Correspondence

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cIAP2 supports viability of mice lacking cIAP1 and XIAP

Kristen N Heard[1], Mathieu JM Bertrand[2] & Philip A Barker[1]

Comment on: **M Moulin *et al*** (April 2012)

Initially classified as potent suppressors of programed cell death, members of the inhibitor of apoptosis (IAP) family have emerged as complex signaling proteins whose functions are not limited to apoptosis regulation, but extend to several signaling pathways involved in the regulation of immunity, inflammation, and cell migration (Gyrd-Hansen & Meier, 2010). Of the eight mammalian IAPs, X-linked IAP and cellular IAPs 1 and 2 (XIAP, cIAP1, cIAP2) show particularly strong structural and functional homology. Genetic approaches have been used to assign precise functions to XIAP, cIAP1, and cIAP2, and initial studies showed that the deletion of XIAP, cIAP1, or cIAP2 resulted in viable animals with relatively subtle phenotypes that may reflect functional compensation between these three family members (Harlin *et al*, 2001; Conze *et al*, 2005; Conte *et al*, 2006).

ciap1 and *ciap2* are located only 15 kb apart on mouse chromosome 9. A conditional approach has been used to selectively delete *ciap1* and *ciap2*, and these mice have been analyzed in wild-type or *xiap* null backgrounds (Moulin *et al*, 2012). $xiap^{-/-}ciap2^{-/-}$ null mice were viable, fertile, and lacked an obvious phenotype, whereas $xiap^{-/-}ciap1^{-/-}$ and $ciap1^{-/-}ciap2^{-/-}$ mice died *in utero* at E12.5. This suggested that cIAP1 could compensate for combined deletion of cIAP2 and XIAP, whereas cIAP2 could not compensate for the combined deletion of cIAP1 and XIAP.

Here, we report that compound *xiap*:*ciap1* null mice generated by simple breeding are viable and fertile and that cells derived from them have essentially normal tumour necrosis factor (TNF) signaling properties. Crosses of mice bearing germline mutations of *xiap*, *ciap1*, or *ciap2* (Harlin *et al*, 2001; Conze *et al*, 2005; Conte *et al*, 2006) resulted in Mendelian distributions of expected genotypes at birth, with no evidence of embryonic or perinatal lethality. Notably, $xiap^{-/-}ciap1^{-/-}$ and $xiap^{-/-}ciap2^{-/-}$ compound nulls developed normally, were fertile, and lacked obvious phenotypes. Reverse-transcription PCR (RT–PCR) performed on mouse embryonic fibroblasts (MEFs) derived from each of the strains confirmed that gene disruptions were complete (Fig 1A). Previous studies from several laboratories have shown that cIAP2 protein levels are dramatically increased in MEFs and tissues lacking cIAP1 (Mahoney *et al*, 2008; Enwere *et al*, 2012) and consistent with this, cIAP2 protein levels are strongly increased in several, but not all, tissues derived from $xiap^{-/-}ciap1^{-/-}$ mice (Fig 1B).

Wild-type, $ciap1^{-/-}$ and $xiap^{-/-}ciap1^{-/-}$ MEFs were compared for their responses to tumour necrosis factor (Fig 1C). In wild-type cells, cIAP2 protein was not detectable and cIAP1 levels were not altered by TNF (10 ng/ml) exposure. In $ciap1^{-/-}$ and $xiap^{-/-}ciap1^{-/-}$ MEFs, cIAP2 protein was readily detected and, interestingly, was sharply reduced by TNF exposure by 15 min, with recovery to control levels only after 6 h of treatment. Otherwise, TNF responses appeared normal across each genotype, with a rapid and complete degradation of IκBα protein that returned to baseline levels within 1 h, and a characteristic molecular weight shift in RIP1 after 15 min of TNF exposure (Fig 1C). TNF-dependent expression of several NF-κB target genes (cIAP2, TLR2, IL6, IκBα) was essentially normal, with identical responses in wild-type and $xiap^{-/-}ciap1^{-/-}$ MEFs and a slightly attenuated response in $ciap1^{-/-}$ MEFs (Fig 1D).

These results differ from those in Moulin *et al* (2012) who reported that TNF-dependent NF-κB activation was absent in $xiap^{-/-}ciap1^{-/-}$ MEFs produced using a conditional gene knockout approach, suggesting that *ciap2* could not compensate for their deletion. However, cIAP2 protein levels in these MEFs did not increase upon *ciap1* deletion, which is at odds with the cIAP2 regulation seen here and by others.

We directly compared cIAP2 mRNA and protein levels in the *ciap1* null MEFs produced by Conze *et al* (2005) to those in the $ciap1^{lox/lox}ciap2^{FRT/FRT}$, $ciap1^{-/-}ciap2^{FRT/FRT}$, and $ciap1^{-/-}ciap2^{-/-}$ MEFs produced by Moulin *et al* (2012). Figure 1E shows that MEFs from all strains produced cIAP2 mRNA, but the $ciap1^{-/-}ciap2^{FRT/FRT}$ MEFs produced by Moulin *et al* (2012) did not produce detectable levels of cIAP2 protein, in contrast to the $ciap1^{-/-}$ MEFS derived from the mice produced by Conze *et al* (2005) (Fig 1F). To determine whether cIAP2 undergoes unusually rapid proteosomal turnover in $ciap1^{-/-}ciap2^{FRT/FRT}$ cells, we exposed cells to epoxomicin, a proteasome inhibitor; this treatment significantly elevated cIAP1 levels in $ciap1^{lox/lox}ciap2^{FRT/FRT}$ MEFs but did not increase cIAP2 levels in the $ciap1^{-/-}ciap2^{FRT/FRT}$ cells (Fig 1G), indicating that cIAP2 production is significantly impaired in $ciap1^{-/-}ciap2^{FRT/FRT}$ MEFs. Although we were unable to detect cIAP2 in the $ciap1^{-/-}ciap2^{FRT/FRT}$ MEFs, these animals are not completely devoid of cIAP2 as Moulin *et al* (2012) were able to immunoprecipitate cIAP2 protein from whole embryo lysates using a biotinylated Smac mimetic compound. We conclude that the mice described in Moulin *et al* (2012)

1 Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, QC, Canada. E-mail: phil.barker@mcgill.ca
2 VIB Inflammation Research Centre and Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
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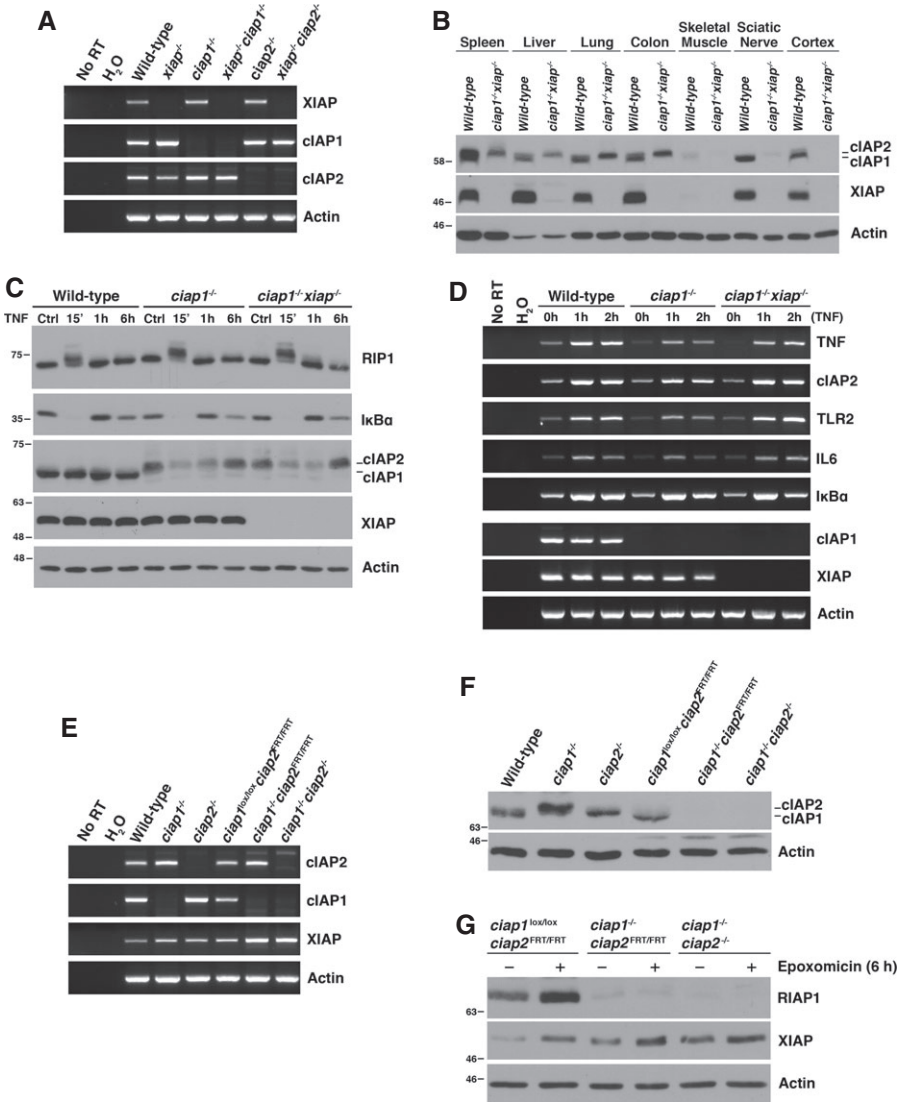

Figure 1. cIAP2 compensates for cIAP1 and XIAP deficiency *in vivo*.
(A) Reverse-transcription PCR (RT–PCR) demonstrating the absence of mRNA transcript in primary MEFs generated from IAP null mice. (B) Immunoblot demonstrating the upregulation of cIAP2 in various *xiap*$^{-/-}$*ciap1*$^{-/-}$ tissues. cIAP1 and cIAP2 protein were detected using a pan-cIAP antibody (RIAP1). (C, D) Western blot (C) and RT–PCR (D) of protein lysates and mRNA collected from MEFs incubated with or without 10 ng/ml recombinant mTNFα for the indicated times. (E, F) Comparison of IAP mRNA transcripts (E) and protein expression (F) in unstimulated MEFs derived from mice generated by classical (*ciap1*$^{-/-}$, *ciap2*$^{-/-}$) and conditional (*ciap1*$^{lox/lox}$*ciap2*$^{FRT/FRT}$, *ciap1*$^{-/-}$*ciap2*$^{FRT/FRT}$, *ciap1*$^{-/-}$*ciap2*$^{-/-}$) knockout approaches. (G) MEFs were treated with proteasomal inhibitor epoxomicin (0.5 μM) for 6 h and harvested for protein detection by Western blot.

have an unanticipated defect in cIAP2 production and as a result, levels of cIAP2 in the *xiap*$^{-/-}$*ciap1*$^{-/-}$*ciap2*$^{FRT/FRT}$ animals are unable to compensate for the loss of cIAP1 and XIAP. Therefore, the divergence in phenotype between the *xiap*$^{-/-}$*ciap1*$^{-/-}$*ciap2*$^{FRT/FRT}$ mice (dead at E12.5) and the *xiap*$^{-/-}$*ciap1*$^{-/-}$ mice produced by simple breeding (viable, fertile, apparently normal) is likely to reflect this difference in cIAP2 expression.

It is important to note that we have previously shown that *ciap1*$^{-/-}$ mice contain an inactivating mutation in the *casp4* gene, due to a 5-bp deletion which originated in the 129-derived ES cell line used to create the cIAP1 knockout strain (Kenneth *et al*, 2012) and that this passenger mutation is also present in the *xiap:ciap1* null strain described here (data not shown). The *casp4* allele produces caspase 11, which has recently been shown to function as a direct innate immune receptor for intracellular lipopolysaccharide and to promote pyroptosis (Shi *et al*, 2014). Although there is no evidence indicating that caspase 11 plays an essential role in development, it is conceivable that the normal survival of the *xiap:ciap1* mice described here may in part reflect absence of caspase 11 activity.

Our data indicate that cIAP2 protein levels are dramatically upregulated in mice lacking cIAP1 and XIAP and that TNF signaling events proceed almost normally in MEFs lacking cIAP1 and XIAP. The *xiap:ciap1* and *xiap:ciap2* compound nulls described here, and cells derived from them, will be useful for isolating cell type- and pathway-specific signaling properties of cIAP1 and cIAP2.

Materials and Methods

Cell culture

Primary mouse embryonic fibroblasts (MEFs) were derived from E12.5 timed pregnant mice and generated in accordance to standard procedures. All MEFs were maintained in 10% fetal bovine serum, 2 mM L-glutamine, and 100 mg/ml penicillin/streptomycin in 5% CO_2 at 37°C.

Antibodies and reagents

The polyclonal XIAP and pan-cIAP antibodies were generous gifts from Dr. Robert G. Korneluk (University of Ottawa, ON). Mouse monoclonal antibodies for β-actin and RIP1 were purchased from MP Biomedicals and BD Biosciences, respectively. Rabbit polyclonal anti-IkBα was purchased from Santa Cruz. Recombinant mTNFα was purchased from R&D Systems. Epoxomicin was purchased from VWR.

Reverse-transcription PCR (RT–PCR)

mRNA was isolated using Qiagen's RNeasy Mini kit as per the manufacturer's instructions. cDNA was produced using the Omniscript RT kit (Qiagen) with random hexamers (GE Healthcare) as primers. PCR primer sequences available upon request.

Sample preparation for SDS–PAGE and immunoblotting

Cells were washed once with PBS then lysed in either 2× Laemmli sample buffer (2% SDS, 50 mM DTT, 60 mM Tris (pH 6.8), 5% glycerol, 0.01% (w/v) bromophenol blue) or NP-40 lysis buffer (1.0% NP-40, 10 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol) supplemented with Complete Mini Protease Inhibitor Cocktail tablets (Roche; Laval, QC). Tissues were dissected from adult mice and homogenized in NP-40 lysis buffer.

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Author contributions

KNH performed the genomic PCR, immunoblots, and RT–PCR shown in Figure 1. MJMB set up initial mouse crosses and performed preliminary analyses of TNF signaling in resulting primary MEFs. KNH compiled the figures and KNH and PAB wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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