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Crystal structure of the trithorax group protein Ash2L reveals a Forkhead-like DNA binding domain

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Abstract

Human ASH2L is a trithorax group (TrxG) protein and a regulatory subunit of the SET1 family of lysine methyltransferases. Here we report that Ash2L binds DNA employing a Forkhead-like helix-wing-helix (HWH) domain. *In vivo*, Ash2L HWH domain is required for binding to the β-globin locus control region (LCR), histone H3 Lys4 tri-methylation and maximal expression of the *β-globin* gene, validating the functional importance of Ash2L DNA binding activity.

The TrxG protein Ash2L is a subunit of several multi-protein complexes associating with the SET1 family of histone H3K4 KMTases. When found in a tri-partite complex with WDR5 and RbBP5, Ash2L stimulates the KMTase activity of MLL1-4, SET1A and SET1B[1]. This activity is essential for regulating gene expression as decreased levels of Ash2L result in a loss of both histone H3 Lys4 tri-methylation (H3K4me3) and expression of specific MLL target genes[2].

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AUTHOR CONTRIBUTIONS STATEMENT

J.-F.C and M.B. designed the experiments. S.S. and V.A were responsible for performing the EMSA and analyzing the crystal structure. V.T. performed the SELEX and validated the Ash2L target genes. C.-P.C. performed the ChIP and qRT-PCR studies. P.Z. and S.L. performed the methyltransferase assays and sequence alignment, respectively. J.S.B. collected the data and calculated the initial phases of the model. A.B performed the analysis of the SELEX data. J.-F.C. provided scientific direction of the project and wrote the manuscript. J.S.B., A.B. and M.B commented on the paper.

ACCESSION CODES

Protein Data Bank; Coordinates for Ash2L N-terminal domain have been deposited with the accession code 3S32.pdb.

Several lines of evidence suggest that Ash2L links genuine transcription factors to histone H3K4 methylation. For example, in myoblasts undergoing terminal differentiation, the p38α-dependent phosphorylation of Mef2d recruits the Ash2L complex to *MyoG* and *Ckm* gene promoters. Ash2L binding to these loci enhances histone H3K4 methylation and maintains *MyoG* and *Ckm* expression[3]. In Neuro2a neuroblastoma cells, the transcription factor Ap2δ directly binds to Ash2L, which tethers Mll2 to the *HoxC8* promoter, thereby maintaining the expression of this gene[4]. In differentiating erythroid cells, the NF-E2 transcription factor complex recruits Ash2L and Mll2 to the hypersensitive site 2 (HS2) of the *β-globin* gene locus control region (LCR)[5]. This recruitment of Ash2L is important as decreased levels of Ash2L diminish β-globin transcription. Overall, these studies suggest that Ash2L plays an important role in linking transcription factors to histone H3K4 methylation; however, the underlying mechanisms remain to be established.

Ash2L is composed of a C4 zinc finger motif located on its N-terminus part ($Ash2L_N$) and a SPla and RYanodine receptor (SPRY) domain located on its C-terminus part ($Ash2L_C$). Two recent studies have shown that the SPRY domain of Ash2L is required for binding to RbBP5 (refs. 6, 7). In contrast, the role of $Ash2L_N$ has remained unexplored. To decipher putative biological role(s) of this domain, we first solved the crystal structure of $Ash2L_N$ at 2.45Å (Supplementary Table 1). As defined by the electron density, $Ash2L_N$ comprises residues 106-269 with one molecule in the asymmetric unit. In brief, the first half of $Ash2L_N$, which includes a C4 zinc finger, is composed two anti-parallel β-sheets intersected by one α-helix (α1) (Fig. 1a). The β–sheets are orthogonal to each other and strands β3 and β4 are connected by a 12-amino acid loop. The other half of $Ash2L_N$ is composed of four consecutive α-helices (α2-α5) and a C-terminal anti-parallel β-sheet (β5-β6) (Fig. 1a). Mapping of the evolutionary conserved residues identified several highly conserved regions including those neighboring β4–α2, the β6–α6 inter-connecting loop and the residues lining the surface of α5-helix (Fig. 1b and Supplementary Fig. 1). To identify structural homologues, we queried the Protein Data Bank using the structural database DALI[8]. The C-terminal half of $Ash2L_N$, which comprises α2-α5 and β5-β6, revealed similarities with the HWH DNA-binding domain (DBD) of the Forkhead transcription factor FOXO4 (z-score of 7.5). Structural alignment of $Ash2L_N$ C-terminal half with FOXO4 DBD revealed r.m.s. deviations of 2.3 Å for Cα-atoms over 75 residues showing several structural similarities including the organization of α2-α5, β5-β6 and the clustering of positively charged residues on the surface of α5-helix. Given that this α-helix is structurally homologous to the canonical DNA binding α-helix of FOXO4 (Fig. 1c–e), these observations suggest that $Ash2L_N$ could directly bind to DNA.

Previous ChIP experiments have shown that Ash2L binds to the HS2 site of the β-globin locus in erythroid cells[5]. To determine whether $Ash2L_N$ could bind directly the HS2 site, we performed electrophoretic mobility shift assays (EMSA) using a probe corresponding to a region neighboring the NF-E2 binding site of HS2 and purified recombinant $Ash2L_N$ (Fig 2a). We found that $Ash2L_N$ binds to HS2 as a clear retardation is observed upon incubation of the probe with the protein (Fig. 2a). Specificity of the interaction between $Ash2L_N$ and HS2 was confirmed as an excess of a non-biotinylated probe competed the shift. Conversely, co-incubation of Ash2L with a mutated HS2 element (HS2mut), modified in a region showing striking sequence homology to one of three DNA sequence motifs recognized *in*

vitro by Ash2L in SELEX experiments (Fig. 2b and Supplementary Methods), failed to compete with the labeled probe. This further underscores the specificity of Ash2L DNA-binding activity. Using EMSA, titration of the HS2 element by $Ash2L_N$ yielded an apparent equilibrium dissociation constant of 3.7μM (Supplementary Fig. 3). While this binding affinity is relatively weaker than that of FOXO1, this may be attributed to dissimilarities in electrostatic potential between the wing regions of these proteins. Indeed, the Ash2L wing region is negatively charged while the FOXO1 and FOXO3 wings are rich in arginine residues[9]. We next sought to verify that $Ash2L_N$ binds specifically to DNA *in vivo*. Flag-tagged Ash2L constructs were ectopically expressed in erythroid cells and chromatin immunoprecipitation (ChIP) was performed with an anti-Flag antibody. As shown in Figure 2c, $Ash2L_{FL}$ and $Ash2L_N$ bind to β-globin HS2 at a similar level while no binding is detected for $Ash2L_C$ (Fig. 2c). This result confirms that $Ash2L_N$, a region dispensable for the stimulation of MLL1 KMTase activity (Supplementary Fig. 4), is specifically dedicated to HS2 binding in erythroid cells.

Close inspection of our amino acid alignment (Supplementary Fig. 1) revealed that one of the evolutionary conserved α-helix in Ash2L shares structural homology with a DNA-binding α-helix of FOXO4 (Fig. 1c–e). In FoxO proteins, hydrophobic and positively charged residues lining this α-helix are key in binding to DNA [10,11]. To determine whether this region of Ash2L plays a similar role, we combined site-directed mutagenesis to EMSA and determined that substitution of Lys225 or Lys229 by glutamic acid residues completely impairs DNA-binding activity on the HS2 probe (Fig. 3a); a result that is not attributable to loss of protein folding (Supplementary Fig. 5). These analyses indicate that Ash2L HWH domain harbors an evolutionary conserved surface that comprises residues conferring DNA-binding activity. To test whether these residues are important in mediating Ash2L binding to the β-globin LCR, we performed Flag-ChIP experiments in erythroid cells stably expressing a doxycycline (Dox) inducible small hairpin shRNA directed against Ash2L[5]. Previous experiments have shown that the Dox-mediated knockdown of Ash2L in this erythroid cell line leads to a decrease of β-globin transcription and the H3K4me3 mark on this gene[5]. ChIP experiments performed on Dox-treated cells transfected with shRNA-resistant Flag-$Ash2L_{WT}$ showed binding of Ash2L to HS2, and revealed a 50% increase in both histone H3K4me3 and β-globin expression compared to cells transfected with an empty vector (Fig. 3b–d). Consistent with our EMSA results, the Flag-Ash2L K225E and K229E mutants failed to bind to HS2, to elicit maximal β-globin expression (Fig. 3b–d) and to rescue the loss of histone H3K4me3.

Collectively, our results suggest that while Ash2L is initially guided to the β-globin HS2 via its interaction with the activator NF-E2 (ref. 5), direct interaction of Ash2L with DNA via its HWH domain is important for stable association to the HS2 site (Supplementary Fig. 6), stimulation of H3K4me3 and activation of β-globin transcription, a mechanism that is likely applicable to the regulation of other genes. Finally, given that Ash2L is a TrxG protein, our observations suggest that Ash2L HWH domain may play an analogous role in the fine balance between Polycomb and Trithorax group proteins occupancy at *Hox* loci during embryonic development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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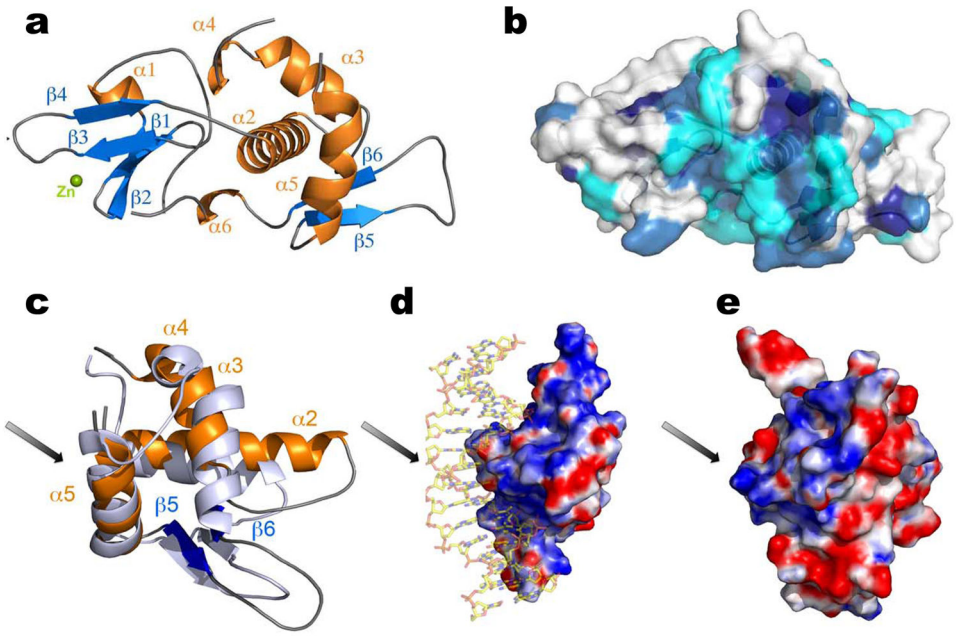

Figure 1.

Ash2L is composed of a C4 zinc finger and a structurally conserved helix-wing-helix domain. **(a)** Overall structure of Ash2L N-terminal domain. β-strands and α-helices are rendered in blue and orange, respectively; the zinc atom is depicted in green. **(b)** Ash2L harbors a structurally conserved helix-wing-helix domain. Evolutionary conserved surface residues colored accordingly to the sequence alignment (Supplementary Fig. 1). (**c**) Superimposition of Ash2L and FOXO4 HWH domains. Ash2L is rendered as in (**a)** and FOXO4 is colored in grey. Electrostatic potential surface of FOXO4 (**d**) and Ash2L (**e)** in which DNA carbon atoms are rendered in yellow. Electrostatic potentials are contoured from $+10k_bTe^{-1}$ (blue) to $-10\ k_bTe^{-1}$ (red). Arrows indicate the canonical and putative DNA binding α-helix of FOXO4 and Ash2L.

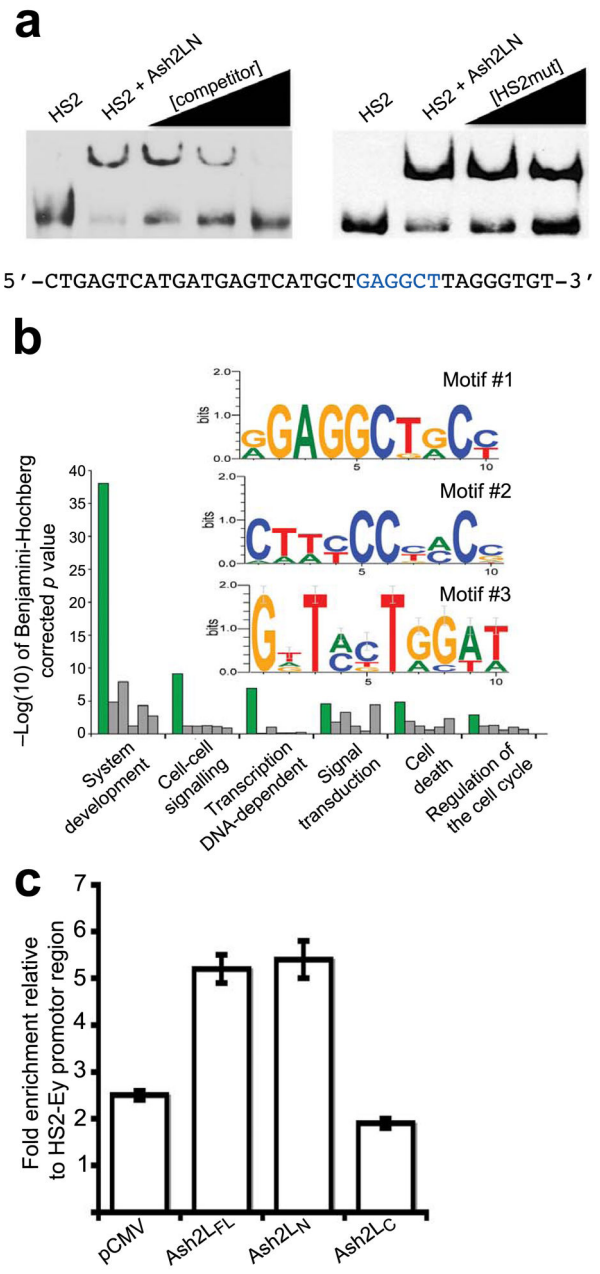

Figure 2.

The N-terminal domain of Ash2L binds DNA. **(a)** Ash2L$_N$ directly binds HS2. EMSA of biotinylated HS2 using 4μM of Ash2L$_N$. Specificity of the binding is confirmed using 10X, 25X and 50X molar excess of non-biotinylated probe (Competitor) or with 10X and 25X fold molar excess of an AGGCT – CTGGG substituted HS2 element (HS2mut). **(b)** Ash2L binds GC-rich DNA motifs. Gene ontology analysis of the human genes containing a good match for the SELEX motif #1 (inset) in their proximal promoters (green histogram bar). Grey bars represent 5 control sets of randomly selected genes. (Enrichment of Ash2L to selected target genes is presented in Supplementary Fig. 2, Supplementary Methods and Supplementary Table 2) **(c)** Ash2L$_N$ is important for the recruitment of Ash2L to HS2. Flag-

tagged Ash2L constructs were ectopically expressed in differentiated mouse erythroleukemia (MEL) cells and ChIP was performed with an anti-Flag antibody. Relative binding of Flag-Ash2L$_{FL}$, Flag-Ash2L$_{N}$ and Flag-Ash2L$_{C}$ was measured by ChIP at the HS2 site of the β-globin LCR. Immunopurified DNA was quantified as previously described[5]. Average values of duplicate qPCR reactions are shown; error bars represent s.d. Each experiment was performed twice with independent chromatin samples.

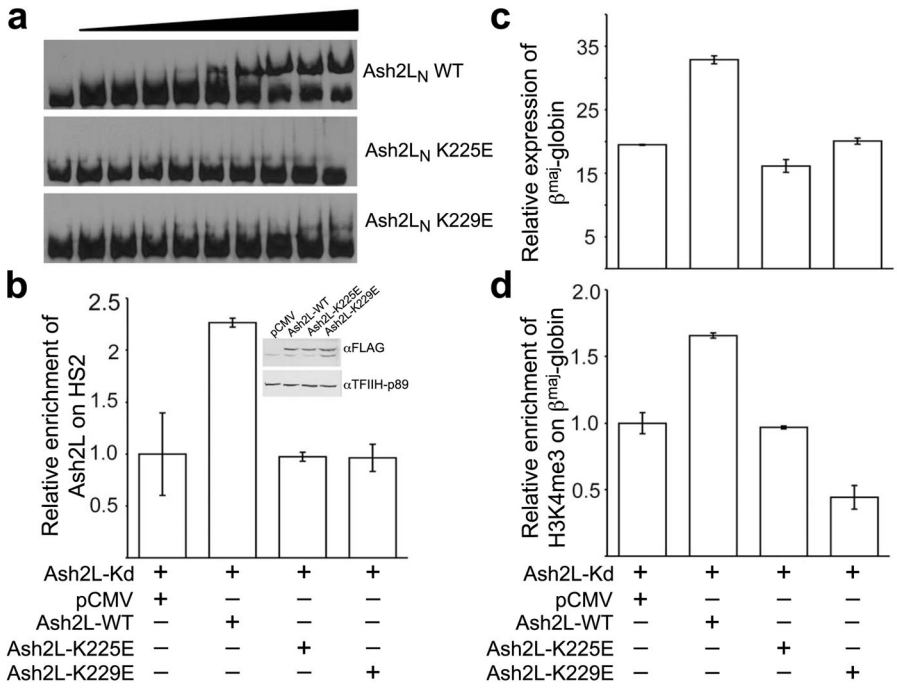

Figure 3.

Ash2L HWH domain is required for histone H3K4me3 and maximal β-globin gene expression. **(a)** Mutation of Ash2L α5-helix impairs binding of HS2 in EMSA. Ash2L α5-helix is essential for Ash2L binding to HS2 **(b)**, maximal expression of β-globin **(c)** and H3K4 tri-methylation **(c)**. Binding of Ash2L and enrichment of H3K4me3 was measured by ChIP using Dox-induced differentiated MEL cells transfected with either the empty vector (CMV), Ash2L-WT, Ash2L-K225E or Ash2L-K229E. The inlet shows the western blots analysis of Dox-inducible knockdown (Kd) of endogenous Ash2L and rescued with shRNA resistant flag tagged Ash2L-WT, Ash2L-K225E and Ash2L-K229E in differentiated MEL cells. $TF_{II}Hp89$ is used as a loading control. β^{maj}-globin transcription relative to GAPDH was assessed by real-time qRT-PCR as previously described[5]. Values were averaged as in fig. 2c.