Programmed cell death, in particular the intrinsic apoptotic pathway, has been shown to play a critical role in the shaping of tissues during embryonic development. The multi-BCL-2 Homology (BH) domain effectors of apoptosis, BAX, BAK, and BOK, are essential for cell killing in the intrinsic apoptotic pathway. It was therefore surprising that we found earlier that a few mice lacking all effectors of apoptosis (Bax;Bak;Bok triple knockout), albeit many fewer than expected based on Mendelian ratios, could reach weaning or even adulthood. This indicated that death receptor induced apoptosis or necroptosis, a lytic form of programmed cell death, may also have roles in embryogenesis alongside the intrinsic apoptotic pathway. To explore this, we generated Bax;Bak;Bok; caspase-8;Mlkl quintuple knockout mice, which lack not only intrinsic apoptosis but also death receptor induced apoptosis (loss of caspase-8) and necroptosis (loss of MLKL). These foetuses exhibited similar defects to the Bax;Bak;Bok triple knockout mice and, intriguingly, a small number of Bax;Bak;Bok; caspase-8;Mlkl quintuple knockout mice could reach weaning or even adulthood. These findings identify the contributions of these three programmed cell death pathways to embryonic development and show that despite the absence of all of them, development to adulthood is possible, albeit very rare.

INTRODUCTION
Programmed cell death plays a critical role in embryonic development by removing cells that are no longer needed, damaged, or infected [1]. There are several distinct pathways to programmed cell death, including apoptosis, which can be activated through the intrinsic (aka mitochondrial or BCL-2 regulated) or the death receptor induced (aka extrinsic) pathway [2], necroptosis, which can be triggered through activation of death receptors when caspase-8 that is essential for extrinsic apoptosis is absent, or pyroptosis, which can be activated by signals from diverse pathogens and requires caspases-1 or -11 and gasdermin D [3]. The intrinsic apoptotic pathway is regulated by the BCL-2 protein family [2]. In healthy cells, pro-survival BCL-2 family members, such as BCL-2, BCL-XL, and MCL-1, restrain the effectors of cell death, BAK and BAK. Stress conditions, such as nutrient deprivation or anoikis (cell detachment) enhance transcription or cause a post-transcriptional increase of the pro-apoptotic BH3-only proteins (e.g., BIM, PUMA). These critical initiators of apoptosis bind with high affinity to and inhibit the pro-survival BCL-2 proteins, thereby unleashing BAX and BAK to cause mitochondrial outer membrane permeabilisation (MOMP), the point-of-no return in apoptosis signalling. MOMP allows the release of apoptogenic factors (e.g., cytochrome c, SMAC/DIABLO) from the intermitochondrial membrane space into the cytoplasm and this leads to the activation of the cascade of caspases that cause the ordered dismantling of the cell [1,2]. BOK structurally resembles BAX and BAK and can also cause MOMP and apoptosis, but it is neither restrained by the pro-survival BCL-2 proteins nor activated by BH3-only proteins [4,5].

The intrinsic apoptotic pathway has long been thought to be the major process of programmed cell death that is critical for embryogenesis [6]. Morphological and histological analyses of Bax−/−Bak−/− and Bax−/−Bak−/−Bok−/− embryos and mice identified those developmental processes that require apoptosis to occur normally. Common abnormalities seen in E18.5 Bax−/−Bak−/− and Bax−/−Bak−/−Bok−/− foetuses include cleft palate/cleft face, aortic arch defects, omphalocele and curled fingers, toes, and tail [5]. Surprisingly, however, many tissues that were thought to depend on apoptosis for development appeared normal in E18.5 Bax−/−Bak−/− and Bax−/−Bak−/−Bok−/− foetuses and some of these animals reached the age of weaning or even early adulthood [5,6]. This raised the possibility that additional programmed cell death pathways, in particular death receptor induced apoptosis and/or necroptosis, might play a role in embryogenesis alongside intrinsic apoptosis. In so-called type 2 cells, death receptor induced apoptosis requires BAX and BAK and would thus be blocked in cells from Bax−/−Bak−/−Bok−/− embryos [7]. However, in so called type 1 cells death receptor induced activation of caspase-8 with consequent activation of the effector caspases suffices for cell killing with no need for engagement of the intrinsic apoptotic pathway by caspase-8 mediated activation of the pro-apoptotic BH3-only protein BID [8]. Thus, death receptor induced apoptosis would be possible in type 1 cells in Bax−/−Bak−/−Bok−/− embryos and can only be abrogated by the loss of caspase-8. Necroptosis, a lytic form of programmed cell
death that is executed by the activation of the pore-forming protein MLKL, is induced when death receptors are stimulated and caspase-8 is absent or inhibited [9]. We examined the impact of the combined absence of both apoptotic pathways and necroptosis on embryonic development.

RESULTS

The observations that many tissues in which apoptosis was proposed to play a role appear surprisingly normal in Bax−/−;Bak−/−;Bok−/− embryos suggested that additional programmed cell death pathways might operate alongside the intrinsic apoptotic pathway to allow the shaping of tissues during embryonic and foetal development. To explore this hypothesis, we generated mice that lacked not only the multi-BH (BCL-2 homology) domain effectors of apoptosis, BAX, BAK and BOK, but additionally were also deficient in caspase-8, which is essential for death receptor induced apoptosis [10], and MLKL, which is needed for necroptosis [11]. Note that loss of caspase-8 causes embryonic lethality ~E11.5 due to aberrant necroptosis that can be prevented by concomitant absence of RIPK3 [12, 13] or MLKL [14], which are both essential for necroptosis. For this we crossed Bax+/−;Bak−/−;Bok−/−;Casp8−/−;Mlkl−/− mice with Bax−/−;Bak−/−;Bok−/−;Casp8−/−;Mlkl−/− mice offering a 1/8 chance to obtain Bax−/−;Bak−/−;Bok−/−;Casp8−/−;Mlkl−/− offspring. 151 offspring reached weaning (~21 days) and even adulthood (~6 weeks of age, Table 1). One Bax−/−;Bak−/−;Bok−/−;Casp8−/−;Mlkl−/− mouse survived for 126 days (Fig. 1a, b). The frequency of Bax−/−;Bak−/−;Bok−/−;Casp8−/−;Mlkl−/− mice was similar to the frequency of Bax−/−;Bak−/−;Bok−/−;Casp8−/−;Mlkl−/− mice at weaning and upon reaching adulthood (3/151 and 2/147 vs 7/392 [5] and 4/444 [5], p = 1 and p = 0.6, respectively; Fisher’s exact test). Similarly, the frequency of Bax−/−;Bak−/−;Bok−/−;Casp8−/−;Mlkl−/− mice was similar to Bax−/−;Bak−/−;Bok−/−;Casp8−/−;Mlkl−/− mice at weaning and upon reaching adulthood (Table 1; p = 0.5 and p = 1, respectively; Fisher’s exact test). This demonstrates that it is possible to obtain adult mice that lack the intrinsic as well as the death receptor apoptotic pathways and necroptosis. Importantly, the loss of the death receptor apoptotic pathway and necroptosis does not appear to affect the frequency of mice reaching weaning or even adulthood that was previously observed in Bax−/−;Bak−/−;Bok−/−;Mlkl−/− TKO mice [5]. To determine whether the additional lack of death receptor induced apoptosis and

| Genotype | Bax+/− | Bax−/− | Total | p valuea |
|----------|---------|---------|-------|----------|
| E18.5−E19 | 13 (15) | 37 (30) | 60 | 0.08 |

aObserved and expected numbers were compared calculating the cumulative probability distribution of being less or equal to the expected value (pbinom; R version 4.0.5 2021-03-31).

bThe frequency of Bax−/−;Bak−/−;Bok−/−;Mlkl−/− mice not significantly different between Casp8−/− and Casp8+/− at weaning (b, 3 weeks of age, Fisher’s exact test p = 0.5) and adulthood (c, 6 weeks of age, p = 1), but less than expected (p value column).
necroptosis could further exacerbate these developmental abnormalities. We generated E18.5 Bax<sup>−/−</sup>Bak<sup>−/−</sup>Bok<sup>−/−</sup>Casp8<sup>−/−</sup>Mlkl<sup>−/−</sup> quadruple knockout (Q5KO) foetuses. Data were presented as mean ± SEM; p, one-way ANOVA followed by multiple comparison and Tukey key correction for multiple testing. Each dot represents an individual foetus. (Fig. 2a, b, c). Percentage of E18.5 Bax<sup>−/−</sup>Bak<sup>−/−</sup>Bok<sup>−/−</sup>Casp8<sup>−/−</sup>Mlkl<sup>−/−</sup> quadruple knockout foetuses exhibiting externally visible developmental defects (numbers above bars represent the percentages of animals) compared to Fisher’s exact test.

DISCUSSION
Since in embryos deficient for the intrinsic apoptotic pathway (Bax<sup>−/−</sup>Bak<sup>−/−</sup>Bok<sup>−/−</sup>) many tissues that were long thought to require apoptosis for development appeared surprisingly normal and since some of these mice could even reach early adulthood [5], we explored whether foetuses deficient in both intrinsic and extrinsic death receptors induced apoptosis and necroptosis (Bax<sup>−/−</sup>Bak<sup>−/−</sup>Casp8<sup>−/−</sup>Mlkl<sup>−/−</sup>) would have more severe abnormalities. Our findings presented here demonstrate that, while the penetrance of the observed defects varied between these two genotypes, overall, the additional absence of death receptor induced apoptosis and necroptosis, due to the loss of caspase-8 and MLKL, had no effect on the survival rate and little effect on the developmental abnormalities caused by the absence of the intrinsic apoptotic pathway (loss of BAX, BAK, and BOK).

Nevertheless, the three defects that were more common in Bax<sup>−/−</sup>Bak<sup>−/−</sup>Bok<sup>−/−</sup>Casp8<sup>−/−</sup>Mlkl<sup>−/−</sup> than in Bax<sup>−/−</sup>Bak<sup>−/−</sup>Bok<sup>−/−</sup> pups, aortic arch defects, extra finger tissue, and curly tails, could indicate some role of the extrinsic apoptotic pathway or necroptosis in restricted tissues. Loss of necroptosis alone [11] or combined absence of death receptor induced...
apoptosis plus necroptosis do not cause any developmental abnormalities [13, 14]. However, inhibition of the intrinsic apoptotic pathway synergises with defects in the death receptor apoptotic pathway in causing lymphadenopathy [15, 16]. We, therefore, assume that, if any additional defects in embryonic development exist at all, it is the loss of death receptor induced apoptosis rather than the absence of necroptosis that causes an increase in some developmental abnormalities in Bax−/−Bak−/−; Bok−/−; Casp8−/−; Mlkl−/− mice. Perhaps some cells that would normally be removed by the intrinsic apoptotic pathway during
embryogenesis but cannot be removed in this manner due to the absence of BAX, BAK, and BOK, could be induced to undergo death receptor induced apoptosis instead, with the ligand(s) for death receptor induced killing (i.e. in type 2 cells, since type 2 cells require the BH3-only protein BID as well). The ratio of offspring obtained from intercrosses of Bax<sup>−/−</sup>:Bak<sup>−/−</sup>:Bok<sup>−/−</sup>:Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> quintuple knockout (Q5KO) foetuses was compared by Fisher’s exact test. Representative images of the large vessels of an E18.5 Bax<sup>−/−</sup>:Bak<sup>−/−</sup>:Bok<sup>−/−</sup>:Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> control foetuses (left panel) and two Bax<sup>−/−</sup>:Bak<sup>−/−</sup>:Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> quintuple knockout foetuses. Schematic outlines of the large vessels are shown below the images. The Bax<sup>−/−</sup>:Bak<sup>−/−</sup>:Bok<sup>−/−</sup>:Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> quintuple knockout foetus in the descending aorta (arrow) and an abnormal origin of the descending aorta from the pulmonary trunk (*). The Bax<sup>−/−</sup>:Bak<sup>−/−</sup>:Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> quintuple knockout foetus showed absence of the right subclavian artery (arrow) and an abnormal origin of the ascending aorta, the ascending aorta connected abnormally to the pulmonary trunk (#). AAo, control foetus (left panel) and a quintuple knockout foetus with curled fingers (arrowhead) and protruding skin tissue in the neck region (arrow). e Image of an E18.5 Bax<sup>−/−</sup>:Bak<sup>−/−</sup>:Bok<sup>−/−</sup>:Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> quintuple knockout foetus with curled toes (arrowhead) and sacral spina bifida (arrow). f Image of an E18.5 Bax<sup>−/−</sup>:Bak<sup>−/−</sup>:Bok<sup>−/−</sup>:Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> quintuple knockout foetus with curled toes (arrowhead) and curled tail (T). g Image of an E18.5 Bax<sup>−/−</sup>:Bak<sup>−/−</sup>:Bok<sup>−/−</sup>:Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> quintuple knockout foetuses with excess toe tissue (arrowhead). 

**MATERIALS AND METHODS**

**Mice**

All experiments with mice were performed with the approval of the Walter and Eliza Hall Institute Animal Ethics Committee. Animals were handled according to the Australian Code of Practice for the care and use of animals for scientific purposes. 

Bax<sup>−/−</sup>;Bak<sup>−/−</sup>;Bok<sup>−/−</sup>;Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> triple knockout (TKO) mice on a C57BL/6 background have been described previously [5]. Bax<sup>−/−</sup>;Bak<sup>−/−</sup>;Bok<sup>−/−</sup>;Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> quintuple knockout (Q5KO) animals were derived by crossing Bax<sup>−/−</sup>;Bak<sup>−/−</sup>;Bok<sup>−/−</sup>;Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> mice [14], also on a C57BL/6 background. Following subsequent rounds of breeding, Bax<sup>−/−</sup>;Bak<sup>−/−</sup>;Bok<sup>−/−</sup>;Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> and Bax<sup>−/−</sup>;Bak<sup>−/−</sup>;Bok<sup>−/−</sup>;Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> mice were obtained and were intercrossed to generate Bax<sup>−/−</sup>;Bak<sup>−/−</sup>;Bok<sup>−/−</sup>;Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> QSKO mice.

For timed matings, noon of the day on which the vaginal plug was first observed was defined as embryonic day 0.5 (E0.5). Mouse foetuses were recovered at E18.5–E19 (just before birth) by Caesarean section. Animals were weaned between 19 and 23 days after birth and deemed adults at 42 days of age. Mouse genotypes by PCR as described previously [5, 14]. All mice with a Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> genotype, regardless of their genotype for Bax, Bak and Bok, that reached >150 days developed lymphanedenaathy, splenomegaly, and/or hepatomegaly, as previously described for Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> mice [10].

**Microscopy**

For detailed phenotypic examination, E18.5 foetuses were euthanised by cooling. Dissections were performed using the Stemi 2000-C dissecting microscope. Pups were photographed with a digital camera (AxioCam HR, Carl Zeiss).

**Quantification and statistical analysis**

The ratio of offspring obtained from intercrosses of Bax<sup>−/−</sup>;Bak<sup>−/−</sup>;Bok<sup>−/−</sup>;Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> or crosses of Bax<sup>−/−</sup>;Bak<sup>−/−</sup>;Bok<sup>−/−</sup>;Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> with Bax<sup>−/−</sup>;Bak<sup>−/−</sup>;Bok<sup>−/−</sup>;Casp8<sup>−/−</sup>;Mlkl<sup>−/−</sup> mice at different stages were analysed using GraphPad Prism software 9.0. The statistical tests used are stated in the figure legend. The number of replicates (n) is defined as number of animals stated in Table 1 or in the figure legends.

**DATA AVAILABILITY**

The authors declare that all data supporting the findings of this study are available within the article and the Supplementary Materials.

**REFERENCES**

1. Green DR. The coming decade of cell death research: Five riddles. Cell. 2019;177:1094–107.
2. Clabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. Nat Rev Mol Cell Biol. 2014;15:49–63.
3. Bedouin S, Herold MJ, Strasser A. Emerging connectivity of programmed cell death pathways and its physiological implications. Nat Rev Mol Cell Biol. 2020;21:678–695.
4. Lammli F, Wang YM, Victor B, Yang M, Schneider DM, Gingras S, et al. BOK is a non-canonical BCL-2 family effector of apoptosis regulated by ER-associated degradation. Cell. 2016;165:421–33.
5. Ke FFS, Vanyai HK, Cowan AD, Delbridge ARD, Whitehead L, Grabow S, et al. Embryogenesis and adult life in the absence of intrinsic apoptosis effectors BAX, BAK, and BOK. Cell. 2018;173:1217–30 e1217.
6. Lindsten T, Ross AJ, King A, Zong W, Rathmell JC, Shiels HA, et al. The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. Mol Cell. 2000;6:1389–99.
7. Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, et al. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature. 1999;400:886–91.
8. Jost PJ, Grabow S, Gray D, McKenzie MD, Nachbur U, Huang DC, et al. XIAP discriminates between type I and type II FAS-induced apoptosis. Nature. 2009;460:1035–9.
9. Vandenabeele P, Riquet F, Cappe B. Necroptosis: (Last) Message in a bubble. Immunity. 2017;47:1–3.
10. Varfolomeev EE, Schuchmann M, Luria V, Chiamnikkulchai N, Beckmann JS, Mett IL, et al. Targeted disruption of the mouse Caspase 8 gene ablates cell death.
induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. 
Immunity. 1998;9:267–76.
11. Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang JG, Alvarez-Diaz S, et al. 
The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. 
Immunity. 2013;39:443–53.
12. Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, 
et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature. 
2011;471:368–72.
13. Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, et al. Catalytic 
activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. 
Nature. 2011;471:363–7.
14. Alvarez-Diaz S, Dillon CP, Lalaoui N, Tanzer MC, Rodriguez DA, Lin A, et al. The 
Pseudokinase MLKL and the Kinase RIPK3 have distinct roles in autoimmune 
disease caused by loss of death-Receptor-Induced Apoptosis. Immunity. 
2016;45:513–26.
15. Hughes PD, Belz GT, Fortner KA, Budd RC, Strasser A, Bouillet P. Apoptosis reg- 
ulators Fas and Bim cooperate in shutdown of chronic immune responses and 
prevention of autoimmunity. Immunity. 2008;28:197–205.
16. Strasser A, Harris AW, Huang DC, Krammer PH, Cory S. Bcl-2 and Fas/APO-1 
regulate distinct pathways to lymphocyte apoptosis. Embo J. 1995;14:6136–47.
17. Baehrecke EH. Autophagy SEPArates germline and somatic cells. Cell. 2009;136:207–8.

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FFSK, AS and AKV designed study, interpreted results, and wrote the manuscript. 
FFSK conducted experiments with help from AKV. KB helped with the analysis of 
some adult mice.

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COMPETING INTERESTS
The authors declare no competing interests.

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