H3K4 Methyltransferase Activity Is Required for MLL4 Protein Stability

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Abstract

Transcriptional enhancers play a key role in cell type-specific gene expression and cell fate transition. Enhancers are marked by histone H3K4 mono- and di-methylation (H3K4me1/2). The tumor suppressor MLL4 (KMT2D) is a major enhancer H3K4 mono- and di-methyltransferase with a partial functional redundancy with MLL3 (KMT2C). However, the functional role of MLL4 enzymatic activity remains elusive. To address this issue, we have generated MLL4 enzyme-dead knock-in (KI) embryonic stem (ES) cells and mice, which carry Y5477A/Y5523A/Y5563A mutations in the enzymatic SET domain of the MLL4 protein. Homozygous MLL4 enzyme-dead KI (*Mll4*$^{KI/KI}$) mice are embryonic lethal and die around E10.5, which phenocopies *Mll4* knockout mice. Interestingly, enzyme-dead MLL4 protein in ES cells is highly unstable. Like *Mll4* knockout ES cells, *Mll4*$^{KI/KI}$ ES cells show reduced levels of H3K4me1/2. Furthermore, we show that ectopic expression of histone H3.3 lysine 4-to-methionine (K4M) mutant, which reduces endogenous H3K4 methylation levels in ES cells, decreases the protein stability of MLL3 and MLL4 but not that of H3K4 methyltransferases SET1A (KMT2F) and SET1B (KMT2G). Taken together, our findings indicate that MLL4 protein stability is tightly regulated by its H3K4 methyltransferase activity.

Introduction

Methylation on lysine 4 of histone H3 (H3K4) correlates with gene activation. H3K4 methylation is catalyzed by the SET domain-containing histone methyltransferase (HMT) Set1 in yeast and Set1-like HMTs in mammals [1]. There are six Set1-like H3K4 HMTs in mammals: MLL1/MLL2 (Mixed-Lineage Leukemia 1/2, also known as KMT2A/B), MLL3/MLL4 (Mixed-Lineage Leukemia 3/4, also known as KMT2C/D), and SET1A/SET1B (also known as KMT2F/G) [1–3]. MLL1 and MLL2 are known as mono- and di-methyltransferases with little tri-methyltransferase activity [4,5]. MLL3 and MLL4 are major mammalian mono- and di-methyltransferases [2]. SET1A and SET1B are major mammalian H3K4 tri-methyltransferases [6]. The H3K4 methyltransferase activity of mammalian Set1-like HMTs requires the common subunits ASH2L, WDR5, RBBP5, and DPY30, which form the WRAD subcomplex [3,7–9] (Table 1). Menin is a unique subunit of MLL1/MLL2 complexes [10,11]. PTIP, PA1, NCOA6, and UTX are unique subunits of MLL3/MLL4 complexes [3]. In yeast, Set1 is responsible for all H3K4 mono-, di-, and tri-methylations [1]. Interestingly, yeast Set1 protein levels are linked to H3K4 methylation levels [12]. However, it is unclear whether protein levels of mammalian Set1-like HMTs are also regulated by H3K4 methylation levels.

We previously showed that MLL4 is partially redundant with MLL3 and is a major H3K4 mono- and di-methyltransferase enriched on enhancers in mouse and human cells [2]. MLL3 and MLL4 are required for enhancer activation and cell type-specific gene expression during cell differentiation [2,13]. MLL3 and MLL4 are dispensable for cell identity maintenance but are required for cell fate transition [13]. *Mll3* knockout (KO) mice die around birth with no obvious morphological abnormalities, whereas *Mll4* KO mice show early embryonic lethality around E9.5 [2]. By tissue-specific KO of *Mll4* in mice, we showed that MLL4 is essential for adipogenesis,

Table 1. Yeast Set1 and human Set1-like complexes methylate histone H3K4

		Human Set1-like Complexes		
	Yeast Set1	SET1A/B	MLL1/MLL2	MLL3/MLL4
Enzymatic subunit	Set1	SET1A/B	MLL1/MLL2	MLL3/MLL4
Common subunits forming the WRAD subcomplex	Bre2	ASH2L	ASH2L	ASH2L
	Swd1	RbBP5	RbBP5	RbBP5
	Swd3	WDR5	WDR5	WDR5
	Sdc1	DPY30	DPY30	DPY30
Distinct subunits	Swd2	WDR82		
	Spp1	CXXC1		
			Menin	PTIP and PA1
		HCF1	HCF1/2	NCOA6
				UTX

myogenesis, and heart development *in vivo* [2,14]. However, the functional role of MLL4 enzymatic activity needs to be explored.

In the present study, we have identified amino acids critical for the H3K4 methyltransferase activity of MLL4 in cells. By generating MLL4 enzyme-dead KI embryonic stem (ES) cells and mice, we found that, surprisingly, MLL4 enzymatic activity is required for MLL4 protein stability in cells and for early embryonic development in mice.

Results and discussion

Identification of amino acids critical for H3K4 methyltransferase activity of MLL4

A previous structural study has revealed that Y3858 and Y3942 in the SET domain of human MLL1 are essential for its H3K4 methyltransferase activity [5]. Both Y3944 and Y3942 are involved in H3 recognition and located in the active center [15]. Y3944 is a more conserved residue than Y3942 across MLL family members [5]. We hypothesized that the corresponding residues, Y5426 and Y5512, in the SET domain of human MLL4 could be critical for MLL4 enzymatic activity (Fig. 1a). To test this hypothesis, we generated FLAG-tagged full-length human MLL4 carrying Y5426A/Y5512A mutations by site-directed mutagenesis. 293FT cells were transiently transfected with plasmids expressing FLAG-tagged wild type (WT) MLL4 or the Y5426A/Y5512A mutant. Ectopic expression of the Y5426A/Y5512A mutant MLL4 did not affect the global levels of H3K4me1, H3K4me2, H3K4me3, and MLL4-associated proteins UTX and RbBP5 in cells (Fig. 1b). For *in vitro* HMT assays, we affinity-purified the MLL4 complex from 293FT cells transiently transfected with plasmids expressing FLAG-tagged WT MLL4 or the Y5426A/Y5512A mutant. Western blot analysis showed that UTX and RbBP5 were present at similar levels between WT and mutant MLL4 complexes (Fig. 1c). In an HMT assay using recombinant histone H3 as the substrate, the mutant MLL4 complex showed a markedly reduced ability to catalyze H3K4me1, H3K4me2, and H3K4me3 (Fig. 1d). These results indicate that the Y5426 and the Y5512 residues are critical for enzymatic activity of human MLL4 *in vitro*.

MLL4 enzymatic activity is essential for early embryonic development

To investigate the biological functions of MLL4 enzymatic activity *in vivo*, we decided to generate MLL4 enzyme-dead knock-in (KI) ES cells and mice. We chose to mutate Y5477 and Y5563 of mouse MLL4, which correspond to Y5426 and Y5512 in human MLL4, to alanine (Fig. 2a). In addition, we mutated mouse Y5523 to ensure that MLL4 enzymatic activity was fully inactivated because the corresponding residue in human MLL3 is important for the methyl transfer from the cofactor *S*-adenosyl-L-methionine to the target histone lysine [15]. Mutating mouse Y5523 to alanine was unlikely to destabilize MLL4 because human MLL4 carrying the corresponding triple mutations (Y5426A/Y5472A/Y5512A) was expressed at similar level as the WT in 293FT cells (Fig. 2b). Also, ectopic expression of the triple-mutated MLL4 did not affect the global levels of H3K4me1, H3K4me2, and H3K4me3 in cells (Fig. 2b). Heterozygous and homozygous MLL4 enzyme-dead KI mouse ES cells were generated by conventional gene targeting methods. Y5477, Y5523, and Y5563 were localized in exon 52, 54, and 55 of the mouse *Mll4* gene, respectively (Fig. 2c). The genotypes of ES cells and the three