The methyltransferase activity of the trithorax group (TrxG) protein MLL1 found within its COMPASS (complex associated with SET1)-like complex is allosterically regulated by a four-subunit complex composed of WDR5, RbBP5, Ash2L, and DPY30 (also referred to as WRAD). We report structural evidence showing that in WRAD, a concave surface of the Ash2L SPRY domain binds to the SPIa and ryanodine receptor (SPRY) domain of Ash2L (Chen et al. 2010; Avdic et al. 2011). Furthermore, mapping analysis has identified that this region of RbBP5 binds to the SPIa and ryanodine receptor (SPRY) domain of Ash2L (Chen et al. 2012); however, the structural basis underlying the interaction of RbBP5 with Ash2L is unknown.

Here, we report the crystal structure of the Ash2L SPRY domain in complex with RbBP5. We show that Ash2L-RbBP5-binding specificity is conferred by several conserved residues on both Ash2L and RbBP5. Structure-
guided mutational analysis reveals that disruption of this binding interface impairs formation of the WRAD complex, stimulation of MLL1 methyltransferase activity, and terminal differentiation of erythroid cells. Interestingly, the structure reveals that a phosphorylation switch on RbBP5 stimulates WRAD complex formation and increases methylation of H3K4 by KMT2 enzymes.

Results and Discussion

Crystal structure of Ash2L in complex with RbBP5

After determining that the Ash2L SPRY domain binds residues 344–364 of RbBP5 (Supplemental Fig. S1), we sought to gain structural insights into the interaction between Ash2L and RbBP5 and solved the crystal structure of Ash2L SPRY\textsubscript{d} in complex with a peptide corresponding to residues 344–357 of RbBP5 at a resolution of 2.20 Å (Supplemental Table S1). The Ash2L SPRY\textsubscript{d} domain adopts a twisted β sandwich composed of two antiparallel β sheets (referred to as A and B). Sheet A is composed of β2, β4, β5, β6, β7, and β11, while sheet B is composed of β1, β3, β8, β9, β10, and β12. The two sheets are linked by several interconnecting loops of varying length that extend out of the β-sandwich fold, and the Ash2L SPRY\textsubscript{d} domain ends with a short α helix [α1] (Fig. 1A).

Simulated annealing omit maps reveal clear electron density for the RbBP5 peptide, including residues 345–354 (Supplemental Fig. S2A). No electron density is observed for the RbBP5 E344 side chain (single letter denotes RbBP5 residues) and residues 355–357, and therefore they are not modeled in the structure. The RbBP5 peptide adopts a chair-like conformation and sits on a shallow surface formed by β4–β5–β6–β7 of sheet A. The N-terminal half of the peptide (residues 344–348) adopts an elongated conformation and protrudes perpendicularly down toward the basic surface of the Ash2L SPRY domain (Fig. 1A, B). In this region of the peptide, the RbBP5 E347 side chain makes van der Waals contacts with the backbone of Ash2L residues forming the β1–β2 loop, while the R348 side chain is solvent-exposed. In stark contrast, the E349 side chain binds in a deep pocket formed by the side chains of Tyr313 and Arg367 (Fig. 1A, C). The main chain carbonyl of E349 makes a hydrogen bond with the Ash2L Tyr313 hydroxyl group, while its carboxylate group engages in several hydrogen bonds with the Ash2L Pro356Ala and Arg367Ala mutants failed with wild-type Ash2L [Fig. 2B; Supplemental Fig. S3B]. Mutation of Pro356 and Arg367, residues interacting with RbBP5, respectively—with alanine severely impaired binding of RbBP5. Accordingly, enzymatic assays performed with wild-type Ash2L and RbBP5, and Arg367, residues interacting with the hydrophobic bulge and E349 of the RbBP5 D/E box, resulted in sixfold and 13-fold reduction in binding, respectively—without alanine severely impaired binding of RbBP5. Accordingly, enzymatic assays performed with the same mutants resulted in an approximately fivefold reduction of MLL1 methyltransferase activity compared with wild-type Ash2L [Fig. 2A, Supplemental Fig. S3A]. Mutation of Pro356 and Arg367, residues interacting with the hydrophobic bulge of the Ash2L SPRY domain binds a D/E box on RbBP5. (A) Cartoon representation of the Ash2L SPRY domain (green) in complex with RbBP5 (yellow) and a zoomed view on the interactions between the Ash2L SPRY domain and RbBP5. Ash2L residues are highlighted in light green and yellow, respectively. Key hydrogen bonds are rendered as red dashed lines. For clarity, only a subset of interactions is shown. [B] Electrostatic potentials are contoured from –10 kT\textsubscript{e} to 10 kT\textsubscript{e} [blue]. [C] Schematic representation of the interactions stabilizing RbBP5 into the Ash2L SPRY peptide-binding pocket. Yellow spheres represent RbBP5 residues. Ash2L residues making hydrogen bonds (filled boxes), hydrophobic contacts, or van der Waals contacts (empty boxes) with RbBP5 are rendered in blue. Hydrogen bonds are highlighted as orange dashed lines. For clarity, some interactions were omitted from the figure.

Disruption of Ash2L/RbBP5 interaction impairs MLL1 enzymatic stimulation and delays erythroid cell terminal differentiation

Following structural analysis of the Ash2L/RbBP5 complex, we first sought to identify Ash2L residues that are key for binding to RbBP5. Using isothermal titration calorimetry (ITC) [Fig. 2A; Supplemental Fig. S3A], we found that replacement of Tyr313 and Arg343—two residues lining the base of the Ash2L SPRY D/E-binding pocket and interacting with RbBP5 E347 and D353, respectively—with alanine severely impaired binding of RbBP5. Accordingly, enzymatic assays performed with the same mutants resulted in an approximately fivefold reduction of MLL1 methyltransferase activity compared with wild-type Ash2L [Fig. 2B, Supplemental Fig. S3B]. Mutation of Pro356 and Arg367, residues interacting with the hydrophobic bulge and E349 of the RbBP5 D/E box, resulted in sixfold and 13-fold reduction in binding, respectively. Accordingly, reconstitution of the complex with the Ash2L Pro356Ala and Arg367Ala mutants failed to stimulate MLL1 methyltransferase activity to the same extent as wild-type Ash2L, demonstrating that an Ash2L positively charged pocket lined by hydrophobic residues is important for WRAD assembly and MLL1 methyltransferase activity [Fig. 2A, B].
Ash2L is essential for maintaining high levels of histone H3K4 trimethylation (Steward et al. 2006; Demers et al. 2007), and knockdown of Ash2L in murine erythroid leukemia (MEL) cells results in a decrease of the H3K4me3 mark at the hypersensitive site 2 (HS2) of the β-globin locus control region (LCR) and a concomitant loss of β-globin gene transcription, a marker of erythroid cell terminal differentiation (Demers et al. 2007). To test the impact of mutations impairing Ash2L/RbBP5 complex formation, we transfected Flag-tagged constructs corresponding to the Ash2L wild type and single-point mutant of residues forming the base of the RbBP5-binding pocket in MEL cells stably expressing a doxycycline (Dox)-inducible shRNA directed against Ash2L (Demers et al. 2007). Treatment of cells with Dox resulted in a 40% decrease of H3K4me3 at the HS2 locus and a corresponding loss of 50% in β-globin gene expression (Fig. 2C,D) and subsequent loss of 50% in β-globin gene transcription (Fig. 2C,D) and subsequent loss of 50% in β-globin gene transcription (Fig. 2C,D). Together, our findings strongly suggest that a functional Ash2L/RbBP5 heterodimer is pivotal for maintaining the differentiation potential of MEL cells.

Phosphorylation of RbBP5 on S350 potentiates WRAD assembly

MLL1 is tightly regulated by various mechanisms, including allosteric regulation by the WRAD complex (Dou et al. 2006), deposition of other post-translational modifications on histone proteins (Southall et al. 2009), and phosphorylation of MLL1 by ATR (Liu et al. 2010). In the RbBP5 D/E box (Supplemental Fig. S4), an evolutionarily conserved serine residue (S350) is found in the center of the Ash2L SPRY concave surface (Fig. 3A). Interestingly, three independent studies revealed that RbBP5 S350 is phosphorylated in vivo (Christensen et al. 2010; Phanstiel et al. 2011; Shiromizu et al. 2013). To determine the impact of RbBP5 phosphorylation on WRAD formation, we ectopically expressed constructs corresponding to either wild-type RbBP5 or an RbBP5 S350A mutant in fusion with a Flag tag in HEK293 cells. While we observed enrichment of Ash2L following immunoprecipitation of wild-type Flag-RbBP5, incubation of Flag-RbBP5 S350A with M2 agarose beads failed to coimmunoprecipitate Ash2L (Fig. 3B). Our findings that S350 does not make significant interactions with Ash2L (Fig. 3C) and that its substitution to alanine impairs WRAD assembly suggest that keeping the hydroxyl group on histone proteins (Southall et al. 2009), and phosphorylation of RbBP5 on S350 potentiates WRAD assembly

Phosphorylation of RbBP5 on S350 potentiates WRAD assembly

With prior studies showing that the Ash2L C4-Winged-Helix (C4-WH) domain is important for binding to DNA (Chen et al. 2011; Sarvan et al. 2011) and ubiquitin (Wu et al. 2013) and that its SDI motif is important for binding to DPY-30 (South et al. 2010; Chen et al. 2012), our results point to a model in which Ash2L acts as a modulatory platform in which events that ultimately lead to the precise regulation of KMT2 methyltransferase activity. Here we report that Ash2L also recognizes the phosphorylated form of RbBP5. Binding and structural studies show that the Ash2L SPRY domain is a novel phospho-reader domain.

RbBP5 phosphorylation: a novel regulatory switch controlling WRAD assembly

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domain binds RbBP5\textsuperscript{phos} with 15-fold more affinity and that the phosphate moiety induces local structural reorganization within Ash2L, suggesting that the Ash2L SPRY domain is a novel phospho-binding domain. However, the recognition of the phosphate moiety by Ash2L SPRY differs from other known phospho-readers. This is particularly apparent for 14-3-3 proteins, which engage in several electrostatic interactions with the phosphate moiety within a well-defined basic pocket (Rittinger et al. 1999). Consistently, Muslin et al. (1996) showed that 14-3-3 can only bind to a Ser259-phosphorylated form of a Raf-1 peptide. Our observations that Ash2L engages in a relatively small number of contacts with the phosphate moiety of S350 and binds to both the unmodified and phosphorylated forms of RbBP5 suggest that this mode of phosphopeptide recognition serves as a rheostat modulating WRAD complex formation rather than an on/off switch assigned to other canonical phospho-readers.

RbBP5 phosphorylation controls histone H3K4 methylation by KMT2 enzymes

Our studies revealed that RbBP5 phosphorylation creates a better epitope for the binding of the Ash2L SPRY domain. However, close inspection of the structure revealed that the RbBP5 phosphate moiety is not entirely buried within the SPRY concave surface (Fig. 4A), suggesting that it may potentially play a direct role in regulating the methyltransferase activity of the KMT2 enzymes. To address this question, we performed pull-down experiments with HistSUMO-tagged MLL3 bound to TALON beads and Ash2L/RbBP5 or Ash2L/RbBP5\textsuperscript{phos}. Following several washes, TALON-bound protein complexes were eluted with sample loading buffer, resolved on SDS-PAGE, and stained with Coomassie. Consistent with previous binding studies (Cao et al. 2010), we observed binding of the Ash2L/RbBP5 heterodimer to the MLL3 SET domain. Interestingly, a fivefold increase in binding was observed when the Ash2L/RbBP5\textsuperscript{phos} complex was incubated with His-SUMO-MLL3 (Fig. 4B), suggesting that the Ash2L/RbBP5\textsuperscript{phos} dimer serves as a better interacting platform for the binding of the MLL3 SET domain.

Based on these observations, we surmised that Ash2L/RbBP5\textsuperscript{phos} might modulate the methyltransferase activity of KMT2 enzymes. To confirm this hypothesis, enzymatic assays were performed with different concentrations of the MLL3 SET domain incubated with stoichiometric amounts of Ash2L/RbBP5 or Ash2L/RbBP5\textsuperscript{phos}. As shown in Figure 4C and consistent with previous studies (Zhang et al. 2012), both complexes stimulated MLL3 methyltransferase activity at 1 \textmu M. However, upon dilution of the complex, Ash2L/RbBP5 failed to stimulate the activity of MLL3, while Ash2L/RbBP5\textsuperscript{phos} retained full activity of MLL3, demonstrating that RbBP5 phosphorylation serves as a rheostat increasing MLL3 kinetics.

After determining the impact of RbBP5 phosphorylation on MLL3 kinetics, we sought to determine the degree of K4 methylation catalyzed by MLL1 and MLL3 in the presence of the Ash2L/RbBP5 heterodimer reconstituted with RbBP5 or RbBP5\textsuperscript{phos}. We conducted enzymatic assays and subjected aliquots of the reactions to electrospray ionization mass spectrometry (ESI-MS). In comparison with the control reactions (Fig. 4D, Supplemental Fig. S5), a shift in the mass from 2346 to 2360 was observed in the presence of the Ash2L/RbBP5\textsuperscript{phos} complex, corresponding to the transfer of a single methyl group to the \textepsilon-amine of K4. However, the increase in H3K4me2 for both MLL1 and MLL3 (Fig. 4D; Supplemental Fig. S4), suggesting that the enhancement of MLL3 catalytic activity, a predominant histone H3K4 monomethyltransferase, by the Ash2L/RbBP5\textsuperscript{phos} complex pushes the reaction further to observe H3K4me2. Intriguingly, methyltransferase
RbBP5 phosphorylation regulates H3K4 methylation

assays performed with a higher concentration of MLL3 reconstituted with the Ash2L/RbBP5 or Ash2L/RbBP5phos showed that both complexes efficiently trimethylate H3K4 but failed to show increased rates of di- and trimethylation of histone H3K4 by the MLL3/Ash2L/RbBP5phos complex (Supplemental Fig. S5). Overall, our observations strongly suggest that RbBP5 phosphorylation couples the assembly of the WRAD complex to the allosteric regulation of KMT2 enzymes.

Enzymatic assays revealed that MLL3 monomethylates H3K4 in the presence of Ash2L/RbBP5 reconstituted with unmodified RbBP5. These observations are consistent with recent studies showing that COMPASS-like MLL3/MLL4 complexes predominantly monomethylate H3K4 at enhancer regions and specific promoter regions (Herz et al. 2012; Hu et al. 2013; Morgan and Shilatifard 2013; Lee et al. 2008). Interestingly, upon incubation of the MLL3 SET domain with the Ash2L/RbBP5 or Ash2L/RbBP5phos complexes by centrifugation, resuspended, and cross-linked as previously described (Demers et al. 2007). After 2 d, cells were pelleted and the cell pellet was bated with equimolar amounts of RbBP5 344–357 for 1 h on ice and crystalized using the sitting drop vapor diffusion method at 18°C. Diffraction-quality crystals were obtained in 0.2 M magnesium chloride hexahydrate, 0.1 M Bis-Tris (pH 5.5), and 25% (w/v) polyethylene glycol. The crystals were sequentially soaked in the mother liquor supplemented with an increasing amount (5%–20%) of glycerol, harvested, and flash-frozen in liquid nitrogen. The structure was solved by molecular replacement, and model building was performed as detailed in the Supplemental Material.

Mel cells

Mel cells were transfected with plasmids expressing Flag-only, Flag-Ash2L wild type, Flag-Ash2L Y313A, Flag-Ash2L R343A, Flag-Ash2L P356A, Flag-Ash2L Y359V, and Flag-Ash2L R367A by electroporation. Twelve hours after transfection, differentiation was induced with DMSO as previously described (Demers et al. 2007). After 2 d, cells were pelleted by centrifugation, resuspended, and cross-linked as previously described (Demers et al. 2007). Chromatin extraction and immunoprecipitation experiments were performed as previously described (Harvan et al. 2011) and quantified as detailed in the Supplemental Material.

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Materials and methods

Protein crystallization and structure determination

Recombinantly purified Ash2LSPRY3Ala [5–10 mg/ml] (see the Supplemental Material) was incubated with equimolar amounts of RbBP5 344–357 for 1 h on ice and crystalized using the sitting drop vapor diffusion method at 18°C. Diffraction-quality crystals were obtained in 0.2 M magnesium chloride hexahydrate, 0.1 M Bis-Tris (pH 5.5), and 25% (w/v) polyethylene glycol. The crystals were sequentially soaked in the mother liquor supplemented with an increasing amount (5%–20%) of glycerol, harvested, and flash-frozen in liquid nitrogen. The structure was solved by molecular replacement, and model building was performed as detailed in the Supplemental Material.

MEC cells

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