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GATA5 SUMOylation is indispensable for zebrafish cardiac development

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ABSTRACT

Background: SUMOylation is a critical regulatory protein modification in eukaryotic cells and plays a pivotal role in cardiac development and disease. Several cardiac transcription factors are modified by SUMO, but little is known about the impact of SUMOylation on their function during cardiac development.

Methods: We used a zebrafish model to address the impact of SUMOylation on GATA5, an essential transcription factor in zebrafish cardiac development. GATA5 SUMOylation was probed by western blot, the subcellular localization and transcriptional activity of GATA5 mutants were examined by immunostaining and luciferase reporter assay. The *in vivo* function of GATA5 SUMOylation was evaluated by *gata5* mutants mRNA microinjection and in situ hybridization in *gata5* morphants and *ubc9* mutants.

Results: Firstly, we identified GATA5 as a SUMO substrate, and lysine 324 (K324) and lysine 360 (K360) as two major modification sites. Conversion of lysine to arginine at these two sites did not affect subcellular localization, but did affect the transcriptional activity of GATA5. Secondly, *in vivo* experiments demonstrated that the wild type (WT) and K324R mutant of *gata5* could rescue impaired cardiac precursor differentiation, while the K360R mutant of *gata5* drastically lost this potency in *gata5* morphant. Furthermore, in SUMOylation-deficient *ubc9* mutants, the abnormal expression pattern displayed by the early markers of cardiac development (*nkx2.5* and *mef2cb*) could be restored using a *sumo-gata5* fusion, but not with a WT *gata5*.

Conclusion: GATA5 SUMOylation is indispensable for early zebrafish cardiac development.

General significance: Our studies highlight the potential importance of transcription factor SUMOylation in cardiac development.

1. Introduction

SUMOylation is a crucial post-translational modification, conserved from yeast through to humans [1,2]. The multistep enzymatic pathway of SUMOylation begins with the activation of SUMO itself by a heterodimer of SUMO-activating enzyme subunits 1 and 2 (SAE1/SAE2). Activated SUMO is then transferred to the unique E2 conjugating enzyme, Ubc9, and together with the specific E3 ligase (PIAS, RanBP2 and Pc2), SUMO is ultimately attached to a specific substrate lysine residue usually located in a consensus Ψ-K-x-D/E motif (ψ represents a hydrophobic amino acid and X represents any residue) [1,3,4]. In vertebrates, three SUMO paralogs exist, namely; SUMO1, SUMO2 and SUMO3. SUMO2/3 show a high degree of similarity to each other and are distinct from SUMO1. Whilst the different SUMO paralogs have a common conjugation function, they also have some specificities, such as subcellular distribution, SUMO chain formation properties and substrate preferences [2–4]. For example, the promyelocytic leukemia protein (PML) can be modified by all three SUMO paralogs, whilst RanGAP1 is preferentially modified by SUMO1, and topoisomerase II is SUMOylated by SUMO2/3 during mitosis [4–7].

Abbreviations: WT, wild-type; UBC9, ubiquitin-conjugating enzyme E2I; SAE1/SAE2, SUMO-activating enzyme subunit 1 and 2; PML, promyelocytic leukemia protein; SENP2, sentrin-specific protease 2; SENP5, sentrin-specific protease 5; ASDs, atrial septal defects; VSDs, ventricular septal defects; Nkx2, Nk2 homeobox; MEF2, myocyte enhancer factor 2; GATA, GATA binding protein; Tbx, T-box protein; Hand, heart and neural crest derivatives expressed; CHDs, congenital heart diseases; BAF, Brg1 associated factor; K282, lysine 281; K324, lysine 324; K360, lysine 360; RT-PCR, reverse-transcription polymerase chain reaction; RT, room temperature; TALEN, transcription activator-like effector nucleases; MO, morpholino; NBT, nitro blue tetrazolium; BCIP, X-phosphate; PIAS1, protein inhibitor of activated STAT 1; hpf, hours post fertilization; ALPM, anterior lateral plate mesoderm; *cmlc2*, cardiac myosin light chain 2; *vmhc*, ventricular myosin heavy chain; OC, outer curvature; GFP, green fluorescent protein; dpf, days post fertilization; PTM, post-translational modification; ZIC3, zic family member 3; ss, somites

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Over a thousand SUMO substrates have since been identified since the discovery of SUMO in the 1990s [8]. Protein SUMOylation is involved in a wide variety of biological processes, including transcriptional regulation, DNA replication, nuclear transport, maintenance of genome integrity and signal transduction [1]. The exact roles of SUMOylation within these diverse biological processes remain an open question.

Accumulating evidence has shown that SUMOylation plays essential roles in developmental processes and diseases including cardiogenesis, hematopoiesis and gametogenesis [9]. Cardiogenesis is a complex process that involves cardiac progenitor specification, differentiation and migration, followed by intricate tissue morphogenesis and remodeling [10]. Perturbation of any of these steps results in abnormal heart development and malformation. Mutations or transgenic alterations of components of the SUMOylation pathway have also been shown to lead to cardiac developmental defects. For instance, both SUMO1 knockout and cardiac-specific SUMO protease 2 (SENP2) transgenic mice, which both manifest reduced levels of SUMOylation in the heart, show atrial and ventricular septal defects (ASDs/VSDs) [11,12]. On the other hand, hyperSUMOylation in SENP2 null mutants result in accumulation of SUMOylated polycomb group protein, leading to hypocellular endocardial cushions and myocardium hypoplasia [13]. However, little is known about exactly how the SUMOylation pathway affects cardiac development.

Heart development is controlled by an evolutionarily conserved network of transcription factors that connect signaling pathways to the genes for muscle growth, patterning, and contractility [14]. The core cardiac transcription factor families, such as Nkx2, MEF2, GATA, Tbx, and Hand, control myocardial gene expression, cardiac cell fate, and morphogenesis [14]. To control the complex development of the heart, the activity of transcription factors and their interactions require intricate regulation. Several cardiac transcription factors have been reported as SUMO substrates. For instance, SUMOylation of Nkx2.5 can stabilize Nkx2.5 containing complexes that boost transcriptional activity and underlie the development of human congenital heart diseases (CHDs) [15]. SUMOylation can also play an important role in controlling MEF2A and MEF2C transcriptional activity [16,17]. GATA4 SUMOylation leads to enhanced transcriptional activity and altered nuclear localization [18]. Although the Nkx2.5 SUMOylation mutant K51R was tested in a mouse model [19], the exact role of *in vivo* SUMOylation of transcription factors during cardiac development remains largely unknown.

The GATA5 transcription factor is expressed in cardiac progenitor cells and the endocardium of both embryo and adult [20]. GATA5's role in cardiac development and congenital heart disease is well established. In humans, GATA5 mutation is relevant to CHD, including ASD and VSD [21,22]. In mice, loss of gata5 leads to bicuspid aortic valves [23]. The role of GATA5 is essentially conserved in zebrafish cardiac development. *Gata5* mutants show a decrease in numbers of cardiac progenitor cells and *gata5* overexpression is sufficient to produce ectopic beating tissue [24]. GATA5 cooperates with the Brg1 associated factor (BAF) chromatin remodeling complex to promote cardiac specification [25]. Recent work suggesting that GATA5 directs the efficient generation of cardiomyocytes in ESC derivatives underscores the upstream role of GATA5 in directing cardiac fate during development [26].

Herein, we show that GATA5 is a novel SUMOylated substrate. Lysine 324 (K324) and lysine (K360) are identified as major SUMOylation sites. K360 is identified as the critical GATA5 functional SUMOylation site *in vivo*, as a GATA5 K360R mutant was severely compromised in its ability to rescue the cardiac defects of a GATA5 zebrafish morphant. Furthermore, a *sumo1-gata5* fusion could partially (but effectively) restore the normal heart phenotype, whilst WT *gata5* was unable to rescue the severe cardiac defects observed in *ubc9*-deficient zebrafish embryos. Taken together, our data highlight the significance of GATA5 SUMOylation in development of the normal heart.

2. Materials and methods

2.1. Zebrafish maintenance and breeding

All experimental procedures followed the rules of the Committee on Animal Care of Shanghai, China. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University. Zebrafish were raised and maintained at 28.5 ˚C in a circulating system that continuously filters, UV treats and aerates the circulating water. The embryos were collected in dishes and reared in an incubator at 28.5 ˚C.

2.2. Generation of constructs

Gata5 and pias1 were amplified by RT-PCR with the indicated primers from the cDNA of WT zebrafish embryos (Tuebingen strain). The *gata5* PCR fragment was ligated into the *EcoR* I and *Xho* I sites, and *pias1* PCR fragment was ligated into the *Xho* I and *Xba* I sites of the pCS2 + vector, respectively. *Sumo1* and *sumo2* expression vectors have been described previously [27]. To generate the *anf* luciferase reporter, the 0.9-kb upstream fragment of the mouse *anf* gene was amplified by PCR from the genomic DNA of a WT mouse (C57BL/6 J strain), and ligated into the *Kpn* I and *Xho* I sites of the PGL3 basic vector (Promega, Maddison, WI, USA). The zebrafish *gata5* serial mutants were generated using a Quick Change Site-Directed Mutagenesis Kit (Agilent, Cedar Creek, TX, USA) with the indicated primers. For the *gata5* morphant rescue experiments, we generated MO-resistant *gata5* mRNA by changing 10 nucleotides at the binding site of translation blocking *gata5* MO to prevent direct interaction with the *gata5* MO, while keeping the GATA5 amino acids unchanged.

To generate *sumo1-gata5* fusion, flag tagged *sumo1* fragment without last 6 amino acids GGCRND was amplified from *sumo1* expression vector by PCR, then ligated in-frame into the *BamH* I and *EcoR* I sites of *gata5* expression vector. To generate *sumo2-gata5* fusion, the *sumo2-cebpa* vector, which have been described previously [28], was digested with *EcoR* I and *Xho* I to remove *cebpa*. *Gata5* was then ligated into the *EcoR* I and *Xho* I sites of this vector. The primers used were as following: *gata5*, forward (5′-ATGTATTCGAGCCTGGCTTTATCTTC-3′) and reverse (5′-GTCTCGGATCACGCTTGAGACAG-3′); *pias1*, forward (5′- AATCTCGAGATGGACTACAAAGACGATGACGACAAGCACAAGATGGCGGAGAG TGC-3′) and reverse (5′-AATTCTAGAAAAGAGCCAGGAGTTCGTCA-3′); mouse *anf* promoter, forward (5′-AAGGTACCGGTGGGACCACC ACATATTTC-3′) and reverse (5′- CACTCGAGGGGGCACGATCTGA TGTTTG-3′); *gata5* K281R, forward (5′-CAAGGCCATTAGCTATGAGAA AAGAAAGCATTCAGAC-3′) and reverse (5′-GTCTGAATGCTTTCTTTTC TCATAGCTAATGGCCTTG-3′); *gata5* K324R, forward (5′- GAAAACGC CTCTACAATAAGAAGTGAACCTAGTATCG-3′) and reverse (5′- CGGAC-ACAGGCAGTCTTCTTATTGTAGAGGCGTTTTC-3′); *gata5* K360R, forward (5′-CCATGTGGACATCAGATATGAAGACTACACATACAC-3′) and reverse (5′-GTGTATGTGTAGTCTTCATATCTGATGTCC ACATGG-3′); *gata5* rescue, forward (5′- CCGAATTCATGTACAGCTCA CTCGCATTGTCTTCCAAC -3′) and reverse (5′-GTCTCGGATCACGCTT-GAGACAG-3′). All constructs were verified by sequencing.

2.3. Cell culture and transfection

Human cardiomyocyte AC16 cells and human embryonic kidney 293T cells were cultured in DMEM/F12 and DMEM medium (Life technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Life technologies, Grand Island, NY, USA) respectively, and subcultured every two days to maintain cells in a proliferative state. For Western blot, 1 μg plasmid was transfected into 293T cells which were 80% confluent in each well of a 6-well plate using 3 μl polyjet reagent (SignaGen Laboratories, Ljamsville, MD, USA) according to the manufacturer's instructions. Lysates were generated by homogenization in 2 × laemmli buffer (Sigma-Aldrich, St. Louis, MO, USA). For the