Nf1 has an essential role in endothelial cells

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Abstract

Neurofibromatosis type 1 (NF1) or von Recklinghausen neurofibromatosis is a genetic disorder that occurs in 1 of 4000 births and is characterized by benign and malignant tumors. Cardiovascular defects also contribute to NF1, though the pathogenesis is still unclear. Deficiency in neurofibromin (encoded by *Nf1*) in mice results in mid-embryonic lethality owing to cardiac abnormalities previously thought to be secondary to cardiac neural-crest defects. Using tissue-specific gene inactivation, we show that endothelial-specific inactivation of *Nf1* recapitulates key aspects of the complete null phenotype, including multiple cardiovascular abnormalities involving the endocardial cushions and myocardium. This phenotype is associated with an elevated level of ras signaling in $Nf1^{-/-}$ endothelial cells and greater nuclear localization of the transcription factor Nfatc1. Inactivation of *Nf1* in the neural crest does not cause cardiac defects but results in tumors of neural-crest origin resembling those seen in humans with NF1. These results establish a new and essential role for *Nf1* in endothelial cells and confirm the requirement for neurofibromin in the neural crest.

Neurofibromin has ras GTPase-activating activity and can downregulate ras signaling. The absence of this activity is thought to underlie some or all aspects of NF1 in humans. NF1 is characterized by proliferation and malignant transformation of neural-crest derivatives. Affected individuals also have disorders that seem unrelated to the neural crest, including hypertension, renal artery stenosis, higher incidence of congenital heart disease, especially valvular pulmonic stenosis, and vascular abnormalities in the central nervous system known as moya-moya[1,2]. *NF1* functions as a tumor-suppressor gene, and loss of heterozygosity in somatic tissues has been associated with tumor formation[3].

Attempts to produce animal models of NF1 have been hampered by the fact that inactivation of *Nf1* in mice leads to mid-gestation lethality from cardiovascular abnormalities. These defects include structural malformations of the outflow tract of the heart and enlarged endocardial cushions[3–5], which are the anlage of cardiac valves. Normally, neural-crest cells migrate to the outflow region of the heart and contribute to the endocardial cushions as early as embryonic day (E) 10.5 (Fig. 1*a,b*). Neural-crest defects can result in abnormal morphogenesis of the heart[6], and cardiac abnormalities in *Nf1*$^{-/-}$ embryos have been attributed to the loss of neurofibromin function in cardiac neural-crest cells[3,4]. But the majority of cells in the forming endocardial cushions are derived from underlying endothelial cells that undergo epithelial–mesenchymal transformation (Fig. 1*c–e*). Hearts of *Nf1*$^{-/-}$ embryos have abnormal epithelial–mesenchymal transformation[5]. This could result from defective signaling by the neural crest or myocardium or from abnormal differentiation of the endothelium.

We used a conditional gene inactivation approach to ablate neurofibromin function specifically in the endothelium. A conditional allele of *Nf1* (*Nf1*$^{\mathrm{tm1Par}}$, herein called *Nf1*$^{\mathrm{flox}}$) is susceptible to Cre-mediated recombination *in vivo* (Fig. 1*f*) and is a phenocopy of the null allele[7]. We crossed *Nf1*$^{\mathrm{flox}}$ mice with *Tek–cre* transgenic mice (also called Tie2-Cre) in which expression of Cre recombinase is driven by an endothelial-specific promoter/enhancer[8]. Endothelial-specific inactivation of *Nf1* (*Nf1*$^{\mathrm{ec}}$) recapitulates the cardiovascular phenotype observed in *Nf1*$^{-/-}$ embryos. We examined cardiac anatomy of wild-type, *Nf1*$^{-/-}$ and *Nf1*$^{\mathrm{ec}}$ embryos from eight litters (Fig. 2 and Table 1). In comparison with wild-type embryos (Fig. 2*a,e*), *Nf1*$^{-/-}$ (Fig. 2*b,f*) and *Nf1*$^{\mathrm{ec}}$ (Fig. 2*c,g*) embryos had enlarged endocardial cushions. Hearts of *Nf1*$^{\mathrm{ec}}$ embryos also had ventricular septal defects (Fig. 2*g*), double outlet right ventricle (data not shown), thinned myocardium (Fig. 2*k*) and pericardial effusions (Fig. 2*c* and data not shown), which were similar to those seen in *Nf1*$^{-/-}$ embryos.

The myocardial defects that we observed were secondary to endothelial dysfunction and were not reproduced by tissue-specific inactivation of *Nf1* in myocardial cells (data not shown). Signaling between the endocardium and myocardium is required for proper myocardial development[9]. Likewise, an endothelial-derived neurofibromin-dependent paracrine signal may be required by neural-crest cells for proper development of neural-crest-derived structures. Nevertheless, our data indicate a genetic requirement for neurofibromin in endothelial cells. These observations may explain why individuals with NF1 have a higher incidence of valvular pulmonic stenosis, hypertension and other cardiovascular disorders[2].

Neurofibromin deficiency in endocardial cells results in altered ras signaling. Immunohistochemistry identified phosphorylated MAPK (P-MAPK) in the hearts of wild-type and neurofibromin-deficient embryos at E11.5. Occasional endothelial cells positive for P-MAPK were present in the region of the endocardial cushions of hearts of wild-type embryos (Fig. 3*a,c*). Expression was more robust and was present in more endothelial cells lining the endocardial cushions in hearts of *Nf1*$^{-/-}$ embryos (Fig. 3*b,d*). Activation of MAPK suggests that *Nf1* deficiency results in elevated ras signaling in endocardial cells, though other regulators of MAPK phosphorylation and other ras-GAPs exist. Using a ras activation assay, we determined that endothelial cell–specific loss of neurofibromin leads to ras activation in *Nf1*$^{\mathrm{ec}}$ embryos (Fig. 3*e*). These data suggest that ras signaling is normally downregulated as endothelial cells mature into valve leaflets. Neurofibromin is required for this downregulation. It has been suggested that defects in erbB3 and PDGFα receptor signaling can lead to developmental defects of the endocardial cushions[10,11]. But the genes encoding these receptors as well as erbB2, erbB4 and neuregulin are expressed normally in *Nf1*-deficient embryos (ref. 5 and M.M. Lakkis and J.A.E., unpublished results). Likewise, mutations in *Sox4*, *Pax3*, *Sema3c*, *Itga4* and members of the Tgfβ family have been

implicated in developmental cardiac defects that include endocardial-cushion abnormalities[12−16]. These genes also seem to be expressed normally in $Nf1^{-/-}$ embryos (E10.5–13.5; M.M. Lakkis, A.D.G., M.M.L. and J.A.E., unpublished results).

Recently, several mouse lines have been generated that exhibit defects in endocardial-cushion morphogenesis[14,17−20]. *Nfatc1* knockout mice die *in utero* with hypoplastic valves[17,20]. Nfatc1 activity is regulated by phosphorylation and sub-cellular localization. The ras signaling pathway can affect nuclear localization of Nfat isoforms in myocytes[21] and T cells[22]. An elevated level of ras signaling in $Nf1^{-/-}$ endocardial cells affects Nfatc1 nuclear localization. Nfatc1 was located in the cytoplasm of wild-type endocardial cells at E9.5 (Fig. 3f). At later time points, it becomes localized to the nucleus[17,20]. Nfatc1 was prematurely localized to the nucleus in $Nf1^{-/-}$ endocardial cells at E9.5 (Fig. 3g). Expression of a constitutively active form of ras in endothelial cells from embryonic endocardial-cushion explants (Fig. 3h,i) or human umbilical vein endothelial cells (Fig. 3j,k) was sufficient to enhance Nfatc1 nuclear localization. We propose that increased ras activity in neurofibromin-deficient endocardial cells leads to Nfatc1 activation. This may contribute to the cardiovascular defects seen in mouse embryos lacking *Nf1* in endothelial cells and to vascular defects observed in individuals with NF1, though our data do not exclude the possible contribution of other downstream pathways regulated by *Nf1*.

These results show that neurofibromin is required in endothelial cells, but do not rule out a simultaneous requirement in the neural crest during cardiac development. Because neural-crest derivatives are affected in individuals with NF1 and in mice with disruption of *Nf1* (refs. 3,4,23,24), we used a *cre–lox* fate-mapping strategy to examine cardiac neural-crest migration in wild-type and *Nf1* mutant embryos[25,26]. Cardiac neural-crest cells populated the outflow tract of hearts from E12.5 wild-type and *Nf1* mutant embryos (Fig. 4a,b). Although the gross morphology of hearts from $Nf1^{-/-}$ embryos is abnormal[3−5], the abundance and location of neural-crest cells was similar to that seen in hearts of wild-type embryos (Fig. 4). Although it is possible that subtle abnormalities of the neural crest exist, cardiac defects in $Nf1^{-/-}$ mice occurred despite apparently normal neural-crest migration.

To determine if *Nf1* function is required in the neural crest, we carried out tissue-specific *Nf1* gene inactivation. We used three independent lines of transgenic mice expressing Cre recombinase in neural-crest cells. In each case, previous fate-mapping experiments have confirmed expression of these transgenes in neural-crest cells fated to populate the heart and other neural-crest derivatives[25−27]. We examined embryos derived from appropriate crosses between *Nf1*^flox mice and *Mpz–cre* (three litters at E13.5), *Pax3–cre* (six litters at E13.5, five newborn litters) and *Wnt1–cre* (five litters at E13.5, six newborn litters). Because we obtained similar results with all neural-crest transgenic *cre* lines, we refer to them collectively as *Nf1*^nc.

Nf1^nc embryos were viable throughout gestation, though $Nf1^{-/-}$ littermates are not viable after E13.5 (refs. 3,4). At birth, 9 of 67 pups (13% versus the expected 12.5%) were *Nf1*^nc. At E13.5, cardiac development in *Nf1*^nc embryos was normal (Fig. 1d,h,l), but newborn *Nf1*^nc pups were not normal. Newborn *Nf1*^nc pups were smaller than wild-type (Fig. 5a,b) and did not initiate normal respiration. Consistent with the tissue-specificity of our Cre-expressing transgenic mice, there was hyperplasia of tissues of neural-crest origin, including massively enlarged sympathetic ganglia (Fig. 5d). These abnormal structures, seen in all five *Nf1*^nc pups examined, consisted of axons that stained for neurofilament and small cell bodies with large nuclei that expressed tyrosine hydroxylase (data not shown). This morphology is consistent with ganglioneuroma or ganglioneurosarcoma, which can develop into malignant peripheral nerve sheath tumors[28]. We observed hyperplastic tissues with similar appearance adjacent to the lumbar spine and infiltrating the abdominal omentum and the uterus. In all

cases, the adrenal glands were enlarged (Fig. 5*f*). There was a marked increase in the number of adrenal medullary cells, suggesting pheochromocytoma, and a thinning and distortion of the adrenal cortex (Fig. 5*e*,*f*). The adrenal medulla is of neural-crest origin, and the abnormally abundant cells stained for tyrosine hydroxylase (Fig. 5*h*). In some cases, we observed cells positive for tyrosine hydroxylase traversing the cortical boundaries (Fig. 5*h*). Hence, *Nf1*[nc] pups showed hyperplasia of tissues that are affected in individuals with NF1 and provide a useful model for the study of *Nf1* and cancer. We conclude that *Nf1* function in the neural crest is not required for normal development of the heart.

Methods

Mouse breeding and genotyping

We genotyped *Nf1*^{+/−}, *Nf1*[flox], *Pax3–cre*, *Wnt1–cre*, *Tek–cre*, *Mpz–cre*, Z/EG and Gt(ROSA)26Sor[tm1Sor] (R26R) mice as described previously[4,7,8,25−27,29,30]. The institutional animal care and use committee of the University of Pennsylvania approved all animal protocols.

Histology and immunohistochemistry

Fixation and immunohistochemistry protocols are available at http://www.uphs.upenn.edu/mcrc. For detection of neurofilament, we used monoclonal antibody 2H3 (Developmental Studies Hybridoma Bank) at a dilution of 1:25. We used Phosphop44/42 MAPK (Thr202/Tyr204) monoclonal antibody E10 (Cell Signaling Technology) at a dilution of 1:100. To detect Nfatc1, we used monoclonal antibody 7A6 at a dilution of 1:50 (BD PharMingen).

Endothelial cell culture

We carried out endocardial-cushion explants from E10.5 mouse embryos and subsequent adenovirus infection as described previously[5]. We cultured human umbilical vein endothelial cells in endothelial growth medium (Clonetics) according to the manufacturer's guidelines and co-transfected them with 1 µg of pCDNA3-Nfatc1 and either 2 µg of pCMV-rasV12 or 2 µg of pCMV using Lipofectin (Invitrogen). We analyzed Nfatc1 localization 48 h after transfection.

Ras activation assay

We used the Ras Activation Assay Kit (Upstate) according to the manufacturer's guidelines and incubated 1 µg of lysate from E11.5 wild-type or *Nf1*[ec] mice with 5 µl of a 50% slurry of Raf-1 RBD (ras assay reagant)–agarose.

Acknowledgments

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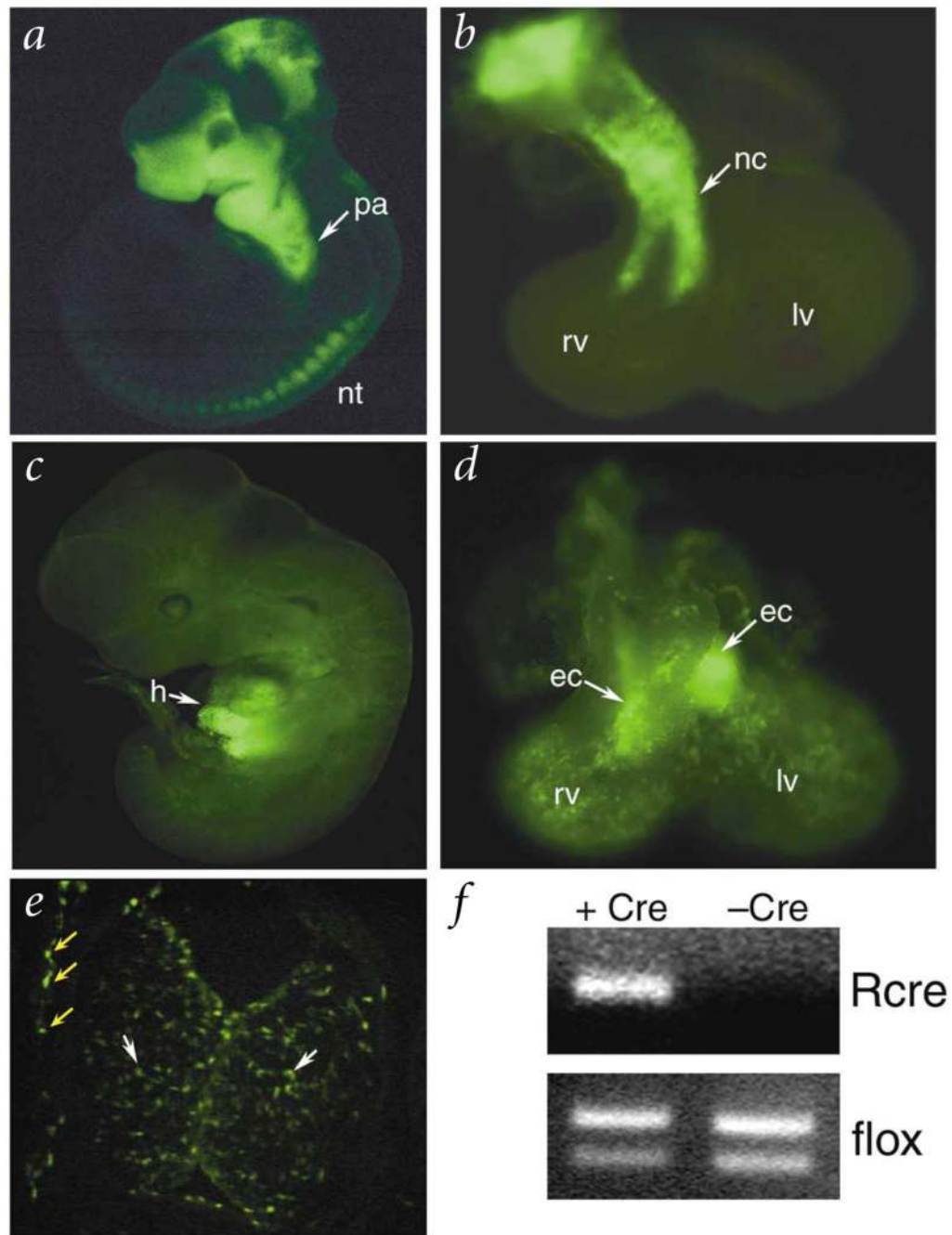
Fig. 1.
Tissue-restricted transgenic expression of Cre recombinase in neural crest and endothelium. E10.5 embryos (*a,c*) and hearts (*b,d*) resulting from a cross of either *Wnt1–cre* (*a,b*) or *Tek–cre* (*c,e*) mice to the Z/EG reporter mouse. Cre activates expression of GFP in derivatives of the neural crest (*a,b*) and of endothelial cells (*c–e*). A transverse section through the heart of a *Tek–cre* × Z/EG embryo (*e*) shows GFP expression in both endothelial cells lining the endocardial cushions (yellow arrows) and the endothelial-derived mesenchymal cells within the cushion (white arrows). *f*, PCR was used to detect the recombined allele (Rcre) as described previously[7] in genomic DNAs isolated from *Nf1*^flox embryonic hearts either positive or negative for *Tek–cre*. PCR amplification of the *Nf1*^flox allele from both samples

is shown below as control. pa, pharyngeal arches; nt, neural tube; rv, right ventricle; lv, left ventricle; nc, neural crest; h, heart; ec, endocardial cushions.
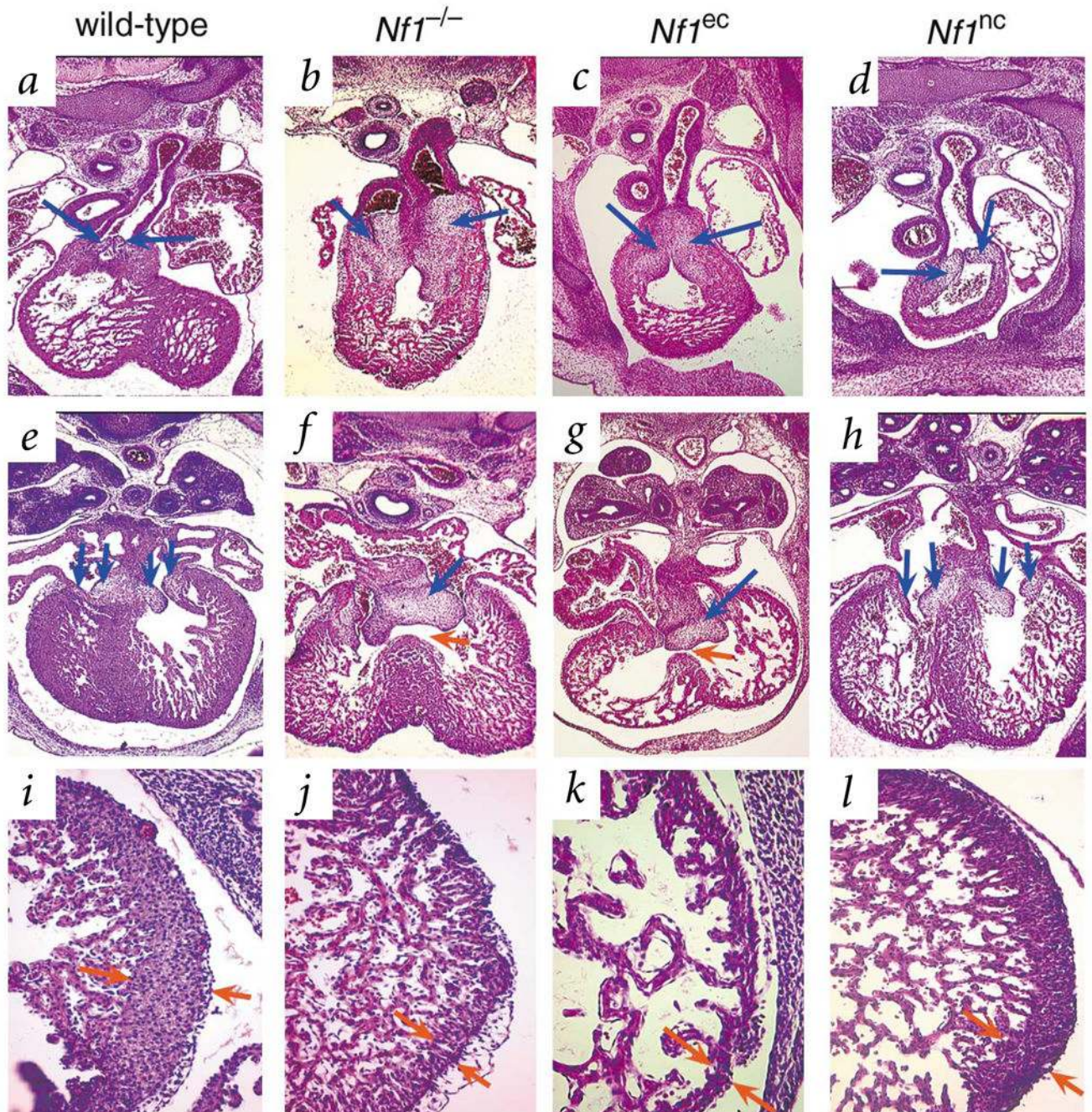
Fig. 2.
Endothelial-specific *Nf1* inactivation disrupts heart development. Sections of hearts from
E13.5 wild-type (*a,e,i*), *Nf1*$^{-/-}$ (*b,f,j*), *Nf1*^{ec} (*c,g,k*) and *Nf1*^{nc} (*d,h,l*) embryos. Outflow tract
endocardial cushions (*a–d*, blue arrows) and atrioventricular cushions (*e–h*, blue arrows) and
high-power magnification of left ventricular myocardium (*i–l*) are shown. The endocardial
cushions of hearts from *Nf1*^{ec} embryos (*c,g*) were enlarged and appeared similar to those of
Nf1$^{-/-}$ embryos (*b,f*). A ventricular septal defect was seen in hearts of both *Nf1*$^{-/-}$ (*f*) and
Nf1^{ec} (*g*) embryos. The compact layer of the myocardium of *Nf1*^{ec} embryos (*k*) was thinned,
as it was in *Nf1*$^{-/-}$ embryos (*j*), whereas *Nf1*^{nc} embryos (*l*) resembled wild-type (*i*).
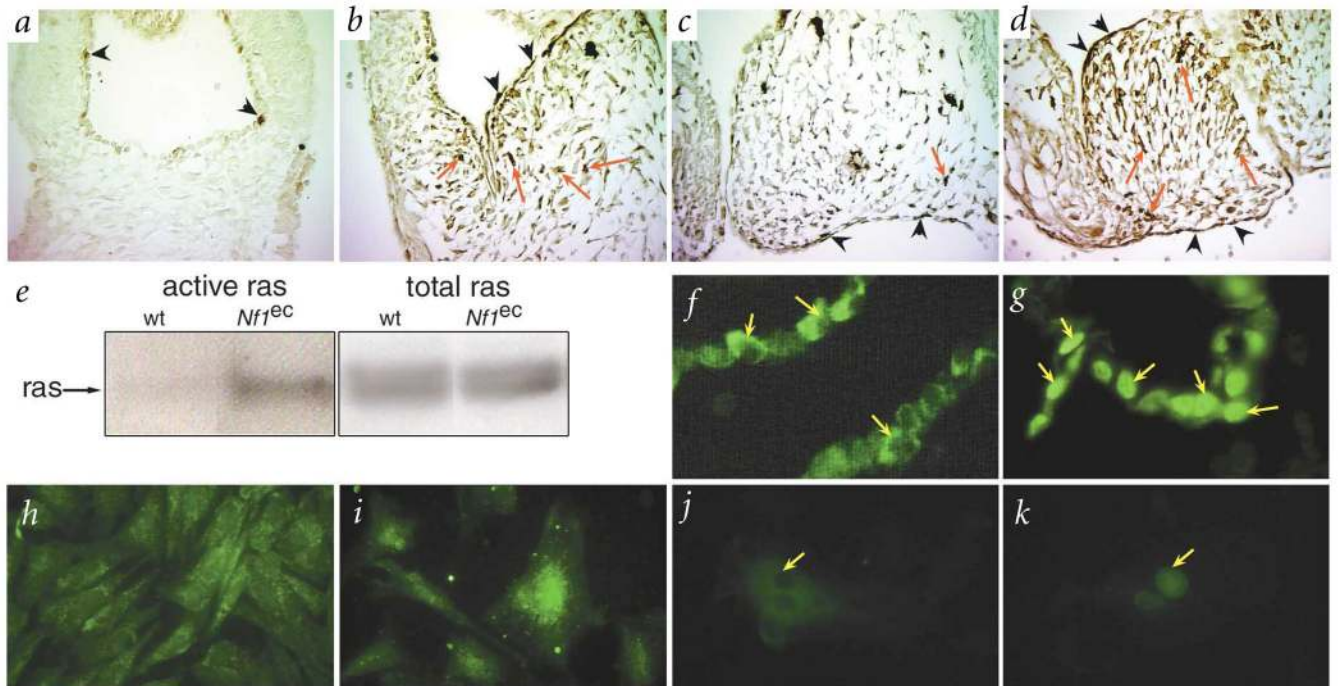
Fig. 3.
Elevated ras signaling and increased Nfatc1 nuclear localization in hearts of $Nf1^{-/-}$
embryos. ***a–d***, Activated MAPK (arrows) detected by immunohistochemistsry in sections of
outflow tract (*a,b*) and atrioventricular (*c,d*) endocardial cushions from E11.5 wild-type (*a,c*)
and $Nf1^{-/-}$ (*b,d*) embryos. ***e***, Higher levels of activated ras were present in $Nf1^{ec}$ embryos as
detected by pull-down assay with the ras binding domain of raf. wt, wild-type. ***f,g***, Nfatc1
was localized to the cytoplasm of endothelial cells from E9.5 wild-type (yellow arrow, *f*)
embryos and to the nucleus in $Nf1^{-/-}$ littermates (yellow arrow, *g*). ***h,i***, Nfatc1 was in the
cytoplasm of endothelial cells cultured from wild-type endocardial-cushion explants infected
with adenovirus expressing *lacZ* (*h*) but in the nucleus of cells infected with rasV12 virus
(*i*). ***j,k***, Human umbilical vein endothelial cells were transfected with Nfatc1 alone (*j*) or
Nfatc1 plus rasV12 (*k*), which resulted in nuclear localization of Nfatc1 (arrow, *k*).
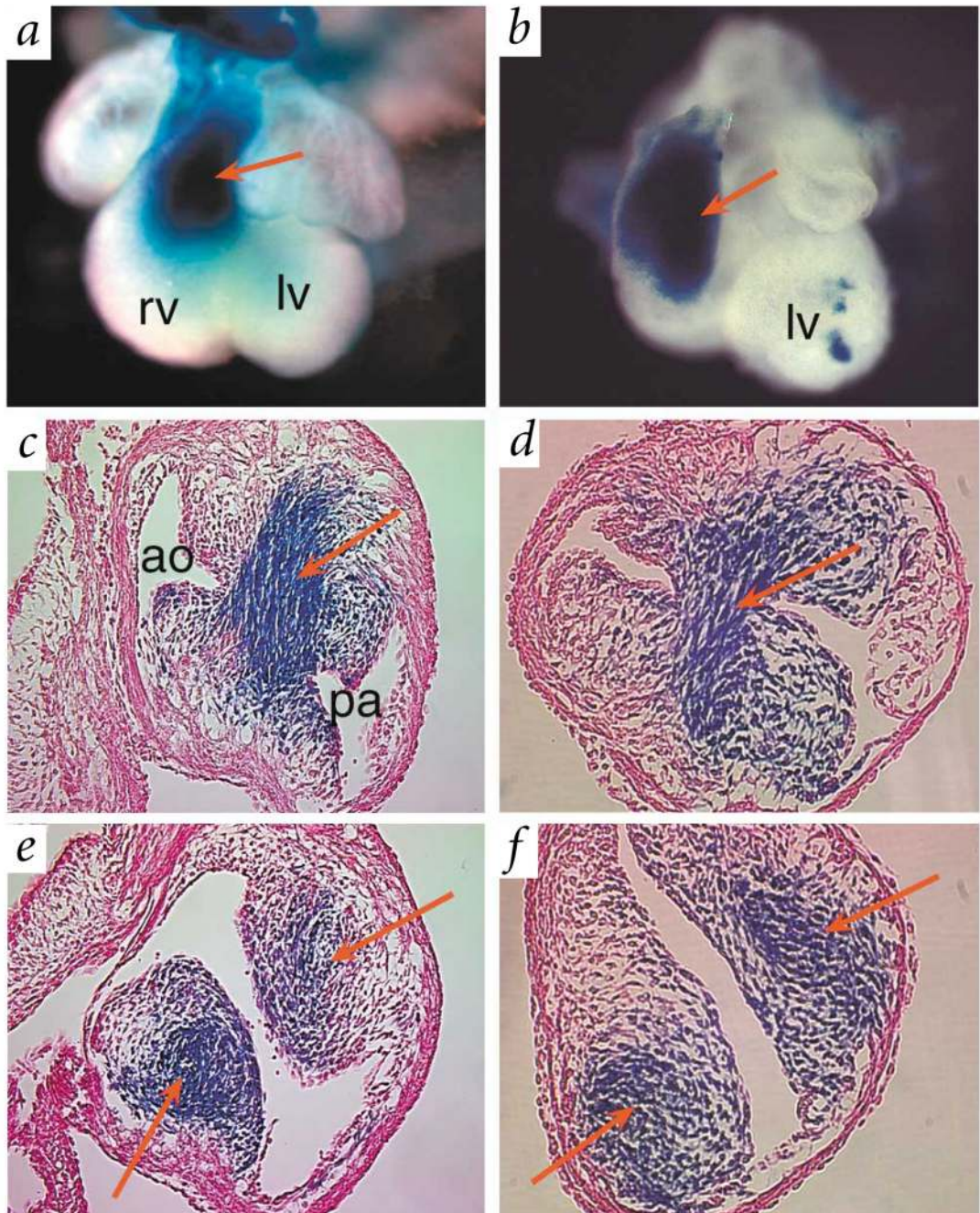
Fig. 4.
Fate mapping of cardiac neural crest. **a,b**, Hearts of E12.5 wild-type (*a*) and *Nf1⁻/⁻* (*b*) embryos stained for β-galactosidase activity. Cardiac neural-crest cells are labeled blue by Cre-mediated activation of *lacZ* in R26R reporter mice30. Red arrows point to labeled cells populating the outflow tract of the developing heart. **c,d**, Cross-sections through hearts at the level of the truncus show labeled blue cells forming the septum between the aorta and pulmonary artery in both wild-type (*c*) and *Nf1⁻/⁻* (*d*) embryos. **e,f**, Red arrows point to labeled neural-crest cells invading the outflow tract endocardial cushions in the conus region of the right ventricular outflow tract in wild-type (*e*) and *Nf1⁻/⁻* (*f*) embryos. pa, pulmonary artery; ao, aorta; rv, right ventricle; lv, left ventricle.
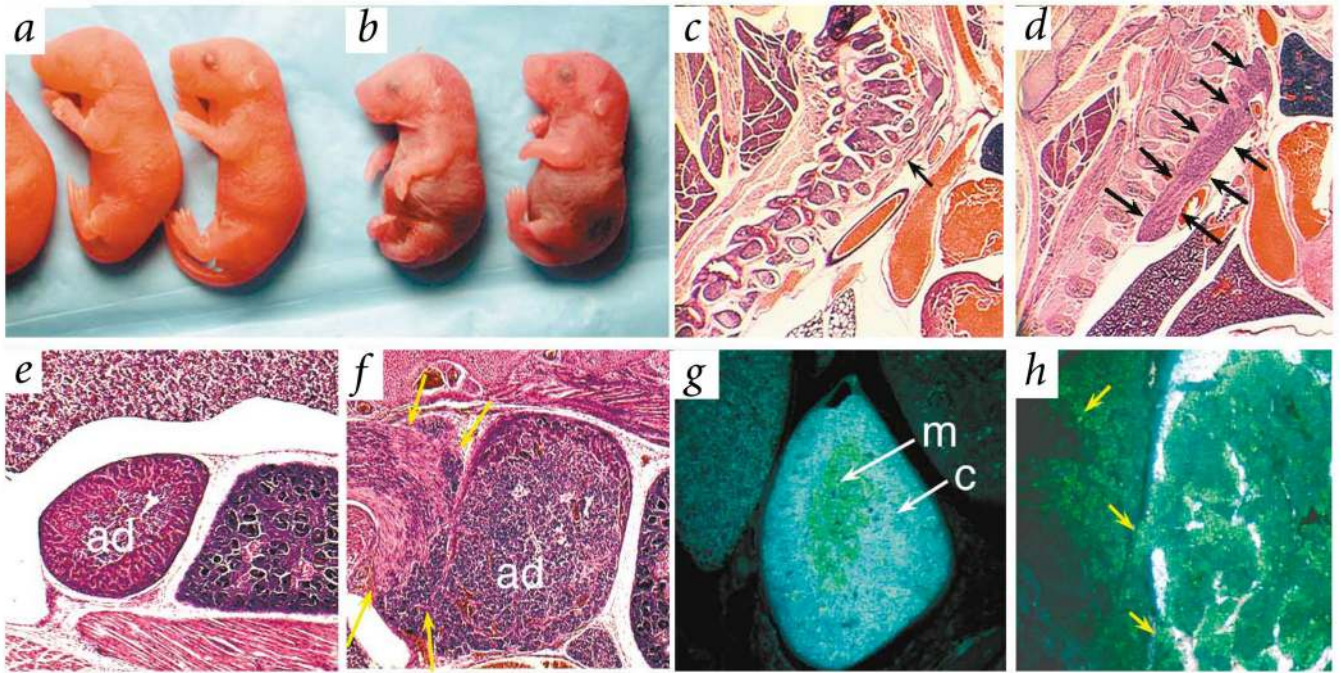
Fig. 5.
*Nf1*nc mice live longer than *Nf1*−/−embryos and develop tumors of neural-crest origin. ***a,b***, Comparison of newborn wild-type (*a*) and *Nf1*nc (*b*) littermates. ***c,d***, Sagittal sections of newborn pups showed normal sympathetic ganglia in wild-type embryos (arrow, *c*) and massively enlarged ganglia in *Nf1*nc embryos (arrows, *d*). ***e–h***, Adrenal gland (ad) of wild-type (*e,g*) and *Nf1*nc (*f,h*) embryos showed hyperplastic adrenal medullary cells reminiscent of pheochromocytoma in *Nf1*nc pups. The cells formed a large mass extrinsic to the adrenal gland (yellow arrows, *f*). Immunohistochemistry showed medullary (m) cells positive for tyrosine hydroxylase surrounded by cortex (c) in wild-type embryos (*g*), whereas cells positive for tyrosine hydroxylase were seen invading the cortex and surrounding tissues in *Nf1*nc embryos (yellow arrows, *h*).

Table 1

Cardiac phenotypes in *Nf1* knockout mice

Genotype	Number analyzed	DORV	VSD	Cardiac phenotype		
				Enlarged AV cushion	Thinned myocardium	
wild-type	10	0	1	0	0
Nf1$^{-/-}$	10	10	10	10	10
Nf1^{ec}	11	8	10	8	8

Frequency of specific cardiac embryonic phenotypes in wild-type, *Nf1*$^{-/-}$ and *Nf1*^{ec} embryos at E13.5. DORV, double outlet right ventricle; VSD, ventricular septal defect; AV, atrioventricular.