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Acetylation of TBX5 by KAT2B and KAT2A regulates heart and limb development

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ABSTRACT

TBX5 plays a critical role in heart and forelimb development. Mutations in TBX5 cause Holt-Oram syndrome, an autosomal dominant condition that affects the formation of the heart and upper-limb. Several studies have provided significant insight into the role of TBX5 in cardiogenesis; however, how TBX5 activity is regulated by other factors is still unknown. Here we report that histone acetyltransferases KAT2A and KAT2B associate with TBX5 and acetylate it at Lys339. Acetylation potentiates its transcriptional activity and is required for nuclear retention. Morpholino-mediated knockdown of *kat2a* and *kat2b* transcripts in zebrafish severely perturb heart and limb development, mirroring the *tbx5a* knockdown phenotype. The phenotypes found in MO-injected embryos were also observed when we introduced mutations in the *kat2a* or *kat2b* genes using the CRISPR-Cas system. These studies highlight the importance of KAT2A and KAT2B modulation of TBX5 and their impact on heart and limb development.

1. Introduction

TBX5 is essential for heart and fore-limb development and mutations in this gene result in defective structures [3,21]. During heart development a number of cardiac transcription factors, most notably GATA4 and MEF2C, physically associate and functionally cooperate with TBX5 in order to control and regulate many processes taking place during cardiogenesis [9,12]. In addition to this, recent studies suggest that overexpression of these three factors is sufficient to reprogram fibroblast cells to cardiomyocyte-like cells [14]. Even though the role of TBX5 in cardiac development and reprogramming has been extensively studied, how TBX5 activity is regulated by other factors remains largely unknown.

Acetylation of histone and non-histone proteins regulates cellular physiology and affects cellular proliferation, growth and differentiation [25]. The acetylation and deacetylation status of histones ultimately controls muscle differentiation and cardiac hypertrophy [1,22]. Their activity on defined non-histone proteins has also been described [6,30]. Two major histone acetyltransferases (HATs) in mammals are KAT2B (PCAF) and p300. Although functionally similar they have distinct roles in muscle differentiation [26]. p300 is essential for embryogenesis and mice lacking a functional p300 gene die between 9 and 11.5 days of gestation. These knockout mice exhibit defects of cardiac muscle differentiation and trabeculation [37]. Furthermore, transgenic expression of p300 in the adult heart results in hypertrophy and heart failure [35]. Kat2a, a paralogue of Kat2b shares similar sequences and enzymatic activity. Although they are 73% identical and possess similar acetyltransferase activity, they have distinct but overlapping roles in mouse embryogenesis. *Kat2a*-null embryos die during embryogenesis, whilst *Kat2b* knockout mice show no obvious phenotype. Combined loss of *Kat2a* and *Kat2b* in mice leads to even more severe developmental defects, suggesting a partial functional redundancy between *Kat2a* and *Kat2b in vivo*[33]. The obvious functional disparity between *Kat2b* and *Kat2a* may be partly due to their differential expression pattern during early embryogenesis, as *Kat2b* is expressed slightly later than *Kat2a* [34]. *Kat2b* and *Kat2a* are ubiquitously expressed and most abundant in skeletal muscle. The former is also highly abundant in the heart, whereas the latter is highly abundant in pancreas [36].

Acetylation of cardiac transcription factors GATA4 and MEF2C is important for the differentiation of cardiomyocytes [18,23]. Indeed, acetylation of GATA4 triggers the differentiation of embryonic stem cells into cardiac myocytes [5]. Recent studies suggest p300 and Hdac3 regulate early cardiogenesis by modulating Tbx5 activity [19]. Here we report that both KAT2A and KAT2B physically associate with and

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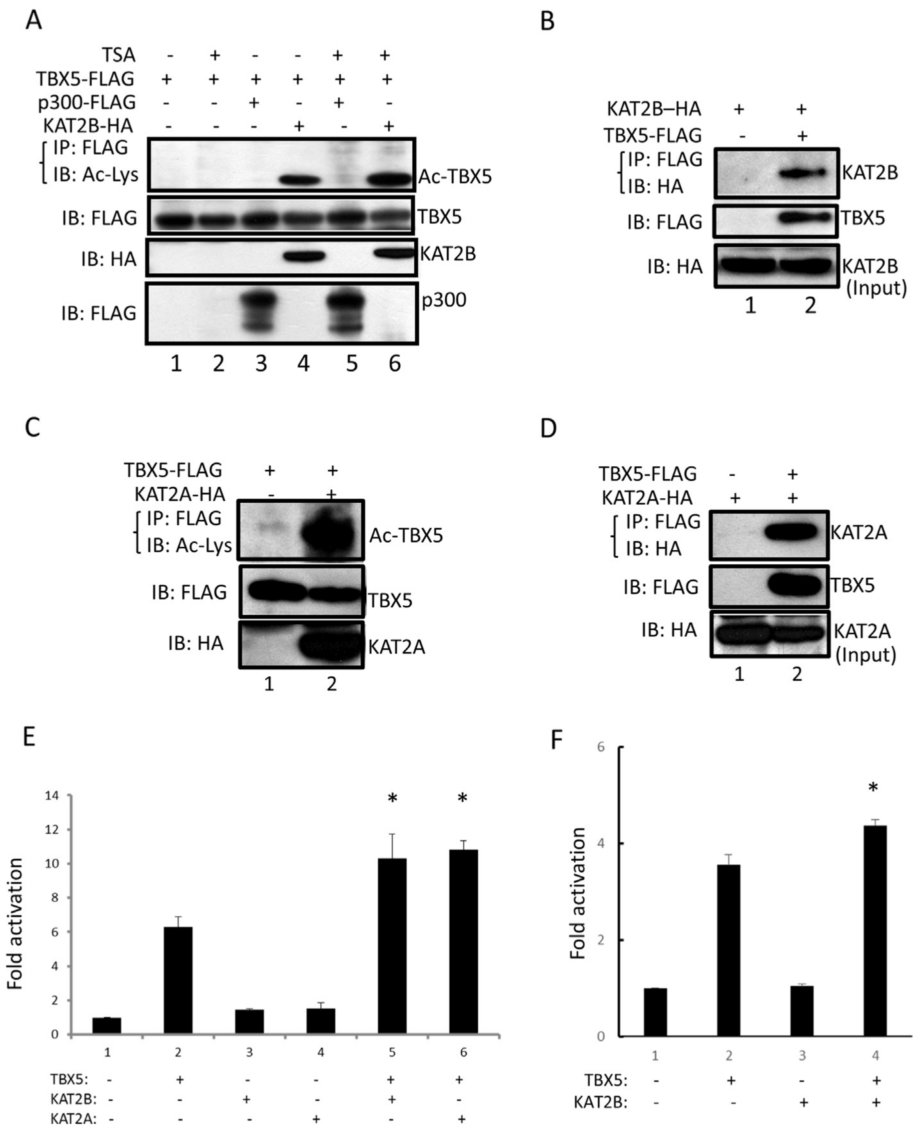

Fig. 1. TBX5 associates with, and is acetylated by KAT2B and KAT2A. (A-D) Western blots of pull down assays show that (A) KAT2B acetylates TBX5 (lane 4 and 6), whereas p300 does not (lanes 3 and 5). TSA enhances the acetylation level of TBX5 (lane 6). (B) TBX5 and KAT2B physically associate. (C) TBX5 is acetylated by KAT2A. (D) Association of TBX5 and KAT2A. (E) and (F) Reporter assays show that KAT2B and KAT2A enhance the TBX5-mediated transcription on a MYH6 promoter (E) and on an ANF promoter (F). Results are from three independent experiments. Error bars represent SD, $^{*}P < 0.05$ (Student's *t*-test). IP- immunoprecipitation, IB-immunoblot and TSA-Tricostatin A.

acetylate TBX5 at Lys339. Acetylation augments its transcriptional activity and is required for its nuclear retention. Morpholino-mediated knockdown and CRISPR-Cas-mediated knockout of *kat2a* and *kat2b* in zebrafish produces both heart and fin phenotypes similar to that observed in *tbx5a* morphants. This study reveals a role for KAT2A and KAT2B and their link with TBX5, in heart and limb development.

2. Results

2.1. TBX5 is acetylated by both KAT2A and KAT2B

In view of the role of p300 in acetylation and transcriptional regulation of GATA4, MEF2C and TBX5 we investigated whether TBX5 could be acetylated and whether this has functional consequences. Cos7 cells were transiently transfected with plasmids encoding TBX5-FLAG along with KAT2B-HA or p300-FLAG. The FLAG-tagged TBX5 proteins were affinity purified from the cell lysates using anti-FLAG-agarose beads. The bound proteins were eluted using sample buffer, fractionated on SDS-PAGE gels and subsequently immunoblotted with anti-acetylated lysine antibody. As shown in Fig. 1A, TBX5 was specifically acetylated by KAT2B, whereas p300 failed to do so. Treatment with histone deacetylase inhibitor Trichostatin A (TSA) enhanced TBX5 acetylation. In order to further confirm the TBX5 acetylation by KAT2B, we also performed *in vitro* acetylation assays in cell-free extracts using purified MBP-TBX5 and KAT2B proteins. The results suggest that MBP-TBX5 is acetylated by KAT2B *in vitro* (Fig. S1).

To demonstrate the association between TBX5 and KAT2B, pull-down experiments were conducted on the cell extracts using anti-FLAG-agarose beads. Fig. 1B shows FLAG-tagged TBX5 specifically pulled down HA-KAT2B from the lysate in which both proteins were expressed suggesting their physical association. In addition we examined whether KAT2A, a functional homologue of KAT2B could also acetylate TBX5. Similar co-expression of KAT2A and TBX5 in Cos7 cells resulted in acetylation of TBX5 (Fig. 1C) and pull-down assays also identified their physical interaction (Fig. 1D).

In promoter-reporter assays, cotransfection of plasmids encoding TBX5, KAT2B and the reporter plasmid pGL3-MYH6-I resulted in significant enhancement of TBX5-mediated transcription. Likewise, KAT2A significantly enhanced the TBX5-mediated transcription from the MYH6 promoter (Fig. 1E). We also performed similar reporter assays on two other TBX5 target promoters: ANF and CX40. The results suggest that the ANF promoter activity is significantly enhanced in the presence of KAT2B and TBX5 (Fig. 1F), whereas Cx40 promoter (-1190/+121-Luc) was not TBX5 responsive in this cell line (data not shown).

Overall, these experiments suggest that the acetylation of TBX5 is a conserved function shared between the KAT2-family of proteins.