SUMOylation inhibits transcription factors FoxC1 and FoxC2

Small Ubiquitin-like Modifier (SUMO) modification mediates the function of the inhibitory domains of the developmental regulators FoxC1 and FoxC2*

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Running title: SUMOylation inhibits transcription factors FoxC1 and FoxC2

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Background: Transcription factors FoxC1 and FoxC2 are involved in development and physiology but their mode of regulation is poorly understood.

Results: SUMO modification at two conserved Synergy Control motifs inhibits transactivation of both factors.

Conclusion: SUMOylation is responsible for the function of key inhibitory domains in FoxC1/C2.

Significance: SUMOylation regulates the function of FoxC1/FoxC2 and may contribute to developmental malformations.

SUMMARY

FoxC1 and FoxC2 are forkhead transcription factors that play essential roles during development and physiology. Despite their critical role, the mechanisms that regulate the function of these factors remains poorly understood. We have identified conserved motifs within a previously defined N-terminal negative regulatory region of FoxC1/C2 that conform to the definition of Synergy Control or SC motifs. Since such motifs inhibit the activity of transcription factors by serving as sites of post-translational modification by SUMO, we have examined whether FoxC1/C2 are targets of SUMOylation and probed the functional significance of this modification. We find that endogenous FoxC1 forms modified by SUMO2/3 can be detected. Moreover, in cell culture, all three SUMO isoforms are readily conjugated to FoxC1 and FoxC2. The modification can be reconstituted in vitro with purified components and can be reversed in vitro by treatment with the SUMO protease SENP2. SUMOylation of FoxC1 and FoxC2 occurs primarily on one consensus synergy control motif with minor contributions of a second, more degenerate site. Notably, although FoxC1 is also phosphorylated at multiple sites, disruption of sites immediately downstream of the SC motifs does not influence SUMOylation. Consistent with a negative functional role, SUMOylation-deficient mutants displayed higher transcriptional activity compared to wild type forms despite comparable protein levels and subcellular localization. Thus, the findings demonstrate that SC motifs mediate the inhibitory function of this region by serving as sites for SUMOylation and reveal a novel mechanism for acute and reversible regulation of FoxC1/C2 function.
FoxC1 is a member of a large superfamily of transcription factors composed of approximately 90 members with orthologs expressed in species ranging from yeast to humans. Members of this superfamily contain an evolutionary conserved 110-amino acid DNA-binding domain known as the forkhead domain (1), and are central to multiple developmental and pathological processes including the maintenance of differentiated cell states, tissue specific gene expression, embryogenesis, and tumorigenesis (2). FoxC1 is highly homologous to FoxC2 which was previously referred to as Mfh1 (3). Animal studies suggest that FoxC1 cooperates with FoxC2 to activate target genes during eye, heart, and kidney development as well as during somitogenesis.

Mutations in the FOXC1 gene in humans are associated with Axenfeld-Rieger Syndrome, a disorder characterized by multiple morphological and functional defects including significant eye abnormalities, predisposition to glaucoma development, craniofacial dysmorphism and dental anomalies (4). Mice homozygous for spontaneous mutations or engineered FoxC1 null alleles die perinatally with multiple abnormalities including hydrocephalus as well as ocular, skeletal and cardiovascular defects (5-7). Mutations in human FOXC2 are responsible for the autosomal dominant syndrome lymphedema distichiasis which is characterized by the obstruction of lymphatic drainage of the limbs and the growth of an extra set of eyelashes or distichiasis (8). Disruption of FoxC2 in mice causes perinatal lethality due to embryonic developmental defects in aortic arch patterning, arterial specification and vessel remodeling (7,9,10). Remarkably, despite ample evidence of their functional importance, the mechanisms that regulate the function of these important factors remain poorly understood.

Post-translational modifications are a widespread mechanism to regulate the activity of sequence-specific transcription factors. Both SUMOylation and phosphorylation are powerful modifications that can regulate the function of a protein by changing its intrinsic activity, subcellular localization or stability as a consequence of alterations in protein-protein or protein-nucleic acid interactions. The mammalian small ubiquitin-like modifier (SUMO) family consists of four genes designated SUMO-1-4. SUMO-2 and -3 are closely related whereas SUMO-1 shares only 48% identity with either SUMO-2 or -3 (11-13). SUMO-4 shares 86% amino acid homology with SUMO-2/3 (14), whether this form is actually conjugated to target proteins however, is unclear (15). Following an initial C-terminal proteolytic processing by SUMO-specific proteases, SUMO is activated in an ATP-dependent manner by the heterodimeric E1 activating enzyme SAE1/SAE2 (16). SUMO is then transferred to the SUMO-specific E2 conjugating enzyme Ubc9, which catalyzes the formation of an isopeptide bond between the C-terminus of SUMO and the amino group of the target lysine (17,18). This step can be enhanced by proteins with SUMO specific E3 activity such as members of the PIAS family of proteins (19).

SUMOylation is a reversible process and mammalian SENP family members are responsible for cleaving the SUMO moiety (20). SUMOylation is now established as an important modification that negatively regulates sequence-specific transcription factors (21). This prominent function was revealed in part by the convergence between the functional definition of synergy control or SC motifs (22) and the consensus for SUMO modification and by the demonstration that SC motifs exert their effects by serving as sites of SUMOylation (23-25). Functionally, SC motifs are short autonomous sequences that limit the transcriptional activity of regulators in a promoter-context dependent manner (22) with the strongest inhibition observed at DNA sequences where regulators bind most stably (26).

Notably, deletion analysis studies have indicated the presence of a functional negative regulatory domain in the central region of FoxC1 (27) and FoxC2 (28) but any mechanistic link to specific post-translational modifications has not been defined. In this study, we have identified conserved motifs within these negative regulatory regions that conform to the definition of synergy control motifs. We have therefore examined whether FoxC1/C2 are targets of SUMOylation and probed the role of this post-translational modification with regards to the mechanism of action of these negative regulatory regions as well as its relation to phosphorylation.
EXPERIMENTAL PROCEDURES

Cell culture and generation of plasmids and lentiviruses – Human kidney epithelial (HEK 293T) and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, (Life Technologies, Carlsbad, CA, USA) and 50 IU/ml penicillin–streptomycin (Life Technologies). Cells were transfected using 25-kDa linear polyethylenimine (Polysciences, Warrington, PA) as described previously (29). The N-terminal, 3×Flag or 6×RGS-Histidine-tagged, mouse FoxC1 and FoxC2 expression plasmids were generated by PCR amplification of FoxC1 (ATCC IMAGE clone: 5720391) and FoxC2 (plasmid kindly provided by Dr. T. Kume University Chicago, IL) cDNA sequences using primers containing EcoRI/XhoI sites and ligation into pcs4-3×flag or pcs4-6×RGS-His. Single and double point mutants within the putative SC motifs of FoxC1 and FoxC2 as well as all phosphorylation site mutations were generated by one-step site-directed PCR-based mutagenesis as previously described (30). Deletion of the FoxC1 inhibitory domain was also accomplished by one-step site directed PCR based mutagenesis using the following primer to delete amino acids 221 to 324 of mouse FoxC1: 5’-CCCGGGCCACAGCCGCCGCCCCTGAGCTCGGTTCC-3’. The CMV-driven (pcDNA3) expression vectors for HA-tagged SUMO-1, -2 and -3 as well as pCMV-β-gal have been described previously (25,31). The reporter plasmid pGL3-6-BS is a derivative of pGL3 harboring six copies of a FoxC1 binding site (32) and was a kind gift from Dr. Michael Walter (University of Alberta, Edmonton, Alberta, Canada). The pΔ6xBSDLO is a derivative of pΔODLO 02 (24) reporter plasmid in which a minimal Drosophila distal alcohol dehydrogenase promoter (-33 to +55) drives the luciferase gene. The oligonucleotides 5’-GATCCAAAGTAAATAAACAAAGGCTAGCCAAAGTAAATAAACAAACAGCAAAGTAAATAAACAACA-3’ and 5’-GATCTGTTGTTTATTTACTTTGCTGTTAATTACCTTGT-3’ containing three FoxC1/C2 binding sites (32) were annealed and ligated into the BamHI and BglIII sites of pΔODLO 02 to yield pΔ3×BSDLO. The binding sites were then duplicated by ligation of independent SalI/KpnI and XhoI/KpnI fragments of pΔ3×BSDLO yielding plasmid pΔ6xBSDLO. Lentivirus overexpression vectors were generated by subcloning 3×flag-FoxC2 and the SUMOylation site mutant from the corresponding pcs4-3×flag vectors into pLentilox-IRES-Puro vector. This vector and the companion vectors for lentivirus production, psPAX2 and pVSV-G were obtained from the University of Michigan Vector Core, (Ann Arbor, MI). For lentivirus generation, HEK 293T cells (10⁶/well) seeded in 6-well dishes were transfected 24 h later with 0.8 µg of lentivirus vectors and 1.6 µg of packaging vectors (0.8 µg each of VSV-G and psPAX2) using PEF. The media was changed and supplemented with 10 mM sodium butyrate (Alfa Aesar, Ward Hill, MA) 24 h post transfection. Supernatants containing mature lentivirus were harvested 48 h after transfection and filtered using 0.45 µm PVDF syringe filters (Millipore, Billerica, MA).

Identification of FoxC1 phosphorylation sites by mass spectrometry – Fertilized Xenopus embryos were obtained and maintained as previously described (33). Embryos were staged according to Nieuwoork and Faber (34). The pcs4-3HA-mFoxC1 construct was linearized and mRNA was transcribed with SP6 RNA polymerase using the mMessage mMachine kit (Ambion). Embryos injected at the two-cell stage with 500 pg of HA-FoxC1 mRNA, were lysed in modified RIPA buffer [(150 mM NaCl, 50 mM Tris (pH 8), 25 mM β-glycerophosphate, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 2 × Complete EDTA-free protease inhibitor mixture (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride), 2 mM EDTA, 1% Nonidet P-40 (NP-40)] at stage 10.5 and subjected to immunoprecipitation using anti HA antibodies. Immunoprecipitates were incubated in the presence or absence of λ-protein phosphatase (λ-PPASE, New England Biolabs). Eluted proteins were resolved by SDS PAGE and detected by immunoblotting with an anti-HA antibody or stained with Coomassie brilliant blue. Bands corresponding to FoxC1 were excised from the stained gel and submitted for analysis to the Taplin Biological Mass Spectrometry Facility (Department of Cell Biology, Harvard Medical School, Boston, MA).
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**Functional assays** – HeLa cells seeded in 24-well dishes (2.4×10^5/well) were transfected using 25-kDa linear polyethyleneimine (Polysciences, Warrington, PA) (29). Cells received 10 ng of pGL3-6×BS, 60 ng of Flag-tagged FoxC1 or control expression vector and 120 ng of pCMV-β-gal per well. FoxC2 assays were similar except for the use of 60 ng of pAODLO 6×BS-Luc as reporter and 100 ng of Flag-tagged FoxC2 expression vector. Cells were lysed 24 h post-transfection and luciferase and β-galactosidase activities were determined as described previously (35). Data represent the average ± standard deviation of the mean of three experiments performed in triplicates. To assess FoxC expression, parallel cultures were lysed in modified RIPA buffer supplemented with 5 mM N-ethylmaleimide (NEM; Sigma). Lysates were centrifuged and supernatants resolved by 7.5% SDS-PAGE followed by immunoblotting as described below. For endogenous gene analysis, 1 ml of lentiviral supernatant and 1 ml of fresh 3T3-L1 medium (DMEM 11995, Life Technologies; 10% Calf Bovine Serum, Denville Scientific) was added to 3T3-L1 cells cultured in 6 well dishes for 24 h in the presence of 8 µg/ml Polybrene (Millipore). The cells were then rinsed thoroughly with PBS and cultured in fresh medium for another 24 h prior to Puromycin (Acros Organics, Thermo Fisher Scientific) selection (4 µg/ml) for two weeks. RNA was isolated and purified using an RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). Oligo d(T) (Applied Biosystems, Foster City, CA) primed cDNA was synthesized from 1 µg of RNA, using Multiscribe reverse transcriptase (Applied Biosystems). Quantitative real-time PCR (qPCR) reactions were carried out in triplicate in a Roche 480 LightCycler using QuantiTect SybrGreen reagents (Qiagen) and primers for mouse genes RPL19 (Forward, 5'-TGTACCTGAAGGTCAAGGGAATGTG-3'; Reverse 5'-TTCTTGGTCTCCTCCTCTTGAC-3') and Hey1 (Forward, 5'-TACCCAGTGCCCTTGAAGAG-3'; Reverse 5'-TCCGATAGTCCATAGGCAGG-3'). Software LinRegPCR (Ver 11.0) was used to estimate mRNA levels of Hey1 relative to levels of RPL19.

**SUMOylation assays** – For endogenous protein SUMOylation, cultured cells were collected ice-cold PBS then lysed in modified RIPA buffer [(150 mM NaCl, 50 mM Tris (pH 8), 25 mM β-glycerophosphate, 200 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride), 2 mM EDTA, 5mM N-Ethylmaleimide, (NEM), 20 nM Calyculin A, 1% Nonidet P-40 (NP-40) and 10µl/ml protease inhibitor cocktail (Sigma)]. Lysates (1 mg) were pre-cleared by incubating 1 h at 4°C gently rocking with 30µl protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Pre-cleared lysates were then incubated with gentle rocking overnight at 4°C with 5 µg/ml FoxC1 antibody (Bethyl Laboratories, Montgomery, TX, USA) or 5 µg/ml normal rabbit Immunoglobulin G (IgG; Santa Cruz Biotechnology; 500 µg per condition). Lysates were then incubated for 1 h at 4°C with gentle rocking with 50 µl protein A/G plus agarose beads. Immunoprecipitates were further washed in SENP buffer (25 mM Tris HCl (pH 8.0), 150 mM NaCl, 0.1% Tween-20 and 2 mM DTT) and incubated at 23°C for 30 min at 50% slurry with 1 nM purified catalytic domain of WT SENP2 or the catalytically inactive C548S mutant (36) before elution in Laemmli sample buffer. In parallel, 15 µg of lysate (pre-IP) was also treated with WT or C548S SENP2 in SENP buffer. Reactions were terminated by addition of Laemmli sample buffer. Samples were resolved by 7.5% SDS-PAGE and processed for immunoblotting with the indicated antibodies. For SUMOylation experiments in cultured cells, HEK 293T cells (1.5×10^6 /well) were seeded onto 6-well dishes and transfected with 0.25 µg of Flag FoxC1 or Flag FoxC2 and 0.5 µg of the indicated HA SUMO expression vector using linear polyethyleneimine (29). When necessary, transfections included an amount of the corresponding empty vector as to maintain an equimolar amount of each vector per well. Cells were harvested 48 h after transfection in modified RIPA buffer [(150 mM NaCl, 50 mM Tris (pH 8), 25 mM β-glycerophosphate, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, Complete EDTA-free protease inhibitor mixture (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride), 2 mM EDTA, 1% Nonidet P-40 (NP-40) and 5 mM NEM] and Flag immunoprecipitation was performed for 3h at 4°C using an anti-Flag affinity agarose gel (Sigma-Aldrich). Beads were washed four times in modified RIPA buffer and once in...
phosphate buffered saline. Immunoprecipitates were eluted in Laemmli sample buffer. Samples were resolved by 7.5% SDS-PAGE and processed for immunoblotting with the indicated antibodies. For samples subjected to SUMO deconjugation, immunoprecipitates were further washed in SENP buffer and treated as indicated above before elution in Laemmli sample buffer. For *in vitro* SUMOylation reactions, HEK 293T cells were transfected as above with 0.5 μg of FoxC1, FoxC2 or empty vector per well. Cells were lysed and immunoprecipitated as above but in the absence of NEM. Beads were washed four times in modified RIPA buffer and once in 50 mM Tris (pH 7.5), 5 mM MgCl2. SUMOylation assays (35) were carried out in the buffer above in the presence (as indicated in the figure legend) of 1 μg of GST-SAE2/SAE1, 5 μg of GST-Ubc9, 5 μg of His-SUMO-1GG, and 20 μl (50% slurry) of immunoprecipitated Flag-FoxC1 WT, its SUMOylation-deficient mutants, or control immunoprecipitates from untransfected cells as indicated. Reactions were initiated by the addition of an ATP regeneration system (final concentrations: 10 units/ml creatine kinase, 25 mM phosphocreatine, 5 mM ATP and pyrophosphatase 0.6 units/ml). Samples were incubated for 30 °C for 90 min, and reactions were terminated by addition of 20 μl of disruption buffer (50 mm Tris-HCl, pH 6.8, 1.67% SDS, 10% glycerol, 0.24 M β-mercaptoethanol, 0.015% bromphenol blue) and boiling for 5 min. Samples were resolved by 7.5% SDS-PAGE, and processed for immunoblotting as described below.

**Immunoblotting and immunofluorescence**—Following SDS PAGE, samples were transferred to nitrocellulose membranes and processed for immunoblotting using anti-Flag (Sigma), HA-11 (clone 3F10, Roche Applied Science), Rabbit anti-FoxC1, (Bethyl Laboratories) or anti-Sumo 2/3 (Abcam, Cambridge, MA, USA) antibodies followed by either goat anti-mouse HRP or goat anti-rabbit secondary antibody conjugates (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) and developed using an enhanced chemiluminescence detection system (Fisher Scientific Research, Pittsburgh, PA). For all figures, images shown are representative of at least three independent experiments. For immunofluorescence, HEK 293T cells transfected with Flag-tagged FoxC1 and FoxC2 constructs were fixed in phosphate-buffered saline (PBS) with 3.7% paraformaldehyde, permeabilized with Triton X-100 (0.05%) in PBS, and incubated with monoclonal anti-FLAG M2-FITC antibody (Sigma, St. Louis, MO, USA), at 10μg/ml in Tris-buffered saline (TBS) for 1 h. Actin staining was then carried out by incubating cells for 30 min in Alexa Fluor 555 phalloidin at a 1:100 dilution (Life Technologies, Carlsbad, CA, USA). DNA counterstain was obtained with 40,6-diamidino-2-phenylindole (DAPI) included in the Vectashield Hard Set mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were obtained using a Leica DMI 6000B fluorescence microscope.

**RESULTS**

**FoxC1 and FoxC2 harbor conserved SC/SUMOylation motifs**—Sequence comparison of FoxC1 proteins from various vertebrate species (Fig.1B) revealed the presence of several conserved clusters within the central negative regulatory region comprising amino acids 215-365 of human FOXC1 (27) (217-366 in mouse). A related pattern of conservation is also evident within an analogous negative region in FoxC2 (Fig.1B) spanning amino acids 204-280 in the human sequence (28) (204-280 of mouse FoxC2). Notably, although the overall homology across these regions between FoxC1 and FoxC2 is rather limited, we have identified that the two N-terminal conserved clusters map to short sequences resembling the consensus SC/SUMOylation motif. The first motif is a perfect match including the presence of flanking Pro/Gly residues whereas the second is significantly more degenerate since the residue immediately upstream of the conserved lysine residue is not a hydrophobic amino acid. The local conservation of these sequences and their presence within the negative regulatory regions argued that they likely play an important role as functional SC motifs as is the case for other regulators.

**FoxC1 and FoxC2 are SUMO-modified in vivo**—To examine whether endogenous FoxC1 is a target of SUMOylation, FoxC1 immunoprecipitates from HEK 293T cells were treated with the SUMO protease SENP2 or with a catalytically inactive...
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mutant. Analysis with an anti FoxC1 antibody revealed the presence of multiple FoxC1 species with a main form with an apparent Mr of ~65 kDa (Fig. 1D closed arrowhead) and several lower forms. Such heterogeneity has been previously reported and tentatively attributed to protein phosphorylation (27). Notably, a FoxC1 immunoreactive species with a lower mobility (apparent MW~80 kDa) that was readily detected in samples treated with the mutant SUMO protease, was absent in those treated with the active form of the protease (Fig. 1D open arrowhead). Parallel analysis of the immunoprecipitates with an anti SUMO2/3 antibody revealed a SUMO2/3 containing band of identical mobility that was also absent in samples treated with the active protease. The ability of WT SENP2 to deconjugate SUMO from cellular proteins was confirmed by analysis of the initial extract with the SUMO2/3 antibody (Fig. 1D, right). Taken together, these data indicate that endogenous FoxC1 is a target of SUMOylation.

To confirm these observations and extend them to FoxC2, we expressed and isolated Flag-tagged FoxC1 and FoxC2 from cells co-expressing HA-SUMO-3. Probing cell extracts with an anti-HA antibody demonstrated the incorporation of SUMO-3 into cellular proteins (Fig. 2, lower panels). Analysis of the immunoprecipitates with an anti-Flag antibody revealed the tagged form of FoxC1 as several species of ~70kDa (Fig. 2A closed arrowheads). Upon co-expression with SUMO3, an additional set of slow migrating bands at ~115 kDa were detected by the anti-flag antibody (open arrowheads). These bands correspond to SUMOylated FoxC1, since they are also detected with an anti HA antibody (Fig. 2A middle panel). Consistent with a covalent linkage, these species (but not the minor ones marked with asterisks) were also recovered in similar experiments where His-tagged FoxC1 was isolated under denaturing conditions via metal chelate chromatography (not shown). To confirm that the slow migrating species correspond to SUMOylated FoxC1, immunoprecipitates were treated in vitro with the SUMO protease SENP2. Both the FoxC1 and SUMO signals of ~115 kDa were readily eliminated by the WT but not by the catalytically inactive mutant SENP2 (Fig. 2A last two lanes). Parallel experiments using FoxC2 yielded analogous results (Fig. 2B). It is clear from this analysis that the migration of the modified species corresponds to an apparent molecular weight significantly larger than that expected for the attachment of a single SUMO moiety. It is important to note however, that due to the branched nature of the conjugate, the migration of SUMO modified proteins deviates significantly from the corresponding linear forms and this deviation is most dramatic when the branching occurs near the center of the polypeptide as is predicted here. Taken together, the data provide strong evidence that both FoxC1 and FoxC2 are targets of SUMO modification.

Conjugation of individual SUMO isoforms requires intact SC/SUMOylation motifs — To examine the ability of different SUMO isoforms to be conjugated to FoxC1 and FoxC2, we performed SUMOylation experiments in cells expressing HA tagged SUMO1, 2 or 3 isoforms. As can be seen in Fig. 3 (open arrowheads), each of the SUMO isoforms can be conjugated to FoxC1 and FoxC2 albeit to different extents. SUMO2 and SUMO3 conjugates are readily detected whereas SUMO-1-modified species are significantly less abundant and required a significantly longer exposure for detection (Fig. 3). It is important to note that although this suggests preferential conjugation, we have observed a similar pattern for other SUMOylation targets (37) and the detection of modified species is likely to reflect in large part the differential expression of the SUMO isoforms rather than an intrinsic property of FoxC1/C2. To determine the role of the motifs identified by sequence comparison in SUMOylation, we replaced the predicted target Lys residues with Arg, which does not support SUMOylation. For FoxC1, and consistent with the very good agreement with the canonical SC/SUMOylation consensus, the K229R mutation in the first motif led to a nearly complete loss of the doublet (~115 kDa) migrating species (Fig. 3A). In contrast, although the K258R mutation in the second, more degenerate motif had little effect on SUMOylation on its own, it eliminated the residual modification observed in the K229R mutant since no SUMOylated FoxC1 could be observed for the double mutant (K229R/K258R). The slower immunoreactive species (marked with asterisks) are unaffected by the mutations and likely represent unrelated SUMO-modified co-
immunoprecipitating proteins since these species are not recovered under denaturing conditions (not shown). Although we have not formally defined the topology of the SUMOylation, the two major SUMOylated species are unlikely to be due to chain formation since they are observed with all three SUMO isoforms. Furthermore, given the extensive and variable heterogeneity of the non-modified forms (Fig. 3A lower panels), the simultaneous loss of the two major SUMO-modified species suggests that they represent similarly SUMOylated forms differing in other aspects.

Notably, analysis of FoxC2 yielded similar results since mutation (K213R) of the canonical first motif led to a substantial loss of the ~102 kDa migrating species (Fig. 3B, open arrowhead) whereas the K226R substitution had little or no effect. No sumoylated FoxC2 could be observed with the double mutant (K212R/K226R). In addition, although Lys residues can be targets for multiple modifications, we did not observe significant changes in the steady-state levels of either FoxC1 or FoxC2 bearing Lys to Arg substitutions, suggesting that the sites are not essential for ubiquitination-dependent degradation. To delineate the contributions of other residues within the first motif of FoxC1, we also examined the effect of substitutions at two additional critical positions (Fig. 4A). Consistent with the key role of the hydrophobic residue upstream of the modification site, replacement of Ile228 by Thr caused a loss in conjugation as severe as mutation of the K229 acceptor lysine. The importance of the downstream Glu231 residue is also evident since a Gly substitution was equally disruptive. Taken together, these findings support the view that the upstream canonical motifs in FoxC1 and FoxC2 serve as the major sites for SUMO conjugation with a minor contribution of the significantly more degenerate downstream motif.

**FoxC1 and FoxC2 are SUMO-modified in Vitro** — To further investigate the mechanism of FoxC1 SUMO modification, we used a reconstituted in vitro SUMOylation assay consisting of purified recombinant SUMO1 as well as E1 (SAE1/SAE2) and E2 (Ubc9) enzymes. FoxC1 Flag-immunoprecipitated from HEK 293T cells was used as an in vitro SUMOylation substrate. Incubation with all SUMOylation machinery components led to the generation of slower migrating bands of ~115 kDa (Fig. 5). Omission of ATP during the reaction prevented the appearance of the slower migrating species indicating that they represent specific covalent modification of FoxC1 by SUMO1. As in the in vivo assays, SUMO conjugates were reduced dramatically for the K229R mutant but not for the K258R. SUMOylation was undetectable for the K229R/K258R double mutant. The results support the in vivo modification data and indicate that the preferential modification of K229 does not depend on additional components and therefore reflects the intrinsic propensity of the motifs to serve as substrates for Ubc9.

**FoxC1 and FoxC2 phosphorylation is not directly coupled to SUMOylation** — transcription factors are often subject to multiple post-translational modifications and SUMOylation can be coupled to phosphorylation in both positive and negative ways. In the case of phosphorylation dependent SUMOylation, proline directed phosphorylation a few residues downstream of the core SUMOylation motif can significantly enhance conjugation of SUMO (38). This arrangement can convert phosphorylation-mediated cellular signals into SUMO based responses. In other cases, exemplified by the transcription factor SF-1, SUMO modification antagonizes phosphorylation since we have shown that loss of K194 SUMOylation enhances CDK7 mediated phosphorylation of the key regulatory S203 site (39). As previously noted (38), the first FoxC2 site (Fig. 1C) matches the phosphorylation-dependent SUMOylation motif (Ψ-K-x-E-x-x-S-P) with potential phosphorylation at S218. To examine the potential role of phosphorylation, we examined the SUMOylation of the S218A mutant in cultured cells. As can be seen in Fig. 6A, this substitution caused only a mild reduction in SUMO conjugates. It is unclear whether this is a direct effect or is a reflection of the mildly reduced levels of this mutant (Fig. 6A lower panel).

In the case of FoxC1, both bioinformatic analysis (http://scansite.mit.edu/) and previous studies argue that FoxC1 is a phosphoprotein (27,40,41) although the complete mapping of phosphorylation sites is not fully defined. Interestingly, a number of residues predicted to be phosphorylated map to the FoxC1 inhibitory domain which harbors the SUMOylation sites and
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Deletion of this domain leads to both enhanced activity and a significant loss in phosphorylation (27). We first confirmed that FoxC1 is phosphorylated in vivo by taking advantage of a Xenopus embryo system. As can be seen in Fig. 6B, expression of FoxC1 generated a species of ~76 kD and treatment with λ protein phosphatase significantly reduced its apparent molecular weight to a value more consistent with its predicted polypeptide mass of 59 kDa. This argues that FoxC1 expressed in this system is a phosphoprotein as previously suggested for tissue culture cells (27). To identify sites of phosphorylation, Flag-tagged FoxC1 expressed in Xenopus embryos was immunoprecipitated and resolved by SDS-PAGE. Following Coomassie staining, the region corresponding to FoxC1 was excised, digested and subjected to mass spectrometry analysis. Using this approach we unambiguously identified phosphorylation at S237, S243, S320 and S521. In addition, phosphorylation was detected in peptides 5-11 (YSVSSPN) and 542-545 (TSGA) (Fig. 6C). Notably, one of these phosphorylation sites (S237) is in close proximity to the main K229 SUMOylation site of FoxC1 (Fig. 1B).

To examine the potential interaction between phosphorylation and SUMOylation, we generated a FoxC1 construct in which all identified phosphorylation sites are mutated to Ala. (For the three Ser in the 5-YSVSSPN-11 motif, we deleted amino acids 5-10). Upon expression in HEK 293T cells and immunoprecipitation, this mutant protein was recovered to the same level as the WT or K229/258R SUMOylation deficient mutant. counterparts (Fig. 6D lower panel). Notably, and consistent with a loss of phosphorylation, the distribution of FoxC1 species in the phosphorylation mutant shifted towards the faster migrating forms whereas a slight shift towards slower forms was detected for the SUMOylation site mutant. Analysis of SUMO conjugates (Fig. 6D, upper panel) revealed that the phosphorylation site mutant was SUMOylated to a comparable level than the WT whereas no SUMOylation was detected for the K229/258R mutant. Notably, the faster migration of the SUMO modified forms of the phosphorylation site mutant suggests that for WT FoxC1, the SUMOylated species are also phosphorylated. These results indicate that phosphorylation at the sites identified in Xenopus embryos, including S237, does not appear to influence SUMOylation under the conditions examined.

Loss of SUMOylation enhances FoxC1 and FoxC2 function — In order to probe the mechanistic link between FoxC1 and FoxC2 SUMO modification and the regulatory function of the negative region in which they are found, we examined the effect of disrupting SUMO modification on the activity of FoxC1 and FoxC2. Since we have previously shown that the inhibitory effects of SC motifs are most pronounced at promoters driving multiple binding sites, we assessed the activity of FoxC1 at pGL3-6xBS, a reporter gene driven by six copies of a previously characterized FoxC1 binding site (32,42). The basal activity of this reporter is relatively low in HeLa cells making them well suited for these experiments. As can be seen in Fig. 7A, disruption of the main SUMOylation site in FoxC1 (K229R), which severely reduces but does not totally eliminate SUMOylation, enhanced activity more than 50%. In contrast, and consistent with the SUMOylation data, the K258R mutant in the secondary site displayed activity (and SUMOylation) comparable to WT FoxC1. Although at first glance this could be viewed as suggesting that the secondary site has negligible effects, its contributions are clearly evident in the combined mutant, which eliminates all detectable SUMOylation and displays much further elevated activity (250% of WT). Notably, this activity was comparable to that observed in the case of a form deleted of the entire inhibitory domain. Furthermore, the combined phosphorylation site mutant, which is not impaired in SUMOylation, had essentially WT activity. A similar analysis of the activity of FoxC2 at the pΔ6xBSDLO reporter (Fig. 7B) revealed a strikingly similar pattern, with the double mutant displaying ~2.5-fold higher activity than WT FoxC2. Consistent with the SUMOylation data, the S218A mutant immediately downstream of the main motif had the same activity as the WT construct, indicating that preventing phosphorylation at this site does not influence activity.

Mechanistically, the enhanced activity of the SUMOylation site mutants do not appear to be due to changes in steady-state levels of FoxC1 or FoxC2 since all forms accumulated to comparable levels (Fig. 7A and 7B lower panels). Since
SUMOylation can influence the function of cellular proteins by altering their subcellular localization, we examined this possibility directly. Notably, the enhanced activity of SUMOylation deficient forms of FoxC1/C2 appears to occur without overt alterations in the subcellular localization since immunofluorescence analysis of the WT and mutant forms of FoxC1 and FoxC2 revealed indistinguishable nuclear localization patterns (Fig. 8).

To examine the functional impact of SUMOylation on the regulation of endogenous genes, we took advantage of recent data indicating that FoxC2 upregulates the expression of the repressor Hey1 in pre-adipocytes and that this is a major mechanism by which FoxC2 inhibits adipocyte differentiation (43). We therefore enforced the expression of FoxC2 and its SUMOylation mutant in 3T3-L1 pre-adipocytes using lentiviral infection and analyzed Hey1 expression by quantitative RT-PCR. Notably, although both forms accumulated to comparable levels (Fig. 7C, left), the SUMOylation-deficient FoxC2 form induced higher Hey1 expression compared to WT FoxC2 (Fig. 7C, right). Thus, the data show that SUMOylation exerts a significant inhibitory effect on FoxC2 driven expression of endogenous genes such as Hey1.

DISCUSSION

Despite the established importance of FoxC1 and FoxC2 in the regulation of both developmental programs and a growing list of physiological processes, defining the mechanisms that control the ability of these factors to regulate their still poorly defined transcriptional targets remains challenging. The current findings provide strong evidence that FoxC1 and FoxC2 are targets of SUMOylation and that this dynamic post-translational modification exerts a significant inhibitory role. Notably, this modification both maps to and accounts for the function attributed to previously defined inhibitory domains within these regulators. Our analysis shows that both FoxC1 and FoxC2 harbor two, closely spaced modification sites. Interestingly, the extent of SUMOylation and the functional contribution of each motif is related to their sequence similarity to the consensus SC/SUMOylation motif. Thus, the upstream and primary motif fully conforms with the SC motif definition whereas the second and weaker motif lacks the bulky hydrophobic residue at the first core position. Our finding that mutation of both motifs is necessary to completely eliminate both SUMOylation and transcriptional inhibition highlights the fact that as in the case of other factors, such as the glucocorticoid or androgen receptor (22), both motifs functionally cooperate and that even relatively modest levels of SUMOylation exert significant inhibitory effects.

A growing body of evidence indicates that SUMOylation, particularly at imperfect sites can be coupled to other modification such as phosphorylation in a positive (38) or negative (39) manner. Although we did not observe effects of disrupting downstream phosphorylation sites on the SUMOylation or activity of either FoxC1 or FoxC2, it is possible that these sites may not be phosphorylated to a significant extent under the current conditions. It remains possible that interactions between these modifications could occur in specific cell types and/or developmental stages. Tools to directly monitor the site specific phosphorylation of these factors would be of great value in this regard.

Recent data indicate that SUMOylation likely mediates its inhibitory effects by facilitating the recruitment of co-repressors bearing SUMO interaction motifs or SIMs. One or more of the several candidate factors that have been proposed (44-47) could be involved in the regulation of FoxC1 and FoxC2. Mechanistic studies indicate however, that such factors preferentially act at genomic sites where the transcription factor binds in a stable manner (26). We therefore anticipate that the regulatory elements responsible for FoxC2 mediated regulation of Hey1 will harbor multiple high affinity sites for FoxC2. From our analysis however, it appears that SUMOylation exerts an inhibitory effect on FoxC1 and FoxC2 without overt changes in the overall stability of the proteins or their subcellular localization.

SUMOylation is likely to play important roles in the biology of FoxC1 and FoxC2 particularly since relatively modest alterations in their function are associated with distinct developmental abnormalities. This is particularly likely since a number of other transcription factors involved in craniofacial development including TBX22 and SATB2 are targets of SUMOylation (48). In addition, although SUMO1 null mice have normal
development (49), natural and engineered SUMO1 alleles have been associated with cleft palate (50). Given the unique role of FoxC1 during the closure of cranial sutures, it is possible that SUMOylation plays a role in the orchestration of FoxC1 function during this temporally narrow developmental process. Examining the spatial and temporal pattern of FoxC1 SUMOylation as well as the potential role of specific SUMO E3 ligases and SUMO proteases will likely be illuminating. In this regard, it is important to note that although we have not examined the contributions that individual SUMO proteases play in the physiological regulation of FoxC1/C2 SUMOylation, the ability of the catalytic domain of SENP2 to carry out this reaction in vitro suggests that this enzyme has the potential to participate in this process.

Interestingly, although there is complete penetrance of disease in Axenfeld-Rieger patients heterozygous for FOXC1 mutations, there is a high degree of variability in the phenotype of affected individuals. One proposed mechanism (41) is that the level or activity of FOXC1 protein produced from the unaffected allele may be reduced through post-translational modifications. Enhanced FOXC1 SUMOylation could therefore make individuals more susceptible to developing diseases associated with the syndrome whereas loss of SUMOylation could attenuate the effects of the mutation. Similarly, chromosomal duplications increasing FoxC1 gene dosage lead to significant anterior segment defects (51) and elevated FoxC1 activity gives rise to ocular abnormalities similar to loss of PitX2, which is a negative regulator of FoxC1 (52). Thus, loss of FoxC1 SUMO modification either through mutations in FoxC1 or alterations in the factors and signals controlling it could be associated with such developmental abnormalities. Since disruption of both motifs would be required, FoxC1 mutations affecting SUMOylation may be more rare than simple loss of function alterations. Mild mutations in the SC motifs of the androgen receptor however, are associated with craniofacial abnormalities. In addition, since enhanced SUMOylation is a specific response to cellular stressors such as heat shock and oxidative stress, some of the teratogenic effects of these stressors (56) could in part be due to alterations in the SUMOylation and hence the function, of critical regulators such as FoxC1 or FoxC2. Only limited information is available regarding FoxC1/C2 target genes. BMP7, SMAD2, TGF-B1, WNT6 (57) as well as FGF19 (58) have been proposed for FoxC1. Although the relevant FoxC1 targets during craniofacial development are not fully defined, Msx2 is an interesting candidate (59,60). In this regard, enhanced FoxC1 activity due to loss of SUMO modification could result in abnormal upregulation of Msx2 and could promote the development of abnormalities such as Boston type craniosynostosis.

In a similar manner, our findings showing that loss of FoxC2 SUMOylation enhances endogenous Hey1 expression in pre-adipocytes suggests that FoxC2 SUMOylation may regulate the ability of this factor to inhibit adipocyte differentiation. Interestingly, we have also shown that SUMOylation inhibits the activity of C/EBP alpha, a key positive driver of adipocyte differentiation (24). Thus, SUMOylation may have opposing effects depending on the particular function of the transcription factor. Although the exact mechanism by which FoxC2 induces Hey1 in pre-adipocytes has not been defined, an interesting parallel exists in the case of FoxC factors and the related factor Hey2 in vascular arterial specification. Hayashi et al. have provided evidence that FoxC2 activates the murine Hey2 promoter by binding to tandem fox binding elements in the proximal promoter and through synergistic interactions with factors bound to a nearby Su(H) site (61). It is therefore likely that FoxC2 SUMOylation influences Hey2 expression, especially since FoxC forms lacking the inhibitory domain are stronger activators in this context (61). It will be also interesting to examine the impact of FoxC2 SUMOylation in other physiological and pathophysiological processes regulated by FoxC2. In this regard, mammary carcinogenesis is particularly interesting in light of recent evidence showing FoxC2 expression in 85% of invasive breast carcinomas and its potential role in invasion and metastasis (62).
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In conclusion, the demonstration that FoxC1 and FoxC2 are targets of SUMOylation and that this modification mediates in large part the functional effects of their inhibitory domains reveals a novel post-translational mechanism that regulates the transcriptional activity of these key developmental and physiological regulators.

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FOOTNOTES

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2 Abbreviations used: FoxC1/FoxC2: forkhead box C1/C2; SUMO: Small Ubiquitin-like Modifier; SENP: sentrin-specific protease; EDTA: Ethylenediaminetetraacetic acid; EGTA: ethyleneglycoltetraacetic acid; NEM: N-ethylmaleimide; SC: Synergy Control.

FIGURE LEGENDS

Figure 1. FoxC1 and FoxC2 harbor conserved SC motifs. A) Consensus sequence for SC/SUMOylation motifs. Note the flanking Pro/Gly residues that precede or follow the boxed core SUMOylation site. B) Schema of human FoxC1 highlighting key structural features including the forkhead (FK) DNA binding domain, two activation domains (AD-1,2), the C terminal inhibitory domain (ID2) and the central negative region (NR) with potential SC motifs highlighted. A plot of the conservation across vertebrate species is shown below the diagram and the sequence in the vicinity of the two putative SC motifs is indicated. The canonical SC motif core is boxed and the second, more degenerate motif is underlined. The location of modified Lys residues as well as a potential phosphorylation site (asterisk) are indicated. C) An equivalent analysis as in B) is applied to FoxC2. D) Whole cell extracts from HEK 293T cells were immunoprecipitated with control IgG or a FoxC1 specific antibody. Samples were then treated with purified WT or catalytically inactive mutant SENP2 and probed with either anti-FoxC1 (left) or anti SUMO2/3 (center) antibodies. The positions of SUMOylated (open arrowhead) or the main non-SUMOylated FoxC1 (closed arrowhead) species are indicated. The ability of SENP2 to deconjugate SUMO is indicated in the right panel.

Figure 2. FoxC1 and FoxC2 are SUMOylated. Samples from HEK 293T cells transfected with expression vectors for FoxC1 A) or FoxC2 B) and the indicated proteins were immunoprecipitated with an anti-Flag antibody and were resolved in parallel by SDS-PAGE and subsequently probed with anti Flag (FoxC) and anti HA (SUMO) antibodies. Samples in the last two lanes were treated after immunoprecipitation with purified WT or catalytically inactive mutant SENP2 but before electrophoresis. Closed arrowheads indicate parental FoxC species whereas open arrowheads indicate SUMO modified forms. SUMO but not FoxC immunoreactive co-immunoprecipitating bands are indicated with asterisks. Bottom panels represent anti-HA (SUMO) probing of the inputs and reveal the overall pattern of SUMO modified proteins.

Figure 3. FoxC1 and FoxC2 are modified by three SUMO isoforms at the predicted SC motifs. A) Samples from HEK 293T cells transfected with expression vectors for the indicated FoxC1 proteins and...
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SUMO1 (left) SUMO2 (center) or SUMO3 (right) were immunoprecipitated, processed and labeled as in Fig. 2. The relative exposure time is indicated in parenthesis. Note the prominent effect of mutation (K229R) of the first motif. B) Similar analysis as in A) applied to FoxC2.

**Figure 4.** FoxC1 SUMOylation requires key residues in the first motif. A) Sequence of the WT (top) and mutant first and second motifs of FoxC1. B) Samples from HEK 293T cells transfected with expression vectors for the indicated FoxC1 proteins and SUMO2 were immunoprecipitated and processed as in Fig. 2. Open and closed arrowheads indicate SUMO modified and parental FoxC1 forms respectively.

**Figure 5.** In vitro SUMOylation of FoxC1. Samples from HEK 293T cells transfected with expression vectors for the indicated WT and mutant FoxC1 proteins were immunoprecipitated and used as substrates for in vitro SUMOylation reactions as described in Experimental Procedures in the presence or absence of ATP as indicated. Samples were subjected to SDS-PAGE and processed for immunoblotting using an anti-Flag (FoxC1) antibody. Open and closed arrowheads indicate SUMO modified and parental FoxC1 forms respectively.

**Figure 6.** Preserved SUMO modification of phosphorylation site mutants. Samples from HEK 293T cells transfected with expression vectors for the indicated WT and mutant FoxC2 proteins and SUMO2 were immunoprecipitated and processed as in Fig. 2. Open and closed arrowheads indicate SUMO modified and parental FoxC1 forms respectively. B) Samples from Xenopus oocytes injected with vehicle or synthetic mRNA encoding HA tagged FoxC1 were immunoprecipitated and treated with vehicle or λ phosphatase. Samples were subjected to SDS-PAGE and immunoblotting with an anti-HA antibody. Note the increased mobility upon phosphatase treatment. C) Location of mass spectrometry defined phosphorylation sites in mouse FoxC1. D) Samples from HEK 293T cells transfected with expression vectors for the indicated WT and mutant FoxC1 proteins and SUMO2 were immunoprecipitated and processed as in Fig. 2. The phospho-mutant lacking all identified phosphorylation sites is described in Experimental Procedures.

**Figure 7.** Loss of SUMOylation enhances the transcriptional activity of FoxC1 and FoxC2. A) HeLa cells were co-transfected as described under Experimental Procedures, with expression vectors for WT FoxC1 or the indicated mutants, a minimal luciferase reporter plasmid driven by six copies of a FoxC1 response element (pGL3-6-BS) and a control pCMV β-gal plasmid. Cells were lysed 24 h post-transfection and luciferase and β-galactosidase activities were determined as described in Experimental Procedures. The data represent the average ± SEM of at least three independent experiments performed in triplicate and are expressed as a percentage of the activity of WT FoxC1 alone. Lysates from the same cells were analyzed by western blot with antibodies against the Flag epitope to examine FoxC1 expression. B) Cells were transfected as in A) but with FoxC2 expression vectors and the pΔ6xBSDLO reporter. Samples were analyzed as in A). C) 3T3-L1 pre-adipocytes were infected with retroviruses for expression of the indicated forms of FoxC2 as described under Experimental Procedures. Protein extracts were analyzed by immunoblotting (left) with anti Flag (FoxC2) or α tubulin as a loading control. Corresponding levels of endogenous Hey1 mRNA are shown on the right.

**Figure 8.** Loss of SUMOylation does not alter subcellular localization of FoxC1 and FoxC2. HEK 293T cells grown in coverslips were transfected with A) control vector or the indicated Flag-tagged WT or SUMOylation deficient forms of B) FoxC1 or C) FoxC2. FoxC1/C2 localization as revealed by anti Flag immunofluorescence is shown in the left panels. Corresponding actin filament (phalloidin, red) and DNA (DAPI, blue) staining are shown on the right.
SUMOylation inhibits transcription factors FoxC1 and FoxC2

Figure 1

A) SC motif/SUMOylation consensus

(P/G)x_{3-4'}ΨKx{E/D}x_{3-4'}(P/G)

B) FoxC1

Conserv.

C) FoxC2

Conserv.

D) Immunoprecipitation

IP IgG FoxC1

SENP


g-FoxC1

α-FoxC1

α-SUMO2/3

α-SUMO2/3

Extract

MAT WT

MAT WT

MAT WT

MAT WT

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Figure 2

SUMOylation inhibits transcription factors FoxC1 and FoxC2
SUMOylation inhibits transcription factors FoxC1 and FoxC2

**Figure 3**
SUMOylation inhibits transcription factors FoxC1 and FoxC2
SUMOylation inhibits transcription factors FoxC1 and FoxC2
Figure 6

SUMOylation inhibits transcription factors FoxC1 and FoxC2

A) Flag-FoxC2
HA-SUMO2

α-HA (SUMO) 150
102

α-Flag (FoxC2) 78

B) HA-FoxC1
λ phosphatase

α-HA (FoxC1) 76
55

C) AD-1 FK AD-2

D) Flag-FoxC1
HA-SUMO2

α-HA (SUMO) 150
102

α-Flag (FoxC2) 76

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Figure 7

SUMOylation inhibits transcription factors FoxC1 and FoxC2
SUMOylation inhibits transcription factors FoxC1 and FoxC2

Figure 8

A) α Flag (FoxC) | Phalloidin/ DAPI
Vector

B) FoxC1
WT
K229/ K258R

C) FoxC2
WT
K213/ 226R

Figure 8
Small Ubiquitin-like Modifier (SUMO) modification mediates the function of the inhibitory domains of the developmental regulators FoxC1 and FoxC2
Theodora E. Danciu, Sergey Chupreta, Osvaldo Cruz, Jennifer E. Fox, Malcolm Whitman and Jorge A. Iñiguez-Lluhí

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