Gain-of-function/Noonan syndrome SHP-2/Ptpn11 mutants enhance calcium oscillations and impair NFAT signaling

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Gain-of-function mutations in SHP-2/PTPN11 cause Noonan syndrome, a human developmental disorder. Noonan syndrome is characterized by proportionate short stature, facial dysmorphism, increased risk of leukemia, and congenital heart defects in ~50% of cases. Congenital heart abnormalities are common in Noonan syndrome, but the signaling pathway(s) linking gain-of-function SHP-2 mutants to heart disease is unclear. Diverse cell types coordinate cardiac morphogenesis, which is regulated by calcium (Ca\(^{2+}\)) and the nuclear factor of activated T-cells (NFAT). It has been shown that the frequency of Ca\(^{2+}\) oscillations regulates NFAT activity. Here, we show that in fibroblasts, Ca\(^{2+}\) oscillations in response to FGF-2 require the phosphatase activity of SHP-2. Conversely, gain-of-function mutants of SHP-2 enhanced FGF-2-mediated Ca\(^{2+}\) oscillations in fibroblasts and spontaneous Ca\(^{2+}\) oscillations in cardiomyocytes. The enhanced frequency of cardiomyocyte Ca\(^{2+}\) oscillations induced by a gain-of-function SHP-2 mutant correlated with reduced nuclear translocation and transcriptional activity of NFAT. These data imply that gain-of-function SHP-2 mutants disrupt the Ca\(^{2+}\) oscillatory control of NFAT, suggesting a potential mechanism for congenital heart defects in Noonan syndrome.

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Abbreviations: NFAT, nuclear factor of activated T-cells; InsP\(_3\), inositol 1,4,5-trisphosphate receptor; RTK, receptor tyrosine kinase; PTP, protein tyrosine phosphatase; SH2, src homology 2; PDGF, platelet-derived growth factor; CPA, cyclopiazonic acid; FDHM, full duration at half maxima.

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Fig. 1. SHP-2 is required for RTK-generated Ca\textsuperscript{2+} signaling. (a–g) Representative single cell Ca\textsuperscript{2+} recordings of Fura-4/AM-loaded WT (SHP-2\textsuperscript{+/+}) (a, c, e, and g) and SHP-2\textsuperscript{Ex3/–} (b, d, f, and g) fibroblasts treated with 10 \( \mu \)M ATP (a and b), 50 ng/ml PDGF (c and d), 100 ng/ml FGF-2 (e and f), and 20 \( \mu \)M CPA (g). Ratio \( F/F_0 \) represents fluorescence intensity over baseline. (h) Immunoblotting of InsP3Rs type-1 (InsP3R1), type-3 (InsP3R3), and SHP-2 in SHP-2\textsuperscript{+/+} and SHP-2\textsuperscript{Ex3/–} fibroblasts. (i) Immunocytochemistry of InsP3R1 and InsP3R3 in SHP-2\textsuperscript{+/+} and SHP-2\textsuperscript{Ex3/–} fibroblasts treated with 10 \( \mu \)M ATP (100 ng/ml). PDGF (22 (81) 76 (285) 2 (7) [373/6]), and 20 \( \mu \)M CPA (96 (192) 3 (6) 1 (3) [201/5]).

Table 1. Characteristics of Ca\textsuperscript{2+} responses in WT (SHP-2\textsuperscript{+/+}) and SHP-2\textsuperscript{Ex3/–} fibroblasts exposed to vehicle, ATP (10 \( \mu \)M), PDGF (50 ng/ml), or FGF (100 ng/ml).

| Treatment | Nonresponding\(^\text{*}\), % | Transient\(^\text{†}\), % | Oscillation\(^\text{‡}\), % | \([n/M]\)^{\text{§}} |
|-----------|----------------|----------------|----------------|----------------|----------------|
| Vehicle   | 93 (153)       | 5 (8)          | 2 (4)          | [165/4]        |
| ATP       | 53 (86)        | 46 (73)        | 1 (3)          | [162/3]        |
| PDGF      | 22 (81)        | 76 (285)       | 2 (7)          | [373/6]        |
| FGF       | 32 (49)        | 18 (28)        | 50 (78)        | [155/4]        |

\(^\text{*}\)Nonresponding cells have no Ca\textsuperscript{2+} increase exceeding 1.25 of the baseline. Values in parentheses are number of cells.

\(^\text{†}\)Transient responding cells have at least three Ca\textsuperscript{2+} peaks exceeding 1.25 of the baseline. Values in parentheses are number of cells.

\(^\text{‡}\)Oscillatory responding cells have at least three Ca\textsuperscript{2+} peaks exceeding 1.25 of the baseline. Values in parentheses are number of cells.

\(^{\text{§}}\)Number of cells/number of experiments.

demonstrate that SHP-2 is required for PDGF-induced, but not ATP-induced, increases in intracellular Ca\textsuperscript{2+}. To further investigate the generality of SHP-2 to regulate intracellular Ca\textsuperscript{2+} signaling by RTKs, SHP-2\textsuperscript{+/+} and SHP-2\textsuperscript{Ex3/–} fibroblasts were exposed to fibroblast growth factor-2 (FGF-2). Unlike PDGF, which caused a Ca\textsuperscript{2+} transient, FGF-2 evoked Ca\textsuperscript{2+} oscillations in SHP-2\textsuperscript{+/+} fibroblasts (Fig. 1e and Table 1), whereas SHP-2\textsuperscript{Ex3/–} fibroblasts were unresponsive to FGF-2 stimulation (Fig. 1f and Table 1). We found that FGF-2 (100 ng/ml) failed to elicit Ca\textsuperscript{2+} oscillations in SHP-2\textsuperscript{Ex3/–} fibroblasts, but \( \approx 50\% \) of SHP-2\textsuperscript{+/+} fibroblasts evoked Ca\textsuperscript{2+} oscillations (Table 1). Hence, SHP-2 is required for the propagation of FGF-2-induced Ca\textsuperscript{2+} oscillations in fibroblasts.

**Intact Intracellular Ca\textsuperscript{2+} Stores and Rescue of Ca\textsuperscript{2+} Signaling in SHP-2\textsuperscript{Ex3/–} Fibroblasts.** A pharmacological approach was applied to determine whether the defect in PDGF- and FGF-2-induced Ca\textsuperscript{2+} responses in SHP-2\textsuperscript{Ex3/–} fibroblasts was due to intracellular defects in Ca\textsuperscript{2+} stores. First, fibroblasts were treated with cyclopiazonic acid (CPA), a sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) inhibitor, which can be used to deplete intracellular Ca\textsuperscript{2+} stores. Both SHP-2\textsuperscript{+/+} and SHP-2\textsuperscript{Ex3/–} fibroblasts showed similar Ca\textsuperscript{2+} responses to CPA (Fig. 1g), indicating that internal endoplasmic reticulum Ca\textsuperscript{2+} stores were equally intact. Thapsigargin, also a SERCA inhibitor, gave comparable responses (see Fig. 5a and b, which is published as supporting information on the PNAS web site). Neither FGF-2 nor PDGF evoked Ca\textsuperscript{2+} responses in SHP-2\textsuperscript{+/+} fibroblasts after internal Ca\textsuperscript{2+} stores were depleted after CPA stimulation (Fig. 5c). Mobilization of intracellular Ca\textsuperscript{2+} is mediated by the inositol 1,4,5-trisphosphate receptor (InsP3R) (20). Preincubation of fibroblasts with the InsP3R inhibitor, 2-aminoethoxydiphenyl borate (2-APB) blocked FGF-2- and PDGF-induced Ca\textsuperscript{2+} responses (Fig. 5d). Thus, PDGF and FGF-2 regulate InsP3R-
mediated Ca\(^{2+}\) increases by controlling the release of internal Ca\(^{2+}\) stores. We also investigated whether SHP-2\(^{+/+}\) and SHP-2\(^{Ex3/−}\) fibroblasts exhibited differences in Ca\(^{2+}\) response as a consequence of altered InsP\(_3\)R levels and/or subcellular distribution. SHP-2\(^{+/+}\) and SHP-2\(^{Ex3/−}\) fibroblasts showed similar levels of expression of the type 1 and 3 InsP\(_3\)R (Fig. 1f); the type 2 InsP\(_3\)R, however, was undetectable. Similar subcellular distribution in SHP-2\(^{+/+}\) and SHP-2\(^{Ex3/−}\) fibroblasts were observed for both type 1 and 3 InsP\(_3\)Rs (Fig. 1f). To determine whether SHP-2\(^{Ex3/−}\) fibroblasts express the FGF receptor to levels equivalent to that of SHP-2\(^{+/+}\) fibroblasts, we used purified GFP-tagged FGF protein (FGF-GFP) to label these cells. When FGF-GFP was exposed to these fibroblasts, SHP-2\(^{E76A}\) expressing fibroblasts exhibited an enhanced oscillatory frequency in response to FGF-2 (Fig. 2f). These results support the interpretation that the impaired Ca\(^{2+}\) signaling in SHP-2\(^{Ex3/−}\) fibroblasts is due to a loss of SHP-2 function rather than defects in the Ca\(^{2+}\)-generating machinery.

To definitively establish that SHP-2 is responsible for FGF-2-induced Ca\(^{2+}\) oscillations, SHP-2\(^{E76A}\) or SHP-2\(^{D61G}\) fibroblasts were transfected with WT SHP-2 (SHP-2\(^{WT}\)) to rescue the Ca\(^{2+}\) signaling defect. We found that SHP-2\(^{WT}\) expressing SHP-2\(^{E76A}\) fibroblasts (SHP-2\(^{Ex3/−}\))/SHP-2\(^{WT}\)) regained their ability to evoke Ca\(^{2+}\) oscillations in response to FGF-2 (Fig. 1f), but vector control transfectants remained nonresponsive (data not shown). The requirement for SHP-2 to generate Ca\(^{2+}\) oscillations in response to FGF-2 depended on SHP-2 catalysis because overexpression of a catalytically inactive mutant of SHP-2 (Table 2), SHP-2\(^{R465M}\) in SHP-2\(^{+/+}\) fibroblasts (SHP-2\(^{+/+}\)/SHP-2\(^{R465M}\)), failed to elicit a Ca\(^{2+}\) response to FGF-2 (Fig. 1f). These results demonstrate that loss of SHP-2 is causally related to the attenuated Ca\(^{2+}\) response and that the catalytic activity of SHP-2 is required to generate FGF-2-induced Ca\(^{2+}\) oscillations.

### SHP-2 Gain-of-Function and Noonan Syndrome Mutations Enhance Ca\(^{2+}\) Oscillatory Frequency.

Because the catalytic activity of SHP-2 is required to mediate Ca\(^{2+}\) signaling in response to FGF-2 (Fig. 1f), we tested the effects of a constitutively active mutant of SHP-2 (Table 2) on FGF-2-induced Ca\(^{2+}\) oscillations. A mutation within the NH\(_2\) terminus SH2 domain of SHP-2 at Glu-76 to Ala-76 (SHP-2\(^{E76A}\)) has been shown to result in constitutive SHP-2 catalysis (3). As compared with vector and SHP-2\(^{WT}\) expressing fibroblasts, SHP-2\(^{E76A}\) expressing fibroblasts exhibited an enhanced oscillatory frequency in response to FGF-2 (Fig. 2a–c; see also Movie 1, which is published as supporting information on the PNAS web site). To quantitate the oscillatory behavior of this response, we performed a spectral analysis (21) of FGF-2-induced Ca\(^{2+}\) oscillations in fibroblasts transfected with vector, SHP-2\(^{WT}\) and SHP-2\(^{E76A}\). This quantitative analysis revealed that vector control and SHP-2\(^{WT}\) expressing fibroblasts exhibited FGF-2-induced Ca\(^{2+}\) oscillations with a frequency of 9.6 ± 1.4 mHz (n = 25) and 12.6 ± 1.0 mHz (n = 25), respectively (Fig. 2c; see also Fig. 7a and b, which is published as supporting information on the PNAS web site). In contrast, SHP-2\(^{E76A}\) expressing fibroblasts increased FGF-2-mediated Ca\(^{2+}\) oscillations with a frequency of 24.2 ± 1.8 mHz (n = 8) (Figs. 2c and 7c and Movie 1). These data indicate that an activated SHP-2 mutant enhances the oscillatory frequency of Ca\(^{2+}\) in response to FGF-2. Similar gain-of-function mutants of SHP-2 cause Noonan syndrome (9, 22); therefore, we tested whether a gain-of-function mutant of SHP-2 found in Noonan syndrome (Table 2) enhances FGF-2-induced Ca\(^{2+}\) oscillatory frequency. Fibroblasts were transfected with a SHP-2 Noonan syndrome mutant in which Asp-61 is mutated to Gly-61 (SHP-2\(^{D61G}\)) (10, 11). We found that when expressed in fibroblasts, SHP-2\(^{D61G}\) also enhanced the oscillatory response evoked by FGF-2 by ~2-fold to 21.7 ± 1.8 mHz (n = 19) (Figs. 2d and e and 7d). To further characterize the oscillatory response we derived the full duration at half maxima (FDHM) for the individual Ca\(^{2+}\) peaks in the oscillatory signal. These calculations showed that the FDMH for FGF-2-triggered Ca\(^{2+}\) oscillations in vector control and SHP-2\(^{WT}\) expressing fibroblasts were 30.9 ± 3.3 s (n = 48) and 30.2 ± 2.6 s (n = 30), respectively (Fig. 2f). The analysis of oscillations in SHP-2\(^{E76A}\) and SHP-2\(^{D61G}\) expressing fibroblasts decreased FDMH to 15.2 ± 1.4 s (n = 36) and 22.5 ± 1.2 s (n = 53), respectively (Fig. 2f). Collectively, these results demonstrate that SHP-2 gain-of-function mutations as well as those found in Noonan syndrome result in enhanced Ca\(^{2+}\) oscillatory frequency and reduced FDMH.

### Gain-of-Function SHP-2 Mutant Enhances Cardiomyocyte Ca\(^{2+}\) Oscillations.

Increases in cytosolic Ca\(^{2+}\) through receptors such as ion channels activate calcineurin, which dephosphorylates NFAT in the nucleus. To determine the consequences of constitutive SHP-2 catalysis on the activity of the Ca\(^{2+}\)/calmodulin-dependent kinase in the cardiomyocyte, SHP-2\(^{E76A}\) was transiently expressed in cardiomyocytes cultured from 10.5-d embyronic rats. Figure 3 shows that vector control cardiomyocytes express Ca\(^{2+}\) oscillations with a frequency of 6.7 ± 0.2 mHz (n = 13) and that expression of SHP-2\(^{E76A}\) increased the oscillatory frequency to 20.4 ± 7.6 mHz (n = 10). Thus, the gain-of-function property of SHP-2\(^{E76A}\) is also present in cardiomyocytes.
cytosol, resulting in its translocation to the nucleus (16). Cardiac morphogenesis relies on Ca\(^{2+}\) and subsequently the precise regulation of the calcineurin/NFAT pathway (14–16). NFAT functions in numerous cellular processes, one of which involves the activation of transcription factors that regulate cardiac development (23, 24). We hypothesized that gain-of-function SHP-2 mutants would enhance Ca\(^{2+}\) oscillations in cardiomyocytes similar to those observed in fibroblasts (Fig. 2 e and f). Moreover, we speculated that altered Ca\(^{2+}\) oscillations in cardiomyocytes could interfere with calcineurin-dependent regulation of NFAT. To test this hypothesis, primary rat cardiomyocytes were first transfected with vector alone. We found that cardiomyocytes exhibited spontaneous Ca\(^{2+}\) oscillations with a frequency of 28.6 ± 5.2 mHz and FDHM of 8.3 ± 0.5 s (Figs. 3 a, e, and f and 7e). Significantly, cardiomyocytes expressing SHP-2 WT exhibited spontaneous Ca\(^{2+}\) oscillations with a frequency of 131.2 ± 17.5 mHz and FDHM of 4.5 ± 0.3 s (Figs. 3 b, e, and f and 7f), whereas SHP-2 WT cardiomyocytes responded with a spontaneous Ca\(^{2+}\) oscillation frequency of 190.5 ± 15.1 mHz and FDHM of 4.3 ± 0.5 s (Figs. 3 c, e, and f and 7g). Importantly, expression of the catalytically inactive mutant of SHP-2, SHP-2 R465M, was without effect as compared with vector control transfected cardiomyocytes (31.3 ± 6.4 mHz and 9.0 ± 1.3 s) (Figs. 3 d, e, and f and 7h). These data imply that constitutive SHP-2 activity gives rise to an increase in cardiomyocyte Ca\(^{2+}\) oscillatory frequency.

**Suppression of NFAT Activation in Cardiomyocytes by Gain-of-Function SHP-2 Mutants.** The oscillatory frequency of Ca\(^{2+}\) has been reported to be a mechanism in which calcineurin/NFAT activation can be “fine-tuned” (17–19). Moreover, NFATc1-deficient mice have defects in the formation of heart valves and the interventricular septum that resemble congenital heart defects seen in humans (23, 24). Therefore, we investigated whether enhanced SHP-2 activity affected NFAT function in cardiomyocytes. When immunostaining for NFATc1 was performed in cardiomyocytes infected with an adenovirus expressing GFP alone, a clear expression of NFATc1 was observed between the nucleus and cytosol (Fig. 4 a and b). In contrast, cardiomyocytes infected with an adenovirus expressing SHP-2 WT showed decreased nuclear expression of NFATc1 (Fig. 4 c and d). Nuclear to cytosolic expression of NFATc1 in cardiomyocytes was quantitated for vector, SHP-2 WT, SHP-2 E76A and SHP-2 R465M (Fig. 4 e). This analysis showed that SHP-2 E76A expressing cardiomyocytes contained ~40% less nuclear localized NFATc1 as compared with SHP-2 WT, expressing cardiomyocytes (Fig. 4 e). Moreover, the catalytically inactive mutant of SHP-2, SHP-2 R465M, displayed levels of nuclear NFATc1 accumulation similar to that of cardiomyocytes infected with vector and SHP-2 WT. These results suggest that a gain-of-function mutant of SHP-2 impairs NFATc1 nuclear localization and by inference NFAT transcriptional activity in cardiomyocytes.

**Fig. 4.** Suppression of NFAT activity by SHP-2 gain-of-function mutant in cardiomyocytes. (a–d) Confocal images of immunostained cardiomyocytes infected with vector expressing GFP alone (green) (a and b) or GFP along with SHP-2 E76A (c and d). Nuclear staining with TO-PRO-3 (blue) (a and c) and NFAT expression (red) (b and d) is shown. (e and f) Statistical analysis of NFAT immunostaining (nucleus/cytosol) (e) and NFAT-luciferase reporter gene in cardiomyocytes (f) infected with vector (VEC), SHP-2 WT (WT), SHP-2 E76A (E76A), and SHP-2 R465M (R465M). (g) Immunoblot of SHP-2 expression from cell lysates used in f and loading controls (extracellular-regulated kinase 2 (Erk-2)). (h) Bidirectional plot of NFAT activity vs. spontaneous Ca\(^{2+}\) oscillatory frequency in cells expressing vector (green circle), SHP-2 WT (blue circle), SHP-2 E76A (yellow circle), and SHP-2 R465M (red circle). Linear regression analysis (black line) with a correlation coefficient r\(^2\) = 0.96. Values are mean ± SEM. * P < 0.05.
tory frequency seen in SHP-2WT as compared with SHP-2E76A was not due to differences in the expression of SHP-2 because immunoblotting of the same lysates used for the NFAT reporter gene experiments showed comparable levels of SHP-2 in SHP-2WT and SHP-2E76A expressing cardiomyocytes (Fig. 4g). Significantly, NFAT activity in SHP-2R465M-expressing cardiomyocytes was not inhibited, although SHP-2R465M was overexpressed relative to vector control-infected cardiomyocytes (Fig. 4f and g). These results suggest that the interpretation that enhanced SHP-2 activity suppresses NFAT activation. Finally, we performed a two-way analysis, correlating NFAT activity in response to the expression of SHP-2WT, SHP-2E76A, and SHP-2R465M with their respective Ca2+ oscillatory frequencies generated in cardiomyocytes. This analysis revealed a correlation coefficient of r = 0.96 for vector, SHP-2WT, SHP-2E76A, and SHP-2R465M vs. their respective cardiomyocyte Ca2+ oscillatory frequencies (Fig. 4h). Thus, SHP-2 catalysis correlates with an increase in Ca2+ oscillatory frequency and suppression of NFAT activation in cardiomyocytes.

Discussion

Our data show that SHP-2 is responsible for evoking distinct Ca2+ responses after stimulation of fibroblasts with either PDGF or FGF-2. We found that PDGF produced a Ca2+ transient whereas FGF-2 generated Ca2+ oscillations, and in both of these cases Ca2+ responses were abrogated in fibroblasts lacking functional SHP-2. Importantly, SHP-2 does not participate in all receptor-mediated Ca2+ signaling pathways because loss of functional SHP-2 in fibroblasts still resulted in Ca2+ mobilization upon activation of the G protein-coupled P2Y receptor. These data demonstrate that SHP-2 is responsible for generating Ca2+ responses downstream of RTKs but not G-protein-coupled receptors. Furthermore, SHP-2 appears to mediate distinct Ca2+ responses among different RTKs.

How SHP-2 regulates Ca2+ transients and oscillations, however, remains to be established. The Src-family kinases can activate phospholipase Cγ1, which mediates the release of InsP3 and subsequently Ca2+ mobilization. The catalytic activity of SHP-2 has been shown to be responsible for the activation of the Src-family kinases (25). Thus, Src-family kinase phosphorylation of phospholipase Cγ1 mediated by SHP-2 in response to RTK activation is one candidate pathway through which SHP-2 may mediate Ca2+ release. Indeed, it was shown recently that SHP-2 is required for the mobilization of Ca2+ in response to interleukin-1 by a mechanism involving phospholipase Cγ1 tyrosine phosphorylation (26). Our data support this idea because we show specifically that the catalytic activity of SHP-2 is required for FGF-2-induced Ca2+ mobilization.

One of the key findings of this work is that enhanced catalytic activity of SHP-2 rendered by a gain-of-function mutation resulted in a significant increase in the Ca2+ oscillatory frequency and a significant FDHM decrease in response to FGF-2 in fibroblasts and primary cardiomyocytes. Gain-of-function SHP-2 mutants have been shown to cause hyperactivation of the extracellular-regulated kinases (ERKs) and to increase cell proliferation (11). Our results now show that the Ca2+ signaling pathway is also a target for aberrant regulation by gain-of-function SHP-2 mutants. Many mutations in SHP-2 that are associated with Noonan syndrome occur at the interface between the PTP domain and NH2-terminal SH2 domain resulting in its constitutive activation (9). We show that in addition to the gain-of-function SHP-2E76A mutant, SHP-2D61G mutation, which is found in Noonan syndrome patients, also resulted in enhanced Ca2+ oscillatory frequency and decreased FDHM in response to FGF-2. Therefore, our results suggest that altered Ca2+ signaling, conferred by SHP-2 gain-of-function mutants found in Noonan syndrome, may be a related molecular mechanism associated with the pathogenesis of this disease. Interestingly, patients with Leopard syndrome, a closely related developmental disorder to that of Noonan syndrome in which SHP-2 mutations have been identified, suffer from electrocardiographic conduction abnormalities (10). Our observation that gain-of-function SHP-2 mutants disrupt the “pacemaker” feature of cardiomyocytes provides insight into potential mechanisms for why Leopard syndrome patients manifest with electrocardiographic conduction defects.

Noonan syndrome patients often present with cardiovascular abnormalities, particularly congenital heart diseases in up to 85% of affected individuals. The most prevalent form of congenital heart disease in Noonan syndrome is pulmonic stenosis, in addition to other lesions such as atrioventricular septal defects, mitral valve abnormalities, and hypertrophic cardiomyopathy (7, 8). A number of signaling pathways have been implicated in cardiac morphogenesis. Notably, the NFAT signaling pathway has been shown to be critical for cardiac development and is regulated by Ca2+ through the actions of calcineurin (13–16). We tested the hypothesis that altered Ca2+ signaling evoked by SHP-2 gain-of-function mutations might disrupt NFAT function. Our data demonstrate that enhanced Ca2+ oscillatory frequency induced by SHP-2 gain-of-function mutants in cardiomyocytes correlates with the inhibition of NFAT translocation to the nucleus and subsequently suppression of NFAT transcriptional activity. These data implicate NFAT as a target of SHP-2 gain-of-function/Noonan syndrome mutants. Provocatively, altered NFAT function in mice through genetic ablation gives rise to valvulogenesis defects (23, 24) that bear some similarity to the congenital heart defects observed in a mouse model of Noonan syndrome (12). There is good evidence that calcineurin which activates NFAT by dephosphorylation is regulated not just by increases in intracellular Ca2+ but also by the oscillatory frequency of Ca2+ (27). After NFAT translocation to the nucleus, it also has been shown that NFAT transcriptional activity is modulated by the frequency of intracellular Ca2+ oscillations (17, 18). The fact that SHP-2 gain-of-function mutations decreased the FDHM also might contribute to the NFAT transcriptional activity. Thus, it is conceivable that altered Ca2+ signaling and/or disruption of the NFAT function by Noonan syndrome mutants may contribute to the development of congenital heart abnormalities.

In summary, we show that the catalytic activity of SHP-2 is required for FGF-2-induced Ca2+ oscillations in fibroblasts. Moreover, constitutive SHP-2 catalysis, as exhibited in gain-of-function/Noonan syndrome SHP-2 mutations, resulted in enhanced Ca2+ oscillatory frequency in response to FGF-2 in fibroblasts and spontaneous Ca2+ oscillations in cardiomyocytes. Both the FGF and the Ca2+/calcineurin/NFAT pathways are critical for cardiac morphogenesis (14, 28). SHP-2 gain-of-function mutants perturb the Ca2+ oscillatory frequency downstream of the FGF receptor, which could contribute to abnormal cardiac morphogenesis. Perturbations in NFAT function through dysregulation of the Ca2+/calcineurin/NFAT axis in cardiomyocytes also appears to be a pathway through which gain-of-function/Noonan syndrome mutants of SHP-2 may disrupt cardiac valvulogenesis (14). Collectively, these results provide a potential mechanistic link between SHP-2 gain-of-function mutants and the development of congenital heart disease seen in Noonan syndrome.

Materials and Methods

Cell Cultures. Fibroblasts derived from mice containing either a deletion within exon 3 of SHP-2 that removes amino acids 46–110 of the NH2-terminus SH2 domain (SHP-2Ex3Δ/Δ) or from WT (SHP-2+/+) littermate controls are described in refs. 4 and 29. Briefly, cells were cultured at 37°C and 5% CO2 in DMEM (Invitrogen) containing 10% FBS (Sigma), 1 mM sodium pyruvate (Invitrogen), 5 units/ml penicillin, and 50 μg/ml streptomycin (Sigma). Cardiac myocytes were prepared as described in ref. 30. Primary cultures of cardiomyocytes were cultured from hearts of 1- to 3-day-old Sprague–Dawley rats seeded on gelatin-coated coverslips and starved in serum-free medium 24 h prior to the experiment. The protocol produces cultures of cardiac myocytes that are at least 95% pure (30).
Plasmids and Adenoviruses. pRES-ΔDsRed plasmids containing either SHP-2 WT or gain-of-function/Noonan syndrome mutants of SHP-2 (SHP-2 WT and SHP-2 ΔE76A) or a catalytically inactive mutant of SHP-2 (SHP-2 ΔE76M) were prepared as described in ref. 31. Transient transfections were performed by using Lipofectamine 2000 (Invitrogen) in OptiMEM. After transient transfections, cells were serum-starved for 16–24 h before the experiments in DMEM containing 0.1% FBS. Purified FGF-2–GF protein was provided by Joseph Schlessinger (Yale University School of Medicine, New Haven, CT). Adenoviruses encoding for the expression of SHP-2 WT and SHP-2 ΔE76A were generated as described in ref. 31.

Calcium Measurements. Cells were incubated (30 min at 37°C in 5% CO2) in Hepes medium containing 5 μM Fluo-4/AM (Molecular Probes) together with 0.1% Pluronic F-127 (Molecular Probes). The Hepes medium contained 130 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl2, 1 mM MgSO4, 1.2 mM KH2PO4, 20 mM Hepes (pH 7.4), and 5 mM dextrose. Coverslips were mounted in a temperature-controlled (37°C) chamber (Warner Instruments, Hamden, CT) and transferred to a Zeiss LSM 510 META scanning laser confocal microscope equipped with a C-Apochromat ×40/1.2 water immersion objective (Zeiss). Images were acquired at 0.2 Hz for the fibroblast cells and 0.5 Hz for the cardiac myocytes. All drugs were bath applied.

Immunocytochemistry. Immunocytochemical staining of NFAT and InsP3Rs was performed according to standard protocol, using fixation by 4% paraformaldehyde in 1 h. After blocking with 1% BSA, cells were incubated with NFATc1 C20 goat polyclonal antibody (Ab) (1:100, Santa Cruz Biotechnology) for 1 h and then with Alexa 488 fluorescent secondary Ab (1:200; Molecular Probes) for 1 h, together with 0.3% Triton X-100. Nucleus was stained with TO-PRO-3 (1:200; Molecular Probes) for 10 min. InsP3Rs were stained by using the same protocol with the following Abs: polyclonal type-1 InsP3R Ab (1:20,000; custom-produced by Research Genetics, Huntsville, AL), polyclonal type-2 InsP3R Ab (1:250; generously provided by F. Leite), and polyclonal type-3 InsP3R Ab (BD Biosciences). Transient transfections were performed by using Lipofectamine 2000 (Invitrogen) in OptiMEM. After transient transfections, cells were serum-starved for 16–24 h before the experiments in DMEM containing 0.1% FBS. Purified FGF-2–GF protein was provided by Joseph Schlessinger (Yale University School of Medicine, New Haven, CT). Adenoviruses encoding for the expression of SHP-2 WT and SHP-2 ΔE76A were generated as described in ref. 31.

Immunoblot Analyses. Cell lysates were prepared and immunoblotting was performed as described in ref. 31. Abs used were as follows: polyclonal SHP-2 Ab (Santa Cruz Biotechnology), polyclonal type-1 InsP3R Ab (custom-produced by Research Genetics), polyclonal type-2 InsP3R Ab (generously provided by F. Leite), and monoclonal type-3 InsP3R Ab (BD Biosciences).

Adenoviral NFAT-Luciferase Infection and Luciferase Reporter Assay. The adenoviral NFAT–luciferase reporter (AdNFAT-luc) construct was generously provided by Jeffery D. Molkentin (Children’s Hospital Medical Center, Cincinnati) and is described in ref. 32. Cardiomyocytes were infected with pAd-GFP, Ad-WT, Ad-E76A, or Ad-R465M and Ad-NFAT-Luc for 2 h before switching to fresh growth medium. Cultures were incubated for 48 h before harvesting. Lysates were generated and luciferase activity was determined according to the manufacturer’s instructions by using the Luciferase Assay System (Promega). Luciferase activity was normalized to total protein concentration for each sample as determined by Coomassie Protein Assay Reagent (Pierce), and results were expressed as relative luciferase units per microgram of protein (RLU/μg).

Chemicals. Chemicals used were as follows: ATP, bradykinin, CPA, thapsigargin, 2-aminoethoxydiphenyl borate (2-APB) (all from Sigma), FGF-2, and PDGF (Calbiochem). All drugs were bath applied.

Data Analysis. Power spectrum analysis was carried out as described in ref. 21. Briefly, a power spectrum of a signal is the squared magnitude of its Fourier transform and describes the contribution to a signal by each of its sine wave components. In the power spectrum, each peak corresponds to a different frequency present in the original data. The peak with highest power spectral density (PSD) indicates the most dominant frequency within the Ca2+ signal. Oscillations were analyzed in a program written in MATLAB (21). The frequency resolution was 3.1 mHz. The time duration for individual peaks in the Ca2+ oscillatory response was analyzed by calculating the FDHM using an algorithm implemented in MATLAB. Briefly, the FDHM value for a Ca2+ peak is given by the time difference between the two points on each side of the peak at which the Ca2+ level reaches half its maximum value. Data are presented as means ± SEM of a minimum of three experiments, unless indicated otherwise. Student’s t test was used, and significance was accepted at P < 0.05. Regression analysis and correlation coefficient r were computed by using SIGMAPLOT (Systat, Evanston, IL).

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