Cell Reports
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MLL-ENL Inhibits Polycomb Repressive Complex 1 to Achieve Efficient Transformation of Hematopoietic Cells

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http://dx.doi.org/10.1016/j.celrep.2013.03.038

SUMMARY

Stimulation of transcriptional elongation is a key activity of leukemogenic MLL fusion proteins. Here, we provide evidence that MLL-ENL also inhibits Polycomb-mediated silencing as a prerequisite for efficient transformation. Biochemical studies identified ENL as a scaffold that contacted the elongation machinery as well as the Polycomb repressive complex 1 (PRC1) component CBX8. These interactions were mutually exclusive in vitro, corresponding to an antagonistic behavior of MLL-ENL and CBX8 in vivo. CBX8 inhibited elongation in a specific reporter assay, and this effect was neutralized by direct association with ENL. Correspondingly, CBX8-binding-defective MLL-ENL could not fully activate gene loci necessary for transformation. Finally, we demonstrate dimerization of MLL-ENL as a neomorphic activity that may augment Polycomb inhibition and transformation.

INTRODUCTION

MLL fusions are highly efficient oncoproteins that transform hematopoietic progenitors and cause aggressive leukemia (Slany, 2009). These proteins are derived from chromosomal translocations that affect the *MLL* locus at 11q23, joining an N-terminal portion of the H3K4 histone methyltransferase MLL with a variety of different partner proteins. These partner proteins replace the original methyltransferase activity contained within the MLL C terminus and create potent transactivators that cause the inappropriate expression of target genes. Trithorax, the MLL homolog in the fly, acts as a positive regulator of the clustered *Hox*-homeobox genes. Analogously, MLL fusions induce a strong overexpression of *HOX, MEIS*, and *PBX* homeobox genes, with the latter two coding for HOX-binding partners. Elevated levels of *HOX/MEIS/PBX* are sufficient to transform hematopoietic progenitor cells, and deregulation of homeobox genes is mainly responsible for the oncogenic activity of MLL derivatives.

Remarkably, MLL chimeras generally do not behave as classical activators in recruiting RNA polymerase II (RNA PolII). Depending on the fusion partner, they seem to either affect chromatin-associated processes or, more frequently, specifically stimulate transcriptional elongation. MLL partners of the ENL (ENL and AF9) and AFF (AFF1–AFF4) families form a higher-order protein complex named EAP (originally standing for ENL-associated proteins and later for elongation-assisting proteins) that was purified from nuclear extracts (Mueller et al., 2007, 2009). In addition to ENL/AF9 and AFF proteins (AFF1 and AFF4 are also known as AF4 and AF5q31 or short AF5), EAP also includes positive transcription elongation factor b (P-TEFb) and the H3K79 histone methyltransferase DOT1L. P-TEFb is a dimer of CDK9 and a cyclinT that phosphorylates RNA PolII at serine-2 within the C-terminal repeat domain. Additional substrates include proteins such as negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF), which help to keep RNA PolII stalled shortly after initiation. These modifications catalyzed by P-TEFb are a crucial prerequisite for efficient elongation of preinitiated transcripts (Peterlin and Price, 2006). DOT1L introduces methylation of lysine-79 in histone H3, a modification associated with actively transcribed chromatin. Interestingly, DOT1L was first discovered in yeast, where H3K79 serves as an "antisilencing" modification that inhibits invasion of heterochromatin into transcribed areas (Nguyen and Zhang, 2011). EAP-related complexes have been isolated by several laboratories (Bitoun et al., 2007; Monroe et al., 2011; Yokoyama et al., 2010) and some studies suggest that EAP can be separated into two subcomplexes with different functions. A super elongation complex (SEC) stimulates elongation by recruiting P-TEFb together with other elongation factors, and a separate DOT1L-complex (DotCom) is responsible for chromatin modification (reviewed in Smith et al., 2011). SEC may be widely involved in transcriptional control because it has also been copurified with the HIV Tat protein, which is known to support viral transcription by stimulating elongation (He et al., 2010; Sobhian et al., 2010). Although EAP is unequivocally connected to active transcription, paradoxically, proteins that are normally associated with Polycomb repressive complex 1 (PRC1) have been repeatedly demonstrated to interact and copurify with EAP components (García-Cuéllar et al., 2001; Hemenway et al., 2001; Monroe et al., 2011; Mueller et al., 2007).

Originally, Polycomb proteins were identified in *Drosophila* as opponents of trithorax function. The balance between trithorax-mediated activation and repression by Polycomb dynamically regulates the transcriptional output of many genes, particularly those involved in self-renewal, differentiation, and developmental decisions (*HOX* genes are a paradigmatic example). Because trithorax as well as Polycomb activities involve chromatin modification, the corresponding marks become heritable and constitute part of what has been termed "epigenetic memory." In mammals, this function has been conserved (reviewed in Margueron and Reinberg, 2011). Major representatives of mammalian Polycomb proteins can be found in two different protein complexes. Polycomb repressive complex 2 (PRC2) contains the conserved histone methyltransferase EZH2 (enhancer of zeste homolog 2), which introduces H3K27 di- and trimethylation, whereas PRC1 catalyzes histone H2A ubiquitination via the RING1/2 E3 ligases. These enzymes are accompanied by a variable set of associated factors that include PCGF (Polycomb group ring finger), PHC (polyhomeotic homolog), and CBX (chromobox) proteins (Gao et al., 2012). Chromobox proteins are chromatin readers that recognize and bind to methylated H3K27. Therefore, a sequential mechanism was suggested whereby PRC2 deposits a repressive mark that is subsequently read and interpreted by PRC1. However, PRC2-independent recruitment of PRC1 has also been demonstrated (Dietrich et al., 2012; Yu et al., 2012). Despite intensive studies, it is not yet completely clear how PRC complexes actually repress. Both chromatin compaction (Eskeland et al., 2010; Gao et al., 2012) and inhibition of transcription by ubiquitinated H2A (Stock et al., 2007; Zhou et al., 2008) seem to be involved.

Here we investigated the reason for the counterintuitive copurification of PRC1 components with EAPs, and demonstrate that Polycomb-mediated repression can be squelched by direct interaction of the ENL and CBX8 proteins. Polycomb proteins have been shown to colocalize with basal transcription factors at loci "poised" for transcription (Oguro et al., 2010; Taberlay et al., 2011), and it was speculated that PRC may block transcription after the initiation step. This would mandate neutralization of PRC function before efficient elongation can occur. We describe a likely mechanism that can achieve this effect and thus may serve as a potential therapeutic target in the context of MLL fusion proteins.

RESULTS

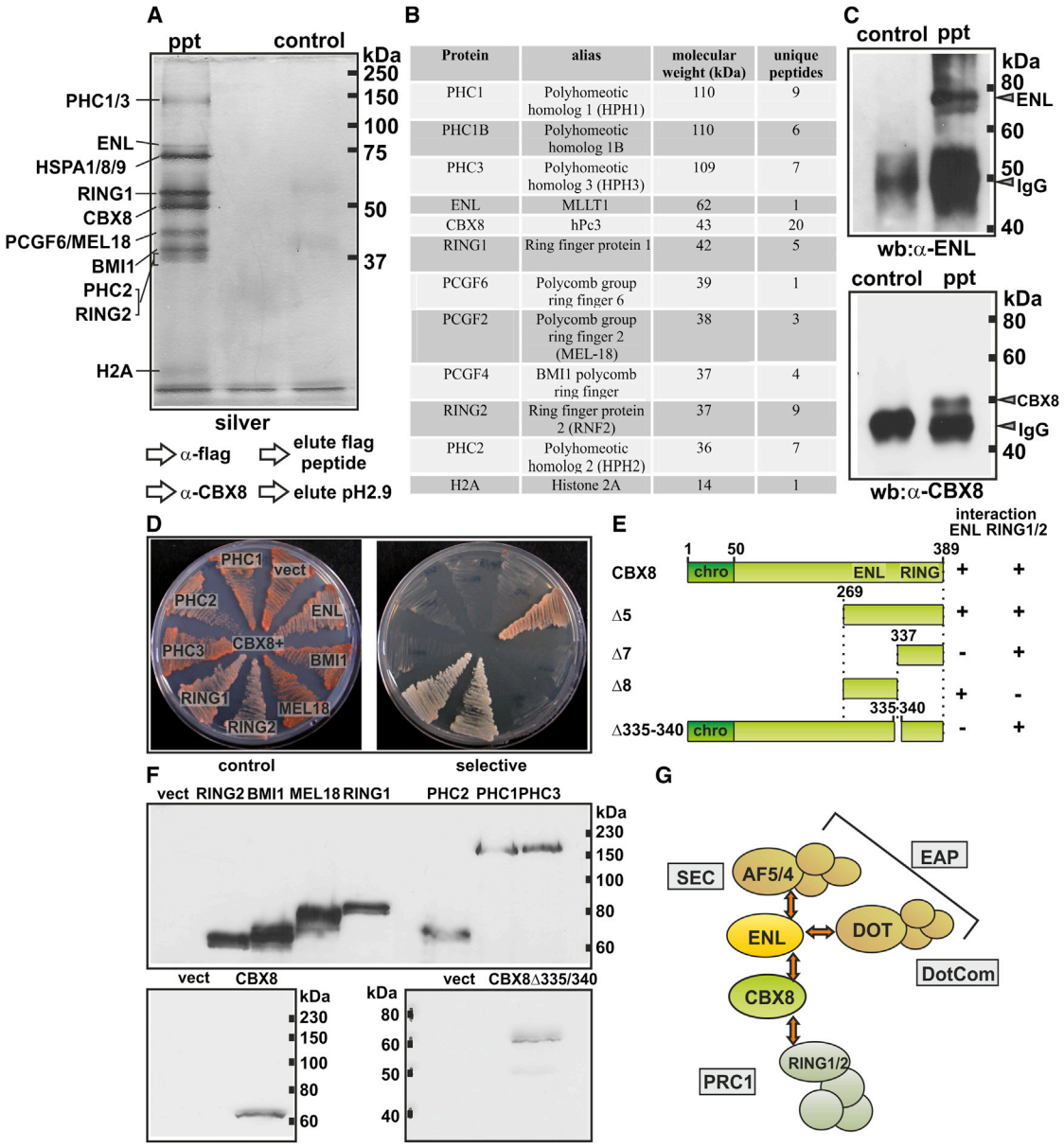
ENL Binds to PRC1 through CBX8

The PRC1 components CBX8 and RING1 were copurified with ENL in a previous study (Mueller et al., 2007). To confirm the data from that study and identify the subtype of PRC1 that interacts with ENL, we purified CBX8 and the accompanying protein complex from HEK293 cells, the source of the original identification of EAP (Figure 1). These cells were transduced with flag-tagged CBX8, and CBX8 interacting proteins were isolated by tandem immunoprecipitation (IP) of nuclear extracts using sequential pull-down with flag- and CBX8-agarose. Nontransduced HEK293 cells served as control. Mass spectrometry identified all classical PRC1 components from eluted bands as visualized by silver staining (Figures 1A and 1B). Consistent with a recent study that classified PRC1 subtypes (Gao et al., 2012), CBX8 occurred mainly in PRC1.2 and PRC1.4 (i.e., in MEL18- and BMI1-containing PRCs). ENL was consistently copurified with PRC1 in three independent experiments, albeit in substoichiometric amounts. The interaction of ENL and CBX8 could also be corroborated by standard IP (Figure 1C). Anti-flag-precipitated material from fCBX8 expressing HEK293 cells contained immunologically detectable ENL and, vice versa, CBX8 could be identified by western blot in fENL precipitates.

Little is known about direct protein contacts within PRC1. To determine how ENL would fit into this interaction network, we identified PRC1 components that make a direct contact with CBX8. Full-length versions of the major PRC proteins were tested in a two-hybrid system, with CBX8 (Figure 1D) or various CBX8 deletion mutants (Figure 1E) used as baits. Constructs were correctly expressed in yeast (Figure 1F) and did not show any endogenous transactivation (not shown). In addition to the known interaction with ENL, direct binding of CBX8 was observed with both RING proteins (RING1 and RING2) but not with any of the other PRC1 components tested. Mapping of the respective interaction domains revealed two separable regions at the CBX8 C terminus that independently mediated binding to ENL and RING (Figure 1E). A small deletion of amino acids 335–340 was sufficient to disrupt ENL interaction, but this did not affect affinity for RING proteins. In summary, CBX8 can establish a connection between ENL and PRC (Figure 1G).

CBX8 Can Bind ENL and RING Simultaneously, but ENL Allows Only Mutually Exclusive Interactions

Because ENL and RING bind to CBX8 at closely neighbored sites, we wanted to know whether these interactions can occur at the same time. It was previously shown that all direct binding partners of ENL (AF5/4 [AFF4/AFF1], DOT1L, and CBX8) contact overlapping or immediately adjacent motifs in ENL (He et al., 2011; Mueller et al., 2009). Therefore, it was not clear whether CBX8 can interact with ENL once it is occupied by other EAP components. To clarify these questions, we performed an elaborate set of IPs (Figure 2). Various combinations of differently tagged proteins were coexpressed in 293T cells to test mutual binding in the presence of a third protein competing for the same binding site. For CBX8, a clear picture emerged indicating that the individual interactions of CBX8 (Figure 2A) with ENL and RING1 can occur simultaneously. CBX8 could bridge ENL with RING, as suggested by coprecipitation of RING1 with ENL (Figure 2A, left panels) and vice versa (Figure 2A, middle panels) in the presence of CBX8. CBX8 itself contacts both proteins (Figure 2A, right panels). In contrast, binding of ENL to its interaction partners was only possible one at a time. Although an association of CBX8 and Dot1l with ENL could be confirmed separately (Figure 2B, right panels), ENL could not "bridge" the two proteins, suggesting that these contacts are not coincident (Figure 2B, left and middle panels). A similar result was obtained with AF5 (Figure 2C). Again, CBX8 and AF5 could associate with ENL individually (Figure 2C right panels) but not simultaneously (Figure 2C, left and middle panels). In summary, these results can be best reconciled with a scaffolding function of ENL that allows only one contact at any given moment, thereby



Protein	alias	molecular weight (kDa)	unique peptides
PHC1	Polyhomeotic homolog 1 (HPH1)	110	9
PHC1B	Polyhomeotic homolog 1B	110	6
PHC3	Polyhomeotic homolog 3 (HPH3)	109	7
ENL	MLLT1	62	1
CBX8	hPc3	43	20
RING1	Ring finger protein 1	42	5
PCGF6	Polycomb group ring finger 6	39	1
PCGF2	Polycomb group ring finger 2 (MEL-18)	38	3
PCGF4	BMI1 polycomb ring finger	37	4
RING2	Ring finger protein 2 (RNF2)	37	9
PHC2	Polyhomeotic homolog 2 (HPH2)	36	7
H2A	Histone 2A	14	1

Figure 1. Purification and Molecular Architecture of PRC1

(A) Silver-stained gel of a typical PRC1 preparation. Nuclear extracts from HEK293 cells transduced with a flag-tagged version of CBX8 were used for tandem IP, as schematically indicated, and nontransduced cells served as controls. The gel is a typical example of three independent experiments.

(B) Mass spectrometric identification of CBX8 copurifying proteins. Not listed are heat shock proteins HSPA1, HSPA8, and HSPA9, which were also present in the immunoprecipitates.

(C) Coprecipitation of CBX8 and ENL. Flag-reactive material was precipitated from nuclear extracts of HEK293 expressing either flag-CBX8 (upper panel) or flag-ENL (lower panel). The presence of ENL and CBX8 was detected by western blotting. WT HEK293 lysates were used as controls.

(D) Typical example of a two-hybrid experiment screening for direct interactions between full-length CBX8 (bait) and PRC1 members (prey). Growth on control and selective (−histidine) plates is shown.

(E) Mapping of the ENL and RING interaction domains in CBX8. CBX8 constructs as indicated were tested as in (D). Growth on selective plates is indicated by +.

(F) Expression of GAL4 constructs used for two-hybrid experiments. Yeast lysates were analyzed by immunoblotting with anti-GAL4 AD antibodies (upper panel) and anti-GAL4 DNA-binding domain reagents (lower panels).

(G) Schematic depiction of PRC1-EAP interactions.

enforcing a sequential order of events. This agrees well with previous results showing that AF5 and DOT1L also exclude each other in binding to ENL (Yokoyama et al., 2010).

PRC1 and MLL-ENL Induce Opposing Activities In Vivo

The in vitro results suggested the possibility that CBX8/PRC1 and ENL/EAP activities may influence each other. There is no

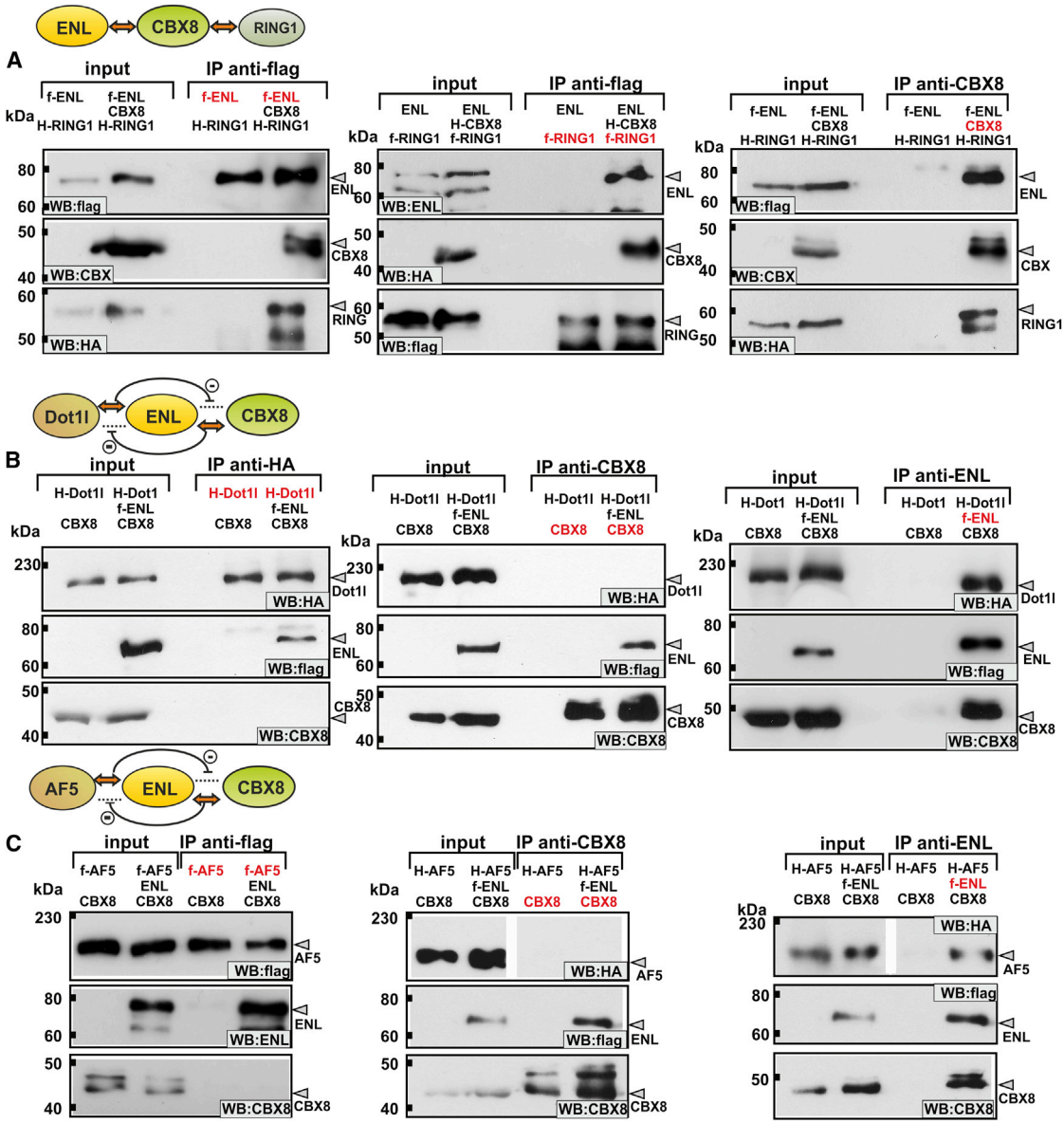


Figure 2. Analysis of Concurrent Protein Interactions

(A) Simultaneous binding of ENL and RING1 to CBX8. Tagged (f, flag; H, HA) and untagged versions of CBX8, ENL, and RING1 were cotransfected as indicated. Proteins targeted for precipitation are marked in red font.

(B) Dot1l and CBX8 cannot bind simultaneously to ENL. No coprecipitation of Dot1l and CBX8 was observed (left and middle panels) despite the fact that ENL binds to both proteins individually (right panels). Tags and proteins are labeled as in (A).

(C) Binding of AF5 (AFF4) and CBX8 to ENL is mutually exclusive. CBX8 could not precipitate AF5 in the presence of ENL (left, middle panels), yet ENL interacts with both proteins in the same lysates (right panels). Labeling as in (A).

direct biological assay for ENL activity in vivo. However, in the context of an MLL fusion protein, ENL function reads out as transformation capability. In order to investigate the biological consequences of CBX8/PRC1-mediated repression for cellular transformation, we overexpressed CBX8 in Mll-ENL immortalized cells. Because artificially high concentrations of MLL-ENL may skew the balance and therefore affect the outcome of this experiment, we chose to use a recently published Mll-ENL-ER knockin model (Meer mice; Takacova et al., 2012) for this test. In Meer animals, an inducible Mll-ENL fusion was created in the germline by knockin of ENL joined to the ligand-binding domain of the estrogen receptor. Therefore, Mll-ENL-ER is expressed under control of the endogenous *Mll* promoter (Figure 3A). Isolated bone marrow progenitors from these mice can be immortalized by the simple addition of tamoxifen, leading to the outgrowth of permanent cell lines. To study the effect of elevated CBX8 on Mll-ENL-mediated transformation, we transduced Meer cells with a pMSCV-based

expression construct for CBX8, which led to a large increase of CBX8 RNA and protein as compared with vector-transduced controls (Figure 3B). The Meer/CBX8 cells were viable and could be propagated for several weeks in culture. In a quantitative assessment by colony-forming cell (CFC) assays, however, they consistently showed a reduced replating capability and formed fewer colonies in methylcellulose (Figure 3C). Phenotypically, these cells displayed a higher level of the differentiation marker Gr-1 on the surface, indicative of a weakened transformation by Mll-ENL (Figure 3D). This was mirrored at the molecular level by lower RNA concentrations of the key Mll-ENL targets *Hoxa9* and *Meis1* (Figure 3E). In contrast to CBX8, overexpression of RING1 elicited only minor effects in Meer cells, indicating that CBX8, but not RING1, is a limiting factor (Figure S1).

To study the molecular events occurring at the respective genomic loci, we performed chromatin IP (ChIP) experiments around the *Hoxa9* and *Meis1* transcriptional start sites (Figures 3F–3H). In cells with active Mll-ENL (+TAM) that overexpress CBX8 (Figure 3F), we observed a drop of four different elongation markers corresponding to the reduced transcription of *Hoxa9* and *Meis1*. H3K79me2, H3K36me2, RNA PolII serine-2 phosphorylation, and ENL were moderately but consistently reduced by 20%–50% compared with vector controls. Interestingly, no significant increase of H2A ubiquitination as a readout for PRC1 activity was observed in CBX8 cells as long as tamoxifen was present (Figure 3G, left panel). H3K27 methylation as one potential recruiting element for CBX8 was equally present in transduced and control cells (Figure 3G, right panel). The observed reduction in *Hoxa9* and *Meis1* transcripts in CBX8 cells was not due to decreased Mll-ENL binding, because this parameter remained unchanged or was even slightly increased after overexpression of CBX8 (Figure 3H, left panel). Rather, the reduction in transcription was correlated with a 2- to 3-fold increase in chromatin-bound CBX8 protein (Figure 3H, right panel).

To investigate this phenomenon, we repeated the ChIP experiments 72 hr after inactivation of Mll-ENL by removal of tamoxifen. As was previously shown (Milne et al., 2005), this led to cellular differentiation and paralleled an exit of Mll-ENL from its target loci. As a consequence, elongation markers were almost completely lost, with the exception of H3K36 dimethylation, which remained detectable at this time point (Figure 3F). Remarkably, inactivation of Mll-ENL led to a large increase of PRC1 and PRC2 activity exclusively in CBX8 cells, whereas this effect was almost negligible in controls (Figure 3G). This was not correlated with the levels of chromatin-bound CBX8 (Figure 3H, right panel), because additional overexpression of CBX8 in differentiating Mll-ENL cells (−TAM) even led to a reduction of ChIP-detectable CBX8 bound to the locus. In contrast, there was an inverse association with remaining Mll-ENL (Figure 3H, left panel) that exited earlier from the *Hox/Meis* loci in CBX8 cells compared with vector controls and corresponding to the more advanced state of differentiation in CBX8 cells. This suggests that Mll-ENL has to fall below a certain threshold before PRC1 can become active, supporting a suppressive role of Mll-ENL in CBX8 and Polycomb function.

CBX8 Bound by ENL Loses Repressor Activity in an Elongation Reporter Assay

Because ENL and MLL-ENL mainly stimulate transcriptional elongation, we wanted to assess the consequences of the ENL/CBX8 interaction with respect to this parameter. Elongation can be specifically measured with a specialized reporter assay (Rev assay) that makes use of the fact that the HIV long terminal repeat (LTR) is known to be controlled mostly after initiation has occurred (Gold and Rice, 1998). A luciferase-based reporter system (Figure 4A) driven by a modified HIV LTR contains an engineered binding motif (SLIIb loop) for the RNA-binding protein Rev. This loop is located within the short RNA that is produced after RNA PolII initiates transcription. In this way, any protein of interest can be recruited to the paused RNA polymerase, allowing readout of either stimulating or repressive activity. A series of Rev-CBX8 mutants, including deletions of the C-terminal RING-binding domain and a CBX8 derivative without the ENL interaction motif (CBX8Δ332-342), were constructed and tested for correct expression (Figure 4B). Coimmunoprecipitation (Co-IP) confirmed that the CBX8Δ332-342 mutant had lost the capacity to bind ENL, whereas the interaction with RING was untouched (Figure 4C). Both CBX8 and CBX8Δ332-342 demonstrated a clear, concentration-dependent repressor activity in Rev assays (Figure 4D, left panel). This indicates a CBX8 intrinsic inhibitory function that does not require binding to ENL. Corroborating a previous study (Grau et al., 2011), the CBX8-encoded repressor function was not contingent on RING binding, as a loss of the RING-binding domain in the CBX8_1-331 construct did not affect its inhibitory activity (Figure 4D, right panel). Rather, the repressor function relied on an extended, highly charged region within the central portion of the protein. Small hairpin RNA (shRNA) experiments were also consistent with a RING-independent repressor activity for CBX8 (Figure S2).

Interestingly, CBX8-mediated repression was largely neutralized by coexpression of ENL (Figure 4E). This phenomenon was absolutely reliant on a direct ENL/CBX8 interaction, because ENL-binding defective CBX8 mutants remained repressors even in the presence of ENL. In an attempt to confirm these results in a reciprocal fashion, we constructed an ENL mutant that has lost CBX8 affinity but keeps all other interactions intact. Because DOT1L and CBX8 bind to coinciding regions in ENL, we chose to test a point mutant that has been found to specifically abrogate CBX8 binding in the homologous AF9 protein (Tan et al., 2011). The corresponding T546A exchange was introduced into ENL and correct protein expression was confirmed by western blotting (Figure 4F). Interactions between Dot1l and ENLT546A were unaffected, but affinity for CBX8 was no longer detectable in co-IP experiments under stringent washing conditions (Figure 4G). Interaction with AF5 also remained intact (Figure 4H). In contrast to wild-type (WT)-ENL, ENLT546A was significantly weaker in "rescuing" transcriptional elongation from CBX8-mediated repression (Figure 4I). The remaining activity of ENLT546A was likely due to residual binding to CBX8 that was not completely abrogated by introduction of the single-point mutation. A complete ablation of the ENL/CBX8 interaction by using an ENL-binding defective CBX8 mutant (CBX8Δ332-342) fully eliminated the ENLT546A-induced effect on CBX8-induced repression (Figure 4J). Because ENLT546A retained some

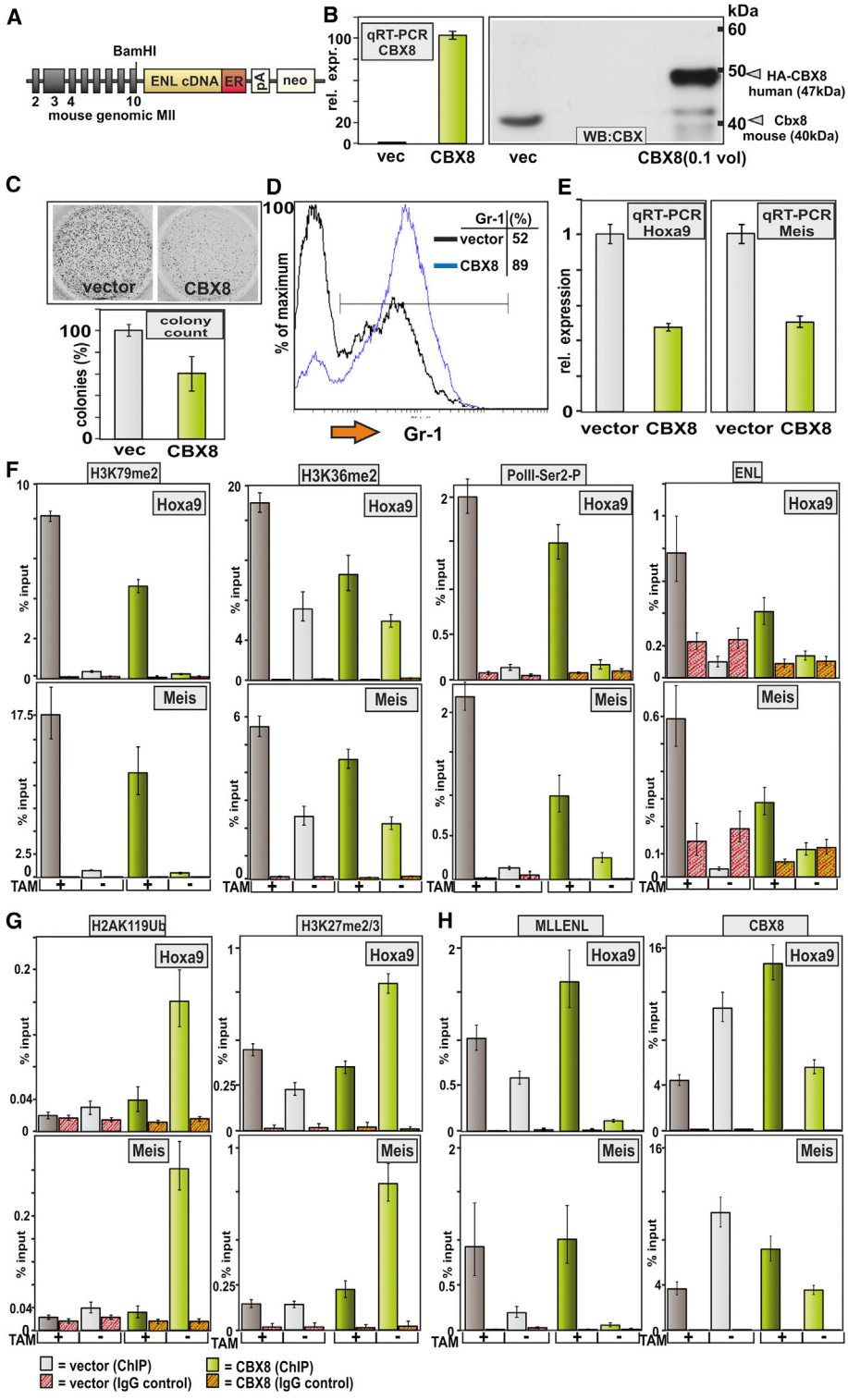


Figure 3. Mutual Inhibition of MLL-ENL and PRC1 In Vivo

(A) Schematic depiction of the Mll-ENL-ER (Meer) knockin construct. Meer bone marrow progenitors can be immortalized by the simple addition of tamoxifen. neo, neomycin resistance; pA, polyA sequence.

(B) Left panel: Detection of CBX8 overexpression by qRT-PCR. Meer cells were infected with either empty viruses or a viral expression construct for CBX8. qPCR primers that amplify the mouse and human CBX8 sequence were chosen. Given are the means and SDs of a triplicate, and these data represent one out of three experiments with similar outcome.

(legend continued on next page)

affinity for CBX8, we tried to introduce an additional amino acid exchange (T534A) that has been demonstrated to be important for CBX8 binding in AF9. The introduction of the second mutation, however, also reduced affinity for Dot1l (Figure S3), and therefore this construct was not tested further.

MLL-ENL Interaction with CBX8 Is Required for Efficient Transformation

To assess the in vivo consequences of interfering with CBX8 binding, we introduced the ENLT546A mutation into an MLL-ENL context (Figure 5A). The single amino acid change did not affect expression of the protein, yet this mutant blunted the transforming capacity of the respective MLL-ENL fusion. Primary hematopoietic progenitors transduced with MLL-ENLT546A formed ~70% fewer colonies than those transformed by the WT counterpart (Figure 5B). In addition, these cells could not be propagated in liquid culture for more than 2 weeks, and terminal differentiation and proliferation arrest eventually prevailed (not shown). Concomitantly with the weaker replating efficiency, endogenous concentrations of *Hoxa9* and *Meis1* transcripts were significantly lower in MLL-ENLT546A cells compared with controls (Figure 5C). H3K79 dimethylation was reduced but nevertheless present at *Hoxa9* and *Meis1* loci, confirming that DOT1L can still interact with the MLL-ENLT546A mutant. H2A ubiquitination was slightly to moderately increased in MLL-ENLT546A cells, and H3K27 methylation was barely detectable in this retroviral overexpression model (Figure 5D). These data can best be reconciled with an inability of MLL-ENLT546A to make the target gene loci sufficiently permissive for transcription. This occurs despite the fact that retroviral transduction leads to overexpression of the MLL fusion.

Global Knockdown of Cbx8 Blunts Transformation by MLL-ENL

Because MLL-ENL must overcome a CBX8 (PRC1)-induced barrier to immortalize cells, we speculated that global reduction of this protein might aid in transformation. To test this assumption, Meer cells were transduced with an shRNA construct targeting *Cbx8* or with a control vector. After antibiotic selection, the cells were screened for surface-marker expression and for gene expression at two different time points. Concordant with our hypothesis, knockdown of *Cbx8* initially (11 days after transduction) caused a shift toward cells with lower Gr-1 levels, indicating a stronger block in differentiation. However, this phenomenon was short-lived and the knockdown effect was lost after further culture (Figure 6A). This transient phenotype was correlated with a rebound of *Cbx8* from ~50% suppression at day 11 to nearly normal levels at day 19 (Figure 6B), indicating outgrowth of cells that have lost shRNA expression. A potential reason for this observation became apparent when we checked the transcripts of the *Cdkn2* (*Ink4*) tumor suppressor family (*Cdkn2a*, *Cdkn2b*, *Cdkn2c*, and *Cdkn2d*), which are known PRC1 targets. In line with previous findings (Dietrich et al., 2007), knockdown of *Cbx8* was inversely correlated to the concentrations of *Cdkn2a* and *Cdkn2b*, two powerful inhibitors of cell-cycle progression that are transcribed from the same locus. Concordant and in parallel with the surge in *Cdkn2a/b* transcription, a pronounced but transient G1 arrest was observed in *Cbx8* knockdown cells (Figure 6C). This phenomenon was correlated to a reduced plating efficiency of the respective cells in methylcellulose (Figure 6D). Thus, derepression of the *Cdkn2a/b* genes is a likely explanation for the growth disadvantage of cells with globally reduced *Cbx8* concentrations.

MLL-ENL Can Dimerize to Allow the Simultaneous Occurrence of Normally Separated Processes

The Co-IP results and the mutational analysis suggested that under normal circumstances, binding of CBX8 and DOT1L/P-TEFb to ENL cannot occur simultaneously. This presumably allows for regulation and may control the extent of transcriptional stimulation. Nevertheless, genes activated by MLL fusion proteins are at the same time hypermethylated at H3K79 (Krivtsov et al., 2008), highly transcribed, and not blocked by PRC1. This suggests that ENL may circumvent normal control mechanisms in the context of the fusion. To investigate the molecular basis of this effect, we probed for dimerization of MLL-ENL because this would allow for recruitment of elongation factors despite the presence of CBX8 (PRC1). In addition, it is known that fusions of MLL with strong dimerization domains are weakly transforming (Martin et al., 2003; Xia et al., 2003). To check for self-association of MLL-ENL, we coexpressed flag- and hemagglutinin (HA)-tagged versions of this protein. Precipitation with a flag-specific antibody (Figure 7A, left panels) clearly brought down HA-tagged MLL-ENL. This was not due to an unspecific

Right panel: Immunological detection of CBX8/Cbx8 in extracts of Meer cells transduced as before. Epitope-tagged human CBX8 is ~7 kDa larger than the endogenous mouse protein. Ten times more total protein was loaded per lane for the vector control.

(C) CBX8 overexpression reduces CFC capacity of Meer cells. Hematopoietic progenitor cells from Meer bone marrow were transduced with CBX8 or control virus. Replating assays were performed in the presence of tamoxifen. The upper panel shows a representative example of third-round colonies; the bar graph charts relative colony numbers as the average and SD of six independent experiments.

(D) CBX8 induces higher levels of the differentiation marker Gr-1 on the cell surface. Meer cells from the experiments shown in (C) were analyzed for Gr-1 expression by fluorescence-activated cell sorting (FACS) analysis. The percentage of Gr-1 positive cells was calculated using the indicated region.

(E) CBX8 overexpression reduces *Hoxa9* and *Meis1* expression in Meer cells. Q-RT PCR was performed on total RNA isolated from Meer cells transduced as indicated. Averages and SDs of a technical triplicate are given, representing a typical example out of three experiments in total.

(F) ChIP for elongation associated chromatin modifications and marker proteins. Meer progenitor cells were transduced with vector (gray bars) or CBX8 (green bars). ChIP for modifications and factors was done as indicated. Experiments were performed in the presence of tamoxifen (dark bars, +TAM) and 72 hr after tamoxifen was removed (light bars, −TAM). Precipitation is given as % input. Unspecific immunoglobulin G (IgG) served as control (red hatched bars). The chart illustrates averages and SDs of PCR triplicates. The ChIP experiment was done on three biological replicates with essentially the same results.

(G) Detection of Polycomb-associated chromatin modifications. ChIP was performed as described in (F).

(H) Presence of CBX8 and Mll-ENL as detected by ChIP.

See also Figure S1.

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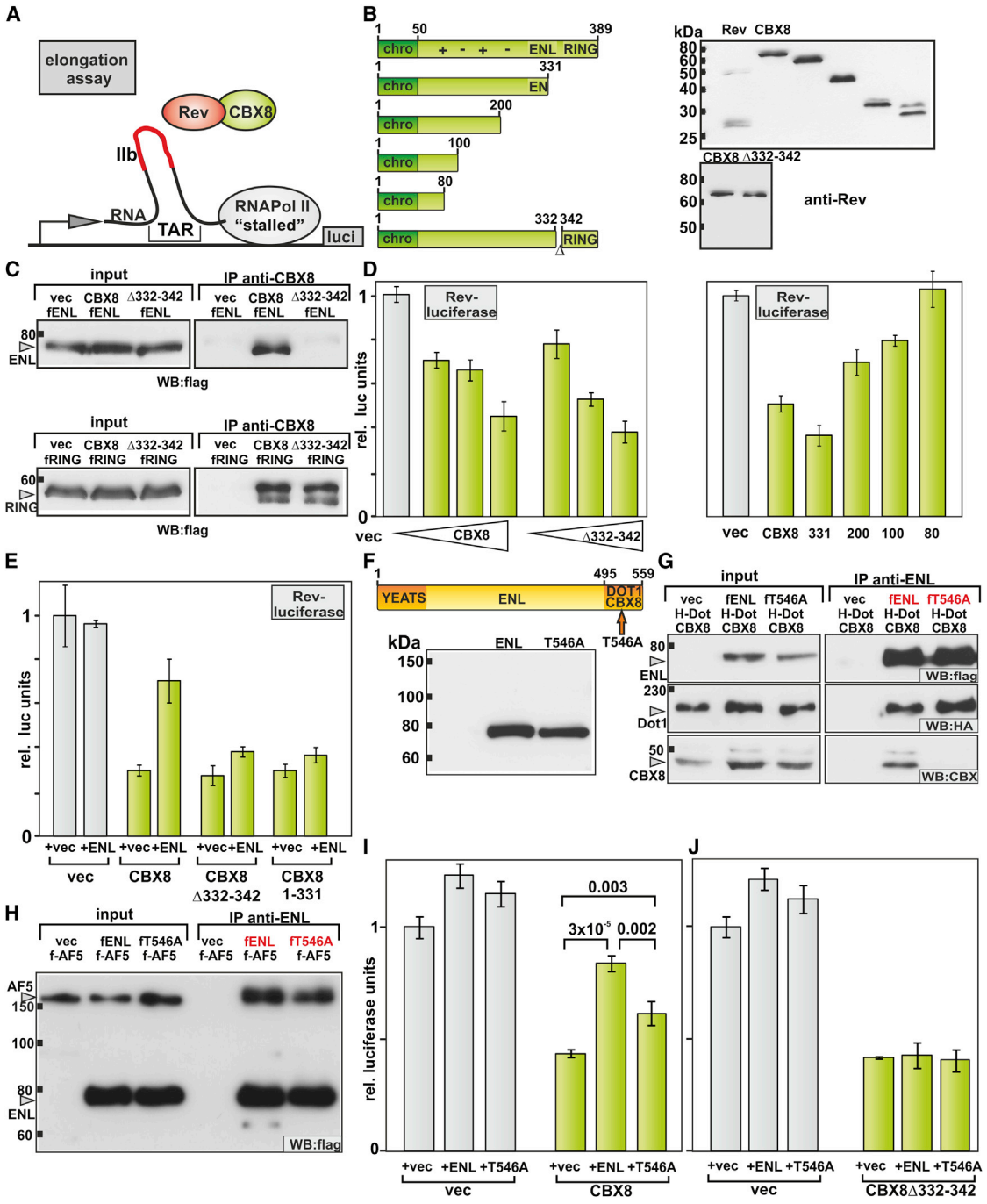

Figure 4. In Vitro Inhibition of Transcriptional Elongation by CBX8 Is Blocked by Direct Contact with ENL

(A) Schematic description of the Rev elongation reporter system. A modified HIV LTR drives a luciferase reporter gene. The Tat-interacting TAR messenger RNA (mRNA) loop is modified to contain an SLIIb recognition site for the RNA-binding protein Rev. Rev-fusion proteins are directed to the RNA-bound, "stalled" RNA PolII. In this way, any influence on elongation can be specifically read out by alterations of luciferase activity.

(B) CBX8 mutants tested in Rev assays. Left panel: Representation of various C-terminal CBX8 deletions. The N-terminal chromobox (chro) and the central charged region (+, −), as well as the ENL and RING binding domains are labeled. Right panel: anti-Rev western blot of Rev-CBX8 derivatives.

(C) An 11 amino acid deletion in CBX8 selectively abrogates ENL binding. CBX8 and a CBX8 mutant missing amino acids 332–342 were tested in Co-IP for their interaction with ENL (upper panel) and RING1 (lower panel).

(D) Repression of transcriptional elongation by CBX8 is independent of ENL and RING and relies on a charged region. Increasing amounts of Rev-CBX8 and Rev-CBX8Δ332-342 constructs were cotransfected together with elongation reporter into 293T cells (left panel). To determine the repressor domain in CBX8, a series of C-terminal deletion mutants was tested in the same assay (right panel).

(legend continued on next page)

association with DNA, as all extracts were extensively digested with benzonase to remove interfering nucleic acids. Unexpectedly, dimerization was not contingent on the ENL fusion partner. The amino-terminal MLL moiety up to the fusion point at amino acid 1444 could replace full-length MLL-ENL as the precipitating agent without loss of efficiency (Figure 7A, right panels). A deletion analysis identified two regions within MLL that were responsible for dimer formation. The first self-association domain coincided with the AT-hook motif at the very MLL N terminus (Figure 7B) and the second comprised the CxxC domain further C-terminal (Figure 7C). Remarkably, both domains could also heterodimerize (Figure 7D). Homo- and heterodimerization capabilities could be separated. N-terminal portions were necessary for homodimerization of the AT-hook and the CxxC motif, whereas the C-terminal parts of the respective peptides were sufficient for heterologous interaction (Figure S4). Homo- and heterodimer formation could also be demonstrated by glutathione S-transferase (GST) pull-down (Figure S4). Purified GST fusions of the AT-hook and CxxC peptides efficiently interacted with their tagged counterparts in nuclear extracts. Dimerization of MLL-ENL with WT-MLL was not possible under identical conditions (Figure 7E). WT-MLL is posttranslationally processed and forms a dimer of the respective MLL^N and MLL^C moieties. In cells coexpressing untagged WT-MLL (= MLL^N and MLL^C after processing within the cell) and flag-tagged MLL-ENL, IP with an antibody that binds to MLL^N brought down the MLL^N-interacting MLL^C (Figure 7E, lower panel). In contrast, the same procedure done with anti-flag precipitated only flagMLL-ENL (Figure 7E, middle panel), and not MLL^C (Figure 7E, upper panel), indicating that the fusion protein does not interact with the MLL^N/MLL^C dimer.

In contrast to monomeric ENL, the MLL-ENL fusion was able to connect CBX8 with both Dot1l and AF5 in precipitation assays. This is in line with a capability for di-/multimer formation that is unique to the MLL fusion protein and is absent in ENL (Figures 7F and 7G).

DISCUSSION

The balance between trithorax/MLL and Polycomb activity is an important factor in determining the output of a transcriptional unit. Here we reveal another aspect of this "ying-yang" relationship and provide evidence that stimulation of transcription by the EAP activator complex and Polycomb-mediated repression are rival activities, with ENL and CBX8 as key regulators. Inhibition of PRC1 adds yet another function to the repertoire of MLL fusion proteins, contributing to their potent transforming potential, and explains the consistent copurification of repressor proteins with ENL.

Because Polycomb complexes encode enzymatic activities that may be potentially "druggable," the role of PRCs in hematological malignancies, and cancer in general, is of great interest. With respect to MLL fusions, two recent studies showed that global reduction of PRC2 function by conditional knockout of essential components impaired MLL-AF9-induced leukemogenesis in vivo (Neff et al., 2012; Tanaka et al., 2012). Loss of PRC2 led to a widespread derepression of genes involved in cell-cycle control and differentiation. This included known tumor suppressors (e.g., *Cdkn2a*) as well as genes that induce maturation (e.g., *Egr1*). In addition, a transformation-associated Myc-gene expression module was suppressed in PRC2 knockout cells. These findings are consistent with our results after general knockdown of *Cbx8*, and similar observations have also been made in solid tumors. In normal cells, CBX8 bypasses senescence by binding directly to the *INK4A-ARF* region (Dietrich et al., 2007). In general, derepression of tumor suppressors is the rationale for clinical attempts to use EZH2 inhibitors as therapeutics. However, it has been known for a long time that the oncogenic *HOX* loci are also under Polycomb control, and here we show that inhibition of Polycomb-mediated repression contributes to transformation in MLL-fusion-induced leukemia. Therefore, the situation in malignant disease is more complex, and artificial interference with repressor activities may have unwanted side effects that could even exacerbate oncogene activity. Reflecting this dualism, it seems logical that EZH2 mutations in hematological malignancies may either enhance or destroy catalytic activity depending on whether tumor suppressors or oncogenes play the major role in the transformation process (Hock, 2012). With respect to novel treatments for MLL-induced leukemia, it would seem more advisable to target the interaction of MLL fusions with CBX8 directly than attempt to inhibit global PRC activity. In particular, patient cells that have lost the *CDKN2* tumor suppressor locus, which occurs frequently in leukemia (Sulong et al., 2009), may respond unexpectedly to such a generalized epigenetic therapy.

Interestingly, MLL fusion proteins have evolved different strategies to overcome Polycomb-induced repression. A recent report (Tan et al., 2011) showed that MLL-AF9 evokes a

(E) Direct interaction with ENL neutralizes the CBX8-induced repression of elongation. CBX8, CBX8Δ332-342 (ENL binding site deletion), and a truncated CBX8_1-331 deleting both ENL and RING1 interaction domains were cotransfected together with reporter and an expression construct for ENL or a vector control as before.

(F) A point mutation that had been shown to disable interaction of the ENL homolog AF9 with CBX8 was introduced at the corresponding residue at the C terminus of ENL, where binding sites for Dot1l and CBX8 overlap.

(G) The ENLT546A mutant abrogates CBX8 binding but leaves interaction with Dot1l intact. ENL, ENLT546A, and a vector-only control were expressed together with Dot1l and CBX8. Precipitation of ENL indicated that the interaction with Dot1l is unharmed, whereas CBX8 association is abrogated in ENLT546A.

(H) Co-IP of WT-ENL and ENLT546A with AF5. Similar affinities of WT-ENL and the CBX8 binding-defective point mutant ENLT546A were observed in these experiments.

(I) ENLT546A does not efficiently rescue CBX8-mediated repression. ENL, ENLT546A, or a vector control were cotransfected with CBX8 and the elongation reporter. Whereas some activity remains for ENLT546A, this mutant is significantly weaker than WT-ENL in rescuing CBX8-mediated repression of elongation (p values were calculated by Student's t test, n = 3).

(J) Combining ENLT546A with the CBX8Δ332-342 mutant completely abolishes any effect of ENL on CBX8-induced repression.

See also Figures S2 and S3.

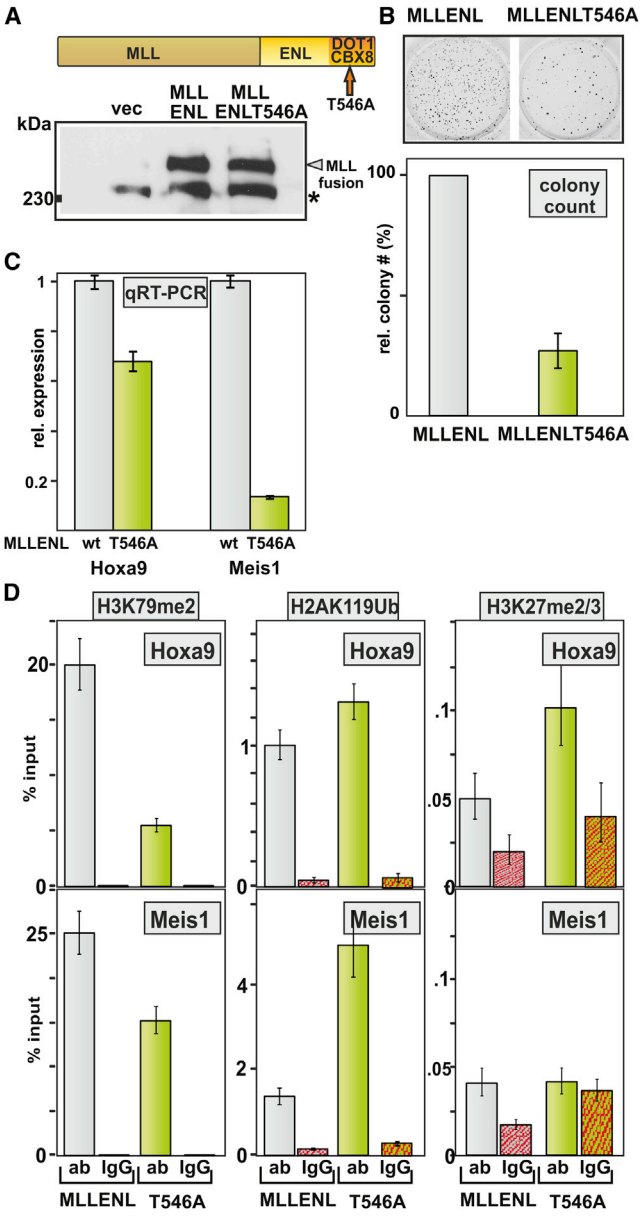

Figure 5. MLL-ENL Needs Interaction with CBX8 for Efficient Transformation

(A) Graphical representation and expression of the MLL-ENL construct containing the T546A mutation in the ENL portion. WT and MLL-ENLT546A were expressed at equal levels in Phoenix packaging cells (*, unspecific band).

(B) Reduced CFC capability of MLL-ENLT546A-transformed cells. Upper panel: Representative example of third-round colonies formed by progenitors transduced by MLL-ENL or MLL-ENLT546A viruses as indicated. Lower panel: Aggregated results (average and SD) of three biological replicates.

(C) Expression of *Hoxa9* and *Meis1* in MLL-ENLT546A-transduced cells is reduced. qRT-PCR was done on RNA isolated from cells after two rounds of replating.

(D) Histone patterns are shifted toward repression in MLL-ENLT546A cells. Chromatin was isolated from cells transduced with either MLL-ENL or MLL-ENLT546A after two rounds of replating and subjected to ChIP as indicated. Enrichment is given as % input. Bars depict the average and SD of a PCR triplicate, and this experiment was done twice with essentially the same result.

"moonlighting" function in CBX8 by using it as an intermediate to recruit the histone acetylase TIP60. In this way, MLL-AF9 induced local histone acetylation that was associated with *HOX* expression and transformation. Our results suggest that MLL-ENL works differently, because we could not find any evidence for an interaction of ENL or CBX8 with TIP60 either by biochemical purification or in direct co-IP attempts (not shown). This is not without precedent, because although ENL and AF9 are homologs, they are not identical. For example, ENL copurifies with CDK9/CYCT2 (Mueller et al., 2007), whereas AF9 associates with the alternative CDK9/CYCT1 conformation of P-TEFb (Monroe et al., 2011).

Unfortunately, not much is known about the molecular details of Polycomb-mediated repression. It would be tempting to speculate that part of the repressor activity of CBX8 may be due to its interfering with ENL function by physically displacing other ENL-bound factors. The structural basis for mutually exclusive binding was explored in a recent study of AF9 (Leach et al., 2012). Interaction of AF9 with various proteins is mediated by an intrinsically disordered domain that adopts different conformations depending on the respective binding partner, thereby restricting interaction to a single protein at any time. However, competition cannot be the sole reason for repression by CBX8, because CBX8 without an ENL-binding domain still acts as a repressor. Therefore, an alternative mechanism(s) must exist. An attractive possibility, as described in a previous study (Grau et al., 2011), is chromatin compaction that can occur in vitro and in vivo. This process has been demonstrated to be a prerequisite for silencing at *HOX* loci (Eskeland et al., 2010). Compaction is independent of histone modification and can be induced by various Polycomb group proteins that carry highly charged regions, such as those present within the central portion of CBX8. In this respect, it is interesting to note that a feature of CBX8, a 16-fold repeat of a dipeptide with alternating charge (DR/ER repeat), is also present in NELF-E. NELF-E is responsible for stalling RNA PolII by binding to nascent RNA, and this block is released by phosphorylation through P-TEFb (Gilchrist et al., 2012).

Finally, we provide further support for a potential role of dimerization in transformation, a feature that has been observed for many oncoproteins (So and Cleary, 2004). ENL complexes also control transcription in normal cells, and the sequential order of events that is enforced by mutually exclusive binding of the ENL interaction partners would be a perfect opportunity for regulation of this process. The high transcriptional output seen at MLL-ENL-controlled loci in leukemic cells suggests a bypass of this mechanism. This could be achieved by dimerization that allows multiple simultaneous interactions. The ability to dimerize seems to be restricted to the fusion proteins, as MLL-ENL did not interact with WT-MLL. Unfortunately, at present, the exact importance of dimerization for MLL-ENL-mediated transformation cannot be experimentally tested because the regions in the CxxC domain that mediate self-association are also responsible for binding to the Polymerase-associated factor (PAF) complex, a necessary cofactor for all MLL-fusion proteins (Milne et al., 2010; Muntean et al., 2010). In this regard, it is important to mention that the add-on of a strong dimerization motif alone is sufficient to convert the MLL N terminus to a weakly transforming protein. This was shown experimentally (Martin et al., 2003; Xia

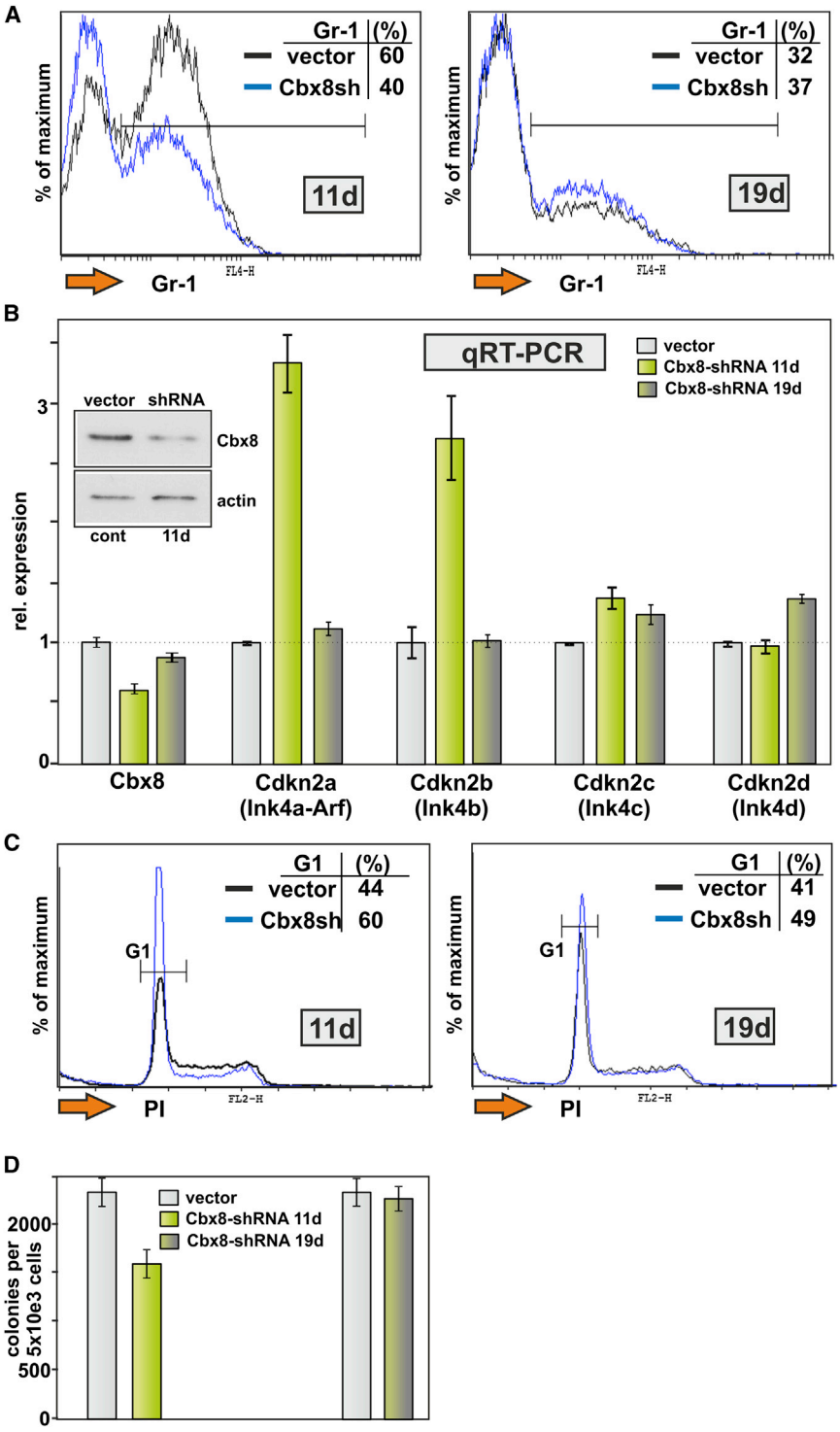

Figure 6. Global Knockdown of Cbx8 Is Incompatible with Transformation by MLL-ENL

(A) Surface Gr-1 marker expression on Meer cells transduced with a control vector or an shRNA plasmid targeting Cbx8. Shown are overlay FACS plots of cell populations after 11 days and 19 days of selective culture, and the percentage of Gr-1 positive cells.

(B) Knockdown of Cbx8 induces tumor suppressor genes. RNA was harvested from control and Cbx8 knockdown cells at 11 days and 19 days of culture as indicated. Expression of *Cbx8* and the *Cdkn2* family was determined by qRT-PCR. The inset shows a Cbx8-specific immunoblot.

(C) Cell-cycle analysis of *Cbx8* knockdown cells. Meer cells containing a *Cbx8* shRNA (blue line) or vector-only cells (black line) were analyzed for the cell-cycle phase by propidium iodide (PI) staining 11 days and 19 days after transduction.

(D) Numerical evaluation of CFC assays performed at early (11 days) and late (19 days) passage. Cells were plated in triplicates into cytokine-supplemented methocel at the indicated time points and colonies were enumerated 4–5 days later.

et al., 2003) and suggested by the numerous rare translocation partners that encode for cytoplasmatic proteins with dimerization domains. Dimerizing MLL fusions would circumvent normal control circuits and recruit more PAF complex. PAF, in turn, interacts with SEC and therefore ENL (He et al., 2011). It is tempting to speculate that fusion partners either increase intrinsic dimerization to offset the indirect ENL interaction or provide direct access to ENL/SEC, which makes endogenous dimerization suffice.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Culture, Animals, and Antibodies

The complementary DNAs (cDNAs) used for cloning are listed in Extended Experimental Procedures. Retroviral packaging was done in the Phoenix-E packaging line (Swift et al., 2001). Protein expression and precipitation were performed in 293T cells. Primary hematopoietic progenitors were isolated from Meer mice that carry a knockin of an inducible ENL-ER fusion that is joined to genomic Mll sequences, reconstituting an Mll-ENL protein analogous to human leukemia-derived samples. For a complete description of the Meer model, see Takacova et al. (2012). The culture conditions for the Meer cells are described in Extended Experimental Procedures.

Purification of PRC1

PRC1 was purified by tandem affinity precipitation of tagged CBX8 essentially as described previously for EAP (Mueller et al., 2007). In short, HEK293 cells were stably transduced with a flag-CBX8 construct. Nuclear extracts from these cells were precipitated with immobilized flag-agarose, bound material was eluted by addition of flag-peptide, and a second precipitation was done with anti-CBX8 agarose. Final precipitates were eluted by acid treatment (100 mM glycine, pH 2.9) and analyzed by gel chromatography, silver staining, and mass spectrometry. Similarly treated extracts from nontransduced HEK293 cells served as controls. Purification was done on three independent biological samples.

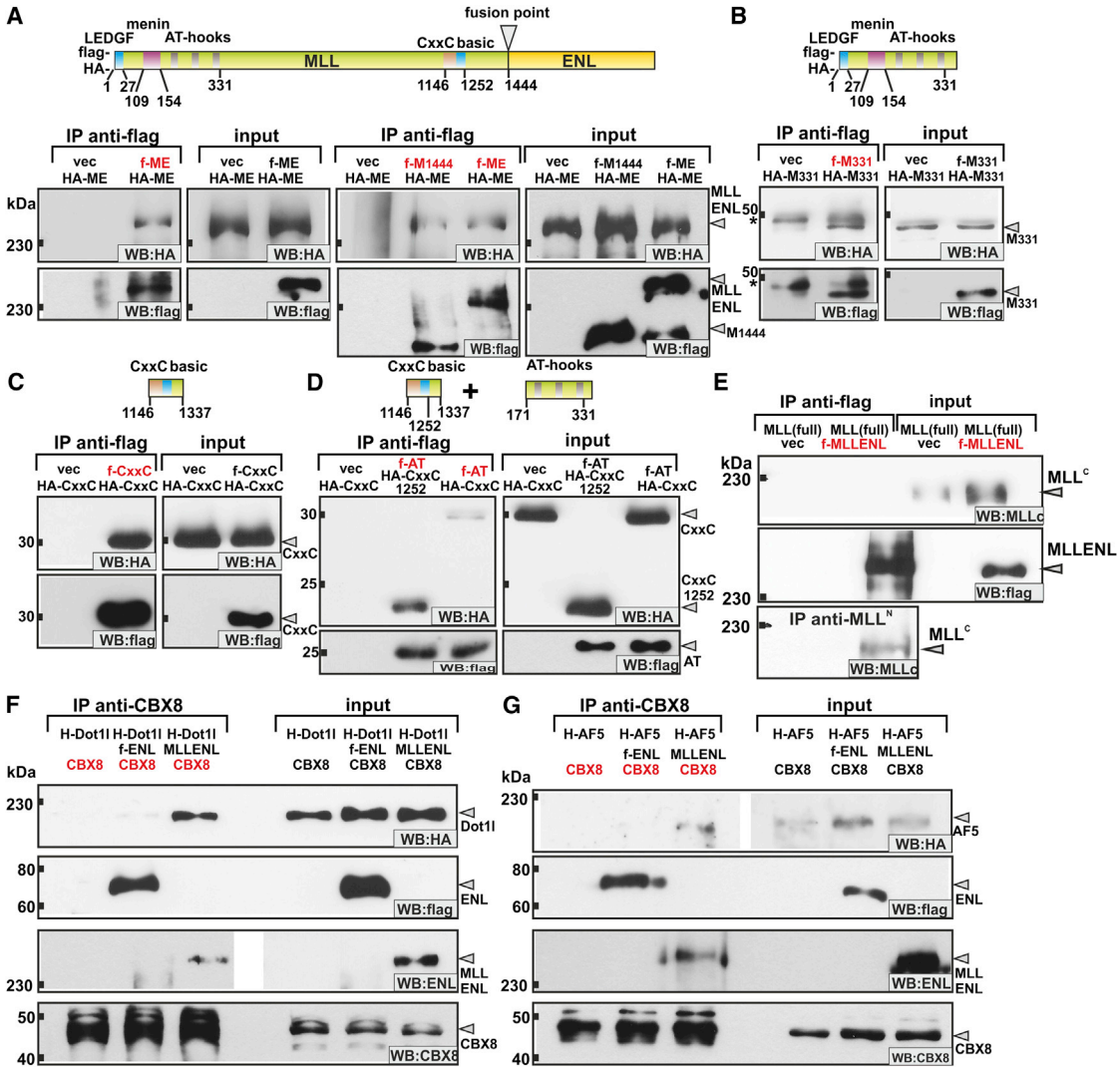

Figure 7. Dimerization of MLL-ENL

(A) MLL-ENL dimerizes through the N-terminal MLL moiety. Flag-tagged and HA-tagged MLL-ENL as depicted were coexpressed and nuclear extracts were precipitated with anti-flag agarose (left panels). Dimerization was not dependent on the ENL fusion partner as an N-terminal MLL moiety (amino acids 1–1444) was sufficient to induce self-association (right panels). f, flag; ME, MLL-ENL; numbers in subscript indicate the last amino acid of C-terminal deletion mutants. The protein/peptide targeted for precipitation is labeled in red font.

(B) The AT-hook motif dimerizes. co-IP was done as in (A) with two differently tagged N-terminal subregions of MLL comprising amino acids 1–331. Note that IgG heavy chain is detected at ~50 kDa.

(C) The CxxC domain contains a second dimerization domain. Amino acids 1146–1337 of MLL containing the CxxC core and flanking regions were differentially tagged and coprecipitation was done as above.

(D) The AT-hooks and CxxC domain heterodimerize. co-IP was done with AT-hooks and either a CxxC peptide as in (C) or a fragment thereof (amino acids 1146–1252) containing only the core CxxC motif and a downstream basic region.

(E) MLL-ENL does not dimerize with WT-MLL. Flag-tagged MLL-ENL was coexpressed together with WT-MLL or empty vector as control. Full-length MLL was subjected to natural posttranslational processing, yielding a stable dimer of MLL^N (300 kDa) and MLL^C (180 kDa) fragments. The MLL^C product can be detected with specific antibodies. anti-flag antibodies brought down substantial amounts of MLL-ENL but no MLL^C (upper and middle panels), whereas antibodies against MLL^N that recognize WT-MLL as well as MLL-ENL successfully coprecipitated MLL^C under the same conditions.

(F) Dimerization of MLL-ENL allows for simultaneous binding of CBX8 and Dot1l. CBX8, Dot1l, and either ENL or MLL-ENL were coexpressed and precipitation was done with a CBX8-specific antibody.

(G) MLL-ENL bridges CBX8 to AF5. The experiment was done as in (F), probing for co-IP of CBX8 and AF5 in the presence of ENL or MLL-ENL.

See also Figure S4.

Interaction Studies: Two-Hybrid, Co-IP, and GST Pull-Down

Two-hybrid analysis was performed according to standard procedures exactly as described previously (Garcia-Cuellar et al., 2009).

For Co-IP studies, tagged and native versions of the proteins were expressed in 293T cells. Both EAP and PRC1 are endogenously present in these cells, and therefore normal interactions should be able to form. When

subregions of proteins without an endogenous nuclear localization sequence were used, a bona fide nuclear localization signal was fused to the N terminus. The detailed procedures for IP and GST pulldown can be found in Extended Experimental Procedures.

ChIP and Quantitative PCR

ChIP was performed with formaldehyde crosslinking according to a standard protocol (Milne et al., 2009), with the modification that magnetic protein G beads (Diagenode, Liege, Belgium) were used instead of agarose-coupled protein G. Evaluation of ChIP was done by quantitative PCR (qPCR) in technical triplicates on at least two different biological samples. The primers used for qPCR of ChIP precipitates were designed to amplify a region immediately downstream of the respective transcription start sites, and they are listed in Extended Experimental Procedures together with the antibody sources.

Rev-Elongation Assays

Elongation was quantified by a special reporter system developed by Gold and Rice (1998). In brief, this reporter uses a modified HIV LTR that has been engineered to contain the sequence of the SLIIb stem-loop Rev-binding structure. Proteins of interest can be recruited through a fusion with Rev to RNA, and thus are brought into the vicinity of an RNA polymerase stalling at the known LTR pause point. Influence on elongation will read out as luciferase activity. Because the elongation machinery is universally expressed, experiments were done in 293T cells.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.03.038.

LICENSING INFORMATION

ACKNOWLEDGMENTS

We thank Renate Zimmermann for technical assistance. This work was supported by research funding from Deutsche Forschungsgemeinschaft (grant SL27/7-1 to R.K.S.), cofinanced by the Bavarian Ministry of Sciences, Research and the Arts within the framework of the Bavarian Molecular Biosystems Research Network. J.L.H. is supported by a Specialized Center of Research Grant from the Leukemia and Lymphoma Society of America. V.D. was supported by the Czech Ministry of Education (NPV2B06077 and MSM6198959205) and in part by Palacky University Institutional Funding (LF_2012_16). E.M., M.P.G.C., C.B., and R.K.S. performed and analyzed experiments; S.T. and V.D. contributed the Meer animals, J.H. provided access to the mass spectrometer and helped to interpret data; and R.K.S. designed the research and wrote the paper.

Received: August 22, 2012
Revised: March 12, 2013
Accepted: March 22, 2013
Published: April 25, 2013

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