Deciphering the histone interactions and reader functions of ASH1L and the PHD-BRD reader domain family in biology and cancer

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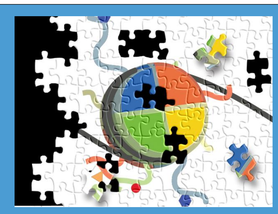


S. Rothbart, 2013

Abstract

Mutation or dysregulation of the proteins that read, write, and/or erase histone post-translational modifications are thought to underlie ~50% of all human cancers. One such family of proteins that are frequently altered in cancer is those containing paired histone reading plant homeodomains (PHD) and bromodomains (BRD). ASH1L is a histone H3 lysine-36 (H3K36) dimethylase which is altered in ~7% of cancers and contains a poorly understood triple histone reader PHD-BRD-BAH domain module. *Although ASH1L and other PHD-BRD proteins contain commonly studied reader domains and have been linked to cancer, how they regulate normal and cancer development is still not well understood.* Thus, there is a critical need to systematically characterize the histone reading interactions and mechanistic processes for ASH1L and the rest of the PHD-BRD family, especially with respect to how these domains in combination can form multivalent interactions with nucleosomes.

Table 1. PHD-BRD family - percent cancer alterations and binding targets. Histone H3 lysine 4 methyl 0 (H3K4me0), acetyl (ac), E3 ligase (E3). Nucleosome studies (*).

Protein Name	Cancer (%)	PHD	BROMO	Refs
ASH1L	7	**Unknown**	Kac	10
BAZ1A	3	DNA	Kac	23
BAZ1B	3	**Unknown**	Kac	23
BAZ2A (TIP5)	3	H3K4me0	H4K16ac	20
BAZ2B	4	H3K4me0	H3K14ac	10
BPTF	5	H3K4me2/3*	H4K16ac*	10,15
BRPF1 (Peregrin)	3	**Unknown**	Kac	10
BRPF2 (BRD1)	4	H3	Kac	10,16
BRPF3	2	**Unknown**	Kac	10
CBP (CREBBP)	6	**Unknown**	Kac, H4K20ac	10,18
EP300 (p300)	5	**Unknown**	Kac*	10,11
KMT2A (MLL1)	6	H3K4me2/3	**Unknown**	10,13
SP100C	3	H3K4me0	Kac	21
SP110	2	**Unknown**	**Unknown**	
SP140	3	BRD (E3)	Kac	10,25
SP140L	2	BRD (E3)	Kac	10,25
TRIM24 (TIF1A)	2	H3K4me0	Kac, H3K23ac	10,14
TRIM28 (KAP1)	2	BRD (E3), H4	SETDB1	12,26
TRIM33	2	H3K9me3	Kac, H3K18ac	16
TRIM66	1	H3K4me0	H3K56ac	24
ZMYND8 (PKCB1)	4	**Unknown**	H3K14ac	22
ZMYND11	3	**Unknown**	H3.3K36me*	19

To address how ASH1L and PHD-BRD readers function, I will employ a suite of innovative histone-binding assays, structural methodologies, and cellular-based analyses. Specifically, I will use modernized histone peptide and nucleosome screening assays, X-ray crystallography and Cryo-EM in parallel, and a knockdown/complementation system of ASH1L in normal and breast cancer cell lines. I hypothesize that ASH1L targets specific acetylated and methylated histone tails via its BRD-PHD-BAH module to regulate homeotic gene expression, but that aberrant expression of ASH1L promotes breast cancer through increased expression of pro-growth and survival genes. Through this examination, this work aims to further decipher the "Histone Code" and provide new insights into cancer biology.

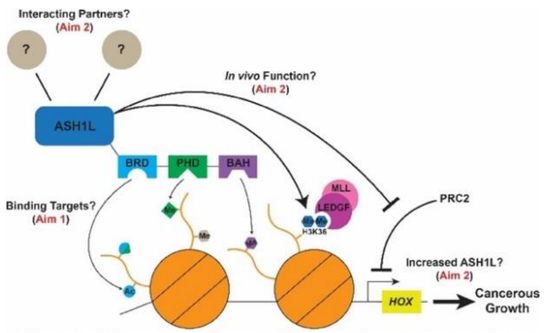


Figure 1. Model for ASH1L function in chromatin.

Results

In vitro characterization of ASH1L histone interactions

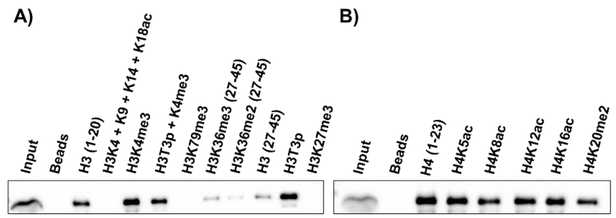


Figure 2. Purified ASH1L GST-BRD-PHD-BAH triple reader protein recognizes unmodified, acetylated (ac), methylated (me), and phosphorylated (p) histone peptides. Peptide pulldown assays with histone A) H3 or B) H4 peptides in 300mM NaCl, 50 mM Tris pH 8, and 0.1% NP-40.

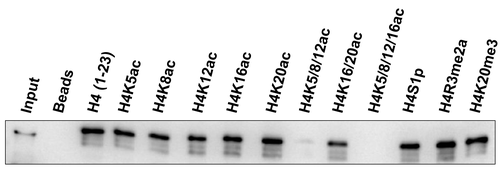


Figure 3. Purified ASH1L GST-DUF (domain of unknown function) recognizes unmodified, acetylated, methylated, and phosphorylated histone H4 peptides, but is sensitive to poly-acetylation. Peptide pulldown assays in 300mM NaCl, 50 mM Tris pH 8, 0.1% NP-40, and 0.5% BSA.

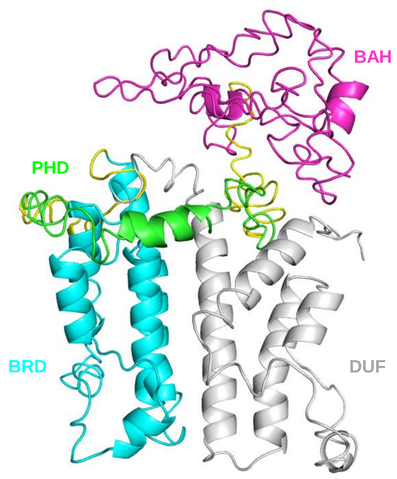


Figure 4. I-TASSER structural model of ASH1L DUF-BRD-PHD-BAH histone reader domain module.

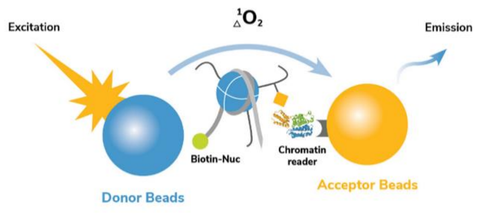


Figure 5. Screen for designer nucleosome binding. High affinity histone binding targets for the ASH1L reader module will be used for structural analyses. Figure adapted from https://www.epicypher.com.

In cellulo characterization of ASH1L function

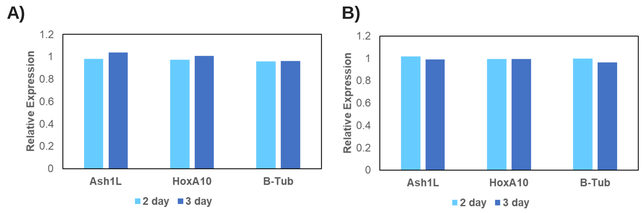
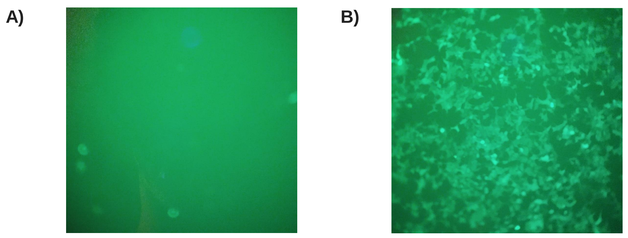


Figure 7. shRNA targeting of the 3' UTR of Ash1L in HEK293T cells results in poor knockdown efficiency. RT-PCR of Ash1L, HoxA10, and loading control β-actin genes from 3' UTR Ash1L targeting SMARTvectors (Horizon Discovery) lipofectamine transfected into HEK293T and induced with 0.75 µg/mL of doxycycline or DMSO for A) 2 or B) 3 days.



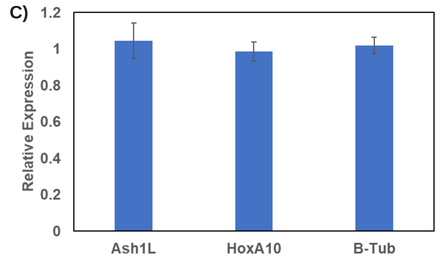


Figure 8. SMART shASH1L-1 isolated clone in HEK293T exhibits high co-expression of GFP, but low knockdown efficiency of Ash1L. GFP fluorescence of shAsh1L-1 transfected HEK293T clone treated with A) DMSO or B) 0.75 µg/mL of doxycycline for 1 day. C) RT-PCR analysis of shAsh1L-1 transfected clone treated with DMSO vs. doxycycline.

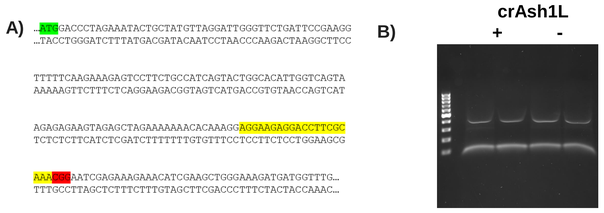


Figure 9. Genomic PCR analysis of CRISPR treated HEK293T cells with sgRNA targeting the N-terminus of Ash1L. A) sgRNA targeting the Ash1L N-terminus (yellow) with PAM sequence identified (red). B) Genomic PCR of Ash1L gene from HEK293T cells treated +) with or -) without 1.25 µg Cas9 + 0.24 µg sgAsh1L using CRISPRMAX Lipofectamine (Thermo) reagent.

Discussion

For *in vitro* analysis of ASH1L binding to histones, I expect to identify the histone PTMs recognized by the ASH1L reader domains as well as the structural determinants behind these interactions. Based on my purified ASH1L analyses, it appears that the triple reader and DUF modules bind to various modified H3 and H4 N-terminal histone regions, but are negatively sensitive to poly-acetylation. Based on poor binding data using purified individual GST-ASH1L reader domains, I have developed point mutants in each of the reader domains but in the context of the triple reader module to test. Furthermore, I will determine the histone binding potential of the ASH1L reader module on EpiCypher's innovative nucleosome screen to better predict *in vivo* binding targets as well as identify optimal substrates for X-ray crystallography and Cryo-EM.

For *in cellulo* analysis of ASH1L function, I intend to develop a system of depleting ASH1L while complementing with recombinant ASH1L to determine the function of various domains with the coding sequence. With a suitable model system in place, I can determine which ASH1L reader domains are vital for histone recognition in cells as well as how ASH1L functions to regulate specific gene expression such as at the HOX gene clusters. Since ASH1L appears to be a critical regulator of cancer survival in myeloid leukemia blood cancers (**Fig. 10**), I will test my model system of regulating ASH1L levels in breast cancer where ASH1L overexpression appears to be promoting oncogenesis.

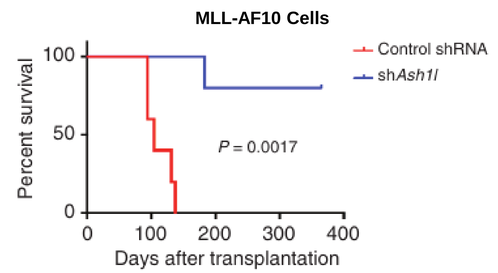


Figure 10. Survival curves of mice transplanted with mouse MLL-AF10 leukemia cells (1 x 10^6) transduced with control or *Ash1L* shRNAs (n=5). Figure adapted from **L. Zhu, 2016**.

Using the toolkit I develop based on ASH1L, I intend to translate this to providing a comprehensive catalog of PHD-BRD tandem reader module histone interactions that will serve as a valuable resource for the epigenetics community. Any novel interactions I uncover would serve as launching points for future investigations and could assist in detecting altered transcriptional regulatory pathways in cancers where these proteins are misregulated. This data repository will guide future investigations into the biological roles of histone PTM readers and writers and expand our understanding of epigenetics and their impact on human diseases such as cancer.

Acknowledgements

We gratefully acknowledge the NIH (R35 GM126900-01; R01 CA198482-01; R61 DA047023), NIH/NCI (T32 CA009156), and Qura Therapeutics for providing funding to supporting this research. I also acknowledge Dr. Kanishk Jain, Dr. Krystof Kryjewski, Dr. Abid Khan, and Dr. Raghuvar Dronamraju for experimental advice and/or materials for the experiments shown.

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