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Loss of Apaf-1 leads to partial rescue of the *HAND2*-null phenotype

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Abstract

HAND2 is an essential transcription factor for cardiac, pharyngeal arch, and limb development. Apoptosis in the *HAND2*-null embryo causes hypoplasia of the right ventricle and pharyngeal arches leading to lethality by embryonic day (E)10.0 from heart failure. In order to investigate the role of apoptosis in inducing the *HAND2*-null phenotype, we generated mouse embryos lacking both HAND2 and Apaf-1, a central downstream mediator of mitochondrial damage-induced apoptosis. In contrast to $HAND2^{-/-}$ embryos, $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos at E10.5–11.0 had well-developed pharyngeal arches, aortic arch arteries, and no signs of cardiac failure. TUNEL analysis through pharyngeal arches of $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos revealed decreased apoptosis and the embryos had clearly patent aortic arch arteries. However, ventricular hypoplasia and cell death were unchanged in these animals compared to $HAND2^{-/-}$ embryos, resulting in growth arrest at E11.0. Our study suggests that loss of HAND2 in the pharyngeal arch mesenchyme leads to apoptosis in an Apaf-1-dependent fashion and that, while loss of aortic arch integrity contributes to the early lethality, the ventricular defects are independent of arch development.

Keywords: Apaf-1; *HAND2*-null phenotype; Pharyngeal arches

Introduction

Cell fate decisions of survival or death play a central role in shaping the developing embryo and its organs. While the triggers and effectors involved in programmed cell death (apoptosis) are well studied (Hengartner, 2000), relatively little is known regarding the transcription factors that influence such decisions. HAND2 is a basic helix-loop-helix (bHLH) transcription factor that is essential for numerous embryologic events (Abe et al., 2002; Charite et al., 2000; Fernandez-Teran et al., 2000; Howard et al., 2000; Srivastava et al., 1997; Thomas et al., 1998; Yamagishi et al., 2000). In mouse, HAND2 is specifically expressed in the developing heart, pharyngeal arch mesenchyme, and posterior limb buds (Charite et al., 2000; Srivastava et al., 1995; Thomas et al., 1998). Embryos lacking HAND2 exhibit hypoplasia of the right ventricle, aortic arch arteries, and pharyngeal arches, all of which are associated with apoptosis of HAND2-expressing cells (Srivastava et al., 1997; Thomas et al., 1998; Yamagishi et al., 2001). However, the cellular pathways through which HAND2 promotes cell survival are unknown.

Pathways triggered by both extracellular and intracellular signals can lead to the proteolytic cascade characteristic of cells undergoing programmed cell death (apoptosis) (Li and Yuan, 1999). The main effectors of apoptosis are cysteine

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proteases known as caspases, which are highly conserved across species (Budihardjo et al., 1999; Cikala et al., 1999; Earnshaw et al., 1999). Signaling through the cell surface death receptor activates initiator caspase-8, which subsequently stimulates the effector caspases (caspases-3, -7, etc.) that are directly responsible for degrading cellular proteins and causing DNA fragmentation (Li and Yuan, 1999). A similar process is activated when mitochondria are damaged leading to release of cytochrome *c* (Kluck et al., 1997; Yang et al., 1997), which together with apoptosis protease-activating factor-1 (Apaf-1) activate caspase-9, an initiator caspase that can activate downstream caspases (Li et al., 1997; Zou et al., 1999).

Targeted deletions of several molecules in the apoptotic cascade have revealed a complex overlap of function and tissue-specific requirement of numerous factors (Ranger et al., 2001). During development, apoptosis can be induced or suppressed by specific pro- or anti-apoptotic stimuli, respectively. This complex interplay between various factors suggests a strict control on their expression or activity (Jacobson et al., 1997). This could be at the level of transcription, translation, or posttranslational modifications in response to specific stimuli. While many mediators of apoptosis undergo proteolytic cleavage in response to stimuli (Li and Yuan, 1999), the transcriptional control of the expression of these factors is poorly understood.

By investigating differentially expressed genes, we had previously found that the hypoxia inducible pro-apoptotic Bcl-2 family member Bnip3 (formerly Nip3), which functions through the mitochondrial damage pathway (Bruick, 2000; Chen et al., 1997; Guo et al., 2001; Regula et al., 2002), was upregulated in the *HAND2*-null embryo prior to signs of heart failure (Villanueva et al., 2002). Bnip3 localizes to the mitochondria (Yasuda et al., 1998) and is implicated in hypoxia-induced apoptosis in cardiomyocytes (Guo et al., 2001; Kubasiak et al., 2002; Regula et al., 2002). Here, we investigated the apoptotic cascades affected by HAND2 during mouse embryonic development. Apaf-1, a downstream mediator of mitochondrial-induced apoptosis (Li et al., 1997; Zou et al., 1997), was required for the apoptosis observed in *HAND2*-null pharyngeal and aortic arch mesenchyme, as in vivo loss of Apaf-1 partially rescued mesenchymal apoptosis in *HAND2*-null embryos and delayed embryonic lethality. Despite prolonged survival until E11.0, ventricular hypoplasia and cell death was unchanged in $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos. These results suggest that HAND2, in addition to regulating differentiation and expansion of specific cell types (Yamagishi et al., 2000, 2001), functions by regulating survival of pharyngeal arch mesenchyme through an Apaf-1-mediated pathway. This study also revealed that the early lethality observed in *HAND2*-null embryos is partly due to loss of pharyngeal arch and aortic arch artery integrity, and that hypoplasia of the right ventricle is independent of the arch artery defects.

Materials and methods

Generation of $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos

Mice heterozygous for the *HAND2* mutation (Srivastava et al., 1997) or *Apaf-1* mutation (Honarpour et al., 2000) were generated and genotyped as described previously on a C57BL/6 or SV129 background, respectively. *Apaf-1* mutant animals in a C57BL/6 background were generated by back-crossing with wild-type C57BL/6 mice for seven generations. Mice heterozygous for the *HAND2* mutant allele or the *Apaf-1* mutant allele were intercrossed and their progeny was genotyped to identify double heterozygous ($HAND2^{+/-}Apaf\text{-}1^{+/-}$) pups. Pregnancies resulting from intercrosses of $HAND2^{+/-}Apaf\text{-}1^{+/-}$ animals were terminated at varying points during gestation by cesarean section and embryos were fixed overnight in 4% paraformaldehyde/PBS. Yolk sac DNA was used to genotype the *Apaf-1* mutant allele by PCR and the *HAND2* mutant allele by Southern to identify embryos that were homozygous for both mutations.

Histology

Wild type, $HAND2^{-/-}$, $HAND2^{-/-}Apaf\text{-}1^{+/-}$ and $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos were embedded in paraffin after fixation. Transverse sections were made through the embedded tissue at 5-μm intervals. Paraffin was cleared with xylene and select sections were counter-stained with hematoxylin and eosin.

Apoptosis and proliferation studies

To visualize apoptotic nuclei in pharyngeal arches, limb buds, and hearts in situ, transverse sections of wild type, $HAND2^{-/-}$, $HAND2^{-/-}Apaf\text{-}1^{+/-}$, and $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos were subjected to terminal transferase-mediated dUTP-biotin nick end labeling (TUNEL) using the ApopTag kit (Intergen Company). Sections were counter-stained with DAPI. Immunohistochemistry using antibodies to pCNA and phosphohistone 3 was performed on sections for assaying proliferative cells using standard methods.

Results

Loss of Apaf-1 leads to partial rescue of the HAND2-null phenotype

TUNEL analysis of $HAND2^{-/-}$ hearts and pharyngeal arches previously revealed increased apoptosis compared to wild type (Thomas et al., 1998; Yamagishi et al., 2001). Furthermore, EM studies on hearts of *HAND2* mutants showed the presence of disrupted mitochondria (unpublished observations). To genetically test the hypothesis that mitochondrial damage and subsequent caspase activation

might contribute to the *HAND2* mutant phenotype, we attempted to block mitochondrial-induced apoptotic signals by generating *HAND2*-null mice in the *Apaf-1*-null background. Apaf-1 is a ubiquitously expressed cytosolic protein that interacts with cytochrome *c* and dATP, and is required for the activation of caspase-9 and subsequent propagation of the apoptotic signal (Li et al., 1997; Liu et al., 1996; Zou et al., 1997). Targeted deletion of *Apaf-1* in mice results in late embryonic or perinatal lethality due to loss of cell death in the nervous system (Cecconi et al., 1998; Yoshida et al., 1998).

Mice heterozygous for the *HAND2* or *Apaf-1* mutant allele were intercrossed to generate trans-heterozygous mice, all of which appeared normal. $HAND2^{+/-}Apaf\text{-}1^{+/-}$ mice were intercrossed and the resulting offsprings were analyzed at E10.5. This stage was chosen for initial analysis because $HAND2^{-/-}$ embryos at this stage are severely malformed with pericardial effusion, a sign of cardiac failure, and many are already dead with their growth having been arrested at E9.5. As expected, all embryos lacking *HAND2* alone were growth retarded, had extremely small pharyngeal arches, and showed severe cardiac failure as evidenced by pericardial effusion (Fig. 1). In contrast, $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos had well-preserved pharyngeal arches and limb buds, and showed no pericardial effusion (Fig. 1), suggesting significant rescue of $HAND2^{-/-}$ embryos. $HAND2^{-/-}Apaf\text{-}1^{+/-}$ embryos showed an intermediate phenotype with respect to preservation of the pharyngeal arches and pericardial effusion (Fig. 1).

Unlike the rescue of the pharyngeal arches, the cardiac phenotype in $HAND2^{-/-}$, $HAND2^{-/-}Apaf\text{-}1^{+/-}$, and $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos was identical at E10.5. Hypoplasia of the right ventricle in $HAND2^{-/-}$ embryos manifests prior to the pharyngeal arch phenotype and is apparent as early as E8.5. Therefore, in an effort to determine whether loss of Apaf-1 might have rescued the early cardiac phenotype, $HAND2^{-/-}$ and $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos were harvested at E9.0–9.5. Even at this early stage, we observed a hypoplastic right ventricle and thin left ventricle in $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos (Fig. 2), suggesting that reduction of Apaf-1 dosage did not rescue the myo-

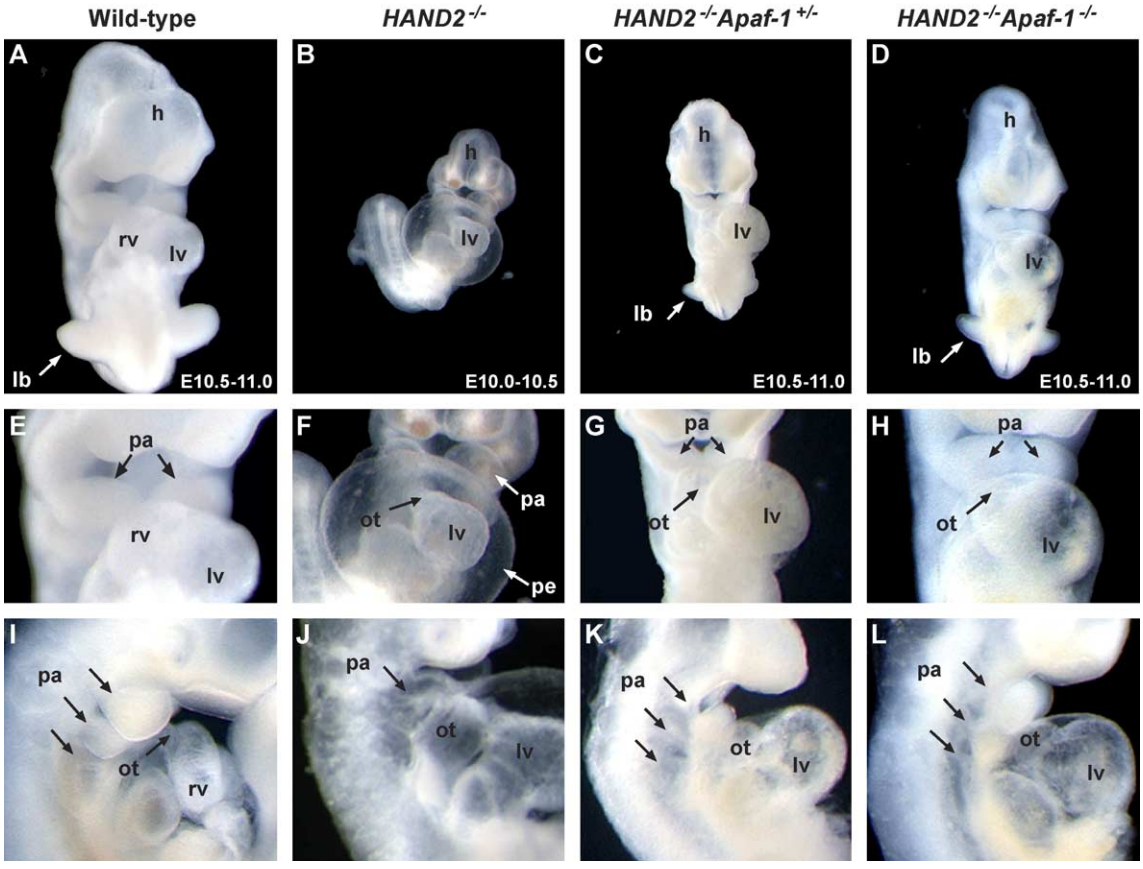

Fig. 1. Decrease in *Apaf-1* gene dosage leads to partial rescue of the $HAND2^{-/-}$ phenotype. Embryos of wild type (A, E, and I), $HAND2^{-/-}$ (B, F, and J), $HAND2^{-/-}Apaf\text{-}1^{+/-}$ (C, G, and K), and $HAND2^{-/-}Apaf\text{-}1^{-/-}$ (D, H, and L) are shown in frontal (A–H) and right lateral (I–L) views. $HAND2^{-/-}Apaf\text{-}1^{+/-}$ and $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos had better developed limb buds (lb) in C and D, respectively, and pharyngeal arches (pa) in G and H, respectively, compared to the $HAND2^{-/-}$ embryo (B and F). However, $HAND2^{-/-}Apaf\text{-}1^{+/-}$ and $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos showed absence of the right ventricle (K and L) similar to the $HAND2^{-/-}$ embryo (J). $HAND2^{-/-}Apaf\text{-}1^{+/-}$ and $HAND2^{-/-}Apaf\text{-}1^{-/-}$ embryos (G and H, respectively) did not exhibit pericardial effusion (pe) as seen in $HAND2^{-/-}$ embryos (F). Images A–D were captured at the same magnification, as were images I–L. Frontal views in E–H are close-up images of A–D, respectively, and focus on the heart and pharyngeal arches. h, head; lv, left ventricle; rv, right ventricle; ot, outflow tract.

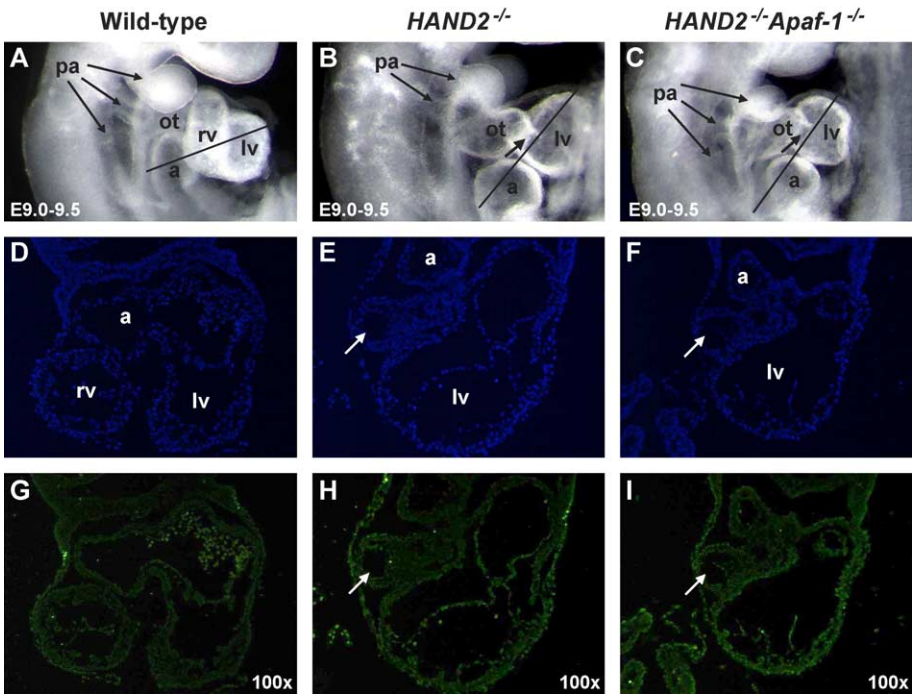

Fig. 2. Cardiac defect in *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos at E 9.0–9.5. Right lateral views of E9.5 wild-type (A), *HAND2*$^{-/-}$ (B), and *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ (C) embryos focusing on cardiac region. Double mutant embryos had a small right ventricle segment (arrow in C), as did *HAND2*$^{-/-}$ embryos (arrow in B). Images are shown at equal magnification. Transverse sections through wild type (D and G), *HAND2*$^{-/-}$ (E and H), and *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ (F and I) are shown after TUNEL assay (G–I) and counter stained with DAPI (D–F). Bright green cells represent cells undergoing apoptosis (TUNEL positive). Arrows in E, F, H, and I indicate the reduced right ventricle. a, atrium; lv, left ventricle; rv, right ventricle; ot, outflow tract; pa, pharyngeal arches.

cardial defect in *HAND2* mutants. Consistent with the persistent ventricular defects, we did not detect any differences in apoptosis upon comparing hearts of *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ to *HAND2*$^{-/-}$ embryos from E9.0 to E9.5 (Fig. 2D–I). *HAND2* mutants do not have any detectable proliferative defects and we also did not detect any changes in cardiac proliferation nor gene expression (e.g., Nkx2.5, Mlc2v, Tbx5, Hand1) in the presence or absence of *Apaf-1* alleles (data not shown), suggesting that the molecular events in the ventricles of *HAND2* mutants were independent of *Apaf-1* dosage. Ultimately, the failure of myocardial development led to growth arrest beyond E11.0.

Because the initial analyses were done in a C57BL6/SV129 mixed background, we asked whether the partial rescue might be due to genetic modifiers in the SV129 background. To answer this question, Apaf-1 mice were back-crossed into the C57BL6 background to achieve greater than 90% pure animals that were then used to generate *HAND2*$^{-/-}$ *Apaf-1*$^{-/-}$ embryos. *HAND2*$^{-/-}$ *Apaf-1*$^{-/-}$ embryos in the nearly pure C57BL6 background at E10.5–11.0 showed the same results as those seen in the C57BL6/SV129 mixed background (Table 1). These results indicated that Apaf-1 function was required to mediate the early hypoplasia seen in the pharyngeal arches of *HAND2*$^{-/-}$ embryos.

To determine the mechanism of partial rescue, sections through the pharyngeal arches and limb buds of *HAND2*$^{-/-}$ and *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ were analyzed using the TUNEL assay to determine the extent of cell death. When embryos were compared at E10.5, the numbers of apoptotic cells were remarkably reduced in the *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos compared to *HAND2*$^{-/-}$ embryos (Fig. 3). E9.5 *HAND2*$^{-/-}$ embryos were used for comparison with E10.5–11.0 *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos because by E10.5 the pharyngeal arches of *HAND2*$^{-/-}$ embryos are severely hypoplastic and the embryo is being reabsorbed. Our results

Table 1
Genotype of embryos from *HAND2*$^{+/-}$*Apaf-1*$^{+/-}$ intercrosses

Genotype	HAND2	+/+	+/+	+/+	+/–	+/–	+/–	–/–	–/–	–/–	Total
	Apaf-1	+/+	+/–	–/–	+/+	+/–	–/–	+/+	+/–	–/–	
E9.5	Observed	6	9	5	10	19	8	3	4	3	67
	Expected	4	8	4	8	17	8	4	8	4	
E10.5–11.0	Observed	16	25	13	24	48	22	2	9	8	167
	Expected	11	21	11	21	42	21	11	21	11	
Expected ratio		1/16	1/8	1/16	1/8	1/4	1/8	1/16	1/8	1/16	

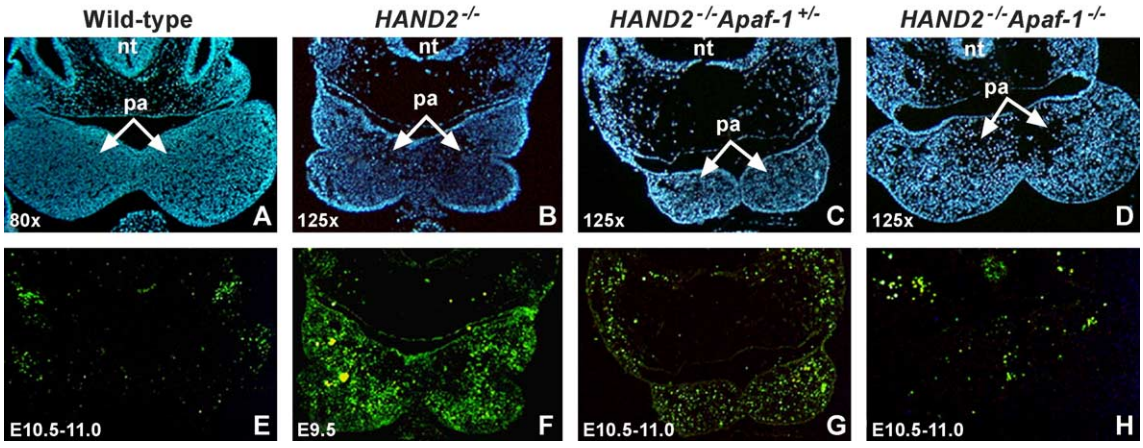

Fig. 3. Decreased apoptosis in pharyngeal arches and limb buds of *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ and *HAND2*$^{-/-}$*Apaf-1*$^{+/-}$ embryos. Transverse sections through wild type (A and E), *HAND2*$^{-/-}$ (B and F), *HAND2*$^{-/-}$*Apaf-1*$^{+/-}$ (C and G), and *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ (D and H) are shown after TUNEL assay (E–H) and counter stained with DAPI (A–D). Bright green cells represent cells undergoing apoptosis (TUNEL positive). nt, neural tube; pa, pharyngeal arch.

show that the pharyngeal arches of E10.5–11.0 *HAND2*$^{-/-}$ *Apaf-1*$^{-/-}$ embryos were still healthy and did not demonstrate excessive apoptosis. We also examined apoptosis in limb buds of wild type, *HAND2*$^{-/-}$, and *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos. As in the pharyngeal arches, we observed a decrease in the apoptosis in *HAND2*$^{-/-}$ *Apaf-1*$^{-/-}$limb buds in comparison to *HAND2*$^{-/-}$ embryos at E9.5 (Fig. 4) but no changes in expression of genes such as sonic hedgehog, thought to be downstream of HAND2 in the limb bud.

Our previous studies demonstrated that *HAND2*$^{-/-}$ embryos have vascular defects with the most prominent being atresia of the pharyngeal arch arteries (Srivastava et al., 1997). The early closure of these vessels that connect the cardiac outflow tract (conotruncus) to the aorta resulted in dilation of the aortic sac (Srivastava et al., 1997; Yamagishi et al., 2000). Because *HAND2* was expressed in the right ventricle and pharyngeal arches, it has remained unclear whether the early lethality was due to the intrinsic myocardial defect or was a result of the pharyngeal arch artery defect. Because *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos survived longer than *HAND2*$^{-/-}$ embryos in spite of similar right ventricular defects, we carefully examined sections through the pharyngeal arches of *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos to determine the patency of the pharyngeal arch arteries. Mice lacking both genes not only survived longer, but also had well-preserved pharyngeal arch arteries (Fig. 5) that were unobstructed. In *HAND2* single mutants, the aortic sac becomes aneurysmally dilated by E10.0, likely secondary to closure of the pharyngeal arch arteries that arise from the aortic sac. Consistent with patency of the pharyngeal arch arteries in *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$, the aortic sac in double mutants was not aneurysmally dilated (Figs. 1K and L compared to Fig. 1J), even at E11. This result suggests that the right ventricular defect in *HAND2* mutants is not a consequence of obstructed blood flow since right ventricular hypoplasia was still observed in *HAND2*$^{-/-}$ *Apaf-1*$^{-/-}$ embryos (Fig. 5). Rather, atresia of the arch

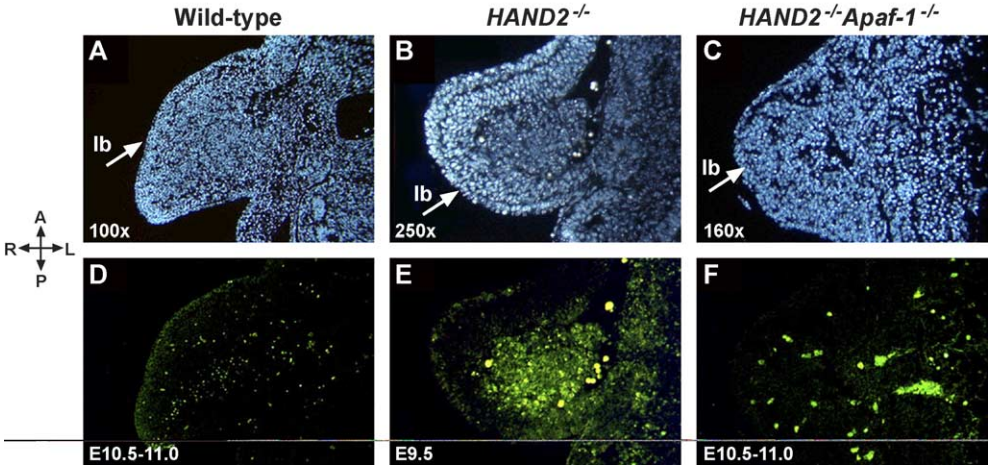

Fig. 4. Decreased apoptosis in limb buds of *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos. Sections through wild type (A and D), *HAND2*$^{-/-}$ (B and E), and *HAND2*$^{-/-}$ *Apaf-1*$^{-/-}$ (C and F) are shown after TUNEL assay (D–F) and counter stained with DAPI (A–C). Bright green cells represent cells undergoing apoptosis (TUNEL positive). lb, limb bud.

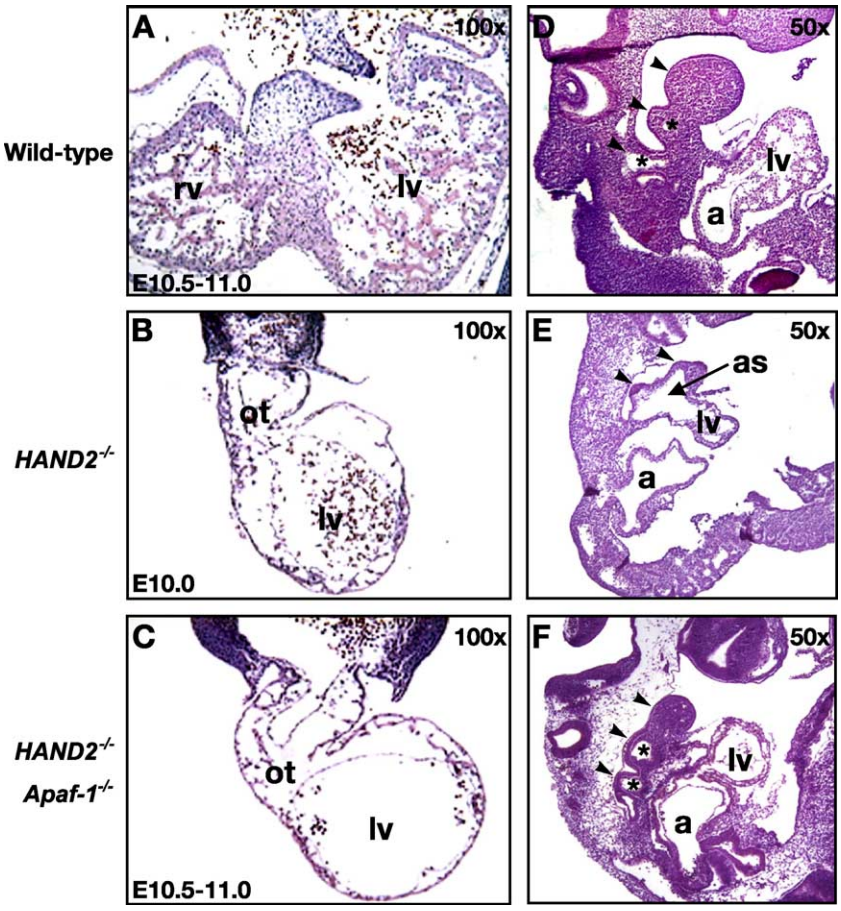

Fig. 5. Histological analysis of *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos. Transverse (A–C) and sagittal (D–F) sections through wild-type (A and D), *HAND2*$^{-/-}$ (B and E), and *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos (C and F) are shown. *HAND2*$^{-/-}$animals showed absence of the right ventricle and a poorly developed left ventricle irrespective of *Apaf-1* dosage (B and C). *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos, however, had better developed pharyngeal arches (arrowheads in F) with patent arch arteries (asterisk in F) compared to *HAND2*$^{-/-}$ embryos (E) at E10.0, which had loss of cells in the arch mesenchyme and atresia of the arch arteries (arrowheads in E) and dilated aortic sac (as). a, atrium; ot, outflow tract; lv, left ventricle; rv, right ventricle.

arteries may be a primary cause of death in *HAND2* mutant embryos.

Discussion

The partial rescue of the *HAND2* mutant phenotype in the *Apaf1*-null background supports the hypothesis that the apoptosis observed in *HAND2*-null embryos occurs in part through nonreceptor-mediated activation of a caspase pathway. Our results also suggest that the atresia of pharyngeal arch arteries might be the primary cause of cardiac failure in *HAND2* mutants and that the right ventricular defect is not secondary to obstruction of blood flow from the cardiac outflow tract.

Apoptosis observed in the right ventricle and pharyngeal arches of HAND2$^{-/-}$ *embryos may occur via different pathways*

HAND2$^{-/-}$ embryos exhibit hypoplasia of the right ventricle at E8.5 and pharyngeal arches at E9.5 due to robust apoptosis (Thomas et al., 1998; Yamagishi et al., 2001). Electron microscopy and comparative gene expression analysis suggested a mitochondrial damage-induced pathway caused this apoptosis. Consistent with this, loss of Apaf-1 blocked apoptosis in the pharyngeal arches and limb bud of *HAND2*$^{-/-}$ embryos. Apaf-1 is a cytosolic protein, which in the presence of ATP and cytochrome *c* can activate pro-caspase-9, an initiator caspase leading to activation of other caspases (Li et al., 1997; Zou et al., 1997). This causes proteolysis and DNA fragmentation in the cell, which is ultimately engulfed by macrophages and cleared. Although most intracellular apoptotic pathways require Apaf-1, other signals transduced by death receptor pathways can also result in apoptosis. Since loss of Apaf-1 only affected pharyngeal arch and limb bud apoptosis, there are likely Apaf-1-independent mechanisms that regulate cell death in the right ventricle of *HAND2* mutants. These could be through death receptors or may represent a default pathway secondary to a differentiation defect in the absence of HAND2, although we cannot distinguish between these alternatives. It is also possible that cells of the cardiac lineage are more sensitive to the necrosis-like cell death

seen in Apaf-1-deficient embryonic fibroblasts (Miyazaki et al., 2001) and in limb buds of Apaf-1-deficient mice (Chautan et al., 1999).

Loss of pharyngeal arch arteries in HAND2$^{-/-}$ embryos is partially responsible for early lethality

In the pharyngeal arches of *HAND2* mutants, apoptosis of the arch mesenchyme coupled with loss of VSMC specification causes hypoplasia of the pharyngeal arch artery (Thomas et al., 1998; Yamagishi et al., 2000). Although these studies were performed in *HAND2*$^{-/-}$ embryos at E9.5 prior to the presence of cardiac distress, there remained the possibility that the pharyngeal arch defect is in some way related to hemodynamic changes caused by the hypoplastic right ventricle and thin walled left ventricle. Therefore, it has been difficult to determine if the early lethality of the *HAND2*$^{-/-}$ embryo was a result of ventricular hypoplasia or if the pharyngeal arch defects might play a causative role in the E9.5 growth arrest of *HAND2*$^{-/-}$ embryos.

We have analyzed pharyngeal arches of *HAND2*$^{-/-}$ embryos with decreased dosage or absence of Apaf-1 at E10.5–11.0. At this stage, *HAND2*$^{-/-}$ embryos with normal Apaf-1 dosage are dead, with a complete absence of cells in the pharyngeal arches and severe pericardial effusion caused by cardiac failure. Loss of Apaf-1 function, however, maintained the integrity of the pharyngeal arch and the arch artery in *HAND2*$^{-/-}$ embryos. These *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos did not exhibit pericardial effusion at E10.5, suggesting that maintenance of the arch arteries reduced cardiac stress leading to longer survival of the animals, consistent with a primary role for the pharyngeal arch arteries in the cardiac failure observed in *HAND2*$^{-/-}$ embryos at E9.5. The failure of myocardial development in *HAND2*$^{-/-}$*Apaf-1*$^{-/-}$ embryos ultimately leads to growth arrest beyond E11.0 when the embryo requires a more vigorous circulation.

In this study, we investigated the apoptotic pathway activated in the absence of HAND2. We have shown that loss of Apaf-1 leads to a partial rescue of the *HAND2*$^{-/-}$ phenotype by blocking apoptosis in the pharyngeal arch mesenchyme, indicating that arch artery defects in *HAND2*$^{-/-}$ embryos contribute to the early lethality. Our results also show that the right ventricular hypoplasia in *HAND2*$^{-/-}$ embryos is independent of the arch defect and possibly results via an Apaf-1-independent mechanism. The identification of distinct enhancers controlling cardiac and pharyngeal arch expression of *HAND2* (Charite et al., 2001; McFadden et al., 2000) provides an important tool for the study of HAND2 function during the development of these two tissues. Deletion of an ET-1- and Dlx6-dependent pharyngeal arch *HAND2* enhancer causes craniofacial defects in mice harboring the homozygous deletion (Yanagisawa et al., 2003); however, these mice continue to express HAND2 in the ventral regions of the pharyngeal arches. Targeted deletion of *HAND2* in specific tissues will more definitively reveal its independent roles in embryonic development.

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