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Notochordal and Foregut Abnormalities Correlate with Elevated Neural Crest Apoptosis in *Patch* Embryos

Paige Snider^{1,*}, Olga Simmons¹, Rhonda Rogers¹, Rachel Young^{1,2}, Mica Gosnell¹, and Simon J. Conway¹

¹Developmental Biology and Neonatal Medicine Program, HB Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Indiana

²University of Ulster, Coleraine, Northern Ireland, BT52 1SA, United Kingdom

Abstract

Although *Patch* mutants show severe abnormalities in many neural crest-derived structures including the face and the heart, there is a paucity of information characterizing the mechanisms underlying these congenital defects. Via manipulating the genetic background to circumvent early embryonic lethality, our results revealed that *Patch* phenotypes are most likely due to a significant decrease in migratory neural crest lineage due to diminished neural crest survival and elevated apoptosis. Homozygous mutant neural crest precursors can undergo typical expansion within the neural tube, epithelial-to-mesenchymal transformation, and initiate normal neural crest emigration. Moreover, in vitro explant culture demonstrated that when isolated from the surrounding mesenchyme, *Patch* mutant neural crest cells (NCCs) can migrate appropriately. Additionally, *Patch* foregut, notochord and somitic morphogenesis, and *Sonic hedgehog* expression profiles were all perturbed. Significantly, the timing of lethality and extent of apoptosis correlated with the degree of severity of *Patch* mutant foregut, notochord, and somite dysfunction. Finally, analysis of Balb/c-enriched surviving *Patch* mutants revealed that not all the neural crest subpopulations are affected and that *Patch* mutant neural crest-derived sympathetic ganglia and dorsal root ganglia were unaffected. We hypothesize that loss of normal coordinated signaling from the notochord, foregut, and somites underlies the diminished survival of the neural crest lineage within *Patch* mutants resulting in subsequent neural crest-deficient phenotypes.

Keywords

mouse embryo; *PDGFA receptor*; neural crest; congenital heart defects; midface clefting; apoptosis; *Shh*; notochord; genetic background

INTRODUCTION

Abnormal neural crest cells (NCCs) morphogenesis is thought to be responsible for a wide variety of congenital defects, including the DiGeorge and Velo-cardio-facial syndromes (Srivastava, 1999), particularly cardiac outflow tract defects (Snider et al., 2007), midface clefting (Morrison–Graham et al., 1992; Trainor, 2010), abnormal eye development (Valleix et al., 1999), piebaldism and pigmentation abnormalities (Wehrle–Hallar and Weston, 1999), Noonan and LEOPARD syndromes (Stewart et al., 2010), and a lack of enteric neural

plexuses in the foregut, resulting in Hirschsprung's disease (Takahashi et al., 1999; Gershon, 2010). Disruption of the neural crest (NC) population by experimental perturbation, including NCC ablation in the chick or mouse embryo, or by numerous classic and targeted genetic mutations (reviewed by Creazzo et al., 1998; Snider et al., 2007) results in phenotypically similar abnormalities affecting NC-derived structures, suggesting that this population of cells is essential to normal development. More recently, NC cells have been shown to interact and modulate signaling in the pharynx during formation of the outflow tract by the secondary heart field (Goddeeris et al., 2007; Hutson and Kirby, 2007). These studies have shown that the NC may not be the direct cause of abnormal cardiovascular development but they are a major component in the complex tissue interactions in the caudal pharynx and outflow tract (Hutson and Kirby, 2007). Moreover, abnormal levels of cell death and/or cell proliferation, changes in cell fate, altered migration, and diminished differentiation have all been proposed to account for the NC-associated abnormal phenotypes. The specific molecular mechanisms responsible for these abnormalities within NC and the role they play during pathogenesis of the congenital defects in the majority of these models remains unclear.

Classic *Patch* (*Ph*; Gruneberg and Truslove, 1960) and *Patch-extended* (*Ph^e*; Truslove, 1977) mouse mutants, and targeted mutations of the platelet-derived growth factor receptor- α (*PDGFR α* ; Soriano, 1997) gene, all exhibit various NC-associated defects and the homozygous mutant embryos usually die around mid gestation. Due to the NC-associated abnormalities present in *Ph* and *PDGFR α* systemic knockout mice, conditional targeting studies have begun to address the role of *PDGFR α* signaling within NC cells (Tallquist and Soriano 2003; Richarte et al., 2007). Significantly, *Wnt1-Cre; PDGFR α* conditional embryos exhibit NC-associated cranial, frontonasal, and aortic arch artery defects, as well as variable penetrance heart defects, suggesting an essential cell autonomous role (Tallquist and Soriano, 2003). However, unaltered NC proliferation, migration, or survival has led to the conclusion that NC-required *PDGFR α* signaling lies in another function such as differentiation or extracellular matrix deposition (Tallquist and Soriano, 2003). Intriguingly, the *Ph* mutation results in the loss of the entire *PDGFR α* gene as well as upstream regions of the adjacent *c-kit* gene (Brunkow et al., 1995; Sun et al., 2000). Moreover, complementation studies have shown that while a human yeast artificial chromosome transgene can transgenically rescue *PDGFR α* systemic knockouts, it is unable to rescue *Ph/Ph* embryos (Sun et al., 2000). As this indicated that genetic defects outside the *PDGFR α* locus may contribute to the embryonic lethality of *Patch* mice and as it has been reported that changing the genetic background enables the *Ph/Ph* mutants to survive longer in utero due to the presence of a putative modifier (Payne et al., 1997); we sought to fully characterize the etiology of *Ph/Ph* NC-related malformations on different genetic backgrounds.

Our results reveal that NC, in both the early lethal and longer surviving *Ph* homozygous mutants, express markers consistent with normal NC expansion within the neural tube, normal emigration from the neural tube, and migration along the expected pathways. However, elevated levels of apoptosis occur during migration resulting in a diminished *Ph/Ph* colonizing NC population. Taken together, these results suggest *Ph/Ph* NC-related malformations are due, at least in part, to increased apoptosis along the NC migratory pathway. Additionally, somitic and related foregut/notochordal abnormalities and alterations in *Sonic hedgehog* expression correlated with the severity of the homozygous *Ph* phenotypes. Combined, these data indicate loss of coordinated signaling from the notochord, foregut, and somites underlies the diminished NC survival within *Patch* mutants resulting in subsequent NC-deficient phenotypes.

MATERIALS AND METHODS

Mice and histologic analysis

Patch (*Ph*) heterozygotes on a *C57Bl/6* background (Jackson Laboratories, Bar Harbor, ME) were backcrossed with Balb/c mice for seven generations to generate *Ph* heterozygotes (Payne et al., 1997). Noon of the day when a vaginal plug was observed was counted as embryonic day (E)0.5. Pregnant dams were euthanized by cervical dislocation from E8.5 to E17.5, uterine horns were removed and transferred to cold phosphate-buffered saline (PBS; 4°C) and embryos were subsequently dissected. Tissue was collected for genotyping, and embryos were fixed overnight in 4% paraformaldehyde/PBS (4°C), dehydrated to 100% methanol through a graded PBT (PBS + 0.1% Tween 20) series, and stored at -20°C until used for either in situ hybridization, apoptosis analysis, or processed for paraffin sectioning and stained with hematoxylin/eosin as described by Conway et al. (1997b).

Polymerase chain reaction genotyping of *Patch* mutants

Two different protocols were used to genotype homozygous *Ph* mutants. First, for embryos on a pure *C57Bl/6* background, Mouse Map Pairs 290 and 201 (D5MIT290 and D5MIT201 Map Pairs; Research Genetics) were used to distinguish between homozygous mutant and wild-type or heterozygote embryos; this method cannot distinguish between heterozygotes and homozygous wild-type embryos. The 201 primer pair (3'-ACCAGTCAAGGAC GAATCCTT-5'; 3'-CCCTTTAGCTTCCTCAGGAG-5') amplifies a segment of *PDGFR α* inside the deletion and the 290 primer pair (5'-ACCCAGGCCACAAAAGAAC-3'; 5'-ACCCCTATTCACTGCCAGG-3') amplifies a region of DNA outside the deletion, thereby serving as a positive control for the presence of genomic DNA. The following program was used for 30 cycles to amplify genomic DNA: denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 30 seconds. PCR products were run on a 4% agarose gel alongside known controls and homozygous mutants were identified by the presence of the 290 amplified band and the subsequent absence of the 201 band. Once the *C57Bl/6* heterozygotes were backcrossed onto a Balb/c background, all three genotypes of embryos could be distinguished from heterozygote matings with PCR primers which localize to the D5MIT135 region of the mouse locus (3'-CGGAGATTAGGTTTTAGAGGGA-5'; 3'-GGGACAGGAAAGGGACACAT-5'; D5Mit135 Map Pairs, Research Genetics; Wehrle-Haller et al., 1996). PCR conditions were the same as listed above and products were run on a 2% agarose gel alongside known controls.

In vitro neural crest cell migration analysis

Ph heterozygotes were mated and embryos collected at E8.5, staged by counting somite number (only embryos having 4-6 somites were used as this is before initiation of NC cell migration) and cultured essentially as described by Conway et al. (2000). Explants were incubated at 37°C with 5% CO₂ and photographed 24 and 48 hours later. Several isolated neural folds (n = 3) were processed for histology, to confirm that the neural folds were explanted without any contaminating mesenchymal tissue. Digital images of the cultures were collected at 24 and 48 hours after isolation using a SPOT camera (Diagnostic Instruments, Sterling Heights, MI). National Institute of Hospital (NIH) Image software was used to measure and analyze the area of outgrowth, as described by Huang et al. (1998) and Conway et al. (2000). Statistical analysis was carried out using ANOVA.

Whole-mount in situ hybridization

Ph embryos were analyzed by whole-mount in situ hybridization (Conway et al., 1997a), (n = 3 pairs of mutants with age-matched control littermates) were subsequently processed for

sectioning on a vibrating microtome also as described (Bundy et al., 1998). Plasmids were linearized with the appropriate restriction enzymes (*Ap2*, Mitchell et al., 1991; cellular retinoic acid binding protein-1 (*Crabp1*), Stoner and Gudas, 1989; *Prx2*, Kern et al., 1994; *Shh*, Riddle et al., 1993; *Wnt-1*, *Wnt-3a*, and *Pax3*, Conway et al., 2000) and used as templates to generate digoxigenin both labeled sense and anti-sense RNA probes according to the manufacturer's directions (Boehringer Mannheim, Phoenix, AZ).

Cell proliferation and apoptosis

Proliferating cells were detected in serial sections of embryos ($n = 4$) labeled for 2 hours by injection of dam with 0.3 mg BrdU/kg body weight and subsequent detection with anti-BrdU-alkaline phosphatase (Boehringer Mannheim, 1:30). The total cell number and positively stained cell number were counted manually in defined areas of tissues under 40 $\times$ magnification. Statistical analysis of cell counts in serial sections and comparison of mutant specimens with controls was performed using one-tailed t tests (p values were assigned, with < 0.05 being significant).

Apoptosis was visualized in wild-type ($n = 2$) and mutant ($n = 3$) whole embryos in parallel, using the TdT-FragEL DNA Fragmentation Detection Kit (EMD Chemicals, San Diego, CA). Washing, developing, and subsequent clearing/dehydration occurred as described for whole-mount in situ hybridization (Conway et al., 1997a). Double labeling of apoptotic cells and *Crabp1* mRNA in situ expression were done using an adaptation of the protocol described by Goh et al. (1997). Briefly, standard whole-mount in situ hybridization using NBT/BCIP (Bundy et al., 1998) was initially used to detect the *Crabp1* mRNA expression (blue stain). Once the signal was developed, the embryos were fixed overnight in 4% paraformaldehyde. After washing in PBS, embryos were then placed in O.C.T. Embedding Compound (Sakura Finetek, Torrance, CA) and cryosectioned (10 μ m thickness). Subsequently, apoptosis was visualized by standard TUNEL using VIP/diaminobenzidine (DAB) detection (Vector Laboratories, Burlingame, CA) to develop the signal.

Immunohistochemistry

Immunostaining was carried out using ABC kit (Vector Laboratories, Burlingame, CA) with DAB and hydrogen peroxide chromogens as described previously (Tang et al., 2010). The following primary antibodies were used to assess NC differentiation: a rabbit monoclonal against β 3-Tubulin (Covance Research Products, Emeryville, CA; TuJ1, diluted 1:1000); a rabbit polyclonal against tyrosine hydroxylase (Millipore, Billerica, MA; TH, diluted 1:500). Negative controls were obtained by substituting the primary antibody with rabbit serum at 1:150 dilution and positive staining within serial sections was examined using at least three individual embryos of each genotype at each developmental stage.

RESULTS

Patch Homozygous and Heterozygous Phenotypes

***Patch C57Bl/6* homozygous phenotype**—The *Ph* heterozygotes used to generate homozygous mutant embryos in this study were originally on a *C57Bl/6* background. Embryos collected displayed the characteristic phenotype associated with this mutation: distorted or kinked neural tube, subepidermal blisters, enlarged heart, hypoplastic branchial arches, and abnormal somites (Fig. 1C), consistent with that reported by others (Orr–Urtreger et al., 1992; Schatteman et al., 1992). Although E8.5 homozygous mutants seemed normal, those at E9.5 and E10.5 showed severe malformations that resulted in 100% of the homozygous mutant embryos dying by E11. These phenotypes are consistent with those observed in systemic *PDGFR α* null embryos, which exhibit variable severity before lethality by E16 (Soriano, 1997). Interestingly, in addition to the enlarged hearts previously

reported in *Ph* mutants but not observed in *PDGFR α* nulls, we observed midline enlarged hearts (where the heart had failed to loop and remains within a midline position; $n = 8$ of 27 homozygous mutants), and cardia bifida (where the two heart primordia remain separated; $n = 6/27$ homozygous mutants) at E9.5 and E10.5. These phenotypes are reminiscent of the abnormal hearts observed after excessive retinoic acid treatment (Dickman and Smith, 1996) and within *Shh* and fibroblast growth factor (*Fgf8*) mouse knockouts (Tsukui et al., 1999; Abu-Issa et al., 2002).

Patch Balb/c homozygous phenotype—To prolong viability, we backcrossed *Ph* *C57Bl/6* heterozygotes with Balb/c, as Balb/c mice are thought to have a modifier present which allows for longer survival (Wehrle-Haller et al., 1996), although there are several conflicting reports of this within the literature. Morrison-Graham et al. (1992) and Schatteman et al. (1992) used mice that carried the mutation on either a Balb/c or *C57Bl/6* background, and reported that 10% of the embryos exhibit the *Ph/Ph* phenotype at E12 and older. Payne et al. (1997) did a single generation backcross on to Balb/c and recovered *Ph/Ph* embryos up to E18. However, Orr-Urtreger et al. (1992) reported that most *Ph/Ph* died by E11.5. After six generations of backcrossing, we found that a significant number of *Ph/Ph* survived up to at least E17.5 (Table 1). However, no homozygous mutants were found postnatally, indicating Balb/c enriched *Ph/Ph* die around birth probably due to respiratory failure (Sun et al., 2000). When Balb/c-enriched *Ph/Ph* were collected at E9.5 or E10.5, they displayed either a subtle or no phenotype (Fig. 1B), suggesting development had occurred up to this point in a grossly normal fashion (Wehrle-Haller et al., 1996). A threshold level of Balb/c background modifier/s appeared to have been reached by the third backcrossing, enabling some Balb/c-enriched *Ph/Ph* to survive until E13.5 (Table 1). Although some embryos survived longer with subsequent Balb/c enrichment, a proportion (~28%) still died by E11 irrespective of the Balb/c generation, possibly due to incomplete penetrance of modifier/s from the Balb/c background. Surviving *Ph/Ph* embryos (past E13.5; Fig. 1E) displayed multiple abnormalities including midline cleft face ($n = \text{all of } 6$), herniation of abdominal organs ($n = 3/6$), and blood-engorged fetal livers ($n = \text{all of } 6$). Further, we observed 100% of these embryos ($n = \text{all of } 6$) exhibited persistent truncus arteriosus (PTA) and ventricular septal defects (VSDs), wherein the aorta and pulmonary trunks had failed to septate, and the right and left ventricles are not fully separated along the midline of the heart (Fig. 1I and K). The frontonasal and mandibular processes are present but undersized and seem to have failed to fuse at the midline (Fig. 1G). Histology also revealed not all the NC-associated structures were abnormal, as surviving *Ph/Ph* thymus (not shown) and dorsal root ganglia (Drg) seemed normal ($n = \text{all of } 6$; Fig. 1G). To assess whether surviving *Ph/Ph* NC can undergo normal differentiation, we examined $\beta 3$ -Tubulin and Tyrosine hydroxylase (TH) expression. The early pan-neuronal $\beta 3$ -Tubulin marker reveals that *Ph/Ph* NC-derived precursors can differentiate into neurons in the sympathetic ganglion primordia and normally populate the Drgs (Fig. 2B and D). Further, surviving *Ph/Ph* NC-derived precursors acquire a noradrenergic phenotype, as they appropriately express TH (Fig. 2F).

Thus surviving *Ph/Ph* embryos (past E13.5) shared many of the same phenotypes as seen in the systemic *PDGFR α* null and *Wnt1-Cre; PDGFR α* NC conditional mutant mice; such as fully penetrant cleft face, blebbing, and normal Drgs (Soriano, 1997; Tallquist and Soriano, 2003). However, there was a notable difference in penetrance of the cardiovascular phenotypes exhibited. Specifically, surviving *Ph/Ph* consistently exhibited PTA with an accompanying VSD, while septation of the *PDGFR α* null heart can proceed normally (Soriano, 1997). Similarly, only ~50% of *Wnt1-Cre; PDGFR α* NC conditional mutants exhibited aortic arch anomalies that are usually associated with defects in cardiac NC and only ~13% exhibited PTA (Tallquist and Soriano, 2003). Given that facial clefting and PTA may be due, in part, to deficiencies in NC-related structures, we sought to determine when

and where along the cranial and cardiac NC-differentiation pathway do *Ph/Ph* NC become abnormal.

Patch Balb/c heterozygous phenotype—Balb/c addition to the *C57Bl/6* background also affected the phenotype of heterozygotes. The *Ph* mutation is semidominant in the adult mouse, as the heterozygotes have a variable-sized midventral white spot (Gruneberg and Truslove, 1960). However, in stark contrast to previous studies, we also observed a grossly abnormal phenotype among some heterozygous *Ph* embryos ($n = 7/84$ embryos examined; and Table 2). The phenotype ranged from mild, which includes such abnormalities as blebs along the neural tube or generalized edema to more severe which included an enlarged heart or a wavy neural tube, similar to that seen in *Ph/Ph*. Only by genotyping of embryos were we able to determine that they were indeed heterozygotes, suggesting that gene dosage may play a role in the *Ph* phenotype. The severity of the abnormal heterozygote phenotype similarly varied with subsequent Balb/c generations. After one generation of Balb/c backcrossing, a proportion of heterozygotes displayed an abnormal phenotype similar to that seen in homozygous mutants. As the genetic background became more enriched for Balb/c, the relative proportion of abnormal heterozygotes decreased, suggesting that the Balb/c background not only enhanced the length of survival, but also decreased the incidence of abnormal phenotypes in heterozygotes (Table 2).

Patch Neural Cell can Migrate In Vitro

Although some NC-derived structures do form and can undergo normal differentiation (Fig. 2), the observed cardiovascular and facial clefting phenotypes suggested a possible deficiency of NC which may be related to problems in NC migration. The extracellular matrix in *Ph/Ph* has previously been shown to have abnormally large proteoglycan-containing granules within the migratory spaces (Morrison–Graham et al., 1992). Thus, we sought to determine if the environment was hindering the NC such that migration was affected, or whether the problem was intrinsic to the *Ph/Ph* NC themselves. To address this question, we isolated the portion of the neural tube from the optic sulcus to the third somite, which contains presumptive cardiac NC from E8.5 *Ph/Ph* and placed the neural tube explant into culture. Upon attachment of the explant to the coverslip, the NCs were able to migrate away from the neural tube, (Moase and Trasler, 1990; Conway et al., 2000). Relative migration distances were measured after 24 and 48 hours.

NC from both wild-type and *Ph/Ph* neural tubes migrated from the explant within hours of placement in culture (Fig. 3). We did not use heterozygous *Ph* embryos, as a proportion have an abnormal phenotype. After both 24 and 48 hours, there was no statistically significant difference in relative NCC migration (after 24 hours $+/+$ cultures [$n = 5$] had a migration index of 0.36 ± 0.19 and *Ph/Ph* cultures [$n = 7$] had a migration index of 0.23 ± 0.11 ; after 48 hours, $+/+$ cultures [$n = 3$] had a migration index of 1.97 ± 0.23 , and *Ph/Ph* cultures [$n = 3$] had a migration index of 1.80 ± 0.29). Mesenchymal cells from either explant could be seen at similar distances, suggesting that *Ph/Ph* NC can migrate away from the neural tube explant in a normal fashion relative to wild-type littermates. As our in vitro approach does not enable us to specifically identify NC, we sought to better distinguish NC from non-NC by utilizing the in vivo approaches outlined below.

Molecular Analysis of Patch Neural Crest In Vivo

Because the in vitro migration assays suggested *Ph/Ph* NC have the ability to migrate in a normal fashion, we sought to molecularly define their migration in vivo, to determine where in their migration or terminal differentiation pathway events they become abnormal. To visualize the NC during different stages, we took advantage of the fact that these cells specifically express a range of different mRNA transcripts during NC expansion,

emigration, migration, and differentiation. We used these molecular markers to determine the relative position of the NC to determine if any aberrations occurred in their molecular expression (Conway et al., 1997a; Conway et al., 2000).

Because *Ph/Ph* display a phenotype consistent with NC-deficiency, we sought to determine if insufficient numbers of NC were present in the *Ph/Ph* neural tube. To do this, we examined expression of *Wnt-1* and *Wnt-3a*, as these winged helix transcripts have previously been shown to be expressed by NC around the time of neural fold closure (Parr et al., 1993) and may be essential for the initial expansion of NC in the neural tube (Ikeya et al., 1997; Dorsky et al., 1998; Conway et al., 2000). To determine if *Ph/Ph* NC were present in the neural tube, and hence could presumably undergo normal cell expansion, we examined E9.5 *Ph/Ph* embryos for the expression of either *Wnt-1* (n = 8) or *Wnt-3a* (n = 7). The relative distribution of *Wnt-1* in both mild looking *Ph/Ph* and severely abnormal *Ph/Ph* appeared similar to that seen in wild-type controls, with expression along the dorsal neural tube in the appropriate regions (Fig. 4B and C). Interestingly, there seemed to be a broader band of expression at the midbrain/hindbrain junction in the mutant with the more severe phenotype (Fig. 4C). To confirm the presence of a normal amount of NC within the neural tube, we also examined embryos for the expression of another known marker of NC expansion, *Wnt-3a*. Consistent with observations for *Wnt-1*, the expression of *Wnt-3a* also seemed unchanged (data not shown). Thus, this data suggests *Ph/Ph* NC reside in the neural tube and undergo normal NC expansion. Therefore, the aberration which results in later *Ph/Ph* NC deficiency most likely lies downstream of this early event.

To monitor the status of NC emigrating from the neural tube and migrating through the extracellular matrix, we examined the expression of the *Crabp1* molecular marker in *Ph* mutants. Normally, *Crabp1* is expressed in the dorsal neural tube and in the migrating NC arising from rhombomeres 2, 4, and 6, which makes expression of this transcript an ideal way to molecularly view NC emigration from the neural tube and during subsequent migration (Maden et al., 1992). When *Crabp1* expression was examined in *Ph/Ph* (n = 15), we observed expression in the neural tube, face, and along the migratory pathway into branchial arches 1, 2, and 3, consistent with that seen in wild-type littermates (Fig. 4E and F). Although *Crabp1* is expressed in the appropriate places, the streams themselves within the arches seem significantly reduced, suggesting a deficiency in the numbers of *Ph/Ph* NC actually reaching the branchial arches.

To analyze NC slightly later along their migration pathway, we examined embryos for the expression of another molecular marker, the *Ap2* transcription factor, which is expressed by NC after emigration from the neural tube and during subsequent migration through the extracellular matrix to the branchial arches (Mitchell et al., 1991). Given that *Ap2* null embryos show some similarities to *Ph/Ph*, we examined whether *Ap2* may be misexpressed in *Ph* mutants (Schorle et al., 1996; Zhang et al., 1996; Nottoli et al., 1998). As seen in wild-type littermates at E9.5, *Ap2* is expressed in three streams of cells, migrating through the branchial arches (Fig. 4G). *Ph/Ph* mutants (n = 5) also demonstrated a similar expression pattern (Fig. 4H and I), but expression within the arches seems significantly reduced (similar to *Crabp1*). Vibratome sectioning did not reveal any *Ap2* expression within the neural tube (data not shown), indicating that unlike the *Wnt-1*; *Wnt-3a* double knockouts (Ikeya et al., 1997), the NC are probably not trapped within the *Ph* neural tube. Since both *Crabp1* and *Ap2* expression was observed at the appropriate place and time in *Ph/Ph*, these data suggests *Ph/Ph* NC are migrating along the appropriate pathways during the appropriate times, but the streams of NC become reduced, the further they migrate away from the neural tube.

To determine if the mutant NC reach their final targets, we analyzed *Ph* embryos for the expression of the Paired-related homeobox gene (*Prx2*) molecular marker, which has been

suggested to maintain or stabilize cell fates in the post migratory cranial mesenchyme (Lu et al., 1999). Although *Prx2* plays a significant role in the craniofacial mesenchyme and 100% of the later surviving *Ph/Ph* ultimately display a midface cleft, we saw no overt difference in expression in mildly affected *Ph* mutants (Fig. 4K). Even in a severely abnormal *Ph/Ph*, we still observed expression of *Prx2* in the branchial arches (Fig. 4L), although there was reduced expression ($n = 3$). Hence, it seems that the reduced numbers of NCs that are able to arrive and colonize the arches within *Ph/Ph* do have the capacity to differentiate – at least as far as the ability to switch on *Prx2* expression is concerned. This is consistent with the data (Fig. 2) indicating that surviving *Ph/Ph* NC-derived precursors can acquire a noradrenergic phenotype and undergo differentiation into neurons in the sympathetic ganglion primordia.

Taken together, the molecular markers suggest *Ph/Ph* NC emigration from the neural tube and subsequent migration into the branchial arches can occur in even severely dysmorphic embryos, but in significantly reduced numbers. This suggests that emigration and initial migration do not account for the observed NC-deficient phenotype in older mutants, per se, and that there must be another mechanism to account for the obvious latter NC-deficiencies. If the *Ph* mutant NCs survive, they are able to undergo normal differentiation. These data are unlike the *Wnt1-Cre* lineage mapping results for PDGFR α -negative NCs, which follow the expected paths of migration (Tallquist and Soriano, 2003). This suggests that in surviving *Ph/Ph*, a reduction of NCs in developing tissues is the most likely cause for the defects observed, while the PDGFR α NC conditional mutant embryo phenotypes are rather due to NC postmigratory differentiation abnormalities rather than reduced NC numbers.

Patch Neural Crest undergo Excessive Apoptosis along the Migration Pathway

As NC expansion, emigration, and initial migration can all occur normally, we sought to determine if a decrease in cell proliferation, an increase in apoptosis, or a combination of both could contribute to the *Ph/Ph* NC-deficient phenotypes. In preliminary experiments, we sought to determine if *Ph/Ph* NC selectively failed to undergo sufficient cell proliferation. Upon comparing serial sections through the branchial arches of wild-type control littermates and *Ph/Ph*, we observed no overt difference in BrdU-labeling, suggesting that an overt decrease in cell proliferation could not account for the NC-deficient phenotype (data not shown).

Because cell proliferation was unaffected, we sought to determine if apoptosis could account for the observed deficiencies in NC-related structures using TUNEL. As seen in Figure 5A to C, apoptosis normally occurs at a low level in arches 1 and 2, among other regions at E9.5. In *Ph* mutant littermates, however, we detected a minor increase in apoptosis within branchial arches 1, 2, and 3 ($n = 6$). Interestingly, apoptosis in the heart was quite low (Fig. 5C). One day later in development at E10.5, we observed a significant elevation of apoptosis along the NC migration pathway into branchial arches 1 and 2, and also within the cardiac NC migrating to the heart in *Ph* mutants ($n = 14$) with either a mild (Fig. 5H) or severe phenotype (Fig. 5I). Moreover, double-labeling of TUNEL and the NC marker *Crabp1* confirmed the existence of a population of apoptotic *Ph/Ph* NC (Fig. 6A and B), however, apoptotic cells were also seen in non-NC mesenchyme between the migratory cardiac NC and the notochord. Indeed, the mesenchyme around the notochord was hypoplastic (Fig. 5J and K). Significantly, elevated levels of apoptosis were also observed in the hypoplastic first arch and the midface region (data not shown), but was not observed within the NC immediately adjacent to the neural tube. This increase in death is an important indication as to the etiology of NC-deficiency, for the NC cells from arches 1 and 2 will help to populate the face, while the cardiac NC will contribute to the aorticopulmonary septum, which are both affected in *Ph/Ph*. Taken together, these results suggest that after *Ph/Ph* NC have left the neural tube and begin to reach their final sites of terminal differentiation, they fail to

survive and undergo apoptosis in both the cranial and cardiac NC regions, likely resulting in the later NC-deficient phenotypes observed. These data are in contrast to the systemic *PDGFR α* null and *Wnt1-Cre; PDGFR α* NC conditional mutants, as neither reduced cell proliferation nor elevated apoptosis were reported (Soriano, 1997; Tallquist and Soriano, 2003).

Notochord and Foregut Abnormalities in *Patch* Mutant Embryos

After whole-mount apoptosis analysis, embryos were embedded and sectioned to enable detailed analysis of internal structures. When wild-type versus *Ph/Ph* were compared in the pharyngeal arch region, we noticed a difference in the pattern of apoptosis in the neural tube. In wild-type embryos, apoptosis was more concentrated in the dorsal neural tube, while in *Ph/Ph* apoptosis was more concentrated in the ventral neural tube (Fig. 5E and F). This alteration in apoptotic pattern suggested that signaling molecules associated with dorsal/ventral patterning may be misexpressed. Previous work by Center et al. (1988) suggested that the notochord was abnormal in *Ph/Ph* due to the presence of the blebs along the neural tube. Given its known importance in patterning, craniofacial, and cardiovascular morphogenesis (Ahlgren and Bronner-Fraser, 1999; Smoak et al., 2005) and its well-characterized expression pattern within the midline notochord, we sought to examine *Ph* mutants for the expression of a key notochordal marker – Sonic hedgehog (*Shh*).

In the early embryo, *Shh* is normally expressed in the notochord, ventral midline of the CNS, branchial arches, and foregut among other structures (Marti et al., 1995). When *Shh* was examined in *Ph/Ph* ($n = 14$), however, we observed both minor and major aberrations, depending on the severity of the *Ph/Ph* phenotype. Relatively mild *Ph/Ph* embryos displayed continuous expression of *Shh* throughout the anterior notochord and floor plate and slightly decreased expression in the branchial arches (Fig. 6E–H). Interestingly, dorsal to the foregut we sometimes observed an ectopic notochordal structure as shown by the ectopic *Shh* expression, lateral to the central notochord (Fig. 6H). *Ph* homozygous mutants with a more severe phenotype, however, displayed a severe alteration in *Shh* expression within the notochord adjacent to the heart. This included regions where both the notochord and *Shh* was completely absent along the dorsal part of the embryo (Fig. 6L and M), along with regions where the notochord appeared to be shifted laterally (Fig. 6K). Further, in other embryos, the notochord seemed compressed and in some cases *Shh* expression appeared laterally expanded across the embryo instead of as a discrete structure (Fig. 6O–Q). Significantly, both mild and severe *Ph/Ph* embryos also exhibit drastically reduced *Shh* expression within the first and second branchial arch endoderm (Fig. 6F) and foregut (Fig. 6G, H, and J). Given the observed disruption of *Shh* expression in regions important for NC-related structure development, the appropriate signals from the notochord and/or foregut may not have been transmitted to the neural tube and surrounding mesenchyme, possibly affecting the patterning signals and/or relationship between migrating NC and the matrix through which they migrate.

Notochordal Abnormalities may Result in Somitic Defects

Both *Patch* (Schattenman et al., 1992; Dickman et al., 1999) and *PDGFR α* null-mutants (Soriano, 1997) display abnormal somitic differentiation, resulting in skeletal abnormalities. Pourquie et al. (1993) have suggested that the dorsoventral patterning of somitic derivatives may be controlled by signals provided by ventral axial structures, including the midline notochord. Thus, given the notochordal abnormalities observed within the *Patch* mutant phenotype, we examined the expression of the paraxial mesoderm marker *Pax3* (Goulding et al., 1994) within *Patch* mutant embryos. Similar to the results reported by Soriano (1997) for the *PDGFR α* null-mutants, the first eight or nine somites are abnormally formed, as illustrated by the abnormal shape and fusions present within *Ph/Ph* (Fig. 7A and B).

Interestingly, the more caudal somites appear relatively remarkably normal, consistent with normal notochordal morphogenesis and intact *Shh* expression (Fig. 6I). Given that the mesodermal somites are thought to control the rostrocaudal patterning of the NC (Bronner–Fraser, 1995), and that the cardiac NC are known to migrate through this rostral somitic region (Creazzo et al., 1998), these results could suggest *Ph/Ph* cardiac NC-associated abnormalities, may be secondary to elevated cardiac NC apoptosis due to the notochordal/foregut and/or somitic abnormalities resulting in abnormal NC patterning and signaling via the adjacent mesenchyme and foregut. The midface defects may also be secondary to abnormal dorsal/ventral patterning of the cranial NC, either within the neural tube or via cell-cell interactions as they migrate and colonize the craniofacial region.

DISCUSSION

Although the phenotypic cardiovascular and craniofacial abnormalities of the *Ph* homozygous mouse mutant had been well characterized (Morrison–Graham, et al., 1992), the underlying etiology of the NC-related defects remained unclear. Via manipulating the genetic background to circumvent early embryonic lethality, our results revealed that *Ph/Ph* phenotypes are most likely due a significant decrease in migratory NC lineage due to diminished NC survival and elevated apoptosis. Our results also suggest that the *Patch* phenotype is due to more than just a deletion of the *PDGFR α* gene. Homozygous *Ph* NC precursors undergo normal NC expansion within the neural tube, undergo epithelial-to-mesenchymal transformation, and initiate normal NC emigration. Moreover, in vitro explant culture demonstrated that when isolated from the surrounding mesenchyme, *Ph/Ph* NC could migrate appropriately. However, robust elevated apoptosis was present both within the migratory NC, as well as the immediate mesenchyme through which the *Ph/Ph* NC migrate. Additionally, *Ph/Ph* foregut, notochord, and somite morphogenesis was perturbed. Significantly, the timing of lethality and extent of apoptosis correlated with the degree of severity of *Ph/Ph* foregut, notochord, and somite dysfunction. Finally, analysis of Balb/c enriched surviving *Ph* mutants revealed that not all the NC subpopulations are affected. Combined, these data indicate that the *Patch* mutation results in problems that may also be extrinsic to the NC themselves.

Contribution of Neural Crest-deficiency to the *Patch* Phenotype

Although the mechanism by which these NC abnormalities arise is not known, studies by Morrison–Graham, et al. (1992) have shown that the extracellular matrix through which the NC migrate is abnormal; and that the mesodermal somites which are thought to control the rostrocaudal patterning of NC are also abnormal (Schatterman et al., 1992; Bronner–Fraser, 1995; Soriano, 1997). Indeed, extracellular proteases are essential for cell movement through extracellular matrix (ECM), and it has been shown that *Ph/Ph* exhibit deficiencies in matrix metalloproteinase (MMP-2) and its activator MT-MMP within the heart and aortic arch arteries (Robbins et al., 1999). MMPs selectively degrade ECM components, and a deficiency results in impaired motility of mesenchymal cells in *Ph/Ph* (Robbins et al., 1999). Our *Crabp1*, *Ap2*, and *Prx2* expression data revealed that *Ph* mutant NCs can express appropriate NC markers during migration and that although the NC streams themselves are significantly reduced in number, the mutant NCs do migrate along the appropriate migration routes. Thus, the finding that *Ph* mutant matrix is abnormal further supports the conclusion that there may be a problem in the environment of the NC and not within the NCs themselves. Whether the environment directly contributes to the observed *Ph/Ph* apoptosis and underlies the lack of apoptosis seen within *PDGFR α* knockouts (Tallquist and Soriano, 2003) requires further analysis. Given the sensitivity of genetic background upon resultant phenotypes, this will necessitate backcrossing appropriate cre/loxP lineage markers for

further whole embryo analysis and cell isolation of the NC lineage for thorough characterization of NC and extracellular matrix expression profiling.

Using TUNEL analysis and double labeling, we determined an increase of apoptosis occurred in a region corresponding to the NC migratory pathway in *Ph*. Although this increase was detected at E9.5, we saw a striking elevation by E10.5, indicating that cell death occurs well after the NCs have left the neural tube. However, not all NC-derived tissues were affected, suggesting apoptosis is only induced in NC subpopulations and there is no wholesale NC deficiency, as seen when NC are genetically ablated (Porras and Brown, 2008). Additionally, NC loss and apoptosis was not induced in emigrating NC but only as the *Ph/Ph* NC are migrating along more distant pathways. Our results indicate that this may result in a significant decrease in the number of NCs that are later able to terminally differentiate, thereby causing a deficiency in subsequent NC-derived structures. For instance, Thomas et al. (1998) observed that extensive apoptosis resulted in the hypoplastic branchial arches observed in *Hand2*-null embryos, possibly due to the necessity of *Hand2* in a pathway important for cell survival. Similarly, *PDGFR α* 's role in cell survival has been well documented and that may be why some of the more severe systemic knockout mutants die by E10.5. Those *Ph/Ph* that survive longer may have compensated (due to something in the Balb/c background that modulates notochord and/or foregut morphogenesis).

Contribution of Notochordal Abnormalities to the *Patch* Phenotype

Significantly, when we examined expression of the key midline patterning morphogen *Shh*, which is known to be involved in patterning in *Ph/Ph*, we noted regions where the notochord was absent, abnormally positioned, or misexpressed *Shh*. We also observed that both severely affected and Balb/c enriched surviving *Ph* mutants failed to normally express *Shh* within the mandibular branchial arch and the foregut endoderm. Finally, we also noted that the rostral somites overlying the abnormal notochord were dysmorphic, but the caudal somites were unaffected (as was the caudal notochord, which expressed *Shh* normally). Given that *Shh* plays a major role in the signaling centers that regulate polarity of the central nervous system (Echelard et al., 1993), that *Shh* is involved in somite patterning (Borycki et al., 1998) and that the notochord may regulate embryonic cardiac lineages (Goldstein and Fishman, 1998), it is possible that some of the NC-related malformations observed in *Patch* are due to either early patterning abnormalities within the neural tube and/or alterations in signaling molecules such as *Shh*. Interestingly, Ahlgren and Bronner-Fraser (1999) have demonstrated that inhibition of *Shh* signaling in vivo in chick embryos results in craniofacial, neural tube, and NC death, which results in phenotypes similar to those observed after cranial NC ablation and within *Ph/Ph*. Similarly, Goh et al. (1997) observed increased numbers of apoptotic migrating cranial NC and discontinuous expression of *Shh* and *Brachyury* within the disorganized notochord of the *$\alpha 5$ integrin* null embryos. Moreover, the posterior half of the somite is known to express contact-repulsive molecules that provide critical cues along the anterior-posterior axis for NC (Pourquie et al., 1993; Kuan et al., 2004). These patterning anomalies are presumably all secondary to the blebbing that occurs within the notochordal region (Center et al., 1988) caused by the *Patch* mutation, and suggests the *Ph/Ph* phenotype is due to more than just a deletion of the *PDGFR α* gene.

The key role of the notochord was elegantly shown via ablation studies, when the notochord was removed from chick embryos, both the Drg's and sympathetic ganglia were able to develop in the absence of the notochord (Telliet and Le Douarin, 1983). However, notochord removal resulted in NC death and it was suggested that dependence of NCs on the notochord parallels the mesenchymal substrate in which they develop (Telliet and Le Douarin, 1983). More recently, notochord ablation has been shown to result in abnormal *Shh* signaling within somites (Resende et al., 2010). Notochord-derived *Shh* is thought to be a

component of the molecular network regulating the pace of the somitogenesis clock (Resende et al., 2010). Mouse studies have also illustrated the importance of *Shh* itself on NC survival and proliferation (Jeong et al., 2004; Smoak et al., 2005). *Shh* null embryos exhibit remarkably increased cell death within the developing arches, forebrain, midbrain, cells adjacent to the neural tube (presumably migrating NC), and presumptive NC entering the outflow tract (OFT) of the heart. These data were interpreted as *Shh* produced from the mesenchyme or endoderm is necessary for guiding NC and loss of this guidance information results in a significant loss of NC via cell death (Smoak et al., 2005). It has also been shown that there is an early requirement for *Shh* from foregut endoderm to ensure cranial NC survival during jaw development (Brito et al., 2006). Similarly, the *Shh*-responsive morphogenetic signal *Fgf8* is also known to be required for foregut (Jung et al., 1999), pharyngeal arch, and cardiovascular development in the mouse (Abu-Issa et al., 2002); OFT and NC development in the chick (Hutson et al., 2006); and as a fibroblast growth factor autocrine loop initiated in second heart field mesoderm to produce ECM and transforming growth factor beta and bone morphogenetic protein signals essential for invasion of cardiac NC within the OFT (Park et al., 2008). Combined, these studies suggest that the loss of *Shh* expression within the *Patch* mutants may explain the widespread midline patterning defects observed and primary cause of the *Ph/Ph* NC cell death.

Differences between the *Patch* and *PDGFR α* Null Mutants

Ph and *PDGFR α* null mice share many similar phenotypes: enlarged heart, wavy or kinked neural tube, cleft face, PTA/VSD, and abnormal somite specification. However, there are also some major differences such as the penetrance of the congenital heart defects, presence of robust apoptosis, notochord, and foregut anomalies. The frequent defect in septum formation within the heart of *Ph/Ph* embryos is mostly absent in *PDGFR α* mutants, illustrating the point that the *Ph* phenotype is multigenic, but that only some and not all of the features are attributable to the loss of *PDGFR α* (Soriano, 1997). These differences may be because the *Ph* mutation not only includes the deletion of the entire *PDGFR α* gene, but also includes regions 3' of the gene which may affect the regulation of the downstream gene *c-kit*. Alternatively, the *Ph* deletion, which encompasses not more than 400 Kb (Nagle et al., 1994) may inactivate another gene/s. These differences may also be because the *PDGFR α* null mice were originally crossed onto a *C57Bl6/J* genetic background (Soriano, 1997) and it would be interesting to test whether the *PDGFR α* null phenotype is altered by crossing them onto a Balb/c background. With the possibility that the loss of *PDGFR β* could exacerbate the previously described partially penetrant phenotype in *PDGFR α* NC-specific conditional embryos, double conditional *PDGFR β* ;*PDGFR α* mice were bred to *Wnt1-Cre* (Richarte et al., 2007). The result was 100% penetrant PTA and VSD in contrast to the variability of heart phenotypes in the *PDGFR α* mutants alone. Although initial NC delamination, migration, proliferation, and differentiation was normal, there was a reduced ability of *PDGFR* cells to populate the OFT, suggesting that combined *PDGFR* signaling directs cardiac NC colonization of the OFT and thus directs NC function specifically in the conotruncal regions (Richarte et al., 2007).

Although the *Ph* deletion does not include any of the *c-kit* coding sequences, the 5' control elements of *c-kit* are affected. Significantly, Duttlinger et al. (1995) have shown that *c-kit* expression is affected in both the *Ph* heterozygotes and homozygotes. At E10.5, wild-type embryos display a low level of uniformly distributed *c-kit* protein within the neural tube while *Ph/+* and *Ph/Ph* embryos showed ectopic *c-kit* protein in the dorsal half of the neural tube and foregut. Although *c-kit* is expressed in the normal embryonic foregut (Reedy et al., 2003), it is not yet clear whether *PDGFR α* itself is ever expressed in the normal notochord during embryogenesis (Sun et al., 2000). Although our data is suggestive that failure of coordinated signaling from *Shh* and the notochord may lead to the NC defects seen in *Ph/Ph*

mutants, we have not ruled out the possibility that these defects are secondary to the severe phenotypes seen in *Ph/Ph*. It also remains to be determined what roles, if any, they may play during pathologic conditions. Future studies will be aimed at determining whether either *PDGFR α* or *c-kit* can directly regulate any of the components of the *Shh* signaling pathway, and if their collective loss is detrimental for NC survival. Collectively, our data support that early embryonic lethality is due to defects outside the *PDGFR α* structural gene.

Influence of Balb/c Genetic Background on the *Patch* Mutation

Given that embryonic *Ph* heterozygotes can have an abnormal phenotype, our data suggests that mammalian embryos need two copies of the missing *PDGFR α* gene and/or the other deleted elements. The issue of haploin-sufficiency has been seen in multiple mouse mutant models and human syndromes (Marino et al., 1999). Also, since further enrichment of the mice with the Balb/c background decreases the incidence of abnormalities in both heterozygotes and homozygotes, it would be interesting to know what specific elements in the Balb/c background are important for its modifying effect.

Balb/c mice have a 5' duplication of the alpha-cardiac actin gene which is associated with abnormally high levels of alpha-cardiac actin and alpha-skeletal actin mRNAs in adult cardiac tissue (Garner et al., 1989) and increased contractility of the Balb/c hearts (Hewett et al., 1994). This could result in increased pools of actin and facilitate a more robust myofibril assembly within the *Ph* mutants on a Balb/c background. A truncated isoform of the cardiac sodium-calcium exchanger has also been shown to present within the Balb/c mouse hearts that is not present in the C57Bl/6 mouse (Shi et al., 1998), although the functional significance of this novel isoform is currently unknown. Whether elevated levels of alpha-cardiac and alpha-skeletal actin within the *Ph*-Balb/c embryos, the expression of the truncated isoform of the cardiac sodium-calcium exchanger, or another undefined element within the Balb/c background prolongs embryonic survival remain to be determined. The variation in expressivity of abnormal phenotypes by changing genetic background has been seen in many other mouse mutants (Kallapur et al., 1999; Doetschman, 2009). Given this strain dependency, further investigations are warranted to determine precisely what additional regulatory elements are deleted and/or created causing a modification of phenotype.

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Figure 1.

Phenotype of mild and grossly abnormal *Patch* (*Ph*) homozygous mutants. (A) Embryonic day (E)9.5 wildtype; (B and C) *Ph* homozygous mutants at E9.5. Note that the phenotype can vary from mild or non-existent (B) to grossly abnormal (C) which includes an enlarged heart (arrowhead), bubbles along the neural tube, and reduced size; (D) E14.5 wildtype; (E) *Ph* homozygous mutant at E14.5 which displays a cleft face (arrow), an engorged liver, and a severe reduction in size. Note, however, that this embryo was still alive at collection. (F–K) Histologic transverse sections of E14.5 wildtype (F, H, J) and *Ph* mutant (G, I, K) littermates. Note that the mandible is not fused (e.g., arrow); the outflow tract has failed to septate (I); and the septum between the right and left ventricles is incomplete (K, arrow). Also, note that the dorsal root ganglia are unaffected in wildtype (F, arrow) and *Ph/Ph* (G, arrow). Ao, aorta; P, pulmonary trunk; RV, right ventricle; LV, left ventricle; PTA, persistent truncus arteriosus. Scale bars: a, b, c = 0.1 mm; d, e = 1 mm.

Figure 2.

Immunohistochemistry of surviving *Patch/Patch* (*Ph/Ph*) on Balb/c background. (**A, C, E**) Embryonic day (E)13 wildtype and (**B, D, F**) *Ph/Ph* mutant transverse sections stained for β 3-Tubulin (**A–D**) and Tyrosine hydroxylase (**E, F**) expression. Note similar β 3-Tubulin in both wild-type and mutant neural tube and dorsal root ganglia (**A, B**), and within sympathetic ganglia (*arrows* in [**C and D**]). Similarly, Tyrosine hydroxylase expression is equivalent in wildtype (**E, arrow**) and *Ph/Ph* (**F, arrow**) sympathetic ganglia. Nt, neural tube; Drg, dorsal root ganglia. Scale bars: e, f = 200 μ m.

Figure 3.

Cardiac neural crest (NC) migration from neural fold explant cultures. Neural folds isolated from the embryonic day (E)8.5 cardiac NC region of wild-type (**A and C**) and *Patch/Patch* (*Ph/Ph*) (**B and D**) embryos were cultured in vitro for 24 and 48 hours. Similar to the wild-type neural tube explant, NC cells migrated away from the *Ph/Ph* explant (*double arrow*), as observed both 24 and 48 hours after placement in culture. The explants have been photographed at the same magnification, and note that there is no significant difference in migration between wild-type and homozygous *Ph* mutant genotypes (schematically represented by *double arrows*). ex, explant. Scale bar = 10 μ m.

Figure 4.

Expression of neural crest cell markers in mutant *Patch* homozygous embryos. (A–I) Embryonic day (E)9.5; (J–L) E10.5. (A) *Wnt-1* expression in wildtype showing a distribution of signal in the midbrain of the embryo along the dorsal neural tube and also in a broadband extending from dorsal to ventral just anterior to the midbrain/hindbrain junction. Note the lack of expression in the rostral hindbrain region, but continuation of signal throughout the length of neural tube. (B and C) *Wnt-1* expression in *Patch/Patch* (*Ph/Ph*) mutants. The expression pattern of *Wnt-1* is similar to that seen in wildtype, even in the more severe mutant (C) which displays an enlarged heart tube (arrow). Note that the signal is strong along the neural tube in the cephalic neural crest (NC) cell region, even though the mutants display a wavy neural tube. The more severe mutant also displays an increase of signal at the midbrain/hindbrain junction (arrowhead). (D) Cellular retinoic acid binding protein-1 (*Crabp1*) expression in E9.5 wild-type embryo; note the high expression in the branchial arches; (E and F) *Crabp1* expression in E9.5 *Ph* mutants. Note that although 3 streams of cells can be seen emigrating from the neural tube, there appear to be fewer cells in the second and third streams within the branchial arches (compare arrowheads in [D] with those in [E and F]). Also, the wavy neural tube can also be seen in these embryos with no apparent affect on expression of *Crabp1*. (G) *Ap2* expression in E9.5 wild-type embryo. Note the strong expression in the branchial arches that will contribute to the facial mesenchyme; (H and I) *Ap2* expression in mild and grossly abnormal E9.5 *Ph/Ph*. Note that the second stream once again appears deficient within the branchial arches relative to the wild-type, consistent with the *Crabp1* results. In (I) the most caudal stream cannot be seen due to a tear in the neural tube; (J) *Prx2* expression in E10.5 wild-type embryos. Note the strong expression in the facial mesenchyme (K and L). *Prx2* expression in E10.5 *Ph* mutants. In both the mild and grossly abnormal homozygote, *Prx2* can be seen in the NC regions. Note that even the mutant with the most severe phenotype still displays a reduced level of *Prx2* expression in the mandible, although this structure is malformed (*).

Figure 5.

Apoptosis in *Patch* homozygous mutants. (A–C) Apoptosis in embryonic day (E)9.5 embryos. (A) Wild-type. Note the normal low endogenous level of apoptosis present in the branchial arches; (B & C) mild and grossly abnormal *Patch/Patch* (*Ph/Ph*). Note that although both have apoptosis occurring in similar regions to the wild-type, there appears to be an increased level rostral to the optic placode (*thin arrow*). This elevation is more pronounced in the more severe mutant with the enlarged heart (*arrow*, D) negative control showing no background staining; (E) Section through wild-type branchial arches. Note the pattern of apoptosis in the dorsal neural tube (*arrowhead*) and in the developing mandible. (F) Section through *Ph/Ph* branchial arches. Note that the ventral portion of the neural tube (*arrowhead*) appears to be enriched in apoptosis as opposed to the enrichment in the dorsal region of the neural tube in the wild-type. (G–I) Apoptosis in E10.5 embryos. (G) Wild-type embryo. (H and I) mild and grossly abnormal homozygotes. Note the elevated level of apoptosis (*arrowhead*) in the mutant with the mild phenotype (H) and in the more severe mutant phenotype (I) with an enlarged heart (*arrow*). This increase is more easily seen at higher magnification (G–I'). Note that in both *Ph/Ph*, there is an enrichment of apoptotic cells in the mandible (*), the first branchial arch ("1"), and in the stream that leads to the heart (*arrowhead*). (J and K) Double labeling of TUNEL (dark purple nuclear stain in [J] but green in [K]) and expression of the neural crest (NC) marker *Crabp1* (blue stain in [J] but yellow in [K]) in cells emigrating from the dorsal neural tube, migrating into the glossopharyngeal vagal NC complex and toward the origin of the third branchial arch of E10 *Ph/Ph* embryo (J). Overlap of the two signals is seen in several cells (shown by *white arrows* in enlarged negative image in [K]) close to the neural tube, but there are also a number of cells that appear to be along the NC migration pathway that do not express *Crabp1* but are apoptotic (indicated via red circle in [J]). The TUNEL cells all appear green in (J), but *Crabp1* mRNA expression (which appears yellow in [J]) is more pronounced in

NC adjacent to the neural tube than in the more distant arch regions and neural tube itself.
op, optic placode; nt, neural tube. Scale bar = 0.1 mm.

Figure 6.

Notochord and foregut abnormalities within *Patch* homozygotes. (A–D) Embryonic day (E)9.5 wild-type embryo. Note strong Sonic hedgehog (*Shh*) expression can be seen along the entire notochord (A). In sections (B–D), expression can also be seen in the floor plate, notochord, branchial arches, and foregut. (E–Q) *Ph/Ph* embryos. In mild looking E9.5 *Ph* mutant, expression can be seen along the notochord (E and G arrows) and in the floor plate (F). Note however that *Shh* expression is absent in the branchial arches (* in F) and foregut (* in both G and H), and that there is an ectopic site of expression lateral to the notochord (H arrowhead). In severe E9.5 *Ph* mutants (I), *Shh* expression can be seen along the notochord, although there are regions where the notochordal signal has shifted laterally (arrow K) or is completely absent (arrows I and M). Note *Shh* signal is also reduced in the floor plate and the foregut. *Ph/Ph* mutants with a midline heart phenotype (n-q) also show *Shh* expression abnormalities. (n and o) Note presence of an abnormal cardia bifida with two separate ventricles (asterisks) and a single atrium, and absent *Shh* in the floor plate. The *Shh* signal becomes compressed in subsequent caudal sections (arrows-p), and the notochord also becomes bifurcated, as visualized by the two streams of *Shh* expression (q-arrowheads).

Figure 7.

Expression of Pax3 in *Ph/Ph* mutants. Wholemount in situ hybridization revealed that the first nine rostral somites are abnormally specified within the homozygous *Ph* mutants (**B** and **C**) when compared to wild-type embryonic day (E)10 embryos (**A**), as they are abnormally shaped and are fused at several sites. However, the more caudal somites (somite 10 onwards) all appear to be normal. Also note, that the heart is enlarged within the mutant embryo in (**B**), and that the neural tube is actually discontinuous within this particular *Ph/Ph* mutant embryo (illustrated in high power image in [**C**]). Scale bar = 0.1 mm.

Table 1 Survival of *Patch* Homozygous Mutants on a Balb/c-enriched Background

	Oldest surviving <i>Ph/Ph</i>	Typical phenotype
Original C57	E10.5 (n = 21)	Enlarged heart, wavy/kinked neural tube
Third generation	E13.5 (n = 5)	Cleft face, PTA, blebs
Sixth generation	E17.5 (n = 17)	Cleft face, PTA, omphalocele, blebs

Patch/Patch, *Ph/Ph*; E, embryonic day; PTA, persistent truncus arteriosus.

Table 2

Incidence of Abnormal^{*} Phenotypes in *Patch* Heterozygotes Relative to Backcrossing onto a Balb/c Background[†]

Heterozygote	Third	Sixth
Phenotype	generation	generation
Normal	19 (86.4%)	58 (93.5%)
Abnormal [*]	3 (13.5%)	4 (6.5%)

Heterozygote *Patch* mice originally on a *C57Bl/6* genetic background were crossed with wild-type Balb/c mice in order to enrich the background for Balb/c. When third generation heterozygotes were mated, 13.5% of the heterozygote embryos had an abnormal phenotype. As the genetic background became more enriched in Balb/c, the incidence of abnormalities in heterozygotes decreased. Note that by the sixth generation, only 6.5% of the heterozygotes embryos (embryonic day 8.5–17.5) were abnormal.

* Abnormal phenotype included an enlarged heart, wavy neural tube, severe growth retardation, facial abnormalities, and bubbles along the ectoderm.

[†] Due to the low incidence of abnormal phenotype, the relative incidence of abnormal phenotype approaches significance, $p = 0.13$ using the chi-square test.