

Ash2l interacts with Tbx1 and is required during early embryogenesis

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Abstract

TBX1 encodes a DNA binding transcription factor that is commonly deleted in human DiGeorge syndrome and plays an important role in heart development. Mechanisms of Tbx1 function, such as Tbx1 interacting regulatory proteins and transcriptional target specificity, are largely unknown. Ash2l is the mammalian homolog of *Drosophila* Ash2 (absent small homeotic 2) and is a core component of a multimeric histone methyltransferase complex that epigenetically regulates transcription via methylation of histone lysine residues. We undertook an unbiased yeast two-hybrid screen to look for functionally relevant Tbx1-interacting proteins and report a physical and functional interaction between Tbx1 and Ash2l. Tbx1 interacts with Ash2l in both yeast and mammalian cells and Ash2l acts as a transcriptional co-activator in luciferase reporter assays. Expression analysis shows that Tbx1 and Ash2l have overlapping mRNA and protein expression patterns during development. By generating an *Ash2l* knockout mouse utilizing gene-trap technology, we show that although *Ash2l* heterozygous mice are normal, *Ash2l*-null embryos die early during gestation. Thus, Ash2l is required for the earliest stages of embryogenesis. Furthermore, our finding of a physical interaction between Tbx1 and Ash2l suggest that at least some functions of Tbx1 may be mediated by direct interactions with a histone methyltransferase complex.

Keywords: DiGeorge syndrome, epigenetics, chromatin, development, transcription

Experimental Biology and Medicine 2010; **235**: 569–576. DOI: 10.1258/ebm.2010.009318

Introduction

DiGeorge syndrome is a common disorder with a high incidence of congenital heart defects and accumulating data suggest that the Tbx1 transcription factor plays a critical developmental role in its pathogenesis. Animal studies using mouse models have implicated Tbx1 as a critical gene within the commonly deleted region of chromosome 22 and three different *TBX1* mutations have been described in families with the classical features of DiGeorge syndrome but without evidence of a chromosomal deletion at 22q11.¹ One of these mutations occurred in the highly conserved T-box DNA binding domain. This domain is not only important for the DNA binding functions of the transcription factor, but is also known to mediate the interaction with transcriptional co-regulators. For example, the homeo-domain transcription factor, Nkx2.5, and the zinc-finger

transcription factor, Gata4, interact with the T-box domain of Tbx20² and Tbx18³ to modulate transcriptional pathways important for cardiac development. The Tbx5 T-box also interacts with Nkx2.5.⁴ In addition, the Tbx18 T-box interacts with the paired-box transcription factor, Pax3, to regulate mesodermal differentiation during somitic development.⁵

Mechanisms regulating the specificity of T-box protein-dependent transcriptional activation are not entirely clear. In part, this may be dependent on specific DNA sequence in regulatory regions of direct downstream targets. The consensus *in vitro* DNA binding site is known for some T-box proteins, including Tbx15, Tbx18 and Brachyury (T).^{3,6–9} Brachyury binds optimally as a dimer to a palindromic DNA sequence containing 24 basepairs,^{7,8} but is also able to bind as a monomer to half of the palindromic DNA

sequence, the T/2 site. Many T-box family members bind to the full T site and the T/2 site with varying affinities,^{3,7} although this binding affinity does not correlate with the ability to mediate transcription. Therefore, DNA binding alone is not sufficient to explain the T-box protein specificity. T-box proteins physically interact with other transcription factors,^{2-5,10-15} mediated by domains outside the T-box, which provides another layer of specificity in their transcriptional regulation. Another exciting observation is that further specificity of T-box protein transcriptional regulation may be conferred through association with epigenetic modification complexes that result in chromatin changes and permissive transcriptional states.¹⁶⁻¹⁸

We undertook a search for functionally relevant Tbx1-interacting proteins to explore mechanisms of transcriptional regulation relevant to the pathogenesis of DiGeorge syndrome and the biology of heart development. In this study, we report a physical and functional interaction between Tbx1 and Ash2l. Ash2l is the mammalian homolog of the *Drosophila* protein Ash2 (absent small homeotic 2). Ash2l is a core component of a multimeric histone methyltransferase (HMT) protein complex^{19,20} and may also function as an oncoprotein.²⁰ HMT complexes modulate transcription by catalyzing the methylation of arginine or lysine residues of N-terminal tails of core histones. In general, lysine methylation functions to modulate transcription and DNA repair, whereas arginine methylation is only known to alter transcription. For example, in the case of dimethylation or trimethylation of core histone 3 lysine 4 (H3K4) residues, these histone modifications functionally result in chromatin unfolding, or euchromatin, and facilitate transcription activation.²¹ These epigenetic modifications play a critical role in myriad biological events from development to oncogenesis.

Materials and methods

Yeast two-hybrid assay

Mouse *Tbx1* was cloned into the pGBKT7 vector (Clontech, Mountain View, CA, USA) and was used as bait to screen a pretransformed mouse cDNA E11 library (Clontech). The AH109 yeast strain was transformed with the pGBKT7-Tbx1 plasmid and mated with the pretransformed Y187 yeast strain. Transformants were screened under high stringency using SD/-Trp/-Leu/-His/-Ade growth conditions. To verify the yeast interactions, individual candidate clones and pGBKT7-Tbx1 were co-transformed into AH109 yeast and grown under the same high stringency conditions with the addition of X- α -gal.

Cell culture and immunoprecipitation

JEG3 cells (HTB-36; ATCC, Manassas, VA, USA) were grown in Roswell Park Memorial Institute-1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. Just prior to transfection, RPMI-1640 media were replaced with OptiMEM (Invitrogen) media. Cells at 60–80% confluence were transfected with 2 μ g of plasmid DNA per 10 cm dish using the FuGENE HD (Roche, Indianapolis, IN, USA) transfection reagent. Transfection complex was prepared

at 4:1 reagent:DNA ratio in 500 μ L OptiMEM per dish. Growth media were replaced with RPMI-1640 18–24 h after transfection and cells were harvested 48 h after transfection. Whole-cell lysates were extracted with lysis buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 20 mmol/L tetrasodium pyrophosphate, 10 mmol/L EDTA and 1% Triton X-100) containing protease inhibitors (Roche, Indianapolis, IN, USA). Cell lysates were incubated with 5 μ g rabbit anti-V5 (Sigma-Aldrich, St Louis, MO, USA) or rabbit IgG antibodies. Immunoprecipitated protein was analyzed by Western blot with mouse anti-myc antibody (1:5000; Invitrogen).

Vector construction and gene targeting

Vector construction and mouse AK7.1 embryonic stem (ES) cell targeting was performed as part of the Soriano Laboratory Gene-Trap Project (<http://www.drbsinai.org/soriano/db5.html>).^{22,23} The *Ash2l*-targeted AK7.1 ES cells correspond to Clone ID# S3-11A1 and utilized the ROSAFARY gene-trap vector.²² ES cell retroviral infection, electroporation, 3' rapid amplification of cDNA ends (RACE) and 5' genomic sequencing have been described previously.²³ The targeted ES cells were injected into C57BL/6J blastocysts and resulting chimeric mice were bred to produce germline offspring that were used for these studies. These mice were maintained on a mixed C57BL/6J;129 genetic background. The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all animal protocols.

Southern blot

Southern blot hybridization was performed on genomic DNA from ES cells digested with *HindIII*, *PstI* and *EcoRI*. The 5' flanking probe used detects a 10.8-, 6.4- or 10.8-kb wild-type band and a 1.5-, 4.5-, or 3.9-kb targeted band, respectively, for each of the three restriction enzymes.

Genotyping

Genotyping of *Ash2l* gene-trap mice was performed using standard polymerase chain reaction (PCR) techniques using an annealing temperature of 60°C for the wild-type allele and 62°C for the mutant allele. The following genotyping primers were used: Ash2l-F5 – 5'-CGTCTTTTCCACCCACA ACTACC-3' and Ash2l-R9 – 5'-ACCAGGACCCGCTCCA GC-3' for the wild-type allele; and Ash2l-F5 and Ash2l-U5R2 – 5'-CTGTTCCCTTGGGAGGGTCTC-3' for mutant allele. The resulting wild-type and mutant PCR products were 572 and 252 basepairs, respectively. For blastocyst genotyping, genomic DNA was amplified by isothermic multiple displacement amplification with the GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Piscataway, NJ, USA).

Reverse transcription PCR

Total RNA was extracted and purified from ES cell colonies with Trizol (Invitrogen) and the RNeasy mini kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized

using random hexamers with the SuperScript III system (Invitrogen). *Ash2l* and β geo* reverse transcription (RT)-PCR primer sequences are indicated in supplemental Tables S1 and S2.

Immunohistochemistry, radioactive *in situ* hybridization

Horseradish peroxidase immunostaining was performed as described previously²⁴ using anti-Ash2 A300-112A (1:100; Bethyl Laboratories, Montgomery, TX, USA). *In situ* hybridization was performed as described previously.^{25,26} Protocols are available at <http://www.med.upenn.edu/mcrrc>.

Luciferase assays

JEG3 cells were grown in six-well dishes to 50–80% confluency. The *TBX1* and *Ash2l* expression constructs were co-transfected with the brachyury (T) half-site luciferase reporter (2xT-tk-GL2)²⁷ and pRL-TK reporter vectors (Promega, Madison, WI, USA) using Lipofectamine transfection reagent (Invitrogen). Firefly and *Renilla* luciferase activity were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's recommended protocol. All results are normalized for transfection efficiency and represent the average of nine experiments.

Results

Tbx1 interacts with Ash2l in a yeast two-hybrid assay

In order to identify novel Tbx1-interacting proteins, we performed a yeast two-hybrid assay using full-length mouse Tbx1 protein as bait and a mouse embryonic (E) day 11 cDNA library as prey. The mouse *Tbx1* coding region was subcloned into the pGBKT7 vector and transformed into AH109 yeast. Yeast mating was carried out between these transformed AH109 yeast cells and the pretransformed cDNA library. Mated yeast cells were grown under high stringency conditions. The interaction screen was performed in triplicate and over 900 colonies grew. Plasmid DNA was isolated from 86 colonies. After sequencing and translation, protein sequence comparison was made between the GAL4 fusion protein of the sequenced clone and public protein databases. The translated proteins aligned to 25 entries in the online databases. Thus, these 25 proteins were subjected to further verification in yeast.

The pGBKT7-Tbx1 vector and the plasmid of candidate clones were co-transformed into AH109 yeast cells and grown on SD/-Trp/-Leu/-His/-Ade plates containing X- α -gal. The pGBKT7 vector alone was used as negative control. Only those plasmid clones that allowed yeast survival when co-transformed with the pGBKT7-Tbx1 plasmid but not with control pGBKT7 vector were considered to interact with Tbx1 (Figure 1a). As a result, 20 proteins were identified to have an interaction with Tbx1 in yeast. Among them, eight colonies with high galactosidase activity were chosen for further analysis. The

clones included Ash2l, Mnat1, Rhox5, Psap, Sox11, Tmem55a, Wdr68 and Tsen54. Ash2l was particularly intriguing, as two non-overlapping interacting domains were independently found to interact with Tbx1 in our yeast two-hybrid screen. Ash2l is the vertebrate homolog of *Drosophila* Ash2 and has been implicated in histone methylation and resulting transcriptional regulation as part of an HMT complex.

Tbx1 physically interacts with Ash2l in mammalian cells

To further show that Ash2l may be an important co-factor for Tbx1, we confirmed the interaction in mammalian cells. V5-tagged full-length Tbx1 and myc-tagged Ash2l mammalian expression constructs were generated. V5-tagged Tbx1 was transiently co-transfected with either myc-tagged Ash2l or with control plasmids into JEG3 chorion carcinoma cells. These cells have previously been shown to allow for robust activation in functional Tbx1 assays.²⁷ This is presumably due to the presence of transcriptional co-factors important for Tbx1-dependent activation. After precipitating with either rabbit anti-V5 antibody or rabbit IgG from whole-cell extracts, samples were analyzed by Western blotting with mouse anti-myc antibody. Full-length Ash2l specifically interacts with full-length Tbx1, while there was no non-specific binding in control experiments (Figures 1b–d). The known functional domains of Ash2l include a conserved plant homeodomain (PHD) finger, a SPLa/Ryanodine receptor (SPRY) motif²⁸ and a bipartite nuclear localization signal.²⁹ PHD domains bind to histone-methylated lysine residues (e.g. H3K4) resulting in conformational changes in chromatin structure and changes in transcriptional activity.³⁰ The SPRY domain is an evolutionarily ancient motif present in many divergent protein families. It may be involved in protein–protein interactions, although its function is largely unknown.³¹ We further characterized the Tbx1–Ash2l interaction by mapping the minimal interacting domain of Ash2l by co-immunoprecipitation (summarized in Figure 1e). We transiently expressed full-length Tbx1 with various truncations of Ash2l in JEG3 cells and performed co-immunoprecipitations with whole-cell extracts. Deletion of the PHD domain (Δ 100–150) has no effect on the Ash2l–Tbx1 physical interaction, but a larger deletion (Δ 6–290) abolishes this interaction, suggesting that Ash2l amino acids 150–289 are required for the interaction with Tbx1 (Figure 1b). The 150–289 interaction domain is sufficient to mediate the interaction with Tbx1 (1–310, 1–405), and when deleted (1–145), the interaction is abolished (Figure 1c). This region does not include the SPRY domain. This observation was confirmed by experiments with SPRY deletion constructs (SPRY del, 1–405) to confirm that this domain is not required for the interaction with Tbx1 (Figure 1c).

Ash2l functionally interacts with Tbx1

To determine the contribution of Ash2l in Tbx1-mediated transcriptional activity, we transiently co-transfected JEG3 cells with Tbx1 and Ash2l in luciferase reporter assays.

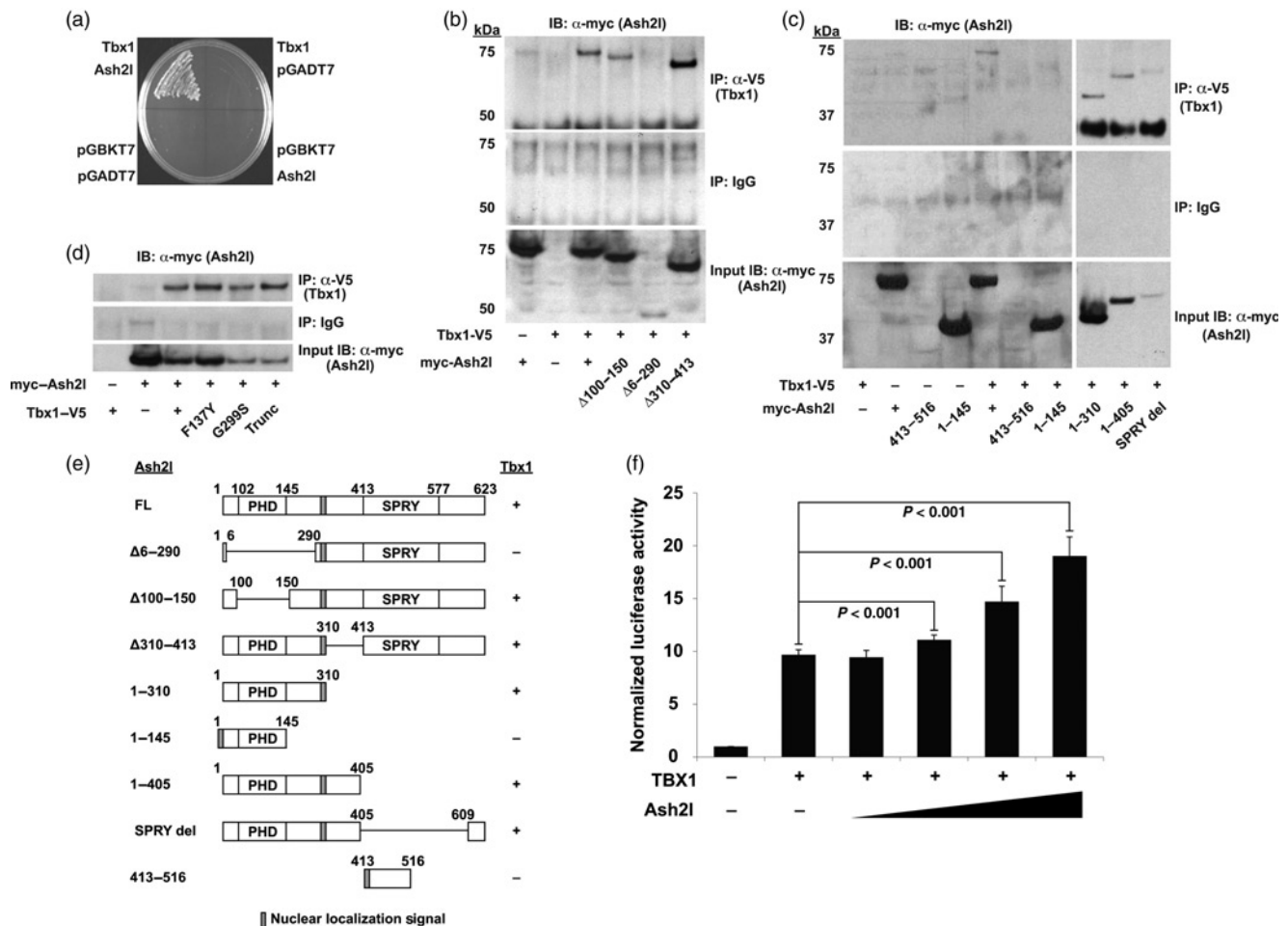


Figure 1 Tbx1 physically and functionally interacts with Ash2l in yeast and mammalian cells. (a) Tbx1 fused to the GAL4 DNA-BD interacts with Ash2l fused to the GAL4-AD allowing for yeast survival. (b) Full-length Tbx1-V5 co-immunoprecipitates with full-length myc-Ash2l and the myc-Ash2l mutants $\Delta 100-150$ and $\Delta 310-413$, but not $\Delta 6-290$. Protein was extracted from transiently transfected JEG3 whole-cell lysates. Immunoprecipitations were performed with anti-V5 or control IgG and complexes were analyzed by Western blot using HRP-conjugated anti-myc antibody. (c) Full-length Tbx1-V5 co-immunoprecipitates with full-length myc-Ash2l and the myc-Ash2l mutants 1-310, 1-405 and SPRY del but not 1-145 or 413-516. Methods were identical to those described in (b). (d) Full-length myc-Ash2l co-immunoprecipitates with full-length Tbx1-V5 and the Tbx1-V5 mutants F137Y, G299S and Trunc (Tbx1delC; Stoller and Epstein³³). Methods were identical to those described in (b). (e) Schematic representation of full-length and mutant Ash2l constructs used to map the Ash2l interaction domain. The ability (+) or inability (-) of full-length Tbx1 to immunoprecipitate the Ash2l proteins is indicated. (f) Transcriptional co-activation of a luciferase reporter by TBX1 and Ash2l. Transient transfection of JEG3 cells with Ash2l augments TBX1-dependent transcriptional activation in a dose-dependent manner. Results are normalized for transfection efficiency and are expressed as mean $\pm$ SD of nine experiments. The statistical significance of differences between groups was analyzed by the paired Student's *t*-test. BD, binding domain; AD, activation domain; HRP, horse radish peroxidase; SD, standard deviation

Transient transfection of Tbx1 in JEG3 cells results in a 10-fold activation of the 2xT-tk-GL2²⁷ over control levels. The Ash2l-containing HMT complex has previously been associated with transcriptional activation,^{20,28,32} and consistent with the hypothesis that Ash2l is a Tbx1 co-factor, we show that co-transfection of Ash2l augments this activation in a dose-dependent manner (Figure 1f). This provides evidence that not only do Ash2l and Tbx1 physically interact, but in addition they functionally act as transcriptional activators.

Known human missense mutations do not affect the physical interaction between Tbx1 and Ash2l

Prior studies in our lab showed that a human frame-shift mutation in *TBX1* results in the absence of a critical

nuclear localization signal.³³ The mechanism by which two other human *TBX1* missense mutations lead to a functional defect was not fully elucidated, although other investigators subsequently reported that these mutations might be gain-of-function mutations.²⁷ To determine if these missense mutations result in an altered interaction with Ash2l, we performed further immunoprecipitation analysis. We transiently expressed myc epitope-tagged Ash2l with either full-length V5-tagged Tbx1 or Tbx1 mutants in JEG3 cells, immunoprecipitated with anti-V5 antibody or IgG, and then detected precipitated Ash2l with anti-myc antibody. As shown in Figure 1d, these mutants retain the ability to interact physically with Ash2l. Although the mutations are associated with human DiGeorge syndrome, the mechanism is not due to an alteration in the interaction of Tbx1 with an Ash2l HMT complex.

Ash2l expression overlaps with areas of Tbx1 expression in the developing mouse embryo

If Tbx1 and Ash2l functionally interact *in vivo*, then they should be co-expressed. Hence, we performed Ash2l expression analysis for comparison with the well-documented expression patterns of Tbx1. Tbx1 is expressed in early embryonic pharyngeal endoderm, the somite, head mesenchyme and in the cardiac outflow tract myocardium (Figure 2).^{34–36} We analyzed wild-type mid-gestation mouse embryos by *in situ* hybridization and by immunohistochemistry to look at overall Ash2l expression patterns. Immunostaining and *in situ* hybridization analysis revealed that Ash2l, both at the mRNA and the protein level, is broadly expressed, including in Tbx1 expression domains (Figure 2). These immunohistochemistry experiments also reveal that Ash2l is a nuclear protein with little or no cytoplasmic expression. Immunostaining experiments utilizing an antigen-specific blocking peptide as negative control supported the specificity of this staining pattern (data not shown). These Ash2l expression data are consistent with prior reports.^{28,29}

Ash2l is critical for the earliest stages of embryonic development

To determine the effect of the loss of Ash2l in the mouse, we generated Ash2l knockout mice utilizing gene-trap targeted ES cells. The Ash2l gene-trap ES cell line harbors a promoter trap whereby a promoter-less cassette (β geo*) comprised of a LacZ-neomycin fusion downstream of an adenoviral splice acceptor site integrates under the control of an endogenous promoter and functions as an artificial 3' terminal exon.²² Analysis of 5' genomic sequence and 3' RACE sequence revealed that the retroviral gene-trap cassette inserted between the alternative exons 1a and 1b of Ash2l (Figure 3a). Blastocyst injections with Ash2l gene-trap ES cells resulted in a chimeric mouse that was capable of germline transmission. The resulting progeny were genotyped by

PCR. To confirm the predicted splicing event between exon 1a and the β geo* cassette, we extracted mRNA from targeted and wild type ES cells derived from healthy heterozygous adult gene-trap mice (see below) and analyzed them by RT-PCR. The predicted exon 1a- β geo* species was present only in targeted cells, but not in wild-type cells (Figure 3b), as predicted. In addition, there was no exon 1b- β geo* species, showing that this gene-trap only directly targeted one isoform of Ash2l. Comparison of the relative levels of isoforms 1a and 1b mRNA in ES cells showed that the 1a isoform is predominant during early development (Figure 3c). In Ash2l targeted ES cells, there was equal mRNA expression of the two isoforms, suggesting a compensatory upregulation of the 1b isoform upon disruption of the transcription of the 1a isoform. We further confirmed the targeting by analyzing Ash2l gene-trap ES cells by Southern blot of genomic DNA digested with three different restriction enzymes. While this Southern analysis of wild-type ES cells revealed a single genomic fragment, there was an additional predicted fragment for each of the restriction enzymes in heterozygous ES cells, thus confirming the targeting at the Ash2l locus (Figure 3e).

Mice heterozygous for Ash2l were born without phenotypic abnormalities in expected Mendelian ratios and survived well past one year of age with normal fertility (data not shown). To determine the phenotype of the homozygous knockout state, we performed multiple crosses of Ash2l heterozygous mice. Of 80 total live born pups 30/80 (37%) were wild type, 50/80 (63%) were heterozygous and 0/80 (0%) were homozygous for the Ash2l gene-trap (Table 1). To establish at what stage of development loss of Ash2l results in embryonic lethality, we crossed heterozygous mice and harvested embryos at progressively earlier stages of development. We were unable to identify null embryos at any postimplantation stage tested (Table 1). We then isolated individual blastocysts, amplified genomic DNA by multiple displacement amplification and performed Ash2l genotyping by PCR. At this early stage of development, we were able to identify null blastocysts at

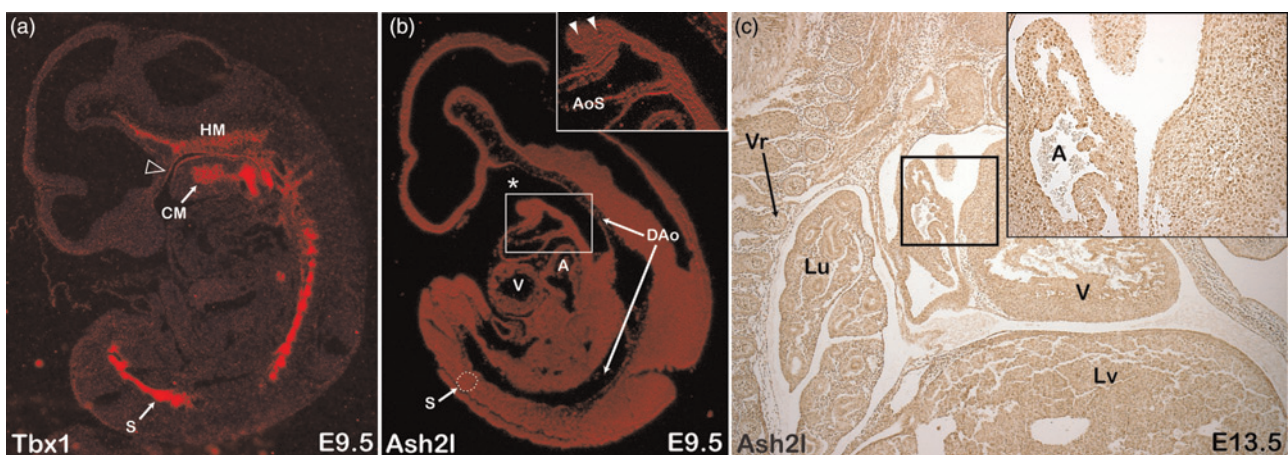


Figure 2 Ash2l is broadly expressed in the mid-gestation mouse embryo. (a) Parasagittal section of E9.5 embryo showing Tbx1 expression by *in situ* hybridization. (b) Parasagittal section of E9.5 embryo showing Ash2l expression by *in situ* hybridization. (c) Parasagittal section of E13.5 embryo showing Ash2l protein expression by immunohistochemistry. Head mesenchyme (HM), pharyngeal arch core mesoderm (CM), pharyngeal endoderm (open arrowhead), ventricle (V), atria (A), somite (S), aortic sac (AoS), dorsal aorta (DAo), pharyngeal region of foregut diverticulum (asterisk), origin of first and second aortic arch arteries (closed arrowheads), vertebra (Vr), lung (Lu) and liver (Lv) are indicated for reference. E, embryonic age

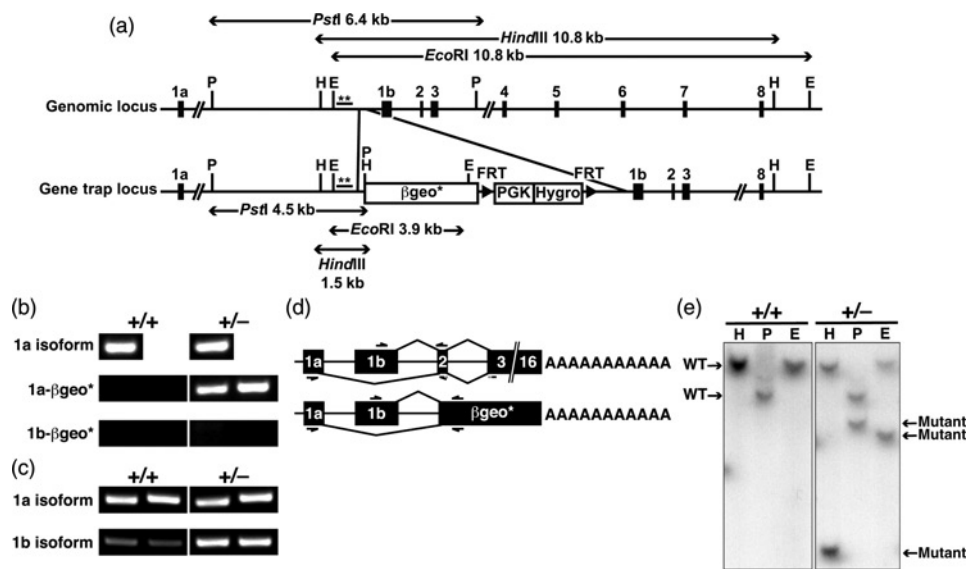


Figure 3 Generation of Ash2l gene-trap knockout mice. (a) Targeting strategy of Ash2l gene-trap. The ROSAFARY retroviral cassette inserted between Ash2l exons 1a and 1b. The expected sizes of the HindIII (H), PstI (P) and EcoRI (E) digests recognized by the indicated probe (**) are shown. (b) Confirmation of predicted Ash2l exon 1a-βgeo* splicing. RT-PCR shows the exon 1a-βgeo* species in heterozygous, but not wild-type (WT) Ash2l ES cells. An exon 1b-βgeo* species is not present in either heterozygous or WT cells. (c) Ash2l isoform 1a is predominant in ES cells. RT-PCR shows that the Ash2l 1a isoform is more highly expressed in WT cells compared with the 1b isoform. In heterozygous Ash2l ES cells, there is equal expression of the two isoforms. (d) Schematic representation of alternative splicing. Ash2l exons 1a and 1b splice with exon 2. In Ash2l gene-trap mice, exon 1a splices with βgeo*. Exon 1b-βgeo* splicing is shown for illustration. Primers used for RT-PCR are indicated. (e) Genomic Southern blot of WT (+/+) and targeted Ash2l gene-trap ES cells (+/-). WT (10.8, 6.4 or 10.8 kb) and mutant (1.5, 4.5 or 3.9 kb) bands are shown, respectively, for the HindIII, PstI and EcoRI restriction enzyme digests. RT-PCR, reverse transcription-polymerase chain reaction; ES, embryonic stem

close to Mendelian ratios (Table 1). We then attempted to generate blastocyst-derived Ash2l null ES cells for functional analysis. Although we were able to derive multiple wild-type and heterozygous ES cell lines (data not shown), we were unable to isolate any null ES cell lines. This underscores the notion that Ash2l is essential for the earliest stages of embryonic development.

Discussion

Epigenetic mechanisms of gene regulation are important during embryogenesis. Some of these epigenetic mechanisms include DNA methylation and histone modifications such as histone acetylation, phosphorylation and methylation. The trithorax group (TrxG) was originally defined in *Drosophila* as a group of proteins required for the positive regulation of *Hox* genes and which functionally oppose the Polycomb group of transcriptional repressors.³⁷ The TrxG proteins are a heterogeneous group of chromatin modifying complexes and include the MLL (mixed-lineage leukemia), NURF, TAC1, Ash1 and SWI/SNF complexes.^{38,39} The specific chromatin modification can vary, but some TrxG

complexes function as HMTs and mediate the methylation of H3K4. The SET (Su(var)3-9, enhancer of zeste, Trx) domain catalyzes the methylation of lysine residues and is present in some TrxG proteins, including *Drosophila* Trx. Many of the TrxG proteins are conserved across species as divergent as yeast and human. The yeast SET domain-containing protein, Set1, was the first protein shown to mediate the methylation of H3K4. Although Set1 is the only H3K4 HMT in yeast, there are many human Set1 homologs including Set1a, Set1b and MLL1-4.⁴⁰ Of the SET domain-containing MLL proteins, MLL1, the human homolog of *Drosophila* Trx, is the best characterized due to its important role in hematopoietic cancers.³⁸ The MLL1 multimeric protein complex includes WD40 repeat domain 5 (Wdr5), retinoblastoma binding protein 5 (Rbbp5) and Ash2l. The triad of Wdr5, Rbbp5 and Ash2l form a core complex that is capable of forming a larger complex through interactions with other SET domain proteins such as Set1, MLL2 and MLL3.⁴⁰ H3K4 methylation, mediated by Ash2l-containing HMT complexes, results in the promotion of cellular differentiation by positively regulating the expression of specific *Hox* genes during development, including *Hoxc8*^{19,28} and *HoxA9*.⁴¹ Interestingly, animal models in which *Hox* genes, such as *Hoxa3*, have been targeted phenocopy many features of mutant *Tbx1* mouse models and human DiGeorge syndrome.⁴²

In this study we show that the T-box transcription factor, Tbx1, interacts both physically and functionally with Ash2l. The multimeric Ash2l HMT complex^{19,20} catalyzes the methylation of histone lysine residues. H3K4 can undergo mono-, di- or trimethylation.²¹ The transcriptional start sites of and genomic regions in close proximity to actively

Table 1 Summary of genotypes in Ash2l heterozygote intercrosses

Genotype	E3.5	E8.5-E11.5	P0
Ash2l ^{-/-}	7	0	0
Ash2l ^{+/-}	28	41	50
Ash2l ^{+/+}	5	21	30
χ ²	P = 0.042	P < 0.001	P < 0.001

E, embryonic age

transcribed genes are highly associated with trimethyl-H3K4 and dimethyl-H3K4.^{43,44} Methylation of H3K4 may be a conserved function of some, if not all, T-box family members. Miller *et al.*¹⁷ have shown that multiple T-box proteins, including Tbx1, are able to induce a dimethyl-H3K4 state, although this is not sufficient for transcriptional activation across family members. The hematopoietic T-box transcription factor, T-bet, is both necessary and sufficient to establish the dimethyl-H3K4 state, but not the trimethyl-H3K4 state at the *CXCR3* and *IFN- γ* promoters. The T-box DNA binding domain directly mediates the T-bet HMT complex interaction.^{17,18} Other T-box proteins, Eomes, Tbx6 and Brachyury, are also able to recruit methyltransferase activity to the *CXCR3* promoter, but do not uniformly result in transcriptional activation. This suggests that events other than lysine methylation are required for factor-specific activation of target genes.¹⁸ Interestingly, the T-bet physically interacts with Rbbp5,¹⁷ which is also a conserved homolog in the Ash2l HMT complex.^{28,45} Other T-box transcription factors have a role in histone biology and chromatin remodeling. Tbx5 directly interacts with BAF (brahma-related gene 1-associated factor) chromatin remodeling complexes¹⁶ and the T-box transcription factor, Tbr-1, directly interacts with CASK (calmodulin-associated serine/threonine kinase) that, by association with the nuclear protein, CINAP (CASK-interacting nucleosome assembly protein), binds histones and facilitates nucleosome assembly.^{46,47}

Ash2l, as part of a structural platform with Wdr5 and Rbbp5,⁴⁰ in concert with SET domain proteins catalyzes lysine methylation in a context-dependent manner. Knockdown of Ash2l as part of an MLL2 HMT complex results in decreased H3K4 trimethylation of the β -globin gene.³² Ash2l, as part of a different MLL2 HMT complex interacting with the paired-box transcription factor, Pax7, regulates *Myf5* by trimethylating H3K4 during myogenesis.⁴⁵ *In vitro* studies of a reconstituted MLL1 complex reveals that the absence of Ash2l results in no change in monomethyl-H3K4, but results in a complete absence of trimethyl-H3K4 and a marked decrease in dimethyl-H3K4. The differential effects on dimethylation and trimethylation may be directly attributable to the PHD domain of Ash2l as the PHD domain has a lower affinity for dimethyl-K4 peptides compared with trimethyl-K4 peptides.³⁰ *In vivo*, chromatin immunoprecipitation experiments in 293 cells reveal that Ash2l knockdown primarily results in decreased trimethyl-H3K4 (and possibly dimethyl-H3K4) at the *HOXC8* locus, but not the *HOXA9* locus.⁴⁸ The absence of an effect on H3K4 methylation at the *HOXA9* locus is consistent with prior reports that Ash2l knockdown has no effect on *HOXA9* transcription in HeLa cells.⁴¹ This *in vivo* finding has been duplicated in HeLa cells in which Ash2l knockdown resulted in both a global reduction in trimethyl-H3K4 as well as a reduction in trimethyl-H3K4 at the *GAPDH*, *E2F2*, and *HOXA11* loci.⁴⁹ Thus, this work supports our findings and the idea that Ash2l-containing HMT complexes are involved in Tbx1 transcription factor-dependent transcriptional regulation.

In addition, we show that, consistent with prior studies, *Ash2l*²⁸ and *Tbx1* expression patterns overlap,^{34–36}

supporting a co-operative role during embryogenesis. Our analysis of targeted Ash2l mutant mice reveals that *Ash2l* expression is absolutely essential during early development. We show that when the *Ash2l* locus is disrupted, no survivors can be identified during mid- or late gestation. *Ash2l*-null embryos reach blastocyst stage but, *ex vivo*, these cells are unable to form ES cells. *Ash2l* is expressed in the developing blastocyst,⁵⁰ and we speculate that maternally derived Ash2l protein is sufficient for epigenetic regulatory function up to the blastocyst stage, but shortly after this stage, the contribution of embryonic Ash2l is required for further development and required for ES cell maintenance and/or proliferation. This requirement underscores the myriad developmental genes that are likely regulated by the Ash2l-containing HMT complexes.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. LH, FH, DDZ, JY and JZS conducted the experiments; CCT and BDG supplied critical reagents (*Ash2l* expression constructs); and JAE and JZS wrote the manuscript.

ACKNOWLEDGEMENTS

We thank the Soriano Laboratory for the gene-trap ES cells. This research was supported by NHLBI K08-HL086633 and the Pediatric Scientist Development Program (NICHD K12-HD00850) to JZS and by NIH 1P01HL075215 to JAE.

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(Received October 19, 2009, Accepted February 3, 2010)