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ROCKs cause SHP-wrecks and broken hearts

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During embryogenesis, the heart is one of the first organs to develop. Its formation requires a complex combination of migration of cardiac precursors to the ventral midline coupled with the fusion of these cardiogenic fields and subsequent cellular reorganization to form a linear heart tube. A finely controlled choreography of cell proliferation, adhesion, contraction and movement drives the heart tube to loop and subsequently septate to form the four-chambered mammalian heart we are familiar with. Defining how this plethora of cellular processes is controlled both spatially and temporally is a scientific feat that has fascinated researchers for decades. Unfortunately, the complex nature of this organ's development also makes it a prime target for mutation-induced malformation, as evidenced by the multitude of prevalent congenital heart disorders identified that afflict up to 1% of the population.

Keywords: Rho, ROCK, Shp2, Noonan syndrome, cardiac morphogenesis

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The gene PTPN11, encoding the SHP-2 phosphatase protein, has been identified as one of the genes wherein mis-sense mutations are strongly associated with congenital heart defects (CHD). Germline mis-sense mutations in SHP-2 are causal for Noonan syndrome (NS) and the associated (albeit less prevalent) disorder LEOPARD syndrome (LS). NS is a disorder mainly characterized by short stature, craniofacial abnormalities (hypertelorism) and heart defects including pulmonary valvular stenosis, septal defects and hypertrophic cardiomyopathy, while LS is primarily associated with pulmonary valve stenosis, hypertrophic cardiomyopathy and cardiac conduction abnormalities.[1,2] SHP-2 is comprised of two tandem N-terminal SH2 domains, a central protein phosphatase (PTP) catalytic domain, and two C-terminal regulatory tyrosine phosphorylation sites. In the resting state, SHP-2 phosphatase activity is negligible due to an intra-molecular interaction between the first SH2 domain and the catalytic domain and SHP-2 is activated by both tyrosine phosphorylation and SH2-mediated protein interactions that are induced by growth factor receptor signaling. Remarkably, nearly 50 distinct SHP-2 mutations are associated with CHD. While the vast majority of mutations map to within the first SH2 domain (including Q79R and D61G), the most prevalent mutation associated with NS occurs within the PTP domain (N308D, observed in approximately 30% of patients). In general, many of the NS-causing mutations tested to date result in enhanced catalytic activity due to relief of SH2-mediated auto-inhibition, while the LS-causing mutations result in loss-of-function SHP-2 variants (including Q510E, Y279C). Interestingly, somatic mutations that occur in these same domains (and also enhance catalytic activity) are often associated with leukemias such as Juvenile myelomonocytic leukemia (JMML).[3] The reason for the distinct pathogenic outcomes of these diverse mutations is currently unknown.

Wild-type SHP-2 imparts tight temporal control of tyrosine-kinase based signaling events by regulating both positive- and negative-feedback loops, often within the same "linear" pathway. SHP-2 has been fastidiously linked to modulation of Ras/extracellular signal-related kinase (ERK) and phosphatidylinositol-3-kinase-AKT

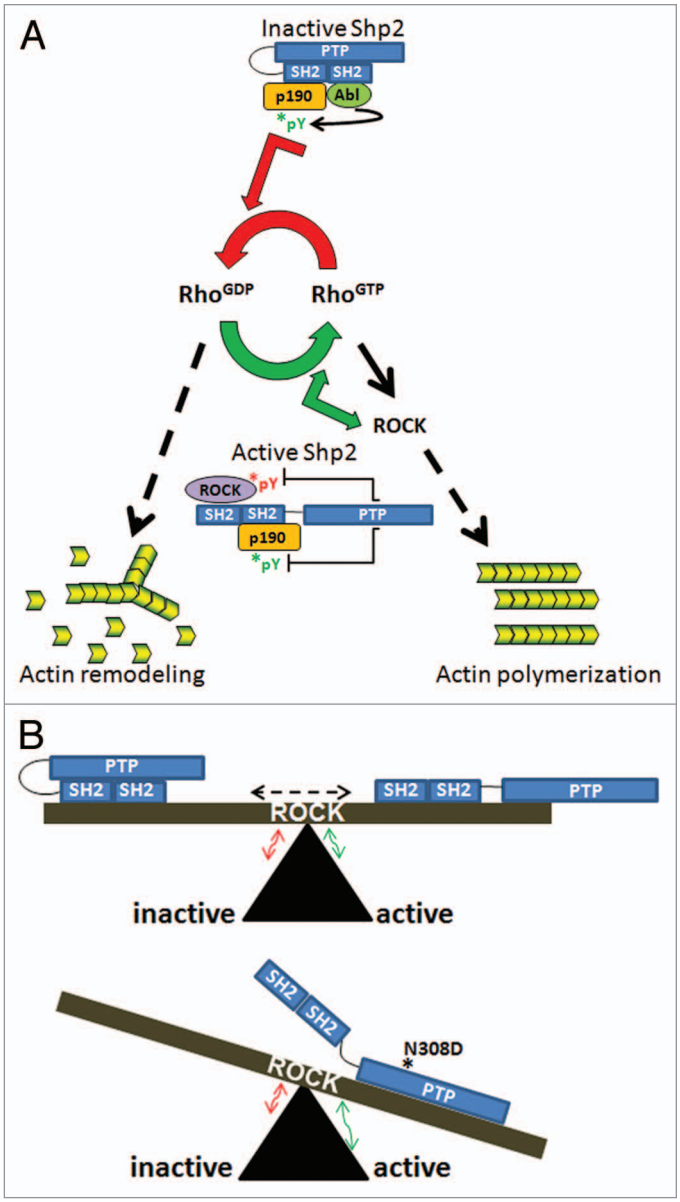

Figure 1. Model for SHP-2 regulation of Rho/ROCK activity and actin remodeling. (**A**) In its basal (inactive) state, SHP-2 serves as an adaptor protein to promote Abl-dependent phosphorylation of an activating tyrosine (pY, green) on p190 Rho GAP (p190). Activation of p190 leads to enhanced Rho GTP hydrolysis and inhibition of Rho/ROCK signaling, which results in actin de-polymerization/remodeling. Upon activation, SHP-2 de-phosphorylates and inactivates p190 to promote Rho signaling. As well, SHP-2 de-phosphorylates an inactivating tyrosine phosphorylation (pY, red) on ROCK and de-represses this kinase; collectively resulting in enhanced actin polymerization and accumulation of filamentous actin. (**B**) Wt SHP-2 maintains balanced ROCK activity that is essential for cell motility. The Noonan's associated N308D SHP-2 mutation tips the balance toward inappropriate actin polymerization and impairs cell motility.

signaling through various binding partners/substrates including GAB1, Grb2, Sprouty, Src and Ras-GTPAse activating protein (GAP).[4-7] The major LS causing loss-of-function variants of SHP-2 are associated with aberrant activation of the hypertrophy-associated AKT and mammalian target of rapamycin (mTOR) pathways.[8,9] Moreover, recent studies revealed that HCM induced by LS variant expression in transgenic mice is fully reversed by rapamycin, indicating that elevated AKT/mTOR signaling is causal for this phenotype.[10,11] In contrast, aberrant fibroblast growth factor receptor-mediated ERK signaling has been implicated in NS-associated SHP-2 cardiac phenotypes in vivo.[6,7] However, the role of SHP-2 is not confined to these growth conduits as more recent data indicates that SHP-2 can influence actin remodeling and cytoskeletal shape by modulating the RhoA-ROCK pathway.[12,13] Indeed, recent mechanistic studies indicate that both Rho-selective GAPs (p190 RhoGAP A and B), and the essential Rho effector, rho-associated kinase (ROCK) are bona fide SHP-2 substrates.[9-11]

Confirming a cause-effect relationship between Noonan's associated SHP-2 mutations and CHD, we recently reported that the global ectopic expression of the NS variants SHP-2^{N308D}, SHP-2^{D61G} and SHP-2^{Q79R} induced cardiac-restricted developmental defects in *Xenopus laevis*.[7,16] Intriguingly, hearts from embryos with ubiquitous ectopic expression of wild-type SHP-2 or a SHP-2 variant associated with JMML (SHP-2^{E76A}) were indistinguishable from un-injected controls. Expression of the NS-related SHP-2 variants led to a delay in cardiac cell cycling[7] and failure of cardiomyocyte progenitors to incorporate into the developing heart. While no difference was noted in the levels or activity of the growth-related ERK pathway, stark changes in cardiomyocyte cytoskeletal organization were evident by transmission electron microscopy. Subsequent studies in cultured cells indicated that SHP-2^{N308D} expression induced hyper-activation of ROCK signaling that led to aberrant actin remodeling. Remarkably, temporal inhibition of ROCK activity by treatment with the pharmacological inhibitor Y27632 completely restored heart formation in the SHP-2^{N308D} expressing embryos. Our studies confirm and extend those of Lee et al. who reported that RhoA-dependent ROCK activation is negatively regulated by Src-induced phosphorylation of ROCK on Y722 and that SHP-2 can activate ROCK by de-phosphorylating this inhibitory site.[14] Thus, we surmise that the SHP-2 NS variants likely uncouple this important negative feedback loop, and lead to un-controlled ROCK-dependent actin polymerization (see **Fig. 1**).

While our studies support that SHP-2 promotes feed-forward signaling through the Rho/ROCK pathway, it is important to note that both loss- and gain-of

function of SHP-2 has been reported to induce Rho/ROCK activity.[15-18] Indeed, the Neel laboratory showed that depletion of SHP-2 in mice led to cardiac dilatation that was associated with elevated Rho-ROCK signaling and they found that the dilated phenotype of cultured SHP-2 null cardiomyocytes was reversed with Y27632.[19] How can SHP-2 act as a negative and positive regulator of the Rho/ROCK pathway? This type of modulation could be explained, in part, by the finding that in its basal state SHP-2 can serve as an adaptor protein to forge an interaction between p190 RhoGAP and its activating kinase, cAbl; thus promoting Rho-GAP activity and blunting Rho activation.[19,20] However, upon activation by receptor signaling, SHP-2 can de-phosphorylate (and inactivate) p190 RhoGAP to promote Rho activity.[19] Collectively, we believe these studies indicate that SHP-2 acts at the level of both Rho and ROCK to impart tight temporal control of this pathway; although it is possible that SHP-2 regulates additional as yet unidentified molecules within the Rho signaling cascade.

Regardless of the specific underlying mechanism(s), our data indicate that the Noonan's associated mutation, N308D, selectively tips the balance toward enhanced/prolonged ROCK signaling in the myocardium. Somewhat surprisingly, we found that ectopic expression of the SHP-2 variants associated with JMML (that exhibit even higher catalytic activity than the NS mutations[21]) did not appear to disturb Rho/ROCK dependent actin remodeling or cardiac morphogenesis. This lack of effect on Rho signaling could be due to myriad potential differences between these variants including differential protein stability, tertiary structure, substrate accessibility, sub-cellular locale or initiation of altered negative feedback control pathways. Nonetheless, it will be of future interest to determine whether Rho/ROCK dys-regulation is involved in cases of NS or LS that result from alternative PTPN11 mutations or even in NS that results from Raf or SOS mutations as aberrant activation of these proteins would also likely impinge on the Rho pathway.[22]

An additional remarkable finding from this study was that regardless of the fact that the N308D SHP-2 variant was overexpressed throughout the entire animal, the only apparent defect was improper heart formation.[13] We surmise that the nature of the specificity of the cardiac defects induced by N308D could relate to the fact that ROCK is particularly abundant in developing hearts[23] and that precise regulation of this pathway is crucial for the appropriate regionalized co-ordination of myocyte growth and migration required for cardiac morphogenesis. We recently showed that despite the fact that cardiomyocytes contain sarcomeric actin arrays, they move in a polarized fashion that (like many cell types) involves protrusion and advancement of a stable leading edge lamellipodium.[24] Whether, these cells are more sensitive to precise changes in ROCK-dependent actin remodeling during translocation of the cell body is an interesting question for future study. We are also pursuing the possibility that the cardiac selective effect of these variants arises from myocyte-specific control of signaling networks that ultimately lead to tissue-restricted dys-regulation of Rho/ROCK signaling.

Given the findings that ROCK inhibition can fully rescue cardiac malformations induced by SHP-2 variants,[13,25] we propose that novel treatment strategies with ROCK inhibitors might be efficacious for NS patients. In this regard, it is promising that the ROCK inhibitor, Fasudil, has been approved for use in adult patients to treat vascular defects and hypertension.[26,27] However, since both ROCK1 and 2 are essential for mammalian development,[28,29] it is clear that further studies are required to define the precise treatment regimen capable of ameliorating cardiac morphogenetic defects without disrupting extra-cardiac organogenesis. It is likely that this will necessitate strict spatial and temporal control of ROCK inhibition.

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