, and total protein lysates were separated on a 10% gel and immunoblotted using anti-RIAP1. NT, nontargeting.

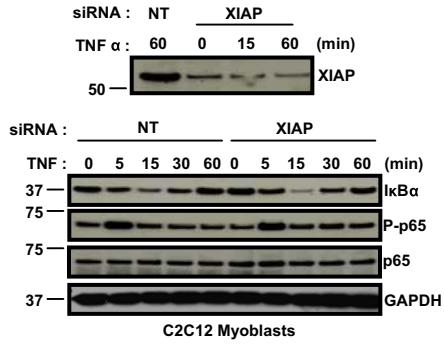

Fig. S2. XIAP is not involved in TNFα-mediated NF-κB activation. C2C12 myoblasts were treated with nontargeting (NT) or XIAP-targeting siRNA for 24 h before TNFα treatment. Protein lysates were collected and immunoblotted for the indicated proteins.

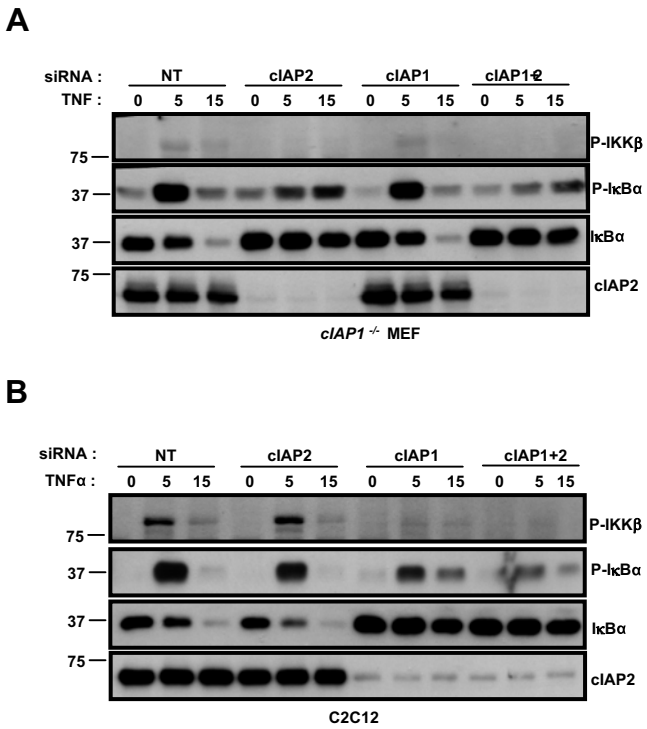

Fig. S3. Either cIAP1 or 2 is required for the phosphorylation of IKKβ and IκBα. (*A*) cIAP1 null MEFs were treated with siRNA for 24 h, and protein levels were assessed at the indicated time points after TNFα treatment. (*B*) C2C12 myoblasts were treated with siRNA for 24 h, and protein levels were assessed at the indicated time points after TNFα treatment. NT, nontargeting.

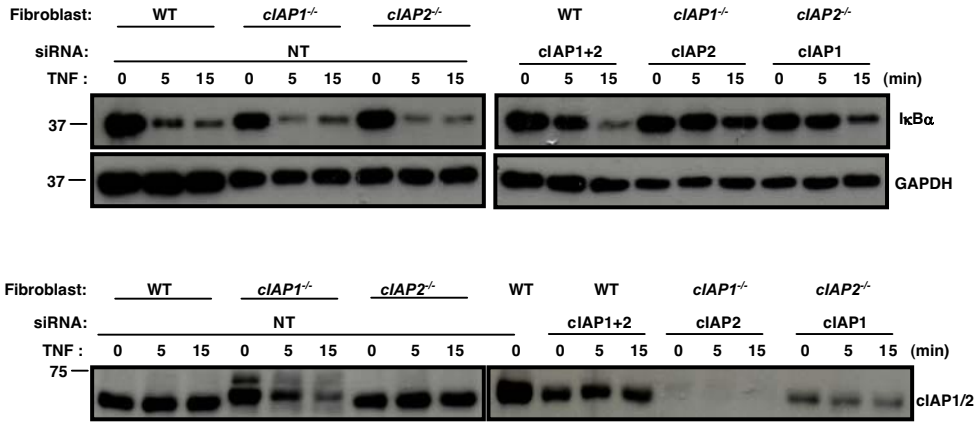

Fig. S4. cIAP1 and 2 redundantly regulate TNFα-mediated IκBα degradation in primary fibroblasts. Primary fibroblasts were extracted from skeletal muscle of WT as well as cIAP1 and 2 null mice, cultured, and treated with the indicated siRNA duplexes. 24 h after siRNA-mediated knockdown, the cells were treated with TNFα (10 ng/ml), and total cell lysates were collected and immunoblots performed. NT, nontargeting.

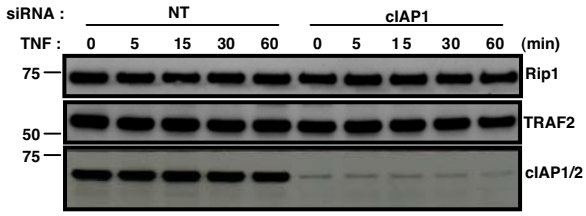

Fig. S5. Rip1 and TRAF2 levels remain unchanged in response to TNFα. C2C12 myoblasts were treated with nontargeting (NT) or cIAP1-targeting siRNA for 24 h. TNFα was given for the indicated time points and protein levels assessed by immunoblot.

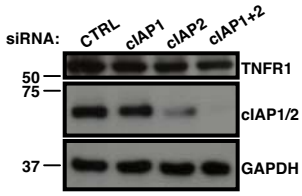

Fig. S6. cIAP1 and 2 knockdown does not alter TNF-R1 protein levels. WT MEFs were grown and treated with siRNA for 24 h, after which time total cell lysates were collected and immunoblotted. CTRL, control.

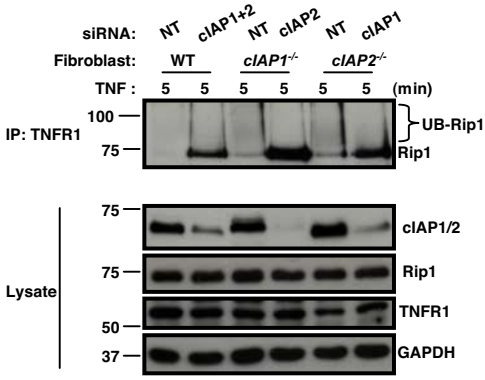

Fig. S7. TNFα-mediated ubiquitination of Rip1 requires either cIAP1 or 2 in primary fibroblasts. Primary fibroblasts were extracted from skeletal muscle, cultured, and treated with siRNA. 24 h after knockdown, the cells were treated with TNFα (10 ng/ml) for 5 min, and TNF-R1 immunoprecipitations (IP) followed by the indicated immunoblots were performed.

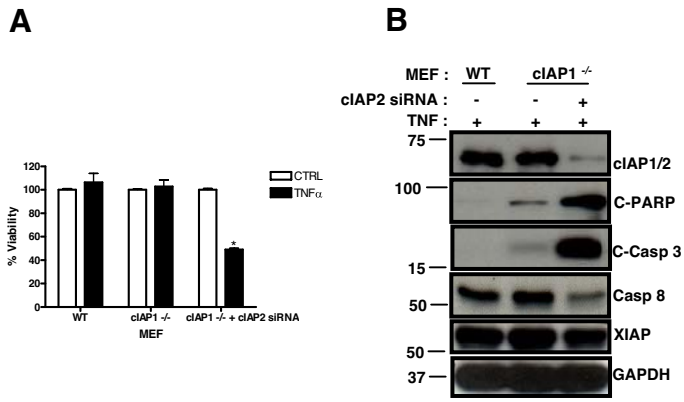

Fig. S8. Either cIAP1 or 2 is required to protect MEF cells against TNFα-mediated apoptosis. (*A*) MEFs were treated with siRNA for 24 h, followed by TNFα for 24 h, and cell viability was measured. Data are expressed as % viability $\pm$ SD relative to no TNF treatment controls (set at 100%), $n = 4$ per condition. (*B*) Protein lysates from MEFS treated with siRNA (24 h) followed by TNFα (24 h) were immunoblotted for various apoptotic proteins.