Defective Sumoylation Pathway Directs Congenital Heart Disease

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Congenital heart defects (CHDs) are the most common of all birth defects, yet molecular mechanism(s) underlying highly prevalent atrial septal defects (ASDs) and ventricular septal defects (VSDs) have remained elusive. We demonstrate the indispensability of ''balanced'' posttranslational small ubiquitin-like modifier (SUMO) conjugation-deconjugation pathway for normal cardiac development. Both hetero- and homozygous SUMO-1 knockout mice exhibited ASDs and VSDs with high mortality rates, which were rescued by cardiac reexpression of the SUMO-1 transgene. Because SUMO-1 was also involved in cleft lip/palate in human patients, the previous findings provided a powerful rationale to question whether SUMO-1 was mutated in infants born with cleft palates and ASDs. Sequence analysis of DNA from newborn screening blood spots revealed a single 16 bp substitution in the SUMO-1 regulatory promoter of a patient displaying both oral-facial clefts and ASDs. Diminished sumoylation activity whether by genetics, environmental toxins, and/or pharmaceuticals may significantly contribute to susceptibility to the induction of congenital heart disease worldwide. *Birth Defects Research (Part A) 91:468–476, 2011.* © 2011 Wiley-Liss, Inc.

INTRODUCTION

Normal cardiovascular development is a complex process that requires highly coordinated collaboration among a variety of transcription factors and signal transduction pathways. Congenital heart defects (CHDs) that are structural malformations present at birth, are the most common of all human birth defects, occurring in approximately 1% of all newborns (Wu and Child, 2004). The two most frequent CHDs are the atrial septal defects (ASDs) and ventricular septal defects (VSDs) (Hoffman and Kaplan, 2002), defined as the presence of a communication between the right and left atria, and the right and left ventricles, respectively. The occurrence of ASDs and/or VSDs can be either isolated, or associated with each other, or with other CHDs, such as Tetralogy of Fallot (Vaughan and Basson, 2000). Although a number of transcription factors, including GATA4, Nkx2.5, and Tbx5, as well as several signaling pathways, including Notch, Wnt, BMP, and Hedgehog have been reported to be involved in septogenesis (Baldini, 2005; Bruneau et al., 2001; Garg et al., 2003; Nie et al., 2008; Niessen and Karsan, 2008; Tanaka et al., 1999), chromosomal and Mendelian syndromes account for only 20% of septal defects (Bentham and Bhattacharya, 2008). Thus, the unifying molecular mechanism(s) accounting for the vast majority of ASDs and VSDs remains elusive.

The fact that SUMO (small ubiquitin-like modifier) conjugation pathway components are abundant in the heart points to the possibility that the SUMO pathway may be implicated in cardiovascular development via modifying transcription factors indispensable for normal

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cardiovascular development (Golebiowski et al., 2003; Watanabe et al., 1996). Indeed, we and others identified several cardiac-enriched transcription factors GATA-4, Nkx2.5, serum response factor (SRF), and Myocardin as SUMO targets (Komatsu et al., 2004; Matsuzaki et al., 2003; Wang et al., 2004; Wang et al., 2007; Wang et al., 2008). Sumoylation is a posttranslational protein modification in which a novel super family of ubiquitin-like proteins (SUMO1-3/Sentrin) is tethered to target proteins, affecting their biologic activity (Johnson, 2004; Yeh et al., 2000). SUMO conjugation is accomplished via the following phases: maturation, activation, conjugation/ligation, and deconjugation (Johnson, 2004; Wang, 2009). At least three functional SUMO protein isoforms have been identified in higher vertebrates (Johnson, 2004). Active SUMO-2 and −3 share over 95% similarity at amino acid level but exhibit only ~50% identity with SUMO-1. SUMO conjugation is accomplished after transfer of the SUMO protein to the unique conjugation enzyme (E2)-Ubc9; thereupon, Ubc9 can transfer SUMO protein directly to its substrates. In vivo SUMO conjugation and ligation can be modulated by a number of E3 ligases. Sumoylation is reversible by deconjugation; SUMO proteins are deconjugated from their conjugation state by Sentrin/SUMO-specific proteases (SENPs), a family of six members of which SENP1 and SENP2 can process all SUMO conjugates (Yeh, 2008).

Proteomic studies have shown that SUMO-1 and SUMO-2/3 can be conjugated to unique target subsets but also show some overlap in target specificity (Rosas-Acosta et al., 2005; Vertegaal et al., 2006; Vertegaal et al., 2004). Other studies have revealed differences in the behavior and dynamics of SUMO isoforms (Ayaydin and Dasso, 2004; Fu et al., 2005; Mukhopadhyay and Dasso, 2007; Saitoh and Hinchey, 2000; Tatham et al., 2005; Zhang et al., 2008b); thus, they are suggestive of distinct functions for SUMO-1 compared with SUMO-2/3. However, there is still no clear understanding of which roles are unique for the SUMO isoforms and to what degree any redundancy exists between them. Sumoylation plays a critical role in many cellular processes, including cell cycle progression and chromatin remodeling. However, no role for decreased sumoylation in cardiovascular development has been reported.

In this study, we demonstrate that hetero- and homozygous SUMO-1 knockout (KO) mice developed CHDs, ASDs, and VSDs, coincident with the dysregulation of genes involved in cell proliferation. SUMO-1 may also play a role in the modulation of chromatin remodeling complexes, whereby inappropriate liver gene expression is suppressed in the developing heart. In addition, sequence analysis of DNA from newborn screening blood spots also revealed a single 16 bp substitution mutation in the SUMO-1 regulatory of a patient displaying both oral-facial clefts and ASDs. Thus, reduced sumoylation activity caused by genetics, environmental toxins, and/or by drugs may significantly contribute to the pervasiveness of congenital heart disease.

MATERIALS AND METHODS

Generation of Transgenic and KO Mice

The α-MHC-flag-SUMO-1 transgene was constructed using PCR-amplified flag-tagged SUMO-1 cloned between the 5.4 kbp mouse α-MHC promoter (provided by Dr. J. Robbins, University of Cincinnati) and the Simian virus 40 (SV40) polyadenylation sequence via *Sal*I sites. The orientation of inserted transgene was confirmed by DNA sequencing. The constructs were microinjected into the pronucleus of fertilized eggs from FVB mice, which were crossed back to C57BL/6 mice for over three generations. Genomic DNA was isolated from tail biopsies performed on weaned animals (about 3-week old pups) and screened by PCR, followed by western blot and/or RT-PCR to verify the expression of the transgene in transgenic (Tg) mouse hearts (SUMO-1-Tg). SUMO-1-Tg mice were born at Mendelian rate, viable, and fertile. No any discernable phenotypes were observed in SUMO-1-Tg mice at age 1 month. SUMO-1 KO mice, derived from gene-trapped embryonic stem cells with lacZ insertion (BayGenomics, San Francisco, CA), were provided by Drs. Richard Maas at Harvard Medical University (designated as SUMO-$1^{Gal/+}$) (Alkuraya et al., 2006) and Michael Kuehn at National Cancer Center (designated as SUMO-$1^{+/-}$ and SUMO-$1^{-/-}$, respectively) (Zhang et al., 2008a). These SUMO-1-KO mice were bred with C57/BL6 mice, and thus were analyzed on a mixed 129P2/OlaHsd and C57BL6/J background. Nkx2.$5^{+/-}$ mouse line were generated previously (Moses et al., 2001). The crossbreeding among various KO and/or Tg mice was performed as needed. Animals were handled in accordance with institutional guidelines with Institutional Animal Care and Utilization Committee approvals.

Plasmids, Transfections, and Antibodies

Human SUMO-1 gene *cis*-regulatory element (−1454 bps from transcription initiation site of human SUMO-1) was cloned and fused into a luciferase reporter construct pGL3-basic from Promega (Madison, WI) at *Kpn*I and *Xho*I sites, and subsequently two mutants, A-1239G and Mut-16, were generated using mutagenesis kit (Stratagene, La Jolla, CA) and designated as Mut-16-Luc and A-1239G-Luc, respectively. Transfections were performed on 24-well/plate containing HL-1 cell line provided by Dr. William Claycomb at Louisiana State University Health Sciences Center (White et al., 2004) using lipofectamine 2000 purchased from Invitrogen (Chicago, IL) based on the protocol provided by manufacturer. Cell extracts were purified for luciferase activity assays 48 hours posttransfection. Anti-SUMO-1 and anti-GAPDH antibodies were purchased from Santa Cruz (Santa Cruz, CA). Anti-flag antibody (M2) was from Sigma-Aldrich (St. Louis, MO).

Whole-mount RNA In Situ Hybridization

In situ hybridization to detect SUMO-1 transcripts in the murine developing hearts was detailed previously (Wei et al., 2001). Briefly, embryos at specified developmental stages were collected and fixed in 4% paraformaldehyde overnight at 4°C, followed by brief wash in diethylpyrocarbonate (DEPC)-treated standard phosphate-buffered saline (PBS), and dehydrated in serial methanol. The samples were then rehydrated in methanol/PBT series, bleach with 6% H2O2 1 hour, and proteinase K (3 μg/ml) treatment. Embryos were soaked in hybridization buffer for 2 hours at 65°C, and with digoxigenin RNA probe overnight at 65°C, followed by block with 1% blocking reagent 2 hours and incubation with antidigoxigenin-AP (1:2000) 6 hours incubation at room

temperature. The color was developed by the solution containing 125 μg/ml BCIP and 250 μg/ml NBT in NTMT.

Western Blot

Protein lysates purified from wild type (wt) and SUMO-1-Tg mouse hearts were subjected to gradient 4–12% NuPage SDS gel and transferred to PVDF membrane, which was subsequently probed with desired antibodies as indicated in the figure legend. The protein bands were revealed by ECL plus (Buckinghamshire, UK).

Histopathology

Mouse neonatal hearts were dissected and fixed in 4% or 10% paraformaldehyde (PFA) for overnight, respectively. Sagittal sections of these hearts at 5 μm thickness were stained with hematoxylin and eosin (H&E) according to standard protocols.

Microarray Assay And Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

RNAs were extracted from wt and SUMO-1 mutant mouse hearts via Trizol and subjected to microarray assays (OneArray Express) provided by Phalanx Biotech (Palo Alto, CA). RT-qPCR was also performed on the RNAs mentioned using specifically designed probes for the particular genes of our interest. One or 5 μg total RNAs were used for reverse transcription reaction using cloned reverse transcriptase purchased from Invitrogen (Chicago, IL), followed by quantitative PCR on machine MX3000 (Strategene). The sequences of oligos used in this study are available on request.

Statistics

The unpaired Student *t* test, chi-square test, or Fisher exact test was applied to determine statistical significance between groups when applicable and shown in each Figure legend. The $p < 0.05$ was defined as significant and $p < 0.01$ as very significant.

RESULTS

SUMO-1 Transcripts Were Detected in the Mouse Developing Hearts During Embryogenesis

SUMO-1 transcripts appeared in the developing embryonic heart, by in situ hybridization assays performed using antisense against SUMO-1 mRNA on murine embryos at developmental stages, E8.5, E9.5, and E10.5 days. As shown in Figure 1, SUMO-1 transcripts were detected virtually throughout the embryos with enhanced staining in the developing heart. Also, SUMO-1 transcripts were present in the craniofacial area (Fig. 1, *arrow*), consistent with the role of SUMO-1 in the development of craniofacial defects. Similarly, LacZ staining of the SUMO-$1^{Gal/+}$ mouse was observed in embryonic heart at E10.5, shown in Figure 1B.

SUMO-1 Haploid and Homozygous KO Mutant Mice Exhibited CHDs, ASDs, and VSDs

To uncover exclusive versus shared functions for SUMO isoforms, we tested SUMO-1 KO mice provided by Dr. Michael Kuehn (Zhang et al., 2008a) on a mixed genetic background of 129P2/OlaHsd and C57BL6/J. We observed a reduced number of expected homozygous SUMO-1 newborn mice (~14%, $p < 0.01$) in comparison to the expected 25% Mendelian genetic rate (Fig. 2A), whereas the birth rate of SUMO-$1^{+/-}$ pups was equivalent to the expected rate of 50%. Also, SUMO-$1^{-/-}$ mice exhibited a high postnatal mortality rate of ~57% versus ~22% for SUMO-$1^{+/-}$ and ~15% for wt mice ($p < 0.01$) (Fig. 2A). Histologic examination of demised pups revealed CHDs, including ASDs and/or VSDs, in both SUMO-1 hetero- and homozygous mice Figure 2B. Even though more than 50% of the newborn SUMO-$1^{-/-}$ mice died immediately after birth, a subset of SUMO-1 null survivors showed growth retardation (data not shown). Incomplete phenotypic penetrance observed in the SUMO-1 null mice may indicate the effects of modifier gene(s) associated with mixed genetic backgrounds.

Similar cardiac phenotypes, ASD and VSD, were also observed in the heterozygous SUMO-$1^{Gal/+}$ mice provided by Dr. Richard Maas (Alkuraya et al., 2006) (Fig. 3A). These SUMO-1 mutant mice were reported to display abnormal palatogenesis and to have immediate postnatal death (Alkuraya et al., 2006). To determine whether the high mortality rates observed in SUMO-1 mutant mice was due to the loss of SUMO-1 in cardiomyocytes, but not in the other tissues and/or organs, we performed rescue experiments in which crosses were arranged between SUMO-$1^{Gal/+}$ mice and SUMO-1-Tg mice. As shown in Figure 3B, sumoylation assays revealed increased free SUMO-1 and SUMO-1 conjugates in SUMO-1-Tg mouse hearts, as expected. Also, SUMO-1-Tg mice did not exhibit any discernable cardiac phenotype(s) before the age of 1 month. The compound SUMO-1-Tg/SUMO-$1^{Gal/+}$ mice presented significantly lower mortality rates in comparison to single SUMO-$1^{Gal/+}$ mice (0 vs 9.35%, respectively, $p < 0.05$). Thus, the overexpressed SUMO-1 corrected the high mortality rate rendered by SUMO-1 mutant mice. Given the fact that SUMO-1 conjugation regulates a variety of factors contributing to cardiogenesis (Wang and Schwartz, 2010), our findings suggest that SUMO-1 conjugation is critical for normal cardiac morphogenesis and highly likely dependent on the levels of SUMO-1. Reduced mortality because of rescue of the SUMO-1 null mice with the cardiac expressed SUMO-1 transgene (Fig. 3C) reinforces the concept that there is an optimal level of SUMO-1 for normal cardiac morphogenesis.

SUMO-1 Mutant Mouse Hearts Exhibited Dysregulation of Genes Critical for Cell Proliferation

Cell proliferation is an indispensable process for the completion of septation in the heart and sumoylation also plays a central role in mitotic chromosome structure, cell cycle progression, kinetochore function, and cytokinesis (Ahuja et al., 2007; Dasso, 2008). For instance, SUMO modification of Topoisomerase II (Topo II),

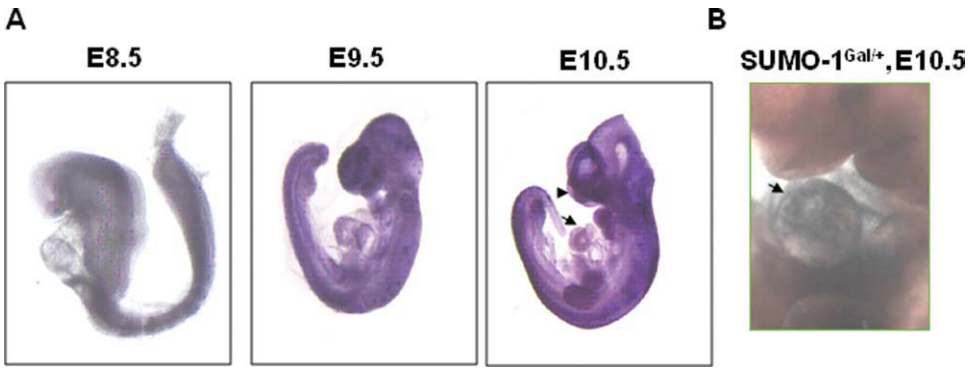

Figure 1. SUMO-1 transcripts were detected in the developing hearts during mouse embryogenesis. **A**. In situ hybridization was performed on mouse embryos at E8.5, E9.5, and E10.5 using antisense of SUMO-1 mRNA. Note that SUMO-1 transcripts were detected on the hearts and craniofacial region (*arrow* and *arrowhead*, respectively). **B**. LacZ staining revealed the endogenous SUMO-1 expression pattern in the heart. LacZ staining was performed on E10.5 of SUMO-$1^{Gal/+}$ mouse embryo. The arrow indicates the positive staining in the heart.

enhanced by E3 ligase RanBP2 during mitosis, is required for proper localization to centromeres, which is essential for normal cell division (Dawlaty et al., 2008), and RanBP2-potentiated sumoylation of RanGAP1 is a prerequisite for k-fiber assembly in mitosis and when suppressed, causes miss-segregation of chromosomes (Arnaoutov et al., 2005). Transcription of RanGAP1 and DNA TopoII, centrally important for cell division as SUMO substrates (Arnaoutov et al., 2005; Dawlaty et al., 2008), were down-regulated in the SUMO-1 mutant

A

Genotyping	WT	SUMO-$1^{+/-}$	SUMO-$1^{-/-}$
Total number of pups at P1 (frequency)	42 (33.6%)	69 (55.2%)	14 (11.2%) **
Total number of demised pups before weaning stage (mortality rate)	6 (14.9%)	15 (21.74%)	8(57.14%) ++

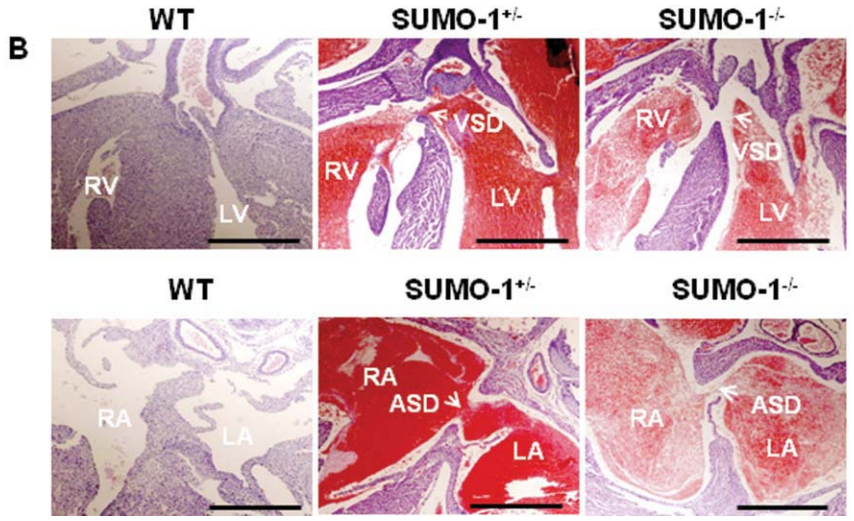

Figure 2. SUMO-1 mutant mice exhibited abnormal septogenesis. **A**. P1 frequency of SUMO-$1^{-/-}$ mice was significantly lower than the expected Mendelian rate, and the mortality rate was higher compared with that of wild type (wt) mice. The data were compiled from 18 litters of 125 animals in total. **, $p < 0.01$, Chi-square test was used; ++, $p < 0.01$, Fisher exact test was used. **B**. Both prematurely demised hetero- and homozygous SUMO-1 mice showed ASD and/or VSD. *Arrows* indicated either an ASD or VSD, respectively. RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium; ASD, atrial septal defect; VSD, ventricular septal defect. Bar = 200 μm.

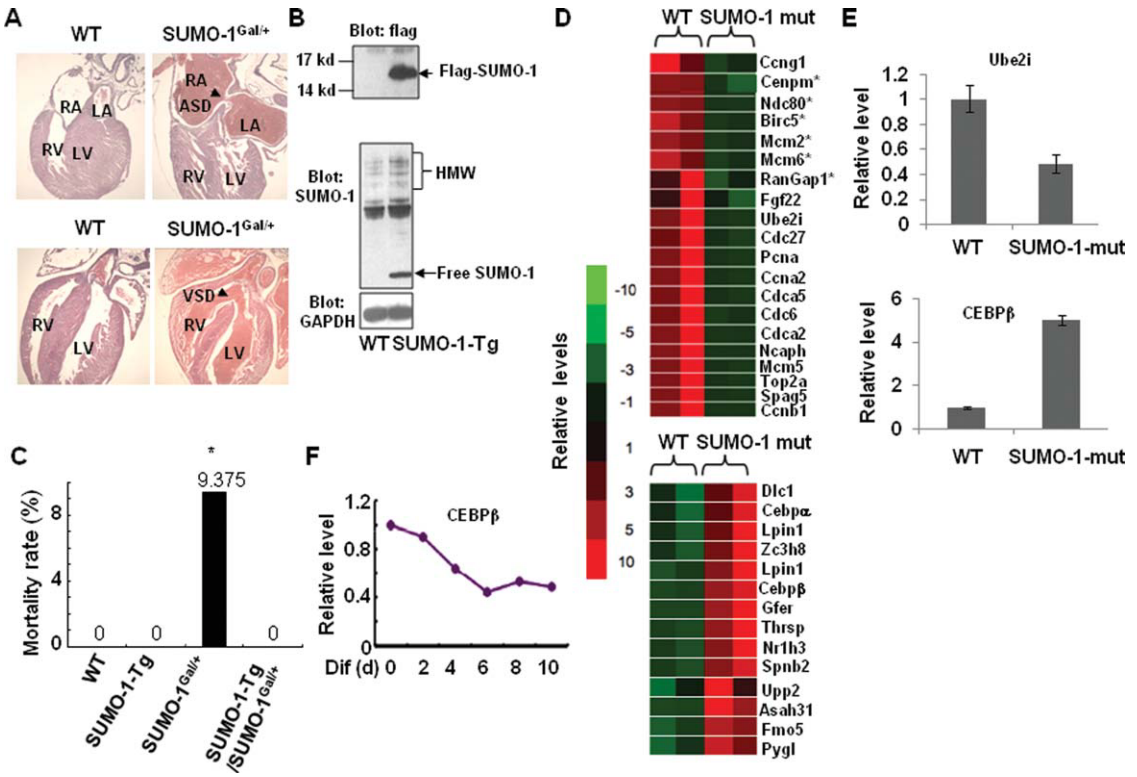

Figure 3. SUMO-1 mutants displayed cardiac dysgenesis. **A**. SUMO-1$^{Gal/+}$ mice exhibited ASDs/VSDs. *Arrows* indicate either an ASD or VSD, respectively. **B**. Generation of flag-tagged SUMO-1 Tg mice under control of cardiac α-MHC promoter (SUMO-1-Tg). Free flag-SUMO-1 (upper panel) and increased high molecular weight (HMW) conjugates of SUMO-1 (middle panel) were detected in SUMO-1-Tg mice. GAPDH (lower panel) served as a control. Western blots were performed on heart extracts from wild type and SUMO-1-Tg mice and labeled with indicated antibodies. **C**. Mortality rate of compound SUMO-1-Tg/SUMO-1$^{Gal/+}$ mice was substantially decreased compared with that of single SUMO-1$^{Gal/+}$ mice. The data were compiled from 17 litters with a total of 140 animals (wt, 30; SUMO-1-Tg, 32;SUMO-1$^{Gal/+}$, 32; SUMO-1-Tg/SUMO-1$^{Gal/+}$, 46). **D**. Down-regulated genes associated with cell proliferation (upper panel) and up-regulated liver-enriched genes (lower panel) in SUMO-1 mutant mouse hearts. Microarray assay on RNAs purified from SUMO-1 mutant and wild type mouse hearts were performed as described in the section of *Materials and Methods*. **E**. Verification of changes in several gene expressions shown in **D** by quantitative RT-PCR. **F**. Liver-enriched transcription factor C/EBPβ was suppressed in ES cells during EB formation.

hearts (Fig. 3D, E; upper panel). It is highly likely that the critical involvement of SUMO pathway in vertebrate cell proliferation may be responsible for defective cardiac septation in SUMO-1 KO embryos.

Ecotopic Expression of Liver-enriched Genes in SUMO-1 Mutant Mouse Hearts

In SUMO-1 defective hearts, a number of liver-enriched transfactors, Cepbα and Cepbβ receptors, liver X receptor alpha (Nr1h3), Lpin1, growth factor, augmenter of liver regeneration (Gfer), and thyroid hormone-inducible hepatic protein (Thrsp) genes were activated (Fig. 3D, E, lower panel). Expression of the liver restricted transcription factor C/EBPβ in embryonic stem cells repressed during embryoid body (EB) formation leading to beating cardiac myocytes may serve as an example of liver genes being silenced during heart cell differentiation (Fig. 3F). Informatics (EBI website CpG Plot program) revealed that 85% of the activated liver restricted genes including Cepbβ, harbor CpG islands that are potential sites for hypermethylation usually coupled with histone H3K27Me3 modification. Because SUMO-1 regulates a number of chromatin remodeling factors, such as the methyltransferase Ezh2 (Riising et al., 2008), and Pc2, a critical component of PCR1 that recognizes H3K27Me3, which is itself a SUMO E3 ligase and a likely target for sumoylation (Kagey et al., 2003). It appears that SUMO-1 may modulate the activity of chromatin remodeling complexes and subsequently inhibiting inappropriate gene activity, such as liver-enriched gene expression in the heart. Also, some of liver-enriched genes, such as CEBPβ, was shown involved in regulating cardiomyocyte proliferation (Bostrom et al., 2010).

Potential Genetic Interaction between Nkx2.5 and SUMO-1

Nkx2.5, a cardiac specific homeodomain transcription factor and a SUMO substrate (Wang et al., 2008), plays a critical for normal cardiac morphogenesis. Because SUMO-1 KO mice developed CHDs similar to those observed in patients whose *Nkx*2.5 gene was mutated, we asked if there was a functional interaction between *Nkx*2.5 and SUMO-1. The compound *Nkx*2.5$^{+/-}$/SUMO-1$^{Gal/+}$ mice were obtained via crossbreeding heterozy-

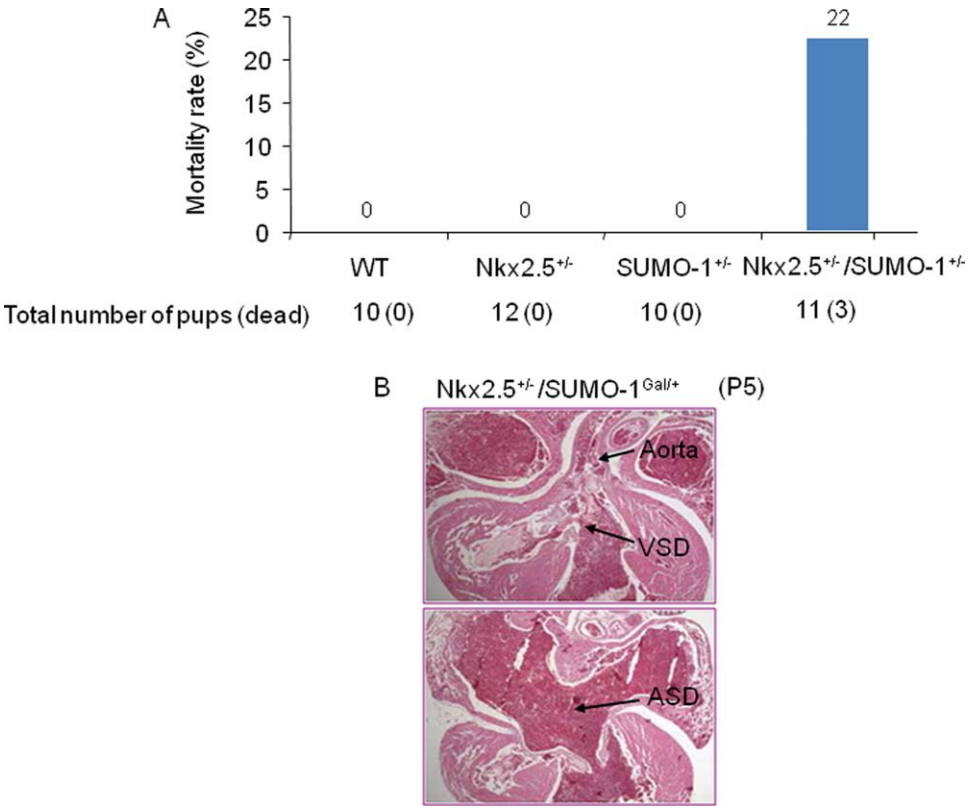

Figure 4. Potential genetic interaction between *Nkx*2.5 and SUMO-1 genes. **A**. Compound Nkx2.5$^{+/-}$/SUMO-1$^{Gal/+}$ mice exhibited higher mortality rates compared with those of single Nkx2.5$^{+/-}$ and SUMO-1$^{Gal/+}$ mice. Total number (dead) of animals for analysis is shown. **B**. Histology of dead compound Nkx2.5$^{+/-}$/SUMO-1$^{Gal/+}$ mice revealed severe ASD/VSD. Representative data are shown.

gous *Nkx*2.5 and SUMO-1 mouse lines and subsequently analyzed. Figure 4A shows a higher mortality rate in the compound *Nkx*2.5$^{+/-}$/SUMO-1$^{Gal/+}$ mouse group than any other genotypic group, although no statistical significance was reached due to the small sample size. The autopsy of these pups revealed the presence of severe ASDs and/or VSDs (Fig. 4B). This finding suggests a potential genetic interrelationship between *Nkx*2.5 and the SUMO conjugation pathway.

Identification of Mutations Within the Upstream Promoter Region of SUMO-1 Gene in Human Patients With Both Cleft Lip and ASDs

Given the findings that in human patients, the deletion of the SUMO-1 gene was associated with an increased prevalence of oral-facial defects, and that defective sumoylation in murine fetuses caused ASDs and VSDs, we asked if SUMO-1 was mutated in human infants born with cleft palates and/or ASDs. We analyzed the genomic sequence of the human SUMO-1 gene in a cohort of newborns who were diagnosed with both ASDs and orofacial clefts among live born (within 1 year after birth), and fetal deaths (≥20 weeks'gestation) delivered to women residing in most California counties. Medical geneticists using detailed diagnostic information from medical records determined case eligibility. Each case was confirmed by review of echocardiography, cardiac catheterization, surgery, or autopsy records. Orofacial clefts cases included infants with cleft palate only (CP) or with cleft lip with or without cleft palate (CLP), confirmed by surgery or autopsy. We analyzed the genomic sequence of human SUMO-1 gene in 87 cases and 100 controls from this California population. As represented in Figure 5A, a A-≥G transition (designated as A-1239G) located 1239 bp upstream of the transcription start site was present in four independent cases (4.6%), but was not found in any of the controls (Fig. 5B, left panel). In addition, a 16 base substitution between 677 and 793 bp upstream of the transcription start site (designated as Mut-16) was present in one case (1.1%), but not in any controls (Fig. 5B, right panel). We believe that the 16 base substitutions between 677 and 793 bp upstream of the transcription start site (designated as Mut-16) is likely to be significant. However, the number of controls sequenced falls short of 3000 control sequences required to conclude whether the nucleotide substitutions are significant.

To further evaluate the biologic consequence of these mutations on SUMO-1 promoter activity, wt human SUMO-1 promoter region (−1454 bps from transcription initiation site of human SUMO-1) was cloned and fused into a luciferase reporter construct pGL3-basic, and subsequently two mutants, A-1239G and Mut-16, were generated and designated as Mut-16-Luc and A-1239G-Luc, respectively. The assays of activity of wt promoter and its two mutants were performed via traditional transfection using transfection reagents in HL-1 cells, which is the only cardiogenic cell line that mimics the properties of differentiated cardiomyocytes (Claycomb et al., 1998;

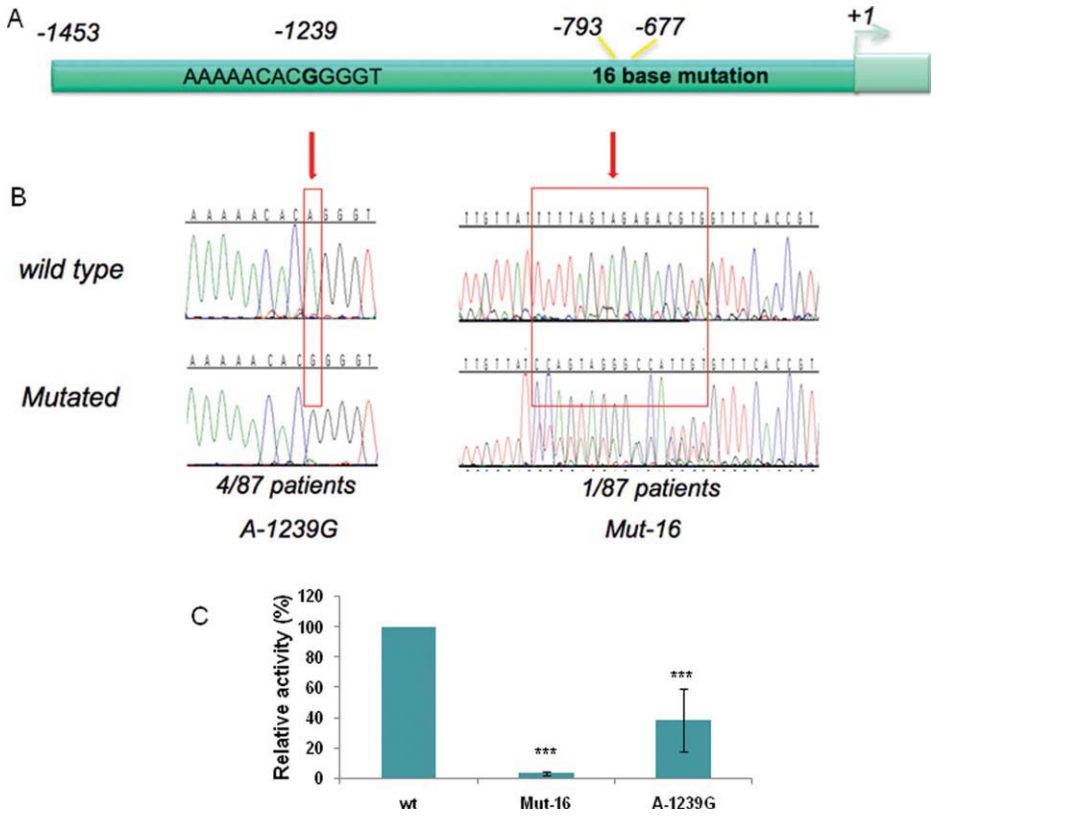

Figure 5. Sequence analysis of DNA from newborn screening blood spots revealed potential SUMO-1 regulatory promoter mutations in infants displaying both oral-facial clefts and ASDs. **A**. Schematic representation of the locations of mutations relative to the transcription initiation site in the human SUMO-1 gene *cis*-regulatory region. **B**. Sequencing graphic revealing mutations in two different human SUMO-1 promoter regions with indicated positive/total number of patients. The observed DNA sequence variants were not detected in a total of 100 control samples. **C**. Luciferase reporter activity assays revealed ~ 95% and ~ 60% decrease in the activity of of mut-16 and A-1239G SUMO-1 promoter, respectively, compared with that of wild type promoter. Data were obtained from four independent assays in HL-1 cell line, each carried out in duplicate. Student *t* test was used for statistical significance analysis. *$p < 0.05$; ** $p < 0.001$, compared with wt group.

White et al., 2004). Although A-1239G mutation decreased the activity of SUMO-1 promoter by ~60% ($p < 0.05$), mut-16 only exhibited ~5% of the wt promoter activity ($p < 0.001$), (Fig. 5C); thus de facto reducing the balance of sumoylated target proteins potentially contributing to abnormal heart and craniofacial development.

DISCUSSION

We showed that reduced levels of SUMO-1 led to premature death and CHD—ASDs and VSD in murine models, indicating an essential threshold level of SUMO-1 for normal cardiac structural development. Our findings were consistent with the previous report of immediate postnatal demise of SUMO-1 mutant mice (Alkuraya et al., 2006), but at the time was contrary to other two observations that stated no discernable phenotype(s) in SUMO-1 KO mice (Evdokimov et al., 2008; Zhang et al., 2008a). The exact mechanisms underlying the phenotypic discrepancy of SUMO-1 KO mice is not well understood, but it is very likely that genetic modifiers associated with various mouse strains may play a role (Bentham and Bhattacharya, 2008; Biben et al., 2000; Winston et al., 2010). The

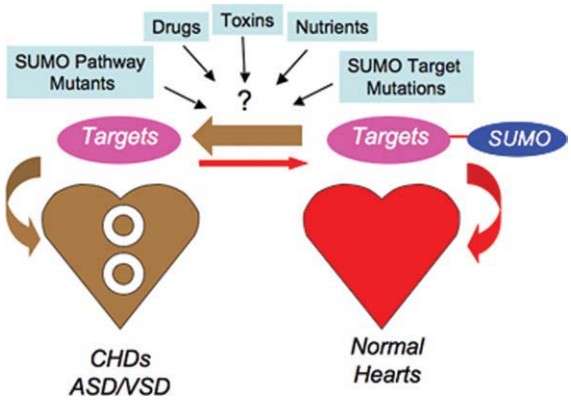

Figure 6. Model of SUMO conjugation-deconjugation pathway is highly complex yet under balance to promote normal cardiac development. Reduction of SUMO-1 by half elicited ASD/VSDs as in SUMO-1 haploid-insufficient mice and may underscore other genetic defects of the individual SUMO conjugation pathway components and even the cardiac transfactor targets causing similar CHD phenotypes. Environmental toxins, nutrients, and drugs may adversely affect the expression of SUMO pathway components.

successful rescue of SUMO-1 mutant mice by reexpressed SUMO-1 in cardiomyocytes indicates the importance of cell autonomous function of SUMO-1 in the high premature death rate and in the development of cardiac structural phenotypes. Although the underlying mechanisms governing the cardiac defects associated with SUMO-1 deficiency is currently unclear, defective cardiomyocyte proliferation may play a significant role. Furthermore, ectopic expression of some of the liver-enriched genes may also contribute to the cardiomyocyte propagation, as evidenced by the recent findings implicating the CEBPβ in cardiac growth (Bostrom et al., 2010).

SUMO conjugation contributes to normal cardiac morphogenesis through maximizing the activity of cardiac muscle-enriched factors via SUMO modification. A functional interaction between Nkx2.5 and SUMO-1 was supported by our observation of the significantly higher mortality of compound $Nkx2.5^{+/-}$/$SUMO\text{-}1^{Gal7/+}$ mice compared with single mutant mice. We showed that sumoylation of two cardiogenic transcription factors Nkx2.5 and SRF enhanced their formation of more stable complexes further achieved by the presence of SUMO-1/PIAS1 (Wang et al., 2008). Our findings indicate that SUMO may modulate Nkx2.5 function via stimulation of complex protein-protein interaction. Thus, Nkx2.5 function appears to be modulated at least partially via sumoylation-induced changes in Nkx2.5-harboring complex formation. Reversible SUMO conjugation and deconjugation regulate the activities of cardiogenic transcription factors controlling cardiac morphogenesis and development. For example, mice with knockdowns of sumoylation pathway components, such as Ubc9, died at an early embryonic postimplantation stage (Nacerddine et al., 2005). More recently, Ubc9 was shown to be required for myotube formation in C2C12 cells and pharyngeal muscle development in *Caenorhabditis elegans* (Riquelme et al., 2006; Roy Chowdhuri et al., 2006); thus, implicating the sumoylation pathway in muscle development. We demonstrated that SUMO modification of GATA4 elicited cardiac muscle-specific gene expression (Wang et al., 2004), and myocardin sumoylation by SUMO-1/PIAS1 showed induced cardiogenic gene expression (Wang et al., 2007). Given the facts that transcription factors such as Nkx2.5, myocardin, SRF, and GATA4 are all SUMO targeted and physically interact with each other, and that all of them are crucial for heart development (Huang et al., 2009; Niu et al., 2008; Oka et al., 2006; Tanaka et al., 1999), taken together, these studies implicate the SUMO-1 conjugation pathway as having a role in cardiac development via altering the activities of cardiac enriched transcription factors.

Epigenetic regulation is essential for gene activation/silencing. A number of chromatin remodeling factors have been identified as SUMO conjugation substrates (David et al., 2002; Kirsh et al., 2002; Ling et al., 2004). In particular is Ezh2, a Histone 3 methyltransferase, which is also SUMO modified (Riising et al., 2008). Pc2, a critical component of polycomb repressive complex 1 (PRC1) that recognizes H3K27Me3, is also a SUMO E3 ligase and, by itself, is also a target for sumoylation (Kagey et al., 2003). Thus, the SUMO pathway governs transcription activation/silencing via modulating the activity of chromatin remodeling factors/complexes which is essential for cell fate determination. Recently, the murine *SENP2* gene KO caused defects in the embryonic heart and reduced the expression of Gata4 and Gata6 (Kang et al., 2010), which are essential for cardiac development (Liang et al., 2001; Zhao et al., 2008). SENP2 regulates transcription of Gata4 and Gata6, mainly through alteration of occupancy of Pc2/CBX4, a PRC1 subunit, on their promoters. Pc2/CBX4 is shown as a target of *SENP2* in vivo. In *SENP2* null embryos, sumoylated Pc2/CBX4 accumulates and Pc2/CBX4 occupancy on the promoters of target genes is markedly increased, leading to repression of Gata4 and Gata6 transcription (Kang et al., 2010). Thus, it is likely that altered sumoylation states in the heart, during either embryonic cardiac development or the maintenance of postnatal heart function, will promote abnormal gene expression leading to cardiac structural malformation and/or dysfunction of chromatin remodeling, such as observed here for the appearance of inappropriate liver-enriched genes in the hearts of SUMO-1 KO mice. Whether and how the dysregulated liver-enriched genes contribute to the cardiac malformations warrants further investigation.

We have demonstrated that balanced sumoylation and desumoylation is required for normal cardiac development as modeled in Figure 6. These dynamic processes demand precise cooperation among a number of distinct signaling pathways and cardiac enriched factors. Our findings support the novel concept that a defective SUMO pathway as exemplified by our SUMO-1-KO mice, may contribute to the high prevalence of congenital cardiac defects. Reduction of SUMO-1 by half was sufficient to elicit in ASD/VSDs in SUMO-1 haploid-insufficient mice. Thus, SUMO conjugation pathway contributes to normal cardiac morphogenesis by maximizing the activity of cardiac muscle-enriched factors via SUMO modification for activation of cardiogenic genes, as well as repression of noncardiogenic tissue activity. The association of the SUMO conjugation pathway with cardiac gene regulation is a relatively new area and the importance of the SUMO pathway for the development and maintenance of a normal cardiovascular system is just beginning to emerge. Hypothetically, genetic mutations of the individual SUMO conjugation pathway components and even the cardiac SUMO targets may generate similar CHD phenotypes accounting for the exceedingly high number of congenital birth defects observed around the world. Also, another important issue is whether environmental toxins, metabolites, and pharmaceuticals that may alter sumoylation gene activity cause heart disease. Any perturbation of signaling components and gene activity of SUMO conjugation pathway are the unknown intangibles that could tilt the balance of the SUMO conjugation pathway and potentially lead to cardiac structural malformations.

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