Construction of hybrid yeast-human histone methyltransferase complexes in *Saccharomyces cerevisiae* clarifies the roles of Bre2 and Ash2L for mixed lineage leukemia

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Abstract

Mixed lineage leukemia (MLL) is an aggressive blood cancer that results from genetic alterations in the MLL1 gene. This gene encodes an enzyme that methylates histone H3 on lysine 4 (H3K4) as a part of the MLL1 multi-protein complex. MLL-related genetic alterations create fusion proteins that render MLL1 incapable of its methyltransferase activity, with particularly devastating effects at homeobox genes. Problematically, higher eukaryotes contain many functionally redundant complexes that complicate the study of MLL1 and associated cancers in a living system. Moreover, the translocations that lead to the formation of MLL1 fusion proteins are variable, generating similarly variable fusion proteins. This inconsistency further complicates the use of MLL1 as a drug target. However, several accessory proteins within the complex are required for catalytic activity, and present possible drug targets themselves. Herein we present an in vivo system for the study of the MLL1 complex in *Saccharomyces cerevisiae*, making use of the homologous Set1/COMPASS complex. We genetically replaced COMPASS members from *S. cerevisiae* with their human homologs using antibiotic resistance cassettes, and subsequently performed phenotypic characterization of chimeric COMPASS/MLL1 complexes, assessing global H3K4 methylation status. Selected chimeric yeast-human methyltransferase complexes conferred catalytic activity at varying degrees, while others did not confer methyltransferase activity. Notably, we observed H3K4 dimethylation levels comparable to wild type when human Ash2L replaced yeast Bre2 but reduced levels of H3K4 trimethylation with this same chimeric complex. Together, these data represent a proof of concept for simplifying the study of this clinically important protein complex in a tractable in vivo system, and also offer mechanistic insight into the functional role of a catalytically essential accessory protein within the MLL1 complex through our model.

Introduction

Genome rearrangements and the MLL1 complex

Drastic genomic rearrangements including chromothripsis, aneuploidy, and translocations are invariably detrimental to all human cells. Faulty regulation of gene expression following such an event often contributes to disease progression, perhaps most notably in cancer. In the case of mixed lineage leukemia (MLL1), a particularly deadly form of cancer, translocations involving the histone methyltransferase gene MLL1 are known to alter gene expression in such a way that normal hematopoiesis is disrupted, leading to leukemia [1,2]. The MLL1 gene encodes the catalytic subunit of a multi-protein complex (termed the MLL1 complex) that performs crucial histone H3 lysine 4 (H3K4) methylation, a modification usually associated with transcriptional activation [3]. Via this epigenetic mechanism, the MLL1 complex exerts regulatory control over many genes, including homeobox (Hox) genes, which are critically important to normal hematopoiesis. In the disease state following a translocation, MLL1 forms fusion proteins with at least 70 partner proteins that alter the MLL1 complex’s catalytic activity, and thereby affect the expression of Hox genes, disrupt normal hematopoiesis, and trigger leukemogenesis [4,5]. MLL1 fusion proteins have also been shown to deleteriously alter p53-mediated response to DNA damage [6].

Therapeutic potential of epigenetic modifiers

Despite the unfortunately poor prognosis of affected individuals, the mechanistic basis of mixed lineage leukemia warrants cautious optimism for the long-term development of targeted therapies. Epigenetic modifiers represent feasible targets for therapeutic intervention, and other such proteins have seen some success as ‘druggable’ targets for cancer treatment as evidenced by the FDA-approved histone deacetylase inhibitor vorinostat used for the treatment of cutaneous T-cell lymphoma [7]. Problematically, MLL1 fusion proteins are structurally inconsistent from one patient to another, making MLL1 itself an impractical drug target. Some of the accessory proteins within the MLL1 complex, however, are required for catalytic activity, affect the specificity and catalytic activity of the

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Results and limitations of prior in vitro studies

Although numerous studies have been published that attempt to elucidate the mechanism of action of the core MLL1 complex in relation to leukemia and H3K4 trimethylation, all such studies have been limited to in vitro models [11,17-19]. Recent in vitro studies were all found that the methyltransferase subunit necessary for the trimethylation of H3K4 is activated by a heterodimer of Ash2L and RbBP5 (two accessory proteins in the MLL1 protein complex) in a two-step process that may potentially be conserved across all SET-containing histone methyltransferases. However, the protein interactions and the degree of their necessity for complex function remain unclear [17]. The extent to which each member regulates and participates in the actions of the methyltransferase are, however, still unclear. In vivo study of the MLL1 protein complex is extremely difficult due to the presence of functionally redundant H3K4 methyltransferase complexes present in human cells such as MLL 2-4, Set1d1A and Set1d1B [20]. These complexes share many of the same accessory proteins; therefore, deconvolution of the specific effects of each accessory protein on MLL1 activity using mammalian cells as a study system is impractical if not impossible [21]. Consequently, most research on these intra-complex interactions has come from in vitro models utilizing recombinant proteins or computer modeling [17,18,22,23]. Several structural studies have been performed yielding insight into conformational changes of MLL as well [12,17,24]. While these studies have provided key insights into the MLL1 complex, exhaustive functional characterization of the accessory proteins is not possible in silico, in vitro, or in bacteria because these proteins are endogenously post-translationally modified. These modifications, in effect, contribute to complex activity and/or specificity. Thus, in vitro studies ignore the interconnectedness of the complexes due to (relatively unknown) endogenous mechanisms and therefore may reach incorrect conclusions from only partial data. To faithfully capture MLL1 complex function in a biologically relevant context, an in vivo model is necessary. S. cerevisiae provides a tractable in vivo model with which to study mis regulation of H3K4 methylation by the MLL complex.

An in vivo model for MLL1 complex function in S. cerevisiae

Previous studies on MLL1 have not been performed in vivo due to the difficulty of the experiment in mammalian cells. Importantly, posttranslational modifications have been identified on MLL1 complex members, which occur only in the context of a eukaryotic cell [10,11,12,24]. As previously mentioned, MLL1 has numerous functionally similar proteins in higher eukaryotes and therefore its isolated study is challenging. However, understanding and analyzing the posttranslational modifications that have been identified on MLL1 complex members are essential to holistically and realistically study the protein complex function.

It has been shown that the SET domain of the MLL1 protein methylate's itself and Ash2L, and this only occurs in the absence of histone H3, whereas unmethylated H3 serves as a stoichiometric inhibitor of auto-methylation [11]. MLL1 auto-methylation appears more prevalent, however, in the absence of the MLL1 core complex members than in their presence [11]. Such modifications likely affect intra-complex interactions that alter catalytic function in vivo, and the effects of these modifications represent key gaps in present knowledge of the MLL1 complex. Additionally, outside of a eukaryotic context, intra-complex regulation dependent on other histone modifications cannot be evaluated. These histone trans-regulatory networks have already been shown as modifying factors for the MLL1 complex [8]. In our study, we sought to analyze the accessory proteins of the human MLL1 complex in a eukaryotic system that lacks the problematic functionally redundant MLL1 homologs present in mammals. The commonly used model organism S. cerevisiae was ideal for this purpose since it is both eukaryotic and contains only a single homolog to the MLL1 complex. The homologous yeast protein complex is termed COMPASS (Complex of Proteins Associated with Set1) [25]. Research centering on COMPASS has begun to define the roles of the accessory proteins associated with yeast Set1, the homolog of human MLL1. Individual COMPASS subunits (Set1, Bre2, Swd1, and Swd3) have been shown to confer proteomic stability and integrity, as well as regulate H3K4 methylation both globally and specifically [3,26,27].

In this study, we have adapted the tools available in yeast genetics to the biochemical study of the clinically important MLL1 complex. We show that specific COMPASS accessory proteins can be replaced by their human homolog and yield varying degrees of global H3K4 methylation. These interspecies chimeric H3K4 methyltransferase complexes have varying functionality based on the specific complex proteins present. Most notably, we show that H3K4 dimethylation levels were comparable to wild type when human Ash2L replaced yeast Bre2. However, this same chimeric complex showed reduced levels of H3K4 trimethylation compared to wild type. This data represents a proof of concept, demonstrating that the study of the MLL1 complex can be assessed in a tractable in vivo model. Moreover, this data also provides mechanistic insight into a functionally important accessory protein within the human complex.

Materials and Methods

Yeast growth assays

Serial dilution yeast growth assays for the indicated strains constructed in this study were performed as previously described [28].

Yeast strains, strain construction and growth media

All S. cerevisiae strains used in this study were W303 and BY4741, both derived from strain S288C (Table 1). All yeast procedures were performed at 30°C and followed as stated in Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual [29]. The genes encoding the four proteins of interest in COMPASS were deleted by a method of homologous recombination wherein an antibiotic resistance cassette is inserted into the gene of interest via transformation (Table 2). Successful transformations were assessed through genotyping with polymerase chain reaction (PCR) and gel electrophoresis.

Gene replacements were performed with one or more of the following resistance genes: kanMX (pUG6), natMX4 (pAG25) or hphMX4 (pAG32) from Euroscarf and then confirmed by PCR genotyping as described previously [30,31,32]. Primers specific for each gene deletion used to amplify the antibiotic resistance cassettes are listed in Table S1. All deletion strains were genotyped by PCR using a primer specific to the 5' or 3' end of the coding region along with a primer specific to the antibiotic cassette insert (“genotyping primers” listed in Table S1). Drug containing plates were prepared using the
Klein D (2019) Construction of hybrid yeast-human histone methyltransferase complexes in *Saccharomyces cerevisiae* clarifies the roles of Bre2 and Ash2L for mixed lineage leukemia

Table 1. *Saccharomyces cerevisiae* strains used in the course of this study

| Yeast Strain | Strain Name |
|--------------|-------------|
| W303: MATa, leu2-3,112 trpl-1 can1-100 ura3-1 ade2-1 his3-11,15, set1::KanMX | set1Δ |
| W303: MATa, leu2-3,112 trpl-1 can1-100 ura3-1 ade2-1 his3-11,15, swd1::KanMX | swd1Δ |
| W303: MATa, leu2-3,112 trpl-1 can1-100 ura3-1 ade2-1 his3-11,15, bre2::KanMX | bre2Δ |
| W303: MATa, leu2-3,112 trpl-1 can1-100 ura3-1 ade2-1 his3-11,15, swd3::KanMX | swd3Δ |
| W303: MATa, leu2-3,112 trpl-1 can1-100 ura3-1 ade2-1 his3-11,15, set1::KanMX, bre2::HphMX, swd3::HphMX | set1bre2Δ |
| BY4741: MATa, leu2-3,112 trpl-1 can1-100 ura3-1 ade2-1 his3-11,15, set1::KanMX, bre2::HphMX, swd1::NatMX | set1bre2swd1Δ |

Table 2. Yeast gene replacements

| Yeast Gene | Deletion antibiotic resistance cassette | Human Gene | Expression Plasmid |
|------------|---------------------------------------|------------|-------------------|
| Set1       | kan'                                  | MLL        | pAG413/His        |
| Bre2       | pat'                                  | Ash2L      | pAG415/Leu        |
| Swd1       | nat'                                  | RbBP5      | pAG414/Tp         |
| Swd3       | hpt'                                  | WDR5       | pAg416/Ura        |

following concentrations: 200μg/mL of Geneticin (Invitrogen), 100μg/mL Nourseothricin sulfate (SIGMA) or 300μg/mL Hygromycin B (SIGMA). Yeast were propagated according to standard procedures either in rich media (YPD), YP media containing 20% galactose or in appropriate selective media.

Constructing the human MLL1 multi-protein complex

All plasmids harboring *loxP* antibiotic marker cassettes (pUC6, pAG25 and pAG32) and yeast expression plasmids (pAG416 GAL-EGFP-ccdB, pAG413 GAL-EGFP-ccdB, pAG414 GAL-EGFP-ccdB and pAG415 GAL-EGFP-ccdB) used in this study are listed in Table S2. Plasmids harboring *loxP* antibiotic marker cassettes used for gene deletions were purchased from Euroscarf. Yeast expression plasmids allowing for expression via a galactose inducible promoter were purchased from Addgene (Alberti, Gitler & Lindquist, 2007). All yeast (*Set1*, *Bre2*, *Swd1*, *Swd2* and *Swd3*) and human (*MLL1*, *Ash2L*, *RbBP5*, *WDR5* and *DPY30*) genes were first cloned into pCR-TOPO (Gateway BP reaction, Invitrogen) according to manufacturer’s instructions. The genes were then ligated into yeast expression plasmids using LR clonase (Invitrogen) according to published method [33]. All constructed plasmids were sequenced by Eurofins Operon. All yeast genes and human *Ash2L*, *RbBP5*, *WDR5* and *DPY30* were cloned as full-length constructs into the expression plasmids. A C-terminal fragment of the human MLL1 gene product (amino acids 3370-3969 harboring the WIN and SET domains) was cloned into the yeast expression plasmid.

Yeast Total Protein Extraction for Western Blots

Overnight 5 mL yeast cultures of indicated strains were grown to an optical density (OD) of 1.5 at 600 nm and subsequently harvested via centrifugation. Pellets were then frozen on dry ice and lyzed with 10 M NaOH and 7.4% Betamercaptoethanol, 50% Trichloroacetic Acid was added as previously described [34], cells were washed with Tris and NaOH and 7.4% Betamercaptoethanol. 50% Trichloroacetic Acid was an ocular density (OD) of 1.5 at 600 nm and subsequently harvested via

Yeast Yeast Growth Assays

For serial dilution yeast growth assays, each yeast strain was plated in a tenfold dilution series to ensure consistent growth pattern and development (i.e. to prove that the genetic alterations have no impact upon the overall yeast growth). In this experiment, the growth assay was as expected, indicating no metabolic or growth-related defects in any yeast strains (Figure 1). Any changes in yeast growth are not attributed to the procedural method involved in specializing the strains and knocking out genes.

Single Gene Deletions and Replacements

Upon deletion of any core COMPASS member, yeast experienced a global loss of H3K4 methylation. This loss of H3K4 methylation was rescued by expression of the human Bre2 homolog, Ash2L, in the yeast *bre2Δ* strain (Figure 2). This rescue was the sole replacement capable of restoring partial methylation. The human homologs for *Set1* and *Swd1* (MLL1 and RbBP5, respectively) were also tested for rescue of H3K4 methylation, but only Ash2L was capable of functioning in place of its COMPASS counterpart, Bre2. MLL1 and RbBP5, the human homologs for *Set1* and *Swd1*, did not rescue di- or trimethylation of H3K4. The Ash2L rescue displayed wildtype-level H3K4 dimethylation and partial trimethylation (Figure 2). Therefore, we have observed that Ash2L can interact with *Set1* and *Swd1* in the place of Bre2 in the COMPASS complex to form a functional H3K4 di- and tri- methyltransferase.

Double and Triple Deletions and Replacements

To understand the MLL1 accessory proteins’ abilities to interact with each other and yeast COMPASS members, we created hybrid yeast-human protein complexes by introducing genes encoding human and yeast genes on plasmids into double and triple COMPASS member deletion strains. These chimeric protein complexes serve to reveal the unstudied interactions between accessory proteins of both complexes and hint at the intricate regulatory mechanism surrounding MLL1’s methyltransferase activity. Selected chimeric methyltransferase complexes conferred methyltransferase activity at varying degrees, while others could not form a functional methyltransferase complex.

Assessing the permutations and combinations of the accessory proteins in all yeast strains constructed (Figures 3 and 4) will reveal functional intricacies about the MLL1 protein, its accessory proteins, and maintenance of proper function.

In a *set1Δbre2Δ* background, rescue of *Set1* and *Bre2*, *MLL1 and Ash2L*, and *Set1* and *Ash2L* restored dimethylation to wildtype levels and trimethylation to partial levels (Figure 3). These experiments suggest that *Set1* and *Bre2* are largely interchangeable with their human homologs, MLL1 and Ash2L. Nevertheless, these experiments again demonstrate the essentiality of *Set1* and *Bre2* to the overall methylation mechanism. Experiments in the double deletion *set1Δbre2Δ* strain have supported the possibility of certain hybrid interactions, as we have observed a complete rescue of H3K4 dimethylation and a significantly weaker rescue of H3K4 trimethylation in *set1Δbre2Δ*+*Set1*+*Ash2L*. Yeast two-hybrid studies in our lab have confirmed that Ash2L binds to *Set1* but their exact interaction remains unknown (data not shown).

Results

Yeast Growth Assays

For serial dilution yeast growth assays, each yeast strain was plated in a tenfold dilution series to ensure consistent growth pattern and
Klein D (2019) Construction of hybrid yeast-human histone methyltransferase complexes in *Saccharomyces cerevisiae* clarifies the roles of Bre2 and Ash2L for mixed lineage leukemia

**Figure 1. Yeast growth is unchanged with COMPASS gene deletions.** Tenfold serial dilution plating showing yeast growth at 24 and 36 hours at 30°C. Each modified strain experienced homogenous growth and therefore growth changes cannot be attributed to the effects of gene deletions.

**Figure 2. Ash2L can functionally substitute for yeast Bre2, while other single COMPASS gene replacements do not restore H3K4 di- or trimethylation.** Yeast strains with individual COMPASS genes deleted were transformed with plasmids encoding either the deleted yeast gene or its human homolog. Total yeast protein extracts from these strains were subjected to Western blotting to detect levels of total histone H3, H3K4 dimethylation and H3K4 trimethylation.

**Figure 3. Hybrid yeast-human protein complexes restore H3K4me2 and H3K4me3 to near-wildtype levels in a set1Δbre2Δ background.** The *set1Δbre2Δ* double deletion strain was transformed with multiple plasmids encoding yeast and human gene homologs (SB = Set1 and Bre2, MA = MLL1 and Ash2L, and SA = Set1 and Ash2L). Total yeast protein extracts from these strains were subjected to Western blotting to detect levels of H3K4 dimethylation and H3K4 trimethylation. Ponceau staining was performed as a loading control.
Klein D (2019) Construction of hybrid yeast-human histone methyltransferase complexes in *Saccharomyces cerevisiae* clarifies the roles of Bre2 and Ash2L for mixed lineage leukemia

Triple rescue of the triple deletion strains resulted in rescue of COMPASS functionality only in the yeast replacement, and not with any combination of human genes (Figure 4).

**Discussion**

Chromosomal translocations of the human *MLL1* gene have been implicated in a variety of leukemias [2]. Exome sequencing has revealed a much broader suite of *MLL1* rearrangements across the range of cancers, suggesting that the identification of a drug target within the *MLL1* complex could have therapeutic ramifications far beyond treatment of leukemias alone [35]. When translocations occur at the *MLL1* locus, the normal function of *MLL1* is altered, leading to misregulation of gene expression, including that of *HOX* genes [1]. Infant acute lymphoid leukemia patients with *MLL1* gene rearrangements show an especially low (15-50%) survival rate compared with those without *MLL1* gene rearrangements (60-80%) [36,37].

In order to construct the human *MLL1* complex in yeast, we deleted genes encoding Set1, Bre2, and Swd1 from the yeast genome, effectively creating single, double, and triple COMPASS deletion strains. Thereafter, we rescued combinations of these deletions’ human effect with either yeast or human homologs (SB = Set1 and Bre2, MA = MLL1 and Ash2L, SBS = Set1, Bre2 and Swd1, MAR = MLL1, Ash2L and RbBP5). Total yeast protein extracts from these strains were subjected to Western blotting to detect total histone H3, H3K4 dimethylation and trimethylation.

Figure 4. Loss of H3K4 methylation associated with triple COMPASS member deletions cannot be rescued to wildtype levels with either human or yeast methyltransferase members. The *set1Δbre2Δswd1Δ* triple deletion strain (abbreviated “ΔΔΔ” in the figure) was transformed with plasmids encoding the three deleted yeast genes or their human homologs (SB = Set1 and Bre2, MA = MLL1 and Ash2L, SBS = Set1, Bre2 and Swd1, MAR = MLL1, Ash2L and RbBP5). Total yeast protein extracts from these strains were subjected to Western blotting to detect total histone H3, H3K4 dimethylation and trimethylation.

The authors declare no conflicts of interest.

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Volume 4: 6-6