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WTIP interacts with ASXL2 and blocks ASXL2-mediated activation of retinoic acid signaling

Farida F. Khan, Yanyang Li, Arjun Balyan, Q. Tian Wang *

Department of Biological Sciences, University of Illinois at Chicago, 900 S. Ashland Ave., Chicago, IL 60607, USA

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ABSTRACT

The Asx-like (ASXL) family proteins are chromatin factors that play dual roles in transcriptional activation and repression. ASXL2 is highly expressed in the heart and is required for proper heart development and function. Here, we identify a novel ASXL2-binding partner, the LIM domain-containing protein WTIP. Genetic and biochemical assays show a direct interaction between ASXL2 and WTIP. In HeLa cells, ASXL2 enhances retinoic acid-dependent luciferase activity, while WTIP represses it. Furthermore, WTIP blocks ASXL2's stimulatory effect on transcription. In addition, we found that ASXL2 and WTIP are expressed in mouse embryonic epicardial cells, a tissue that is regulated by retinoic acid signaling. Together, these results implicate ASXL2 and WTIP in regulation of retinoic acid signaling during heart development.

1. Introduction

The *Drosophila* Asx protein is a chromatin factor that plays dual roles in transcriptional activation and repression [1]. Asx has three mammalian homologs, Asx-like 1, 2 and 3 (ASXL1, ASXL2, and ASXL3). Mutations in ASXL family proteins have been implicated in a wide range of myeloid malignancies [2–4] and in Bohring–Opitz syndrome [5,6]. Using a mutant mouse model, we have previously shown that ASXL2 is highly expressed in the heart [7] and is involved in maintaining proper cardiac function [8]. More recently, we demonstrated a requirement for both ASXL1 and ASXL2 during normal heart development [9].

A number of studies have shown that Asx and ASXL proteins regulate chromatin configuration through functional interactions with two histone-modifying complexes, Polycomb repressive deubiquitinase (PR-DUB) [10] and Polycomb repressive complex 2 (PRC2) [11,12]. PR-DUB removes ubiquitin from mono-ubiquitinated histone H2A lysine 119 (uH2A). The deubiquitinase activity of PR-DUB was shown to be required for proper *Hox* gene repression in *Drosophila* [10]. PRC2 contains histone methyl-transferase activity and methylates lysine 27 of histone H3 (H3K27) [13]. Trimethylated H3K27 (H3K27me3) is a well-known mark of gene silencing.

The full-length ASXL2 protein contains several conserved domains separated by sequence-divergent regions. These include the HARE helix-turn-helix (HARE-HTH) predicted DNA-binding domain (aa10-88), the Asx-homology (ASXH) domain (aa215-344), a nuclear receptor (NR) binding motif (aa896-901), and the plant homeodomain (PHD) (aa1335-1366) [14–16]. In *Drosophila* Asx and mouse ASXL1, an N-terminal fragment containing HARE-HTH and ASXH was shown to mediate interaction with the histone deubiquitinase Calypso/BAP1 [10]. Consistent with this finding, our lab has shown that ASXL2 and BAP1 interact *in vivo* to regulate deubiquitination of uH2A [11]. We and others have also shown that ASXL1/2 interact with PRC2 and regulate PRC2 recruitment to target loci [11,12]. However, it is unclear which region of ASXL mediates this interaction.

There is also evidence that ASXL proteins interact with nuclear receptors. For example, ASXL1 has been shown to interact with the retinoic acid receptor RARα via an NR binding box and act as either co-activator or co-repressor of RA signaling in a cell type-dependent manner [16,17]. RA signaling plays important roles during heart development by regulating chamber formation and cardiomyocyte proliferation and maturation [18–20]. Since our lab has previously shown that ASXL2 is predominantly expressed in the heart [7] and required for heart morphogenesis [9], we hypothesize that ASXL2 may regulate RA signaling.

Here we present a study aimed at a better understanding of the functional mechanism of ASXL2. First, we screened for proteins that interact with ASXL2 outside the N-terminal region. We discovered

Abbreviations: Asx, additional sex comb; ASXH, Asx-homology; ASXL, additional sex comb-like; ATRA, all *trans*-retinoic acid; HARE-HTH, HARE helix-turn-helix; H3K27me3, trimethylated histone H3 lysine 27; NR, nuclear receptor; PHD, plant homeodomain; PRC2, Polycomb repressive complex 2; PR-DUB, Polycomb repressive deubiquitinase; RA, retinoic acid; RAR, retinoic acid receptor; uH2A, mono-ubiquitinated histone H2A; WTIP, Wilms tumor 1-interacting protein.

* Corresponding author.
E-mail address: qtwang@uic.edu (Q.T. Wang).

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genetic and biochemical evidence for a direct interaction between ASXL2 and Wilms Tumor 1-Interacting Protein (WTIP), a member of the ZYXIN family of LIM domain-containing proteins [21]. Secondly, we investigated the effect of ASXL2, with or without WTIP, on RA signaling.

2. Materials and methods

2.1. Plasmids

For yeast two-hybrid experiments, the following ASXL2 cDNAs were cloned in frame into pGBKT7 vector (Clontech), and expressed as GAL4-DBD fusion proteins: full-length ASXL2, N-terminal region ($ASXL2_{1-599}$), C-terminal region ($ASXL2_{600-1370}$), C-terminal region missing the PHD domain ($ASXL2_{600-1295}$), and PHD region alone ($ASXL2_{1313-1370}$). The prey plasmid isolated from the initial screen, pACT2-$hWTIP_{245-430}$, was used in all confirmation matings.

For co-immunoprecipitations (co-IPs) and luciferase assays, FLAG-$ASXL2_{720-1370}$ was inserted into pcDNA3 vector (Invitrogen). Myc-WTIP, Myc-NΔWTIP, and Myc-NΔZYXIN [22] were generous gifts from Dr. John R. Sedor (Case Western Reserve University). The WTIPΔLD2,3-HA construct [23] was a gift from Dr. Sigmar Stricker (Berlin, Germany). The LD2,3-HA construct was generated by inserting WTIP cDNA into pCS2 destination vector (gift from Dr. Hua Jin, University of Illinois at Chicago). The following reporter constructs were used in luciferase assays: RARE-tk-luciferase (gift from Dr. Rene Bernards, Netherlands Cancer Institute) and pSV-β-gal (Promega).

For GST-pulldown assays, GST-NΔWTIP [22] was a gift from Dr. Sedor (Case Western Reserve University). GST-PHD was constructed by inserting the PHD region of ASXL2 (aa1332-1370) into pGEX-6P-1 vector (GE Healthcare). For radiolabeling, $ASXL2_{600-1370}$, $ASXL2_{600-1295}$, and pcDNA3-WTIP [23] (gift from Dr. Stricker, Berlin, Germany) were used.

2.2. Yeast two-hybrid assays

The initial yeast two-hybrid screen was conducted using the Matchmaker System (Clontech). Bait plasmid pGBKT7-$ASXL2_{600-1370}$ was used to screen a pretransformed human heart cDNA library in pACT2 vector (Clontech). Mating and colony selection was performed according to the user's manual (Clontech). For mapping of interacting domains, bait and prey constructs were individually transformed into AH109 and Y187 yeast strains, respectively, and mated.

2.3. Co-immunoprecipitation

For co-IP experiments, mammalian HEK293 cells cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone) were transiently transfected with equal amounts of DNA using branched polyethylenimine (Sigma–Aldrich). Cells were harvested 48 h later and lysed in lysis buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 3% Triton-X 100) supplemented with protease inhibitor cocktail (Calbiochem). After centrifugation, the supernatants were used as input for IP and incubated with anti-FLAG M2 magnetic beads (Sigma) or anti-Myc agarose beads (Clontech). Immunoprecipitates were washed extensively in wash buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl) and bound proteins were eluted. Whole cell lysate, input, and IP fractions were used for SDS–PAGE followed by Western blot analysis.

2.4. GST-pulldown assay

GST and GST-fusion proteins were expressed in *Escherichia coli*. Radiolabeled proteins were obtained *in vitro* using the TNT Coupled Reticulocyte Lysate System (Promega) in the presence of [^{35}S]-methionine (Perkin–Elmer). [^{35}S]-labeled proteins were incubated with GST or GST-fusion proteins immobilized on glutathione-Sepharose 4 Fast Flow beads (GE Healthcare) supplemented with protease inhibitor cocktail (Calbiochem) and 100 μM ZnCl (Sigma). After incubation, the beads were washed extensively and bound proteins were separated via SDS–PAGE and visualized by autoradiography.

2.5. Luciferase reporter assays

HeLa cells were seeded in 24-well plates and transfected with RARE-tk-luc, pSV-β-gal, and constructs for ASXL2, WTIP or both using branched polyethylenimine (Sigma–Aldrich). Total amount of DNA was kept constant using pcDNA3 empty vector. After 48 h, cells were fed with DMEM containing 5% charcoal-stripped FBS (Life Technologies) and incubated overnight with or without 0.2 μM ATRA (Sigma). Cells were lysed using 1× passive lysis buffer (Promega) and luciferase activity was measured according to the Luciferase Reporter Assay (Promega). β-Galactosidase activity was assayed to normalize for transfection efficiency.

2.6. Immunostaining

Primary embryonic epicardial cells were isolated from E11.5 mouse hearts and cultured in DMEM supplemented with 10% FBS and primocin as described previously [24]. After 48 h, cells were fixed in methanol and stained with anti-WTIP polyclonal antibody (sc-241738; Santa Cruz Biotechnology) and visualized with Texas-Red-conjugated secondary antibody. Cells were double stained with Hoescht 33342 nuclear stain (Life Technologies).

E18.5 mouse heart frozen sections were fixed in methanol and stained with anti-WTIP antibody as mentioned above.

2.7. Reverse transcription (RT)-PCR

Total RNA was extracted from immortalized mouse embryonic epicardial cells [24] using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized and amplified from 200 ng of total RNA using OneStep RT-PCR Kit (Qiagen) with primers specific to *Asxl2*: forward, 5′-CGAGCACTGATCAACAAGCAC-3′, reverse, 5′-TCTTGTCGAATTCT-CACCTGC-3′ or *Actin* control: forward, 5′-TCACCCACACTGTGCC-CATCT-3′, reverse, 5′-TGGTGAAGCTGTAGCCACGCT-3′.

3. Results

3.1. Genetic interactions between WTIP and ASXL2 in yeast

To identify protein partners that interact with ASXL2 outside of the N-terminus, a yeast two-hybrid screen was conducted using a cDNA corresponding to the C-terminal region ($ASXL2_{600-1370}$) (Fig. 1A). One of the positive clones isolated encoded a fragment of the human WTIP (Fig. 1B). WTIP contains an N-terminal proline-rich region with putative SH3 binding sites and a C-terminal region with three LIM domains [22]. The $ASXL2_{600-1370}$-interacting clone, hereafter referred to as $hWTIP_{245-430}$, encoded partial LIM 1 but complete LIM 2 and 3 of WTIP.

Confirmation matings showed that $hWTIP_{245-430}$ strongly interacts with $ASXL2_{600-1370}$, but not $ASXL2_{1-599}$ or full-length protein (Fig. 1C and D). Furthermore, strong interaction was detected with