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Phosphatase-Dependent and -Independent Functions of Shp2 in Neural Crest Cells Underlie LEOPARD Syndrome Pathogenesis

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SUMMARY

The tyrosine phosphatase SHP2 (*PTPN11*) regulates cellular proliferation, survival, migration, and differentiation during development. Germline mutations in *PTPN11* cause Noonan and LEOPARD syndromes, which have overlapping clinical features. Paradoxically, Noonan syndrome mutations increase SHP2 phosphatase activity, while LEOPARD syndrome mutants are catalytically impaired, raising the possibility that SHP2 has phosphatase-independent roles. By comparing *shp2*-deficient zebrafish embryos with those injected with mRNA encoding LEOPARD syndrome point mutations, we identify a phosphatase- and Erk-dependent role for Shp2 in neural crest specification and migration. We also identify an unexpected phosphatase- and Erk-independent function, mediated through its SH2 domains, which is evolutionarily conserved and prevents p53-mediated apoptosis in the brain and neural crest. Our results indicate that previously enigmatic aspects of LEOPARD syndrome pathogenesis can be explained by the combined effects of loss of Shp2 catalytic function and retention of an SH2 domain-mediated role that is essential for neural crest cell survival.

INTRODUCTION

The nonreceptor tyrosine phosphatase SHP2 (*PTPN11*) plays a key role in signaling by receptor tyrosine kinases (RTKs), cytokine receptors, and integrins (Feng, 1999; Neel et al., 2009). A ubiquitously expressed molecule with two N-terminal SH2 domains, a catalytic (PTP) domain, and a C terminus with tyrosyl phosphorylation sites and a prolyl-rich stretch, SHP2 is regulated via an elegant mechanism that couples intracellular localization to catalytic activation (Barford and Neel, 1998; Hof et al., 1998). In the absence of cell stimulation, SHP2 exists in an inactive "closed" conformation with its N-terminal SH2 domain (N-SH2) wedged into the catalytic cleft, blocking substrate access. Upon receptor activation, SHP2 is recruited via its SH2 domains to specific cellular phosphotyrosyl (pTyr) proteins, which include some RTKs themselves, scaffolding adapters, or immune inhibitory receptors (Feng, 1999; Neel et al., 2009). Binding of an appropriate pTyr-protein to the N-SH2 domain of SHP2 abrogates inhibition of the PTP domain, resulting in an "open" structure and phosphatase activation.

Although its key substrates remain controversial, much evidence has established that appropriate localization of SHP2 and its catalytic activity are required for full activation of the RAS/ERK cascade (Neel et al., 2009). In tissue culture cells, catalytically inactive SHP2 mutants have dominant-negative effects on multiple RTK and integrin signaling pathways, inhibiting RAS/ERK activation, cell proliferation, focal adhesion turnover, and cell spreading and migration (Neel et al., 2009). Mutations in the *Drosophila* SHP2 ortholog *corkscrew* also impair RTK signaling, and are rescued by gain-of-function mutants in Ras/Erk cascade components (Allard et al., 1996; Perkins et al., 1992). Dominant-negative shp2 blocks fibroblast growth factor-evoked erk activation, mesodermal gene induction, and gastrulation in *Xenopus* (O'Reilly and Neel, 1998; Tang et al., 1995). Gastrulation is also defective in mouse embryos homozygous for a hypomorphic *Ptpn11* mutation, and cells from these embryos show impaired Ras/Erk activation in response to multiple stimuli (Saxton et al., 1997; Shi et al., 2000; Zhang et al., 2004). In contrast, homozygous null *Ptpn11* mutation leads to peri-implantation lethality due, at least in part, to defective Erk activation and trophoblast stem cell death via a Bim-dependent pathway (Yang et al., 2006). SHP2 has cell type- and receptor-specific roles in PI3K, Rho, NFκB, and NFAT activation, but in those cases analyzed carefully, SHP2 catalytic activity also appears to be required (Neel et al., 2009).

Improper regulation of SHP2 can lead to disease. Germline *PTPN11* mutations cause ~50% of Noonan syndrome (NS)

cases and the vast majority of LEOPARD syndrome (LS) cases (Tartaglia and Gelb, 2005). NS displays some combination of cardiac (most often valvuloseptal) abnormalities, proportional short stature, and facial dysmorphia (e.g., ocular hypertelorism) and a variety of less penetrant defects (e.g., cognitive, genitourinary, auditory abnormalities). LEOPARD is an acronym for multiple lentigines, electrocardiographic abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness. Somatic *PTPN11* mutations are the most common cause of juvenile myelomonocytic leukemia and occur more rarely in solid tumors (Mohi and Neel, 2007).

Because LS and NS share several features, they are generally viewed as overlapping syndromes. Other evidence suggests that their pathogenesis is distinct. Lentigines (dark freckle-like lesions containing melanocytes) are characteristic of LS, but not NS (Tartaglia and Gelb, 2005). Hypertrophic cardiomyopathy is common in LS, yet rare in *PTPN11*-associated NS (Digilio et al., 2006; Ogata and Yoshida, 2005). NS patients often show transient myeloproliferation and rarely develop juvenile myelomonocytic leukemia (Bader-Meunier et al., 1997). LS patients may be predisposed to other malignancies, such as acute leukemia and neuroblastoma (Merks et al., 2005; Ucar et al., 2006).

Most importantly, the biochemical properties of disease-associated PTPN11 proteins are distinct. Nearly all *PTPN11* mutations identified in NS and human tumors affect residues at the interface between the N-SH2 and PTP domains, resulting in enhanced SHP2 catalytic activity and RAS/ERK activation in vitro (Fragale et al., 2004; Keilhack et al., 2005; Niihori et al., 2005; Tartaglia et al., 2006) and in vivo (Araki et al., 2004). Gain-of-function alleles of *KRAS* (Schubbert et al., 2006), *SOS1* (Roberts et al., 2007; Tartaglia et al., 2007), or *RAF1* (Pandit et al., 2007; Razzaque et al., 2007) also cause NS, providing genetic evidence that this syndrome results from inappropriately high RAS/ERK pathway activity. In contrast, LS mutations target the PTP domain, typically involve catalytic residues, and result in variants with substantially decreased/absent phosphatase activity that act as dominant-negative mutants in transfection assays (Hanna et al., 2006; Kontaridis et al., 2006; Tartaglia et al., 2006). These findings pose two related questions: How do syndromes with overlapping features result from mutations with opposite effects on the catalytic activity and, apparently, the biological function of SHP2? And are LS mutations pure dominant-negative alleles or do they also have phosphatase-independent activities that mediate LS phenotypes?

Many LS features (e.g., altered pigmentation, craniofacial defects, semilunar valve disorders) could involve defects in the neural crest. Zebrafish provide an excellent system for studying neural crest development because of their transparency and highly conserved molecular pathways. Therefore, we compared the effects of antisense morpholinos (*shp2* MO) and LS mutant mRNAs on zebrafish neural crest development.

RESULTS

LS Mutations Have Dominant-Negative Effects on Zebrafish Gastrulation

Zebrafish Shp2 is highly similar (92% identical) to its mammalian orthologs and is expressed ubiquitously during gastrulation (see Figures S1A and S1B available online; Jopling et al., 2007). If LS mutants only have dominant-negative effects on development, then these should be qualitatively similar to the effects caused by Shp2 deficiency. We compared zebrafish embryos injected with mRNAs for LS mutants (engineered into zebrafish *shp2*) with those injected with *shp2* MOs to block Shp2 expression (Figure 1A). This experiment tested three LS alleles (Y280C, A462T, and T469M, corresponding to the human LS alleles Y279C, A461T, and T468M; Figure S1A) as well as two *shp2* MOs that block either translation or splicing (Figures S1C and S1D). As reported earlier (Jopling et al., 2007), LS (A462T) mRNA or *shp2* MO injections caused similar gastrulation defects, which were rescued by coexpression of wild-type (WT) human *SHP2* (Figure 1A and Figure S1E). Both MOs, but not a control "mismatch" MO (mmMO), depleted Shp2 and impaired Erk activation (Figure 1B). Increasing amounts of *shp2* LS mRNA caused a dose-dependent decrease in Erk activation (Figure 1C). In contrast, overexpressing WT zebrafish (or human) *shp2* did not affect Erk activity or development (Figures 1A and 1B; see Experimental Procedures). Thus, LS mutants and *shp2* deficiency have similar effects on phospho-Erk levels and early embryonic events, consistent with LS mutations acting as dominant-negative alleles.

Neomorphic Effects of LS Mutants on Neural Crest Development

We next compared the effects of LS mRNAs or *shp2* MOs on neural crest development. At 6 days postfertilization (dpf), >90% of embryos injected with *shp2* MOs had markedly abnormal craniofacial skeletons, including incomplete fusion and posterior displacement of the first and second arch and loss of the third to seventh branchial arches (Figure 1D, middle panels). LS mRNAs caused craniofacial dysmorphia in ~50% of embryos, although these defects were milder, with arch elements preserved but reduced in size and the first and second arches posteriorly displaced (Figure 1D, bottom panels). The remaining LS embryos (50%) had severe gastrulation phenotypes that prevented analysis, mild "hammerhead" phenotypes, as described previously (Jopling et al., 2007), or no obvious phenotype.

In contrast, the effects of *shp2* MOs and LS mRNA on pigment cell development were quite distinct (Figures 1E–1G). At 2 dpf, morphants had significantly fewer pigment cells, with iridophores reduced by ~70% and melanophores by ~35% (Figure 1E and Figure S1F). Conversely, LS mRNA-injected embryos with neural crest defects displayed an ~45% increase in iridophores and an ~20% increase in melanophores. The increase in melanophores was even more evident at 6 dpf (Figure 1F); indeed, the pigmentation phenotype of LS mRNA-injected embryos (increased melanophores) resembled multiple lentigines, a hallmark of LS and a major phenotypic difference between NS and LS (Figures 1E and 1F and Figure S1F). Pigment cells from LS mRNA- and MO-injected embryos also showed delayed migration over the yolk toward the ventral stripe (Figure 1G, arrow).

Peripheral sympathetic nervous system development also differed in LS mRNA- and MO-injected embryos. There was an ~40% increase in tyrosine hydroxylase (*th*)-positive sympathetic neurons at 4 dpf in LS embryos, whereas morphants had an ~60% decrease in these cells (Figure S1G). Morphants, but