IAPs limit activation of RIP kinases by TNF receptor 1 during development

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Inhibitor of apoptosis (IAP) proteins cIAP1, cIAP2, and XIAP (X-linked IAP) regulate apoptosis and cytokine receptor signalling, but their overlapping functions make it difficult to distinguish their individual roles. To do so, we deleted the genes for IAPs separately and in combination. While lack of any one of the IAPs produced no overt phenotype in mice, deletion of cIap1 with cIap2 or XIap resulted in mid-embryonic lethality. In contrast, Xiap−/−cIap2−/−mice were viable. The death of cIap2−/−cIap1−/− double mutants was rescued to birth by deletion of tumour necrosis factor (TNF) receptor 1, but not TNFR2 genes. Remarkably, hemizygosity for receptor-interacting protein kinase 1 (Ripk1) allowed Xiap−/−cIap1−/− double mutants to survive past birth, and prolonged cIap2−/−cIap1−/− embryonic survival. Similarly, deletion of Ripk3 was able to rescue the mid-gestation defect of cIap2−/−cIap1−/− embryos, as these embryos survived to E15.5. cIAPs are therefore required during development to limit activity of RIP kinases in the TNF receptor 1 signalling pathway.

The EMBO Journal (2012) 31, 1679–1691. doi:10.1038/emboj.2012.18; Published online 10 February 2012

Subject Categories: signal transduction; differentiation & death
Keywords: apoptosis; IAP; NF-κB; RIP kinase; TNF

Introduction

The cellular inhibitor of apoptosis (IAP) proteins cIAP1 (Birc2), cIAP2 (Birc3), and XIAP (X-linked IAP, Birc4) were initially identified by their similarity to baculoviral IAP genes, and, in the case of cIAP1 and cIAP2, also because they bound to tumour necrosis factor (TNF) receptor-associated proteins (TRAFs) 1 and 2 (Rothe et al, 1995; Duckett et al, 1996; Liston et al, 1996; Uren et al, 1996). Cellular IAPs and XIAP are very similar proteins and belong to the BIRC (baculoviral IAP repeat containing) protein family, as they all bear three BIR domains, a ubiquitin-associated domain and a carboxy-termin

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Figure 1  Deletion of cIap1 plus cIap2, or cIap1 plus Xiap, results in embryonic lethality at ~E10. (A) Generation of a genetically modified cIap locus. Exons 2 and 3 of cIap2 were flanked by frt sites and a neomycin phosphotransferase expression cassette and exon 1 of cIap1 was flanked by loxP sites with an hygromycin cassette. (B) Incidence of genotypes of weaned mice or embryos derived from indicated intercrossed mice; * represents embryos without heartbeat or reabsorbed and # represents embryos with minimal heart contractile activity. Expected numbers of each group shown in brackets (based on Mendelian ratios). (C) Detection of cIap1, cIap2 and Xiap expression during development (E10.5) by in situ hybridization as compared with sense control probe. (D) Expression of IAPs during mouse development detected by western blotting. A WT E10.5 embryo was lysed in DISC lysis buffer supplemented with protease inhibitors, NEM and Pefabloc. IAPs were trapped using biotinylated SM and precipitated with streptavidin beads. Lysates (lys), unbound fractions (unb), glycine elutions (ge), and boiled beads (bb) were separated using SDS/PAGE and blotted with antibodies to cIAP1, cIAP2, and XIAP. 293T cells transfected with a plasmid encoding mouse cIAP2ΔC6 were used as a positive control for detection of cIAP2. See also Supplementary Figure S1. Figure source data can be found in Supplementary data.
were subsequently crossed with mice bearing alleles. Both the copies of the gene for and sufficient for viability. Similarly, mice bearing one or two genotypes were present in a ratio of 2 to 1 (Figure 1B; Supplementary Figure S1A), consistent with previous reports of independently generated clp2 or clp1-deficient mice (Conze et al., 2005; Conte et al., 2006). Among >200 progeny of 40 clp2+/−/clp1+/− intercrosses, no homozygous clp2−/−/clp1−/− mice were found, whereas double heterozygote and wild-type (WT) genotypes were present in a ratio of 2 to 1 (Figure 1B; Supplementary Figure S1B). This indicates that the absence of both clp1 and clp2 results in lethality at or before birth. Therefore, at least for development, clp1 and clp2 are redundant, but presence of at least one allele of either gene is required and sufficient for viability. Similarly, mice bearing one or two copies of the gene for clp1 (i.e., Xiap−/−/clp1+/− and Xiap−/−/clp1−/− mice, respectively) were found in a ratio of 2 to 1, indicating lethality of embryos lacking both clp1 and Xiap (Figure 1B). In contrast, mice that lacked genes for both XIAP and clp2 were viable and fertile without any obvious phenotype (Figure 8, Supplementary Figure S1A, and data not shown). Together, these results show that clp1 is sufficient for development in the absence of clp2 and XIAP, but that presence of both clp2 and XIAP is needed if clp1 is absent.

All three IAPs are expressed during early mouse development

To determine the expression and distribution of the three IAPs during development, we analysed clp1, clp2, and XIAP embryonic expression by in situ hybridization using sections of WT embryos from E9.5 to E15.5. Dark field photographs are shown in Figure 1C. Ubiquitous expression of clp1, clp2, and XIAP was observed at all stages, with the strongest expression being from E9.5 to E12.5. We also determined levels of IAP proteins by lysing whole E10.5 embryos, and immunoprecipitating using a biotinylated IAP antagonist/Smac mimic (SM) (compound A, TetraLogic Pharmaceuticals) (Vince et al., 2007). Consistent with the in situ hybridization data, all three IAPs could be pulled down with the biotinylated compound at E10.5 (Figure 1D and data not shown). These results showed that clp1, clp2, and XIAP are all expressed during mouse development, and of these three proteins, only clp1 is sufficient to act alone to allow normal development.

Cardiovascular defect in clp2−/− clp1−/− and Xiap−/− clp1−/− DKO embryos

To determine the cause of death of the double IAP-deficient embryos, we examined them from E9.5 to E14.5. At each stage, no differences were observed between WT, double heterozygote clp2+/−/clp1+/−, Xiap+/−/clp1+/− and Xiap−/−/clp1−/− embryos. At E9.5, all clp2−/−/clp1−/− and Xiap−/−/clp1−/− embryos were viable and could not be distinguished by overall morphology from WT or Xiap−/−/embryos. However, between E10.5 and E11.5, we observed that the pericardial cavities of double homozygous mutant embryos were frequently swollen and filled with blood (Figure 2A and B). In most cases, mutant embryos were found with minimal or no contractile activity of the heart. Placenta appeared normal by gross morphology and histology (data not shown). Transverse serial sections of the mutant embryos showed that most tissues appeared normal, except for the heart, which showed sporadic discontinuities, usually in the atrial chamber. These discontinuities were often in association with pyknotic nuclei, suggestive of apoptosis (Figure 2C and D). However, elsewhere in the embryos we saw no major differences in the overall pattern of apoptosis between WT, Xiap−/−, clp2−/−clp1−/− and Xiap−/−clp1−/− embryos (head and eye sections are shown as examples in Figure 2E and F). We conclude that the lethal phenotype of clp2−/− clp1−/− and Xiap−/−clp1−/− embryos is not due to generalized growth retardation or widespread failure to control cell death, but is due to haemorrhages and cardiovascular failure at ~E10.5.

In mouse embryonic fibroblasts, only clp1 is necessary, and provides sufficient IAP function, for normal induction of canonical NF-kB by TNF

Previous studies using clp1−/− mouse embryonic fibroblasts (MEFs) and siRNA have shown that clp1 is required for TNF to efficiently activate canonical p65/RelA NF-kB in cultured cells (Mahoney et al., 2008; Varfolomeev et al., 2008). To further investigate the roles of the IAPs in NF-kB activation, we generated MEFs from clp2−/−clp1−/−, Xiap−/−clp1−/− and Xiap−/−clp2−/− DKO embryos and immortalized them with SV40 large T antigen. We used western blotting to determine the levels of the three IAPs in each DKO MEF line. As expected, clp1 was absent from the clp2−/−clp1−/− and Xiap−/−clp1−/− lines, and was present at similar levels in WT and Xiap−/−clp2−/− lines (Figure 3A). Similarly, XIAP was absent from the Xiap−/−clp1−/− and Xiap−/−clp2−/− lines, and present in the clp2−/−clp1−/− line. In contrast, clp2 was not only absent from clp2−/− lines, but was also not detectable in WT MEFs or Xiap−/−clp1−/− MEFs, presumably reflecting either low levels of transcription in unstimulated MEFs, protein instability, or low sensitivity of the antibody. Because the same antibody readily detected clp2 in embryos that was immunoprecipitated by a biotinylated SM compound, as well as overexpressed clp2 (Figure 1D), we believe unstimulated MEFs normally contain very low levels of clp2.

Previously, we have found elevated levels of NIK, and processing of NF-kB2 to p52, in unstimulated clp1−/− and clp2−/−clp1−/− MEFs, suggesting that a normal function of clp1 is to promote degradation of NIK in cells that have not been treated with cytokine (Vince et al., 2007; Feltham et al., 2010). Consistent with these observations, we found high levels of NIK and activation of non-canonical NF-kB and processed p100 NF-kB2 in untreated Xiap−/−clp1−/− MEFs (Figure 3A). In contrast, the amounts of NIK, p100 and p52 in Xiap−/−clp2−/− MEFs were similar to those in WT MEFs. Therefore, the presence of clp1 is both necessary and sufficient to cause degradation of NIK and prevent spontaneous activation of non-canonical NF-kB in untreated cells. Furthermore, the presence of clp1 was also both necessary and sufficient for TNF to promote phosphorylation ofcanonical p65/RelA NF-kB, and trigger degradation of IkBz (Figure 3B). These results also show that, on its own, XIAP is not able to activate canonical NF-kB following TNF stimulation of MEFs, and that clp2 is also not able, or is
not present at high enough levels, to signal activation of canonical NF-κB, confirming that cIAP1 is the most important IAP for regulation of canonical and non-canonical NF-κB both in MEFs and for development.

Lack of cIAP1 sensitizes MEFs to TNF-induced apoptosis, but loss of both cIAP2 and XIAP does not

Low levels of cIAP1, whether due to gene deletion or treatment with SM, sensitized MEFs to TNF-induced cell death (Varfolomeev et al., 2007; Vince et al., 2007). Consistent with this, WT MEFs were not killed by TNF, but the cells died when TNF was combined with SM (Figure 4A). Compared with WT MEFs, those lacking both cIAP1 and cIAP2, or both cIAP1 and XIAP, were also much more sensitive to killing by CD95L (Fas ligand), or the topoisomerase II inhibitor etoposide, but not the DNA alkylating agent cisplatin (Figure 4A). Surprisingly, Xiap−/− cIap2−/− MEFs were similar to WT MEFs, because they were not killed by TNF alone, but could be significantly sensitized by addition of SM, which depletes the cells of cIAP1.

To investigate further the ability of cIAPs to regulate TNF-induced cell death, we complemented the cIap2−/− cIap1−/− and Xiap−/− cIap1−/− MEFs with WT cIAP1, EGFP-tagged cIAP1, Flag–cIAP2, or XIAP, expressed from a tamoxifen-inducible lentiviral vector. EGFP-tagged cIAP1 and XIAP proteins were functional because they bound TRAF2 and Smac/DIABLO, respectively (Supplementary Figure S2). Induction of cIAP1 or Flag–cIAP2 by 4-hydroxy-tamoxifen (4-HT) in cIap2−/− cIap1−/− MEFs, and induction of EGFP–cIAP1 in Xiap−/− cIap1−/− MEFs made cells almost as resistant to TNF-induced death as WT MEFs (Figure 4B and D). Even the low background levels of cIAPs expressed from the uninduced lentiviral vector (Figure 4C and E) gave partial protection against TNF killing (Figure 4B and D). Moreover, reconstitution with cIAP1 or XIAP was able to give partial protection to Xiap−/− cIap1−/− MEFs against CD95L-induced cell death, but cIAP1 seemed to be more potent than XIAP (Figure 4D). Similarly, overexpression of XIAP in cIap2−/− cIap1−/− MEFs provided no protection, and reconstitution of XIAP into Xiap−/− cIap1−/− MEFs provided only modest protection from killing by TNF (Figure 4B and D). These data demonstrate that unlike cIAP1, XIAP is neither
KO mice. Unlike the lacking cIAP1. SV40 large T antigen-immortalized MEFs derived from WT, Tnfr2 and the generated MEFs from two different TNF receptors in death induced by TNF, we (Figure 5A). To further analyse the relative contributions of the birth. We therefore generated no overt phenotype, but none of the cIap2 mice were found at weaning. In contrast, deletion of TNFR2 was not able to prolong survival of mice were found at weaning. In contrast, deletion of TNFR2 are all very sensitive to induction of apoptosis by TNF alone, and all show deficits in activation of canonical (p65/RelA) NF-kB in response to TNF, we hypothesized that deletion of TNFR1 would allow clap2−/−clap1−/− embryos to survive until birth. We therefore generated clap2−/−clap1−/− Tnfr1−/− triple KO mice. Unlike the clap2−/−clap1−/− embryos, most of the clap2−/−clap1−/− Tnfr1−/− pups were born alive (Figure 5A and B). Some of them survived until day 2 with no overt phenotype, but none of the clap2−/−clap1−/− Tnfr1−/− mice were found at weaning. In contrast, deletion of TNFR2 was not able to prolong survival of clap2−/−clap1−/− embryos (Figure 5A). To further analyse the relative contributions of the two different TNF receptors in death induced by TNF, we generated MEFs from clap2−/−clap1−/− Tnfr1−/− and clap2−/−clap1−/− Tnfr2−/− embryos and immortalized them with SV40 large T antigen. For comparison, we generated WT, Tnfr1 KO, and Tnfr2 KO MEFs. As expected, none except the clap2−/−clap1−/− Tnfr2−/− MEFs was killed by TNF alone (Figure 5C). Moreover, all MEF lines lacking Tnfr1 were resistant to TNF even in the presence of SM. These results demonstrate that death of clap2−/−clap1−/− MEFs in response to TNF, and death at E10.5 of clap2−/−clap1−/− embryos, are both dependent on the presence of TNFR1 but not TNFR2.

**TNF can activate both caspase-dependent and caspase-independent, RIPK1 kinase-dependent cell death mechanisms**

The amount of cell death induced by signalling through TNFR1 is greatly increased when IAPs are absent, in clap2−/−clap1−/− embryos as well as in MEFs, whether cIAPs are absent due to gene deletion or treatment with SM. To determine if TNF causes cell death via a caspase- or a RIPK1-dependent mechanism, we analysed the effect of adding a pan-caspase inhibitor, QVD-OPh (QVD) (Caserta et al., 2003), and a specific RIPK1 kinase inhibitor, necrostatin (Degterev et al., 2008). In all types of cells tested (WT, Xiap−/−clap2−/−, clap2−/−clap1−/−, Xiap−/−clap1−/−), TNF caused death when cIAP1 was genetically deleted, or was reduced by treatment with SM (Figure 6A). Moreover, in every case, TNF-induced cell death could be almost entirely blocked when QVD and necrostatin were added together (Figure 6A, black columns). When added alone, the degree of protection provided by necrostatin (Figure 6A, light grey columns) appeared to correlate with the amount of remaining cIAP1. In contrast, the extent of protection by QVD was greater when cIAP1 was genetically deleted than when it was reduced by SM treatment. These results suggest a larger role for caspasess, but a lesser role
for RIPK1 kinase activity, in TNF-induced death in the absence of cIAP1. Furthermore, in agreement with previous results (Haas et al., 2009), ubiquitylation of RIPK1 was reduced in the absence of both cIAP1 and cIAP2, but the amount of unmodified RIPK1 bound to the TNFR1 signalling complex was increased (Figure 6B). Findings were similar in XIap−/−clap1−/− cells, whereas normal levels of RIPK1 ubiquitylation were observed when only cIAP1 was present (i.e., in XIap−/−clap2−/− MEFs) (Figure 6B). These experiments indicate that in MEFs, TNF mainly activates a caspase-dependent death process, but provide some evidence, suggesting that it can also activate a caspase-independent, RIPK1 kinase-dependent death mechanism, which is more apparent when cIAP1 levels are reduced by SM, than when cIAP1 is eliminated by gene deletion.

clap2−/−clap1−/− E10.5 cardiac defects are absent if Ripk1 or Ripk3 genes are deleted, and heterozygosity for Ripk1 allows XIap−/−clap1−/− mice to survive embryogenesis

The ability of necrostatin to increase survival of MEFs with reduced levels of IAPs when exposed to TNF raised...
the possibility that the death of clp2/− clp1/− and Xiap/− clp1/− clp2/− DKO embryos at day E10.5 might also involve RIPK1 or RIPK3. To test this, we generated Xiap/− clp1/− Ripk1/−, clp2/− clp1/− Ripk1/−, and clp2/− clp1/− Ripk3/− mice. Xiap/− clp1/− Ripk1/− embryos were viable to E14.5, but by E15.5, they had died. Surprisingly, Xiap/− clp1/− Ripk1/− mice were born alive (Figure 6C). While most Xiap/− clp1/− Ripk1/− mice died within the first 10 days of birth, two runted males even survived for 6 weeks (Figure 6C; Supplementary Figure S3B and C). Unlike the clp2/− clp1/− embryos that never survive past E10.5, clp2/− clp1/− Ripk1/− and clp2/− clp1/− Ripk3/− embryos survived to E12.5, and clp2/− clp1/− Ripk1/− and clp2/− clp1/− Ripk3/− embryos survived to E15.5 (Figure 7A and C). Six out of seven clp2/− clp1/− Ripk1/− embryos were viable at E12.5 and three embryos were found dead at E15.5 (Figure 7B and D). Four clp2/− clp1/− Ripk3/− embryos were viable at E13.5 and three embryos were found dead around E15.5 (Figure 7C). These results show that the absence of RIPK3 or loss of only one allele of Ripk1 can overcome the mid-embryonic lethality in clp2/− clp1/− or Xiap/− clp1/− mutant mice, respectively. Moreover, the presence of clp2 allowed the further development of Xiap/− clp1/− Ripk1/− to birth, but Xiap was not sufficient for clp2/− clp1/− Ripk1/− embryos to survive beyond E15.5. Together with the data presented above, these results indicate that of the IAPs, only cIAP1 is sufficient on its own to prevent RIP kinases from acting in the TNF receptor signalling pathway to cause mid-embryonic lethality.

**Discussion**

Consistent with earlier reports, we found that mice lacking a single IAP, either clp1, clp2, or Xiap, are viable and fertile and exhibit relatively subtle phenotypes (Harlin et al., 2001; Conze et al., 2005; Conte et al., 2006). To study the functional redundancy of the IAPs, we examined compound IAP mutant
three IAPs are expressed, but cIAP2 protein is present at comparatively low levels. These low levels of cIAP2 protein, together with the easily detectable mRNA revealed by in situ hybridization, are consistent with cIAP2 having a shorter half-life than cIAP1, which might be due to an increased propensity for cIAP2 than cIAP1 to spontaneously dimerize, autoubiquitylate, and be degraded (Feltham et al., 2006). Under these conditions, the RIPK1 kinase inhibitor necrostatin and the caspase inhibitor QVD protect IAP gene deleted MEFs from killing by TNF. MEFs derived from null, lacZ mutant for their single cIAP gene (Santoro et al., 2007). In marked contrast, clp2–/–clp1–/– and Xiap–/–clap1–/– DKO mice died in utero after 10 days of development.

The phenotype of the compound mutants implied that all three IAPs are expressed during early embryogenesis. However, Shafey et al. (2006), who used clp2 null, lacZ reporter mice, concluded that clp2 was not expressed before E11.5. To resolve this, we performed immunoprecipitations using biotinylated SM, and analysed them by western blot. Using this technique, we were able to detect cIAP2 from E10.5, which suggests that at this stage of embryogenesis all three IAPs are expressed, but cIAP2 protein is present at comparatively low levels. These low levels of cIAP2 protein, together with the easily detectable mRNA revealed by in situ hybridization, are consistent with cIAP2 having a shorter half-life than cIAP1, which might be due to an increased propensity for cIAP2 than cIAP1 to spontaneously dimerize, autoubiquitylate, and be degraded (Feltham et al., 2011).

The double IAPs-deficient embryos and placentas appeared normal with the exception of the heart. The appearance of these embryos bore similarities to zebrafish appearing normal with the exception of the heart. The appearance of these embryos bore similarities to zebrafish...
by severe haemorrhage and vascular regression during development. A similar phenotype is also seen in mouse embryos lacking other genes in the death receptor signalling pathway (Figure 8A), such as Fadd, Casp8, and Flip mutants (Varfolomeev et al., 1998; Yeh et al., 1998, 2000). This raised the possibility that there might be common mechanisms for the developmental deaths of the embryos, even though some of the proteins (e.g., FADD and caspase 8) are generally thought to be pro-apoptotic, whereas others (e.g., FLIP and IAPs) are generally thought to promote survival or inhibit apoptosis. Interestingly, apoptosis (as revealed by TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling) staining) was unremarkable in the clap2+/−/clap1+/−/Ripk1−/− embryos, just as it was in the Fadd−/−, Casp8−/−, and Flip−/− mutants, raising the possibility that some other cell death process, such as necroptosis (Hitomi et al., 2008) might be involved.

Depending on the cell type and environment, TNFR1 signalling can result in a variety of responses in living cells, or death of cells by apoptosis or necroptosis. We found that in untreated cells, cIAP1 played a much more important role than cIAP2 and XIAP in limiting levels of NIK, and cIAP1 was the most critical IAP for activating canonical p65/RelA NF-κB to prevent TNF-induced cell death. In MEFs, only cIAP1 was necessary and sufficient to stop cells dying when they were exposed to TNF, or to allow TNF to rapidly activate p65/RelA NF-κB. cIAP1 was necessary and sufficient to cause degradation of NIK, and prevent the spontaneous activation of the non-canonical p100/p52 NF-κB2 pathway. In contrast, in the absence of cIAP1, neither cIAP2 nor XIAP allowed TNF to activate NF-κB.

In MEFs, TNF mainly activated a caspase-dependent death process, but it could also activate a caspase-independent, RIPK1 kinase-dependent death mechanism. The differences in the ability of QVD and necrostatin to block TNF-induced cell death in cIAP1 gene deleted MEFs compared with MEFs depleted of cIAP1 by SM suggest a complex interaction between cIAP1 and RIPK1. One possibility is that the amount of ubiquitilation of RIPK1 by cIAP1 determines the outcome following TNF treatment. In normal cells, TNF triggers cIAP1 to poly-ubiquitylate RIPK1, which efficiently activates p65/RelA NF-κB, but RIPK1 is then degraded and no cell death

Figure 7 Prevention of E10.5 lethality of clap2−/−/clap1−/− embryos by deletion of Ripk1 or Ripk3 genes. (A) Incidence of genotypes of born pups or embryos derived from intercrosses of clap2−/−/clap1−/−/Ripk1−/−/− mice. (B) Representative photographs of E12.5 embryos from a clap2−/−/clap1−/−/Ripk1−/−/− male cross. (C) Incidence of genotypes of embryos derived from crosses of clap2−/−/clap1−/−/Ripk3−/−/− females with clap2−/−/clap1−/−/Ripk3−/−− males. In (A, C), * represents embryos without heartbeat or reabsorbed and # represents embryos alive but with a small liver. (D) Representative photographs of E13.5 and E14.5 embryos from a clap2−/−/clap1−/−/Ripk3−/−− female with clap2−/+clap1+−/Ripk3−/− male cross.
occurs; if cIAP1 levels are reduced by SM, RIPK1 is ubiquitylated to a small extent, which increases its ability to cause cell death by a necrostatin blockable, caspase-independent mechanism; and if no cIAP1 is present, there is no RIPK1 ubiquitylation, and most of RIPK1 remains within the TNFR1 signalling complex to help recruit and activate caspase 8. Recently, deletion of RIPK1 was shown to rescue the embryonic lethality of Fadd-null embryos to birth (Zhang et al., 2011), and deletion of RIPK3, which is thought to act downstream of RIPK1 in the necroptotic pathway, was shown to allow Casp8 KO mice to develop into normal, fertile adults (Ch’en et al., 2011; Kaiser et al., 2011; Oberst et al., 2011) (Figure 8A). However, later in life, mice lacking both RIPK3 and caspase 8 develop a progressive lymphoaccumulative disease, a phenotype similar to Fas deficiency (Kaiser et al., 2011; Oberst et al., 2011). These experiments imply that FADD and caspase 8 are required to inhibit RIPK1 and/or RIPK3, which, when left uncontrolled, cause lethal developmental defects at E10.5. Our observations of the clp2−/− clp1−/− Ripk1+/− and clp2−/− clp1+/− Ripk3−/− mice suggest that cIAPs inhibit RIPK activity during development, presumably through ubiquitylation (Bertrand et al., 2008), whereas FADD and caspase 8 limit RIPKs by proteolysis (Pastorino et al., 1999; Feng et al., 2007). Furthermore, inhibition of RIPKs by

Figure 8 Roles of cIAPs in signalling and cell death during development. (A) Diagram of extent of viability of single, double, and triple gene deleted mice. (B) Speculative model to account for the phenotypes of gene deleted mice. Binding of TNF to TNFR1 triggers (large blue arrow) formation of complexes that can culminate in cell death by apoptosis or necroptosis, or lead to cell survival. Activating interactions are indicated with blue arrows; inhibitory interactions are indicated with orange lines; transcriptional induction is indicated with grey arrows. Merging of lines (such as those from Casp8 and FLIP to RIPK1, or IAPs and RIPK1 to p65/RelA) indicate proteins that can act together. cIAPs limit levels of NIK, and inhibit cell death mediated by RIPK1 and RIPK3, but cooperate with RIPK1 to activate p65/RelA. RIPK1 has both pro-death and pro-survival functions, by promoting necroptosis via RIPK3, apoptosis via FADD and Casp8, and cell survival via p65/RelA. Casp8 inhibits cell death by necrosis, but promotes cell death by apoptosis. FLIP inhibits cell death by both pathways. This model is not complete, and does not include other important proteins, such as TRADD, TRAF2, CYLD, A20, TAK1, HOL, HOIP, or Sharpin, just to name a few. For further details, see Discussion.
Regardless, the pathway activated by TNF during development of mouse embryos that lacked both copies of \(cIap1\) and \(cIap2\) (Vince et al., 2007). The embryonic lethality unless it is controlled by IAPs, or, IAPs signalling occurs as early as E10.5, and this will lead to surviving beyond birth. This was confirmed by deleting genes for RIPK1 or RIPK3. Early activation of RIPK-dependent embryonic lethality, rather, both by cIAP-dependent ubiquitylation, and by FADD/FLIP/caspase 8 and cIAP1/2 are required to control RIP kinases.

Promisingly, the absence of both \(cIap1\) and \(cIap2\) (through targeted mating and timed mating of \(cIap2^{FRT/FRT}\) and \(cIap1^{lox/lox}\) mice by gene targeting and gene expression, which were transcribed into BRUCE embryonic stem cells, which were inserted into C57BL6 mice (Koentgen et al., 1993; Hughes et al., 2007). The \(cIap2^{FRT/FRT}/cIap1^{lox/lox}\) mice were crossed to transgenic mice expressing FLPe recombinase to generate the \(cIap2^{FRT/FRT}/cIap1^{lox/lox}\) line, and to mice expressing Cre recombinase to generate the \(cIap2^{FRT/FRT}/cIap1^{lox/lox}\) line. The \(cIap2^{FRT/FRT}/cIap1^{lox/lox}\) mice were subsequently crossed to FLPe mice to generate the \(cIap2^{FRT/FRT}/cIap1^{lox/lox}\) mice then used for targeted matings. For all timed matings, a single male was housed with 2–5 females. The females were checked each morning for vaginal plugs. When a vaginal plug was detected, the female was separated from the male. The embryonic days were counted starting at E0.5 on the day the plug was detected. The embryos were taken on the embryonic days described throughout.

Materials and methods

Generation of \(cIap2^{FRT/FRT}\) and \(cIap1^{lox/lox}\) mice by gene targeting and timed mating

Animal experimental procedures were approved by the Animal Ethics Committee of La Trobe University. \(cIap1^{lox/lox}\) and \(cIap2^{FRT/FRT}\) constructs are described in Gardam et al. (2011). In order to generate the \(cIap2^{FRT/FRT}/cIap1^{lox/lox}\) mice, both constructs were inserted into BRUCE embryonic stem cells, which were derived from C57BL6 mice (Koentgen et al., 1993; Hughes et al., 2007). The \(cIap2^{FRT/FRT}/cIap1^{lox/lox}\) mice were crossed to transgenic mice expressing FLPe recombinase to generate the \(cIap2^{FRT/FRT}/cIap1^{lox/lox}\) line, and to mice expressing Cre recombinase to generate the \(cIap2^{FRT/FRT}/cIap1^{lox/lox}\) line. The \(cIap2^{FRT/FRT}/cIap1^{lox/lox}\) mice were subsequently crossed to FLPe mice to generate the \(cIap2^{FRT/FRT}/cIap1^{lox/lox}\) mice then used for targeted matings. For all timed matings, a single male was housed with 2–5 females. The females were checked each morning for vaginal plugs. When a vaginal plug was detected, the female was separated from the male. The embryonic days were counted starting at E0.5 on the day the plug was detected. The embryos were taken on the embryonic days described throughout.

In situ hybridization

In situ hybridization was performed as described previously (Thomas et al., 2000). Briefly, sections were dewaxed, rehydrated through graded concentrations of alcohol, incubated for 10 min at room temperature in 10 mg/ml proteinase K, fixed in 4% paraformaldehyde 10 min, then dehydrated through graded concentrations of alcohol. Sections were air dried, and hybridization solution containing 5 × 10⁶ counts/min/ml of intron transcribed cRNA probe was placed over the section. Slides were incubated overnight at 56°C and then washed as described (Hogan et al., 1986).

Histological analysis

Embryos were dissected between E10.5 and E11.5, fixed in 4% paraformaldehyde, paraffin embedded and serially sectioned (7 µm). Sections were stained with haematoxylin and eosin or used unstained for TUNEL and immunofluorescence, which were conducted as described previously (Thomas et al., 2000).

Generation of MEFs and lentiviral particles

Generation of MEFs has been previously described in detail (Vince et al., 2007). Briefly, primary MEFs were generated from E9.5/E10.5 (\(cIap2^{FRT/FRT}/cIap1^{lox/lox}\)), E10.5/E11.5 (\(Xiap^{−/−}/cIap1^{lox/lox}\)), E10.5/E11.5 (\(cIap2^{FRT/FRT}/cIap1^{lox/lox}\)), E11.5 (\(cIap2^{−/−}/cIap1^{−/−}/Xiap^{−/−}\)). E13.5 (\(WT, cIap2^{−/−}/cIap1^{−/−}/Tnfrf^{−/−}, Tnfr2^{−/−}\) and \(Xiap^{−/−}/cIap2^{−/−}/cIap1^{−/−}\) embryos, using standard protocols, and then infected with SV40 large T antigen expressing lentivirus to generate immortal cell lines.

Generation of lentiviral particles has recently been described (Vince et al., 2007; Geserick et al., 2009). Briefly, to generate \(cIap2^{−/−}/cIap1^{−/−}/Xiap^{−/−}/cIap2^{−/−}/cIap1^{−/−}\) MEFs expressing inducible mouse \(cIap1\), EGFP-\(cIap1\), Flag-c\(Iap2\), mouse \(XIAP\), or EGFP-XIAP, 293T cells were transfected with packaging constructs pCMVΔR8.2, VSV-G and the relevant lentiviral plasmid. DKO MEFs were infected with packaged lentivirus and polyclonal MEFs were obtained after puromycin (2–5 µg/ml; pF 5xUAS selection) and hygromycin selection (100–500 µg/ml; G418 selection). Cells were subsequently tested for expression of the respective proteins after 24 h of induction with 10 or 50 nM 4-HT.

Death assay

Cells were seeded on 12-well tissue culture plates at ~ 40% confluency and were allowed to adhere for 24 h. Cells were incubated in the presence or absence of 10 nM 4-OH-tamoxifen (4-HT) for 24 h, then 100 ng/ml Fc-TNF or 20 ng/ml Fc-CD95L (Bosson et al., 2006) plus or minus 500 nM SM (compound A), and/or 10 µM QVD were added to cells for 24 h and cell viability measured by propidium iodide (PI) exclusion using flow cytometry. In each sample, 10,000 events were measured and the percentages of PI-negative (viable) cells are shown.

Western blotting

Samples were lysed in DISC lysis buffer containing 1% Triton X-100 supplemented with protease inhibitor cocktail (Roche), N-Ethylmaleimide (NEM) and pefabloc on ice for 30 min and clarified by centrifugation. Samples were separated on precast 4–12% polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes for antibody detection. The blots were stained with Ponceau S to confirm the uniformity of protein loading in each lane. All membrane blocking steps and antibody dilutions were
performed with 5% skim milk in PBS containing 0.1% Tween 20 (PBS-T). Antibodies were used as follows: monoclonal anti-β-actin (Sigma), polyclonal mouse anti-TRAF2 1:1000 (Santa Cruz), monoclonal anti-cIAP1 1:500 (Alexis), monoclonal anti-cIAP2 1:300 (in house), monoclonal anti-XIAP 1:1000 (MBL), anti-polyclonal RIPK1 1:1000 (BD Biosciences), monoclonal anti-Pgk 1:2000 (Sigma), monoclonal anti-phospho p65 1:000 (Cell Signaling), monoclonal anti-p100–p52 1:1000 (Cell Signaling), monoclonal anti-IkBx 1:1000 (Cell Signaling), monoclonal anti-NIK 1:1000 (Cell Signaling). Washing steps were performed with PBS-T. After incubating with HRP-coupled secondary antibodies, western blots were visualized by ECL (GE Healthcare, Rydalmere, NSW, Australia).

**Immunoprecipitation of EGFP-cIAP1 and EGFP-XIAP**

Xiap
c

EGFP–cIAP1 and EGFP–XIAP were precipitated using protein A beads overnight. The beads were washed four times. Samples then were separated on 4–12% polyacrylamide gels and transferred to Hybond C nitrocellulose membrane as described in western blotting section (see above).

**TNFR1 signalling complex precipitation**

Cells were seeded on 15 cm tissue culture plates and treated for the indicated time with 1 μg/mL of Fc-TNF. Cells were lysed in DISC lysis buffer at 4°C for 30 min. The lysates were centrifuged for 1 h (Chromotek). The beads were washed four times. Then, the beads were washed four times. Samples then were separated on 4–12% polyacrylamide gels and transferred to Hybond C nitrocellulose membrane as described in western blotting section (see above).

**References**


**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

**Acknowledgements**

This work was funded by a Centre grant from the Leukemia and Lymphoma Society, and NHMRC grants and fellowships 433063, 461221, 541901, 575512, 1003435, and was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIISS. We thank Michelle Kellieher for providing Ripk3<sup>+/−</sup> mice; Vishva Dixit for the Ripk3<sup>−/−</sup> mice; Heinrich Kornier for the Tnfr1<sup>−/−</sup> and Tnfr2<sup>−/−</sup> mice; Mark McKinlay for biotinylated and non-biotinylated Smac mimetic compounds (TetraLogic Pharmaceuticals). We thank CAH staff at Latrobe University, especially Jose Ramos, Samantha Kelly, and Melinda Boulton for mouse husbandry, and Frank Koentgen (Oxygen) for generating the clp<sup>C</sup>/*<sup>C</sup>C<sup>C</sup>C<sup>C</sup> mice.

**Author contributions:** MM performed most of the experimental work; HA was responsible for mouse breeding, husbandry, and genotyping; AKV and TT analysed the embryos; WWW, AB, RF, DC, and WDC performed tissue culture experiments, made constructs and viruses, the anti-cIAP2 mAb, and performed western blot analysis. MM, AKV, TT, and DLV wrote the manuscript. WDC and WWW provided critical comments. MM, JS, and DLV designed and directed the experiments.

**Conflict of interest**

DLV and JS are on the Scientific Advisory Board of TetraLogic Pharmaceuticals.

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