

# Regulation of Cardiac Specific *nkx2.5* Gene Activity by Small Ubiquitin-like Modifier\*

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The cardiac specific homeobox gene *nkx2.5*, a member of the *nk-2* class family, plays a central role in cardiogenesis and is a target of the small ubiquitin-like modifier (SUMO). Nkx2.5 was modified by SUMO on its 51st amino acid, a lysine residue conserved across species but absent in other *nk-2* members. Conversion of this lysine to an arginine (K51R) substantially reduced Nkx2.5 DNA binding and also its transcriptional activity. Unexpectedly, mutant K51R was targeted by ubiquitin. E3 ligase PIAS proteins PIAS1, PIASx, and PIASy, but not PIAS3, enhanced SUMO-1 attachment to Nkx2.5 on the primary SUMO acceptor site. SUMO-2 linkage to Nkx2.5 was catalyzed only by PIASx and not by other PIAS proteins. SUMO conjugation stabilized the formation of Nkx2.5-containing complexes that led to robust transcriptional activation. Thus, SUMO modification serves as a positive regulator for Nkx2.5 transcriptional activity.

Cardiac specific homeobox gene *nkx2.5* (1, 2), a member of the *nk-2* class of homeodomain (HD)<sup>2</sup> factors, is required for early heart development and morphogenesis (3, 4). Nkx2.5, recognized as one of those earliest known markers for cardiac progenitors, has important roles in tissue patterning and lineage determination. Homozygous *nkx2.5* null mice died between 9.5 and 11.5 days post coitus (d.p.c.), after the initial heart looping was completed (5, 6), supporting the notion that *nkx2.5* played a central role for early cardiac development. Nkx2.5 bound the NKE DNA sequence motif, TYAAGTG, with high affinity via its unique homeodomain that contains a tyrosine at the 54th position in the helix (7). In addition, Nkx2.5 also bound homeodomain target sequences TTAATT, allowing for transactivation of ANF and cardiac  $\alpha$ -actin promoters (8, 9). Nkx2.5 transcriptional activity may be augmented in combination with other co-factors, such as SRF, GATA4, and Tbx5 (9–12). Also, posttranslational modification, such as phosphorylation on its serine 163 by casein kinase II, elevated Nkx2.5 activity via increased DNA binding (13). However, little is known about Nkx2.5 functional regulation by other post-translational modifications.

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<sup>2</sup> The abbreviations used are: HD, homeodomain; WT, wild type; AD, activation domain; aa, amino acids; E3, ubiquitin-protein isopeptide ligase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Small ubiquitin-like modifiers (SUMOs) are small molecules that can be covalently and reversibly conjugated to the specific lysine(s) mostly localized in SUMO-targeting sequence  $\psi$ KXE (where  $\psi$  represents a bulky hydrophobic amino acid, and X represents any residue) within its targets (14, 15). Three functional isoforms of SUMO family members have been recognized in vertebrates, of which active SUMO-2 and -3 share high similarity with each other but only ~50% cross-homology with SUMO-1. Also, these SUMO isoforms displayed preferences for different targets. For example, RanGap1 was a preferred substrate by SUMO-1 but poorly modified by SUMO-2 or -3 (16). Although the activities of many targets were depressed after SUMO conjugation (14), sumoylation also enhanced the transcriptional activity of some substrates (17–20). Consequently, SUMO modulates a variety of cellular processes under both physiological and pathological states via regulating the functions of its targets (21–23).

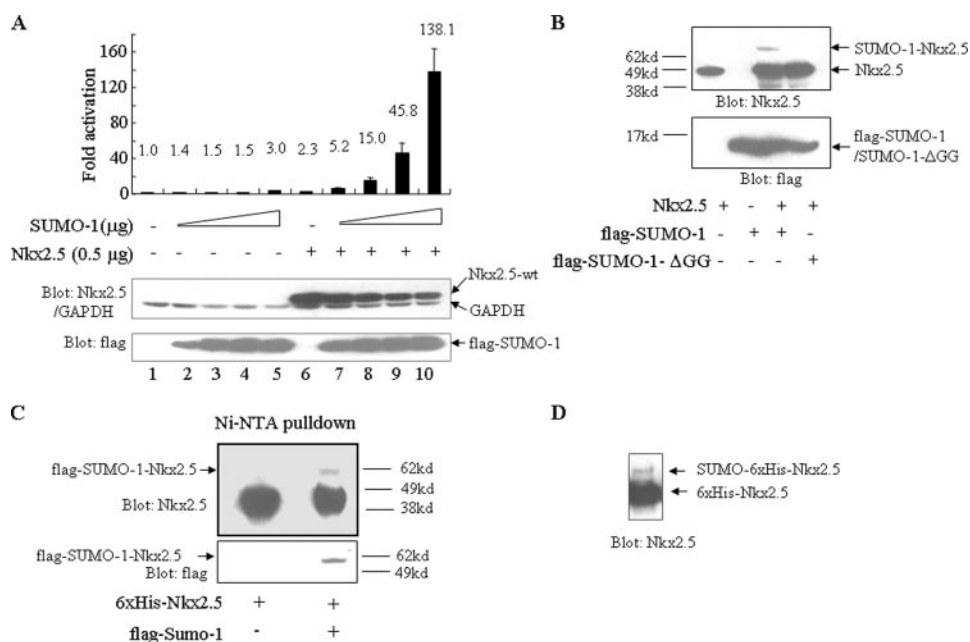
The SUMO conjugation pathway is similar to that of ubiquitination in the way that both processes require catalytic enzymes to accomplish the covalent linkages. However, they also differ in several significant ways, one of which is the functional consequence after conjugation. In general, polyubiquitination directed its targets toward proteasome-associated degradation, but SUMO modification rarely fragmented its targets; instead, SUMO modulated its targets' functions via changes in nuclear-cytoplasmic shuttling (24), protein-DNA binding affinity (18), protein-protein interaction, or stabilizing the targets by antagonizing ubiquitination on the same lysine residue in the substrates (25, 26).

Our previous work showed robust transcriptional activities for GATA4 and myocardin caused by SUMO addition (17, 20), factors centrally involved in cardiovascular development. Bioinformatics unveiled a potential SUMO acceptor site in Nkx2.5, another critical factor for early heart formation. Nkx2.5 was targeted by SUMO-1 on lysine 51, and mutation to an arginine (K51R) reduced its DNA binding. SUMO linkage to Nkx2.5 enhanced transcriptional activity through multiple mechanisms, including the formation of a stable complex containing Nkx2.5 and SRF, another SUMO target (27).

## EXPERIMENTAL PROCEDURES

**Plasmid Constructions**—The cardiac  $\alpha$ -actin promoter-driven luciferase reporter construct (Ca-actin-Luc) and Nkx2.5 expression vector were described previously (9). PIAS proteins, the PIAS1 RING domain mutant, and PIAS1 serial deletions were recently described (20, 28). Nkx2.5 mutant K51R was generated by a two-step PCR protocol using one pair of primers

## Nkx2.5 Sumoylation



**FIGURE 1. SUMO-1 up-regulated Nkx2.5 transcriptional activity via covalent modification.** *A*, Nkx2.5 expression vector (0.5  $\mu$ g) was co-transfected into CV1 cells in the absence or presence of increasing doses of a plasmid-based SUMO-1 expression vector (0.25, 0.5, 0.75, and 1  $\mu$ g, respectively) together with a Ca-actin-Luc reporter construct, as indicated. Data were expressed as mean  $\pm$  S.E. from at least two independent assays, each carried out in duplicate. The values shown on each group inside the panel indicate the -fold activation. The lower two panels showed the expression levels of transfected Nkx2.5 and FLAG-tagged SUMO-1 in each corresponding group in 12-well plates. GAPDH served as an internal control. *B*, Nkx2.5 was modified by SUMO-1 *in vivo*. The Western blot was executed on cell lysates from HeLa cells transfected with Nkx2.5-encoding vector in the presence or absence of SUMO-1 or SUMO-1  $\Delta$ GG as indicated. *Top*, blotted with anti-Nkx2.5 antibody; *bottom*, blotted with anti-SUMO-1 antibody. *C*, Ni<sup>2+</sup>-NTA assay confirmed the presence of SUMO-1-conjugated Nkx2.5. His<sub>6</sub>-tagged Nkx2.5 was precipitated with Ni<sup>2+</sup>-NTA. Note that the position of the protein band revealed by anti-FLAG antibody (*bottom*) was equivalent to the retarded migratory band revealed with anti-Nkx2.5 antibody (*top*). *D*, exogenous Nkx2.5 was modified by endogenous SUMO proteins. Ni<sup>2+</sup>-NTA was performed on COS-7 cells transfected with His<sub>6</sub>-tagged Nkx2.5 expression vectors. The representative blot was revealed with anti-Nkx2.5 antibody. The upper and lower arrows point to the SUMO-conjugated and free His<sub>6</sub>-tagged Nkx2.5, respectively.

overlapping the conversion site and a second pair covering each end of cDNA. The PCR fragment that contained K51R was subcloned into PCGN at XbaI and BamHI sites. Nkx2.5 serial deletion mutants were generated by PCR using corresponding oligonucleotides covering the desired segments of cDNA, which were thereafter subcloned into pcDNA4B-V5/His vector with EcoRV and HindIII. All mutants were confirmed by sequencing. Encoding vectors for hemagglutinin-tagged ubiquitin (MT123) and His<sub>6</sub>-tagged ubiquitin (MT107) were generously provided by Dr. Dirk Bohmann (Rochester, NY) (29).

**Antibodies**—All antibodies used in the present study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), unless otherwise stated.

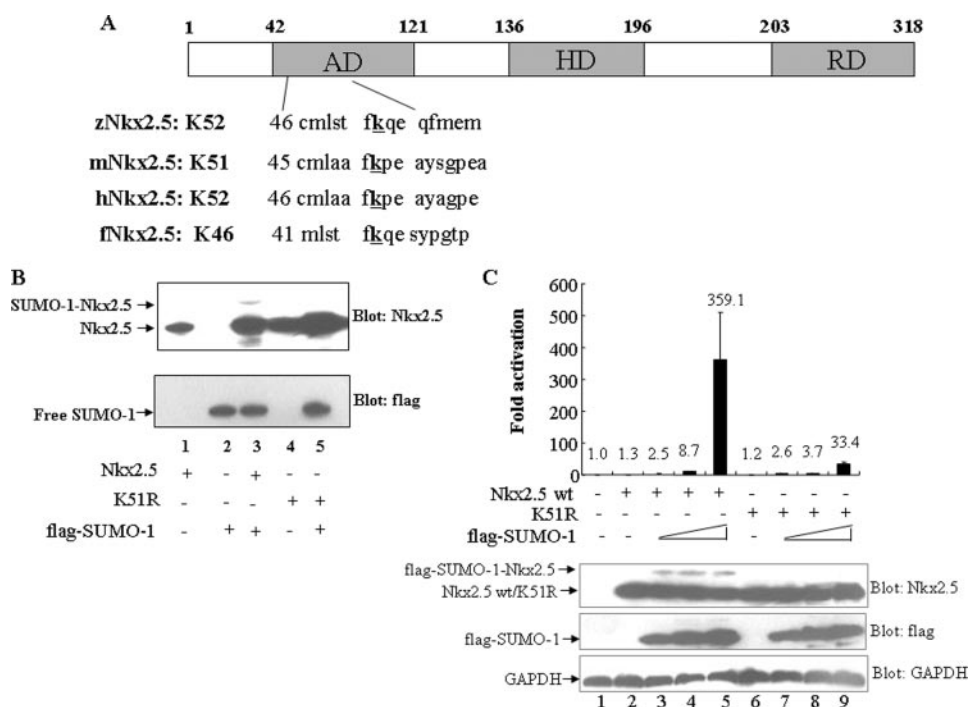
**Cell Culture and Transient Transfection**—CV1, HeLa, or COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Reporter transactivation assays were performed in 12-well plates, whereas transient transfections for Western blotting were carried out in 6-cm plates, using Lipofectamine 2000 according to the protocol described by the manufacturer. Reporter constructs (200 ng) and expression vectors were transfected into CV1 cells in the Opti-MEM I medium, balanced with empty expression vector to maintain the total amount of DNA constant per well. CV1 cells were harvested 48 h post-transfection, and then luciferase activity assays were executed with Monolight<sup>TM</sup>3010 (Amer-

sham Biosciences). Promoter activity was expressed as the ratio of luciferase activity induced by the presence of specific factor(s) to the control group with only the presence of empty vector. Data shown were expressed as mean  $\pm$  S.E. from at least two independent assays, each carried out in duplicate.

**In Vivo Sumoylation and Ubiquitination Assays and Ni<sup>2+</sup>-NTA Chromatography**—Wild type Nkx2.5 or K51R mutant was transfected into HeLa cells with FLAG-tagged SUMO-1 wild type (WT) or its mutant FLAG-SUMO-1- $\Delta$ GG. To determine whether PIAS1 potentiated the sumoylation of Nkx2.5 *in vivo*, the transfection assays were also done in the presence of PIAS1 or its RING finger mutant expression vectors. The whole procedure for protein blotting and visualization was detailed previously (17, 20). Ni<sup>2+</sup>-NTA chromatography was used to confirm the SUMO conjugates as described (20). Ni<sup>2+</sup>-NTA pull-downs for ubiquitination assays were performed by either transfecting His<sub>6</sub>-tagged Nkx2.5 WT or K51R in the presence or absence of ubiquitin expression vector or transfecting Nkx2.5 WT or K51R with or without His<sub>6</sub>-epitoped ubiquitin, as indicated in the corresponding figure legends. To determine if exogenous Nkx2.5 was modified by endogenous SUMOs, Ni<sup>2+</sup>-NTA was performed on COS-7 transfected with His<sub>6</sub>-tagged Nkx2.5, and protein blots were tested with anti-Nkx2.5 antibody.

**Co-immunoprecipitation and GST Pull-down Assays**—Plasmid-based expression vectors encoding hemagglutinin-tagged Nkx2.5 and FLAG-tagged PIAS1 were co-transfected into HeLa cells. Cell lysates were collected 48 h post-transfection, and Nkx2.5 antibody was used to precipitate Nkx2.5. The precipitate was washed, subjected to SDS-PAGE, and transferred to a polyvinylidene difluoride membrane, and PIAS1 was revealed by FLAG antibody (M2; Sigma). To visualize Nkx2.5, the membrane was stripped and reprobed with anti-Nkx2.5 antibody. The GST pull-down assay was detailed previously (20).

**Electrophoretic Mobility Shift Assays**—Two different doses of whole cell proteins (10 and 30  $\mu$ g, respectively) purified from HeLa cells transfected with encoding vectors for either Nkx2.5 WT or K51R mutant were used in binding assays performed at room temperature, as described in detail (17). Binding buffer consisted of 120 mM KCl, 25 mM MgCl<sub>2</sub>, 20 mM Tris-Cl, pH 7.9, 2 mM dithiothreitol, 2 mM EDTA, 5% glycerol, 50,000 cpm of double-stranded synthetic oligonucleotides. In supershift assay, the protein was incubated for 30 min in the presence of



**FIGURE 2. Lysine 51 was the primary SUMO-1 conjugation site.** *A*, schematic structure of Nkx2.5 consensus SUMO-targeting sequence conserved across species. The *underscored letters* indicate the SUMO linkage site. *B*, mutation on lysine 51 abolished SUMO-1 modification of Nkx2.5. Western blot was conducted on cell lysates containing Nkx2.5 wild type or K51R mutant in the absence or presence of SUMO-1 expression vector, as indicated. *Top*, the slower migrating band (SUMO-1-conjugated Nkx2.5) was present in the group containing Nkx2.5 wild type and SUMO-1 but absent in the group containing K51R mutant and SUMO-1 (compare *lanes 3* and *5*). The *lower panel* showed comparable SUMO-1 expression in each corresponding group. *C*, K51R mutant exhibited lower transcriptional activity in the presence of SUMO-1. Luciferase activity assays were conducted on whole cell lysates from CV1 cells transfected with WT Nkx2.5 or K51R (0.5  $\mu$ g each) in the absence or presence of increasing doses of SUMO-1 expression vector (0.25, 0.5, and 1  $\mu$ g, respectively), as indicated. Data shown were expressed as mean  $\pm$  S.E. from at least two independent determinations with each carried out in duplicate. The *numbers shown above each bar inside the panel* indicate the -fold activation for each group. The *lower three panels* showed the expression levels of transfected Nkx2.5 WT, K51R, and FLAG-tagged SUMO-1 in each corresponding group in 12-well plates. GAPDH served as an internal control.

specific Nkx2.5 antibody. Hot probes were end-labeled by using [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol; Amersham Biosciences) and T4 polynucleotide kinase and purified through chromatography columns purchased from Bio-Rad. The bindings were revealed by autoradiography. The two oligonucleotides used in the electrophoretic mobility shift assays were as follows: consensus Nkx2.1 binding sequence, 5'-TCGGGATCGCCAGT-CAAGTGC-3'; Nkx2.5 binding sequence localized in cardiac  $\alpha$ -actin promoter, 5'-CGACCTGCCATTTCATGGCCGCG-3'. All of these sequences and their functions with Nkx2.5 were described previously (7, 9).

**Size Exclusion Chromatography**—Complex formation was determined by gel filtration performed on the high pressure liquid chromatography equipped with a Superdex 200 10/300GL column. The column was equilibrated with buffer A containing 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol. Molecular weight markers (MW-GF-200; Sigma) generated a calibration curve obtained with the method provided by the manufacturer. Cell extracts (100  $\mu$ l; 5 mg/ml) were loaded onto the column in buffer A at 0.75 ml/min. The fraction collector was set at 1 ml/fraction after 7.0 ml had eluted out. Proteins in each fraction were precipitated with 10% trichloroacetic acid and subjected to SDS-PAGE followed by Western blotting.

## RESULTS

**SUMO-1 Targeted Nkx2.5 via Modification of Lysine 51**—We asked if sumoylation influenced Nkx2.5 activity by first evaluating the cardiac  $\alpha$ -actin promoter (Ca-actin-Luc) activity (7) in a co-transfection experiment with the increasing doses of SUMO-1 encoded expression vector alone or together with Nkx2.5 expression vector. While expressed, SUMO-1 or Nkx2.5 alone had no significant effect on the tested promoter (Fig. 1A); together, SUMO-1 potentiated Nkx2.5 activity in a dose-dependent fashion to a maximal activation of  $\sim$ 150-fold. To determine if Nkx2.5 was a SUMO-1 target, protein blots were performed on extracts from HeLa cells transfected with Nkx2.5 in the presence or absence of SUMO-1 WT or SUMO-1- $\Delta$ GG, a defective SUMO mutant. Although SUMO-1 WT and SUMO-1- $\Delta$ GG were expressed to the same extent (Fig. 1B, *bottom*), an additional slower migrating band was observed only in the presence of Nkx2.5 and SUMO-1 and not in the presence of Nkx2.5 alone or Nkx2.5 with SUMO-1- $\Delta$ GG (*top*). Ni $^{2+}$ -NTA pull-down assays confirmed that the upper band was SUMO-1-con-

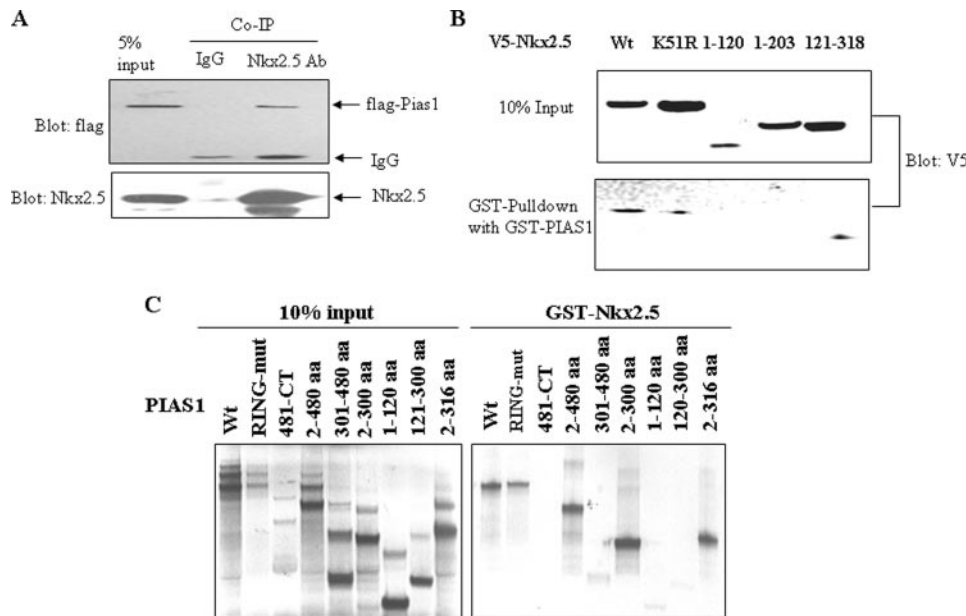
jugated Nkx2.5, since it reacted with both anti-Nkx2.5 antibody and anti-FLAG antibody against FLAG-SUMO-1 (Fig. 1C, *top* and *bottom*, respectively). Next, Ni $^{2+}$ -NTA chromatography further confirmed that the exogenous His $_6$ -tagged Nkx2.5 was modified by endogenous SUMO proteins (Fig. 1D).

SUMO modification usually occurs on the consensus sequence  $\psi$ KXE (where  $\psi$  represents a bulky hydrophobic amino acid, and X represents any residue), and one potential targeting sequence was identified: lysine 51, localized within the Nkx2.5 activation domain (AD) and conserved (Fig. 2A). Conversion of lysine 51 to arginine abrogated Nkx2.5 sumoylation (Fig. 2B, compare *lane 3* with *lane 5*), indicative of lysine 51 as the principal SUMO-1 conjugation site. In co-transfection assays, the Nkx2.5 mutant K51R blunted reporter Ca-actin-Luc activity by 90% (Fig. 2C) in the presence of SUMO-1 as compared with that by Nkx2.5 WT and SUMO-1. These data indicated strong enhancement of Nkx2.5 transcriptional activity via SUMO-1 conjugation to lysine 51.

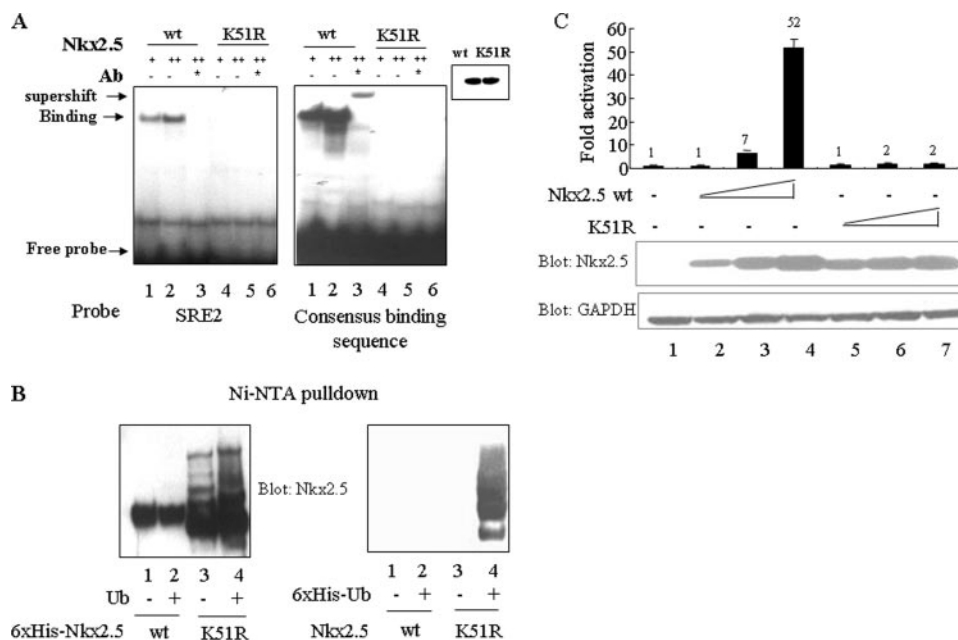
**PIAS1 Stimulated SUMO-1 Conjugation to Nkx2.5**—One of the SUMO E3 ligases, PIAS1, was shown to be effective in linking SUMO-1 to the cardiac muscle-enriched transcription factors GATA4 and myocardin (17, 20). To test whether PIAS1 could also act as an E3 ligase in SUMO-1 modification of







**FIGURE 4. Physical association of Nkx2.5 with PIAS1.** *A*, co-immunoprecipitation revealed the presence of physical association between PIAS1 and Nkx2.5. Co-immunoprecipitation was performed on cell lysates containing both FLAG-tagged PIAS1 and Nkx2.5 using either IgG or Nkx2.5 antibody. *B*, *C* terminus of Nkx2.5 interacted with PIAS1. GST-fused PIAS1 immobilized to GST beads was incubated with cell lysates containing encoding vectors for various Nkx2.5 versions, as indicated. *C*, N terminus of PIAS1 interacted with Nkx2.5. GST-fused Nkx2.5 immobilized to GST beads was incubated with [<sup>35</sup>S]methionine-labeled PIAS1 WT or its various mutants, as indicated.



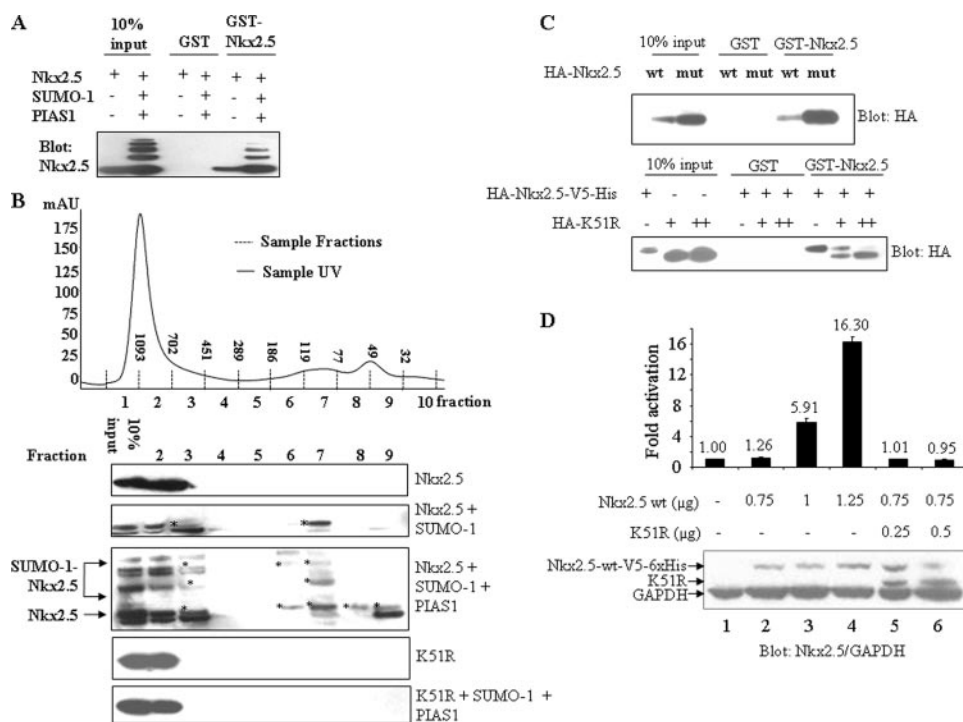
**FIGURE 5. SUMO attachment site regulated Nkx2.5 activity via multiple mechanisms.** *A*, lysine 51 was critical for Nkx2.5 DNA binding. An electrophoretic mobility shift assay was performed using whole cell lysates containing either wild type Nkx2.5 or K51R mutant on two Nkx2.5 DNA binding sequences as indicated. The *right small panel* showed equivalent expressions of Nkx2.5 wild type and K51R. *B*, K51R mutant was targeted by ubiquitination. *Left*, His<sub>6</sub>-tagged Nkx2.5 wild type or K51R co-transfected with ubiquitin expression vector was pulled down by Ni<sup>2+</sup>-NTA and revealed by anti-Nkx2.5 antibody in the Western blot. *Right*, Nkx2.5 wild type or K51R mutant co-expressed with His<sub>6</sub>-epitoped ubiquitin was pulled down by Ni<sup>2+</sup>-NTA and revealed by anti-Nkx2.5 antibody in the Western blot. *Ub*, ubiquitin. *C*, K51R exhibited lower activity compared with wild type Nkx2.5. Luciferase reporter activity assays were executed on cell lysates containing Ca-actin-Luc reporter together with increasing doses of either Nkx2.5 wild type or K51R mutant, as indicated (three increasing dosages: 0.5, 0.75, and 1 μg). The *numbers shown above each bar inside the panel* indicate the -fold activation for each group. The *lower two panels* showed the relative expression levels of transfected Nkx2.5 WT and K51R in each corresponding group. GAPDH served as an internal control. *Ab*, antibody.

with lane 3, in the *left* and *middle panels*), confirming the binding specificity.

To explore whether sumoylation of Nkx2.5 antagonized ubiquitination of Nkx2.5, pull-down assays were performed with Ni<sup>2+</sup>-NTA, followed by Western blotting on extracts from HeLa cells transfected with His<sub>6</sub>-epitoped Nkx2.5 WT or K51R in the absence or presence of ubiquitin expression vector. As shown in Fig. 5*B* (*left*), a clear single band was visualized in the presence of Nkx2.5 WT alone or together with ubiquitin expression vector (compare *lanes 1* and *2*); however, ladders were observed in the presence of K51R alone or together with encoding vector for ubiquitin, indicating that the K51R mutant was targeted by ubiquitin. To further corroborate this, Ni<sup>2+</sup>-NTA pull-down followed by Western blotting was carried out on HeLa cells transfected with encoding vector for Nkx2.5 WT or K51R alone in the presence or absence of His<sub>6</sub>-tagged ubiquitin. As shown in Fig. 5*B* (*right*), the high molecular weight band/smear was observed only in the presence of both K51R mutant and His<sub>6</sub>-ubiquitin and not in the other groups. Thus, we concluded that the substitution of lysine 51 by arginine turned Nkx2.5 into a polyubiquitination target. Reporter transactivation assays revealed that K51R exhibited much lower transcriptional activity than wild type Nkx2.5 (Fig. 5*C*). These data supported the critical involvement of the SUMO conjugation site in regulating Nkx2.5 transcriptional activity.

One way to modulate Nkx2.5 activity is through homodimerization, which enhanced Nkx2.5 transcriptional activity (31). To determine whether SUMO-1-conjugated Nkx2.5 formed dimers with Nkx2.5, SUMO-1-conjugated Nkx2.5, produced by Nkx2.5/SUMO-1/PIAS1 in whole cell lysates, was precipitated with either GST or GST-Nkx2.5 immobilized to GST beads. GST-Nkx2.5 retained both free and SUMO-1-conjugated Nkx2.5,

## Nkx2.5 Sumoylation



**FIGURE 6. SUMO-1 conjugation remodeled Nkx2.5-containing complex formation.** *A*, SUMO-1-conjugated Nkx2.5 homodimerized with free Nkx2.5. SUMO-1-conjugated hemagglutinin-tagged Nkx2.5 obtained from whole cell lysates containing various expressed proteins as indicated was incubated with GST or GST-fused Nkx2.5 immobilized to GST beads. The blot was revealed with Nkx2.5 antibody. *B*, Western blot was conducted on fractions collected from size exclusion chromatography using cell lysates containing various combinations of proteins as indicated. The asterisks indicate the altered complexes bearing Nkx2.5 (free or SUMO-1-attached or both). *C*, K51R heterodimerized with wild type Nkx2.5 via competition. *Top*, K51R physically interacted with GST-Nkx2.5 but not GST alone. *Bottom*, K51R heterodimerized with wild type Nkx2.5 via competition against Nkx2.5 homodimerization. *D*, K51R did not exhibit any synergy with WT Nkx2.5. Luciferase activity assays were conducted on cell lysates transfected with various doses of WT Nkx2.5 or K51R mutant as indicated. Please note that the replacement of a certain amount of WT Nkx2.5 by K51R failed to activate the tested promoter compared with the one without replacement. Data shown were expressed as mean  $\pm$  S.E. from at least two independent assays with each carried out in duplicate. The numbers shown above each bar inside the panel indicate the -fold activation for each group. The lower panel shows the relative expression levels of transfected V5-His<sub>6</sub>-Nkx2.5 WT and K51R in each corresponding group in 12-well plates. GAPDH in the same blot served as an internal control.

whereas GST alone failed to sequester either of them (Fig. 6A). To examine if SUMO modification may remodel formation of the Nkx2.5-containing complex, conventional size exclusion chromatography was conducted on whole cell lysates transfected with encoding vectors for wild type Nkx2.5 or K51R alone or in combination with SUMO-1 or SUMO-1/PIAS1, respectively. As shown in Fig. 6B, Nkx2.5 alone appeared in a complex with a size ranging between 702 and 1093 kDa. However, in the presence of SUMO-1, Nkx2.5 also appeared in a complex with a size ranging between 77 and 119 kDa (lane 7). In the presence of SUMO-1/PIAS1, SUMO-1-conjugated as well as unconjugated Nkx2.5 showed up in several complexes with a size ranging from 49 to 186 kDa (lanes 6, 7, 8, and 9), which were not observed in the combination of K51R/SUMO-1/PIAS1. These observations suggested that SUMO-1 modification may modulate Nkx2.5 function via altering Nkx2.5 interaction with itself and/or with its associated proteins. Surprisingly, K51R maintained the capacity to physically associate with wild type Nkx2.5 (Fig. 6C, top). Increasing doses of K51R competitively blocked homodimerization of wild type Nkx2.5 by forming heterodimers (Fig. 6C, bottom panels). The results from reporter activity assays indicated that K51R did not dramatically syner-

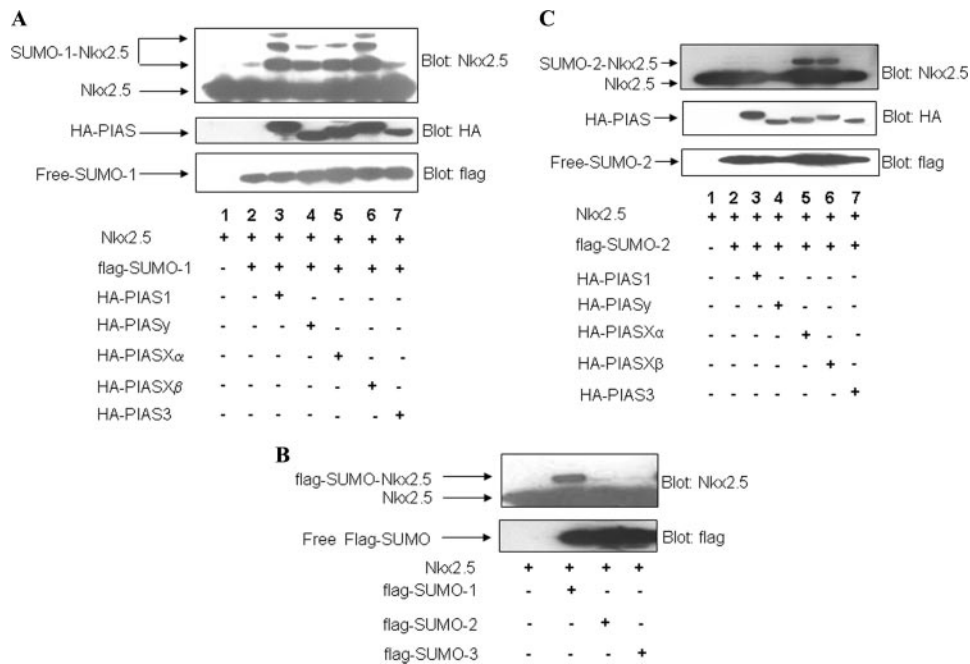
gize with wild type Nkx2.5 (Fig. 6D). These data indicated that SUMO-1 modification remodeled Nkx2.5-containing complex formation and that K51R did not function the same way as wild type Nkx2.5.

*PIAS Family Members Exhibited both Substrate and SUMO Specificity*—The PIAS family is composed of at least five members: PIAS1, PIAS3, PIASy, PIASx $\alpha$ , and PIASx $\beta$ . These members may share some redundant functions but may also perform unique particular roles independent of each other (32, 33). To examine which PIAS enhanced Nkx2.5 sumoylation by SUMO-1, *in vivo* sumoylation assays were complemented in cell lysates from HeLa cells transfected with plasmid-based expression vectors encoding wild type Nkx2.5 alone or in combination with SUMO-1 and PIAS isoforms, as indicated in Fig. 7A. Although all PIAS isoforms were comparably expressed (middle), clearly, the presence of PIAS3 was unable to promote SUMO-1 conjugation to Nkx2.5 (compare lane 2 with lane 7); all other PIAS proteins (PIAS1, -x $\alpha$ , -x $\beta$ , or -y) facilitated SUMO-1 linkage to Nkx2.5 (compare lane 2 with lanes 3–6).

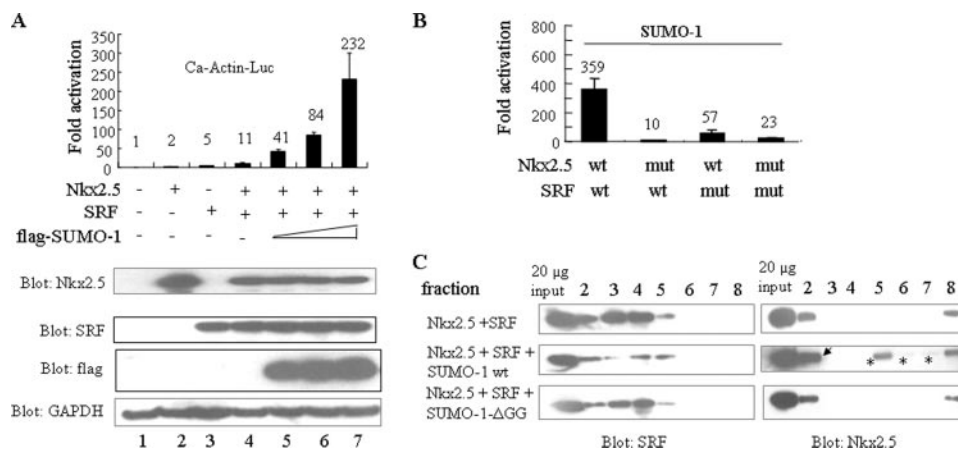
The SUMO family contains three functional members, among which highly identical SUMO-2/3 are generally weaker modifiers than SUMO-1. Indeed, SUMO-2/3 was also able to modify Nkx2.5 but to a much lesser extent in comparison with SUMO-1 (Fig. 7B). Surprisingly, only PIASx $\alpha$  and -x $\beta$ , and not the other PIAS proteins, catalyzed SUMO-2 attachment to Nkx2.5 (Fig. 7C), supporting the ideas that PIAS proteins promoted linking specificity for both SUMO proteins and their substrates and that SUMO family members were preferentially linked to substrates.

*SUMO-1 Potentiated Synergy between Nkx2.5 and Its Co-factor SRF*—Nkx2.5 may act alone but may also synergize in combination with its co-factors, such as SRF, which was also reported to be SUMO-targeted on lysine 147 (27). To test if SUMO-1 exerted any effect on the functional interaction between Nkx2.5 and SRF, transactivation assays were performed on Ca-actin-Luc in the presence of SUMO-1 with the combination of wild type Nkx2.5/SRF, as shown in Fig. 8A. Although a synergy was observed between Nkx2.5 and SRF, SUMO-1 further stimulated the activity of the reporter to ~232-fold, which was repressed by the mutations on the SUMO sites of these two trans-factors (Fig. 8B), indicating the critical role of SUMO sites in driving transcriptions. Next, we asked if SUMO-1 affected the formation of Nkx2.5-SRF-con-





**FIGURE 7. PIAS1 family members directed multiple specificities for both substrates and SUMO isoforms.** A, PIAS1,  $-\alpha$ ,  $-\beta$ , and  $-\gamma$  but not PIAS3 stimulated SUMO-1 conjugation to Nkx2.5. *In vivo* sumoylation assays were performed on whole cell lysates containing Nkx2.5 alone or together with SUMO-1 in the absence or presence of one of five PIAS family members, as indicated. *Top*, the existence of multiple SUMO-1-conjugated Nkx2.5 with PIAS1,  $-\alpha$ ,  $-\beta$ , and  $-\gamma$  but not with PIAS3. *Middle and bottom*, comparable expression levels of PIAS proteins and SUMO-1, respectively. B, Nkx2.5 was a weak target by SUMO-2/3. *The top* shows much reduced Nkx2.5 modification by SUMO-2/3 versus SUMO-1. *Bottom*, comparable expressions of SUMO-1, -2, and -3 in each group. C, PIAS $\alpha$  and  $-\beta$  but not other PIAS proteins catalyzed Nkx2.5 modification by SUMO-2. *Top*, Nkx2.5 antibody revealed strong stimulation of SUMO-2 modification by PIAS $\alpha$  and  $-\beta$  (compare *lane 2* with *lanes 5* and *6*). *The middle and bottom* show comparable expression of PIAS proteins and SUMO-2 in each group.



**FIGURE 8. SUMO-1 stimulated functional association between Nkx2.5 and SRF.** A and B, Ca-actin-Luc activity was assayed in the presence of various combinations of Nkx2.5, SRF, and dosed SUMO-1 expression vectors transfected into CV1 cells (A) or various combinations of wild type Nkx2.5 and SRF or their SUMO site mutants transfected into CV1 cells along with SUMO-1 expression vector (B). A, the *bottom three panels* show the expression levels of transfected Nkx2.5, SRF, K51R, and FLAG-tagged SUMO-1 in each corresponding group. GAPDH in the same blot served as an internal control. The *numbers shown above each bar* indicate the  $\pm$ -fold activation for each group. C, gel filtration revealed a different pattern of Nkx2.5-SRF-containing complexes formed in the presence of SUMO-1 from those formed in the absence or presence of SUMO-1 $\Delta$ GG. *Right and left*, the levels of SRF and Nkx2.5 in each corresponding fraction, respectively. The *asterisks* indicate the appearance of Nkx2.5 in the complex that was absent in the absence of wild type SUMO-1. The *arrowhead* indicates more accumulation of Nkx2.5 in fraction 2 in the presence of SUMO-1, whereas transfected proteins were comparably expressed in each group, as shown by the 20- $\mu$ g input of cell lysates.

taining protein complexes. Gel filtration was conducted on HeLa cell lysates containing various combinations of desired proteins, as indicated in Fig. 8C. In this complex, SRF mainly stayed in fractions 2–5, whereas Nkx2.5 stayed in fractions 2

and 8 in the absence of SUMO-1 or the presence of SUMO-1 $\Delta$ GG. However, the addition of wild type SUMO-1 promoted the accumulation of Nkx2.5 in the higher molecular weight fraction 2 and also introduced the existence of Nkx2.5 in the lower molecular weight complex (fractions 5–7). Thus, SUMO-1 potentiated synergy between Nkx2.5 and SRF via enhancing the stability of Nkx2.5-SRF-containing complexes and the appearance of Nkx2.5-containing low molecular weight complexes.

## DISCUSSION

In the past decade, great advances have been made toward identifying SUMO target proteins as well as understanding the mechanisms of regulating the activities of those substrates through SUMO modification (14, 34); however, little is known regarding how SUMO may target cardiac muscle-enriched factors in cardiogenesis. Following our previous two publications (17, 20), we recently identified cardiac specific homeobox gene *nkx2.5*, as a *de novo* SUMO target, and this modification positively modulated Nkx2.5 transcriptional activity.

*Lysine 51, the SUMO Attachment Site, a Distinct Character in Nkx2.5 Protein, Is Critical for Its Activity*—Nkx2.5 is a critical transcription factor involved in defining cardiac progenitors and heart development. Like other transcription factors, such as GATA4 and SRF, the functional regulatory network involves protein-protein interaction and posttranslational modification (10, 35–37). Our study provided a novel regulatory mechanism of Nkx2.5 activity by SUMO modification. SUMO targeted lysine 51 of Nkx2.5, which is about 70 amino acids away from the N-terminal HD, the DNA binding domain in NK-2 proteins (38). Although mutation of lysine 51 to arginine did not significantly alter Nkx2.5 nuclear occupancy (data not

shown), it dramatically reduced its DNA binding (Fig. 5A). We speculate that this site regulated Nkx2.5 DNA binding ability in the context of whole protein structure, since single HD isolated from Nkx2.5 without C- and N-terminal domains still pos-

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sessed the capacity to contact DNA as well as the full-length wild type Nkx2.5 (7). In other homeodomain proteins, such as *Drosophila* engrailed protein, a phosphorylation site, which was outside the HD, was also implicated in modulating its DNA binding (39). Given the fact that K51R was still able to dimerize with Nkx2.5 as well as wild type protein, we do not believe that mutation on Lys<sup>51</sup> inhibited its DNA binding via dramatic conformational changes. We noted the localization of lysine 51 in Nkx2.5 activation domain and gathered that this site probably provided a central regulatory role in conferring Nkx2.5-DNA contact.

Lysine residues in proteins are not only the potential SUMO moiety acceptors but also the potential modification sites for other covalent posttranslational modifications, such as methylation, acetylation, and ubiquitination (40–42). Under certain conditions, SUMO conjugation may antagonize ubiquitination or acetylation by competing for the same lysine(s) in the targeted proteins (25, 43, 44). The presence of lysine 51 appeared to protect Nkx2.5 from ubiquitination, since K51R was targeted by polyubiquitin, whereas wild type Nkx2.5 was not, indicative of the existing different acceptor sites for SUMO and ubiquitin. It also implied that mutation of lysine 51 may expose additional site(s) for polyubiquitination. However, how sumoylation of Nkx2.5 was involved in antagonizing Nkx2.5 ubiquitination still needs to be uncovered, since in the overexpression system, only ~10–20% of Nkx2.5 was SUMO-modified in the absence of E3 ligase. Also, depending on transfection efficiency and even in the absence of SUMO-1/PIAS1, ubiquitination of wild type Nkx2.5 was not observed under our assay conditions (Fig. 5B). Furthermore, K51R targeted by ubiquitin was as stable as wild type Nkx2.5 in the first 8 h in cycloheximide chase pulse assays (data not shown). Apparently, polyubiquitination did not target K51R for degradation. Actually, not all polyubiquitinations cause degradation. For instance, polyubiquitination on Lys<sup>63</sup> in ubiquitin led to changes in protein function but not degradation (45). Probably, polyubiquitination of K51R blocks its DNA binding. The physiological relevance of Nkx2.5 sumoylation to ubiquitination requires further investigation.

Although *nkx2.5* belongs to the *nk-2* class that was characterized by the presence of tyrosine 54 in HD, the SUMO-targeting consensus sequence, including lysine 51, is conserved only in Nkx2.5 and not in any other NK-2 class members. Correspondingly, other NK-2 class members, such as Nkx2.2, were not targeted by SUMO (data not shown). Our findings indicate that lysine 51 is a distinct character of Nkx2.5 proteins and performs a unique regulatory role in Nkx2.5 function.

**SUMO Conjugation Pathway and Nkx2.5 Transcriptional Activity**—The transcriptional activity of Nkx2.5 was governed via several mechanisms. For instance, phosphorylation by casein kinase II on serine 163 increased its DNA binding (13), and homodimerization properties or physical interaction with other factors (GATA4, SRF, etc.) via its homeodomain enhanced its activity (9, 10, 31). We showed that Nkx2.5 sumoylation enhanced the formation of Nkx2.5 containing complex achieved by the presence of SUMO-1/PIAS1 (Fig. 6B and Fig. 8C). Our findings indicated 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.

The SUMO family is composed of three identified functional isoforms, which possess differential substrate specificity. Nkx2.5 was poorly targeted by SUMO-2/3 *versus* SUMO-1. On the other hand, PIAS proteins, as one of the extensively studied SUMO E3 ligases, have shown substrate specificity as well. Although all PIAS family members appeared to substantiate SUMO-1 attachment to RET (46), PIAS1 and  $\alpha$ , but not PIASy and  $-\beta$ , stimulated sumoylation of androgen receptor (47), regardless of the fact that two alternatively spliced forms, PIAS $\alpha$  and  $-\beta$ , only differ in their C-terminal domains. In addition, PIASy was able to facilitate SUMO-1 conjugation to NEMO and SUMO-2 attachment to Topoisomerase-II, respectively (48, 49), and PIAS3 also acted as E3 ligase for SUMO-1 conjugation to IRF-1 (50). In the case of Nkx2.5 sumoylation, except for PIAS3, all other PIAS family members (PIAS1,  $-\alpha$ ,  $-\beta$ , and  $-\gamma$ ) were capable of catalyzing the covalent conjugation by SUMO-1, whereas only PIAS $\alpha$  ( $\alpha$  and  $\beta$ ), but not other PIAS proteins, stimulated SUMO-2 conjugation to Nkx2.5. In addition to the stimulated SUMO-1 modification of Nkx2.5 by PIAS1, we also noticed that PIAS1 WT catalyzed SUMO-1 modification of itself (Fig. 3C). Thus, the observed elevated activity of Nkx2.5 by PIAS1 in the presence of SUMO-1 was obtained probably via 1) increased SUMO-1 modification of Nkx2.5 by PIAS1 or 2) the presence of SUMO-1 modified PIAS1 or both. These findings demonstrate that PIAS family members, acting as SUMO E3 ligases, bear selectivity for both SUMO isoforms and target proteins.

In conclusion, Nkx2.5 transcriptional activity was positively regulated by the SUMO pathway, presumably via SUMO conjugation, since mutation on the SUMO acceptor site (lysine 51) abrogated more than 90% activation achieved by SUMO modification on wild type Nkx2.5. Further elucidation of the biological role of sumoylation in regulating Nkx2.5 function in cardiogenesis will require a K51R “knock-in knock-out” model *in vivo*.

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