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Response to Heard *et al*

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Reply to: **KN Heard *et al*** (in this issue)

Heard *et al* (2015) generated *cIap1*$^{-/-}$ *Xiap*$^{-/-}$ mice and were surprised to find them to be viable and fertile, because we had reported (Moulin *et al*, 2012) that *cIap1*$^{-/-}$*Xiap*$^{-/-}$ mice died by day E12.5 of embryogenesis (Moulin *et al*, 2012 Figs 1B and 2B and Supplementary Fig S1A). We are working with Heard *et al* (2015) in an attempt to determine why.

It is, however, clear that failure of our *cIap2*$^{FRT/FRT}$*cIap1*$^{-/-}$*Xiap*$^{-/-}$ mice to survive past E12.5 is not due to non-functional *cIap2*$^{-/-}$ genes. Three types of cross indicate that the *cIap2*$^{FRT/FRT}$ locus, which is carried by our *cIap1*$^{-/-}$ mice, does produce functional cIAP2. Firstly, comparison of the phenotypes of the *cIap2*$^{FRT/FRT}$*cIap1*$^{-/-}$ mice, which are viable and fertile, with the *cIap2*$^{-/-}$*cIap1*$^{-/-}$ mice, which die at E12.5, indicates that the *cIap2*$^{FRT/FRT}$ locus can function, at least to the extent needed to allow normal development when *Xiap* is present (Moulin *et al*, 2012). Secondly, when specific deletion of *cIap1* in B cells was combined with whole body *cIap2* deletion, it led to more profound B-cell expansion than deletion of either IAP alone (Gardam *et al*, 2011). Thirdly, deletion of *cIap1* in myeloid cells on either a *cIap2*$^{-/-}$ or *cIap2*$^{-/-}$ *Xiap*$^{-/-}$ background triggered splenomegaly, increased neutrophils and monocytes, inflammatory cytokine production and spontaneous inflammatory arthritis, whereas deletion of *Xiap*, *cIap1* or *cIap2* alone did not (Wong *et al*, 2014; Lawlor *et al*, 2015).

In addition to the differences in viability of the *cIap1*$^{-/-}$*Xiap*$^{-/-}$ mice, Heard *et al* (2015) found much higher levels of cIAP2 protein in their *cIap1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) than we reported in our *cIap1*$^{-/-}$ MEFs. Furthermore, Heard *et al* (2015) confirmed this difference: when they directly compared our *cIap1*$^{-/-}$ MEFs with their *cIap1*$^{-/-}$ MEFs, they saw that ours had very low to undetectable levels of cIAP2 protein (like wild-type MEFs), whereas theirs had much higher levels of cIAP2 (Fig 1F, compare lanes 1, 2 and 4).

Consistent with their finding that levels of cIAP2 rise in the absence of cIAP1 in MEFs, they also found elevated levels of cIAP2 protein in several tissues of *cIap1*$^{-/-}$ *Xiap*$^{-/-}$ mice.

Although we did not observe elevated cIAP2 in our *cIap2*$^{FRT/FRT}$*cIap1*$^{-/-}$ MEFs, their finding of increased cIAP2 in their *cIap1*$^{-/-}$ MEFs is consistent with data from several laboratories (including our own) showing that absence or depletion of cIAP1 leads to activation of non-canonical NF-κB and cIAP2 up-regulation (Varfolomeev *et al*, 2007; Vince *et al*, 2007; Darding *et al*, 2011). Indeed, as Heard *et al* (2015) show, our *cIap2*$^{FRT/FRT}$ *cIap1*$^{-/-}$ MEFs have elevated *cIap2* mRNA expression when compared with their *cIap1*$^{loxP/loxP}$ *cIap2*$^{FRT/FRT,}$ cIAP1-proficient counterparts. This indicates a potential defect in translation or stability of the cIAP2 protein in our MEFs.

Note, however, that in our hands, immortalised MEFs are highly genetically variable, with a tendency to lose the expression of proteins, often seemingly at random (Cook *et al*, 2014). Thus, it remains possible that the particular line of immortalised MEFs that we shared with Heard *et al* (2015) are not truly representative of the situation elsewhere in the mice.

Why might MEFs derived from our *cIap2*$^{FRT/FRT}$*cIap1*$^{-/-}$ and *cIap2*$^{FRT/FRT}$*cIap1*$^{-/-}$ *Xiap*$^{-/-}$ mice have much lower levels of cIAP2 than the MEFs from their *cIap1*$^{-/-}$ and *cIap1*$^{-/-}$*Xiap*$^{-/-}$ mice? If the differences in cIAP2 levels in the MEFs are reflected *in vivo*, one reason their *cIap1*$^{-/-}$*Xiap*$^{-/-}$ mice are viable, whereas our *cIap1*$^{-/-}$*Xiap*$^{-/-}$ mice die in mid-embryogenesis, might be differing levels of cIAP2 present during embryogenesis. In a number of molecular pathways minimum threshold levels of protein are required for normal development. As we have only observed one morphological anomaly, namely defects in the integrity of the atrial walls of the heart (Moulin *et al*, 2012), it is possible that in one experimental system there is enough IAP2 protein to avoid this lethal defect, whereas in another there is not. Furthermore, if this is the case, is the amount of cIAP2 aberrantly low in our mice, or is it aberrantly high in theirs, or both?

If there are differences in the production of cIAP2 protein, it might be due to the way the closely linked *cIap1* locus was deleted in each of the strains. Heard *et al* (2015) used *cIap1*$^{-/-}$ mice as described in Conze *et al* (2005). These were generated from 129/Sv E14 embryonic stem (ES) cells by homologous recombination of a neomycin (Neo) resistance gene in reverse orientation in place of the transcription initiation start codon and the first BIR domain of *cIap1* (see Fig 1A of Conze *et al*, 2005). These mice were backcrossed to C57BL/6 mice for multiple generations. We generated *cIap2*$^{FRT/FRT}$*cIap1*$^{loxP/loxP}$ mice by sequentially targeting the same chromosome in BRUCE embryonic stem cells, which were derived from C57BL/6 mice (Koentgen *et al*,

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1993). In these mice, an FRT site is inserted 5′ of the ATG of *cIap2*, and an FRT-flanked Neo gene is inserted into the intron between exons 3 and 4 (see Fig 1A of Moulin *et al*, 2012).

Because the *cIap2* gene is so close to the *cIap1* gene, in some circumstances, the Neo gene or the promoter driving it in Heard *et al*'s $cIap1^{-/-}$ mice might enhance the expression of the linked *cIap2* gene, or it is possible that *cIap2* regulatory sequences were inadvertently altered during homologous recombination. On the other hand, in our $cIap2^{FRT/FRT}cIap1^{-/-}$ mice, it is possible that the intronic Neo gene and *Pgk* promoter sometimes decrease the expression of *cIap2* or the efficiency with which its mRNA is spliced.

Another possible explanation for the differences between the two sets of $cIap1^{-/-}Xiap^{-/-}$ mice is the presence or absence of 129/Sv versus C57BL/6 polymorphic genes, especially those physically linked to the *cIap2-cIap1* locus. In our mice, the genes are of C57BL/6 origin, as the mice were generated from C57BL/6 BRUCE ES cells, whereas even with extensive backcrossing, the genes linked to the *cIap2-cIap1* locus in Heard *et al*'s mice will be of 129/Sv origin. In addition to the mutation in *caspase-11* already described (Kenneth *et al*, 2012), there is a very high probability that the *Mmp1a* gene is also mutated in these strains of mice (Vanden Berghe *et al*, 2015). We know that even minor differences in the expression of other genes can have a major effect on the survival of $cIap1^{-/-}Xiap^{-/-}$ embryos. For example, when we crossed our $cIap1^{-/-}Xiap^{-/-}$ mice onto a heterozygous $Ripk1^{+/-}$ background, rather than dying at E12.5, some survived until weaning (Moulin *et al*, 2012, Fig 6C and Supplementary Fig S3).

There are several lines of experimentation that might reveal why Heard *et al*'s $cIap1^{-/-}Xiap^{-/-}$ mice are viable, whereas our $cIap1^{-/-}Xiap^{-/-}$ mice die by day E12.5. Sequencing the *cIap1-cIap2* locus in the two $cIap1^{-/-}$ lines might reveal unexpected changes in the parental $cIap2^{FRT/FRT}cIap1^{-/-}$ or $cIap1^{-/-}$ mice. Using CRISPR/Cas9 technology to mutate the $cIap1^{-/-}$ gene in cell lines or C57BL/6 zygotes might show if our $cIap2^{FRT/FRT}cIap1^{-/-}$ mice have aberrantly low levels of cIAP2, or their $cIap1^{-/-}$ mice have aberrantly high levels of cIAP2. These mice could be crossed with $Xiap^{-/-}$ knockouts to test their viability. We welcome any other suggestions for experiments and are happy to provide mice or cell lines to other investigators.

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