Supplemental Inventory

A higher-order complex containing AF4- and ENL-family proteins with P-TEFb facilitates oncogenic and physiologic MLL-dependent transcription

Akihiko Yokoyama, Min Lin, Alpana Naresh, Issay Kitabayashi and Michael L. Cleary

Supplemental Data

Figure S1, related to Figure 1. Supporting evidence for AEP and MLL/AEP hybrid complex compositions.

Figure S2, related to Figure 2. Supporting evidence for the co-localization of MLL fusion proteins and AEP components on chromatin.

Figure S3, related to Figure 3. Supporting evidence for preferential hetero association of AF4 and AF5q31.

Figure S4, related to Figure 5. Supporting evidence for the mutually exclusive association of ENL with DOT1L and AF5.

Figure S5, related to Figure 6. Supporting evidence for the indirect recruitment of AEP to MLL-AF6-occupied or wt MLL-occupied loci.

Supplemental Experimental Procedures

Purification of the AEP complex

Antibodies used in this study (Table S1)

Primers used in this study (Tables S2 and S3)

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Supplemental Figure 1. Yokoyama et al.

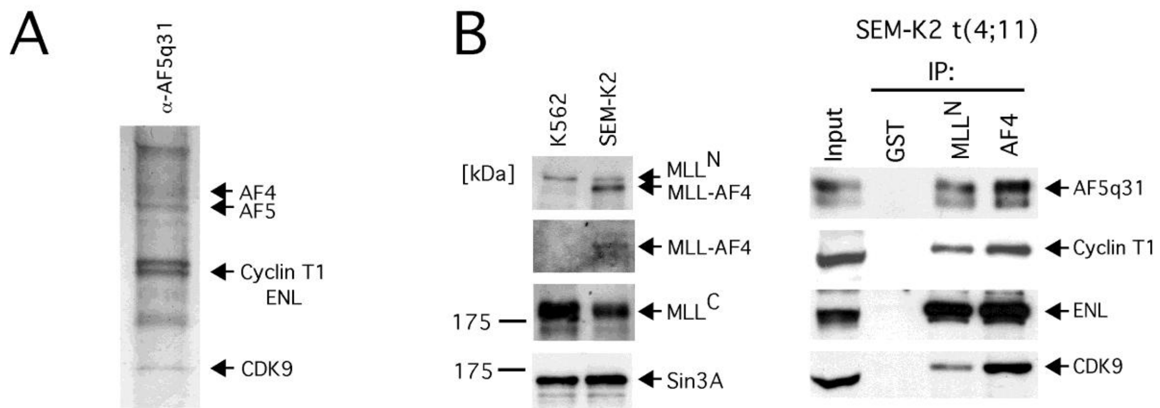

Figure S1, related to Figure 1. Supporting evidence for AEP and MLL/AEP hybrid complex compositions.

(A) Purification of AEP from ML-2 cells with an anti-AF5q31 antibody. A silver-stained image is shown. Mass spectrometry identified the indicated proteins.

(B) MLL-AF4 forms an AEP-like complex in SEM-K2 cells. SEM-K2 cells express MLL-AF4 visualized by anti- MLL^N (top) and anti-AF4 (upper middle) antibodies. In SEM-K2 cells, anti-MLL^C detected ~ 50% decreased amount of MLL^C (lower middle) versus similar levels of Sin3A (bottom) compared to K562 cells. In right panel, IP was performed with the antibodies indicated at the top and precipitates immunoblotted with the antibodies indicated on the right. Anti-GST antibody served as a negative control.

Supplemental Figure 2. Yokoyama et al.

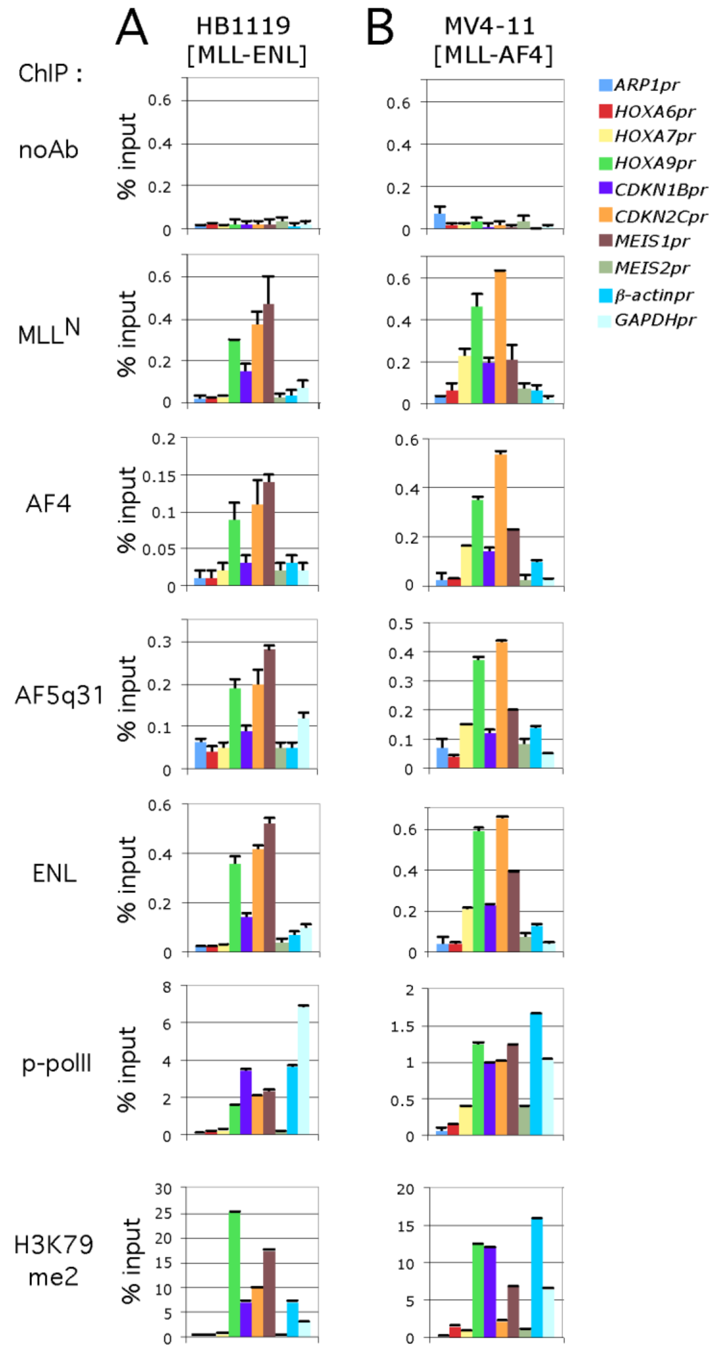

Figure S2, related to Figure 2. Supporting evidence for the co-localization of MLL fusion proteins and AEP components on chromatin.

(A & B) Unprocessed chromatin IP data are shown for HB1119 (A) and MV4-11 (B) cells. IP signals relative to the input are shown for the promoter adjacent regions indicated by the color key (right). Error bars represent standard deviations of triplicate PCRs.

Supplemental Figure 3. Yokoyama et al.

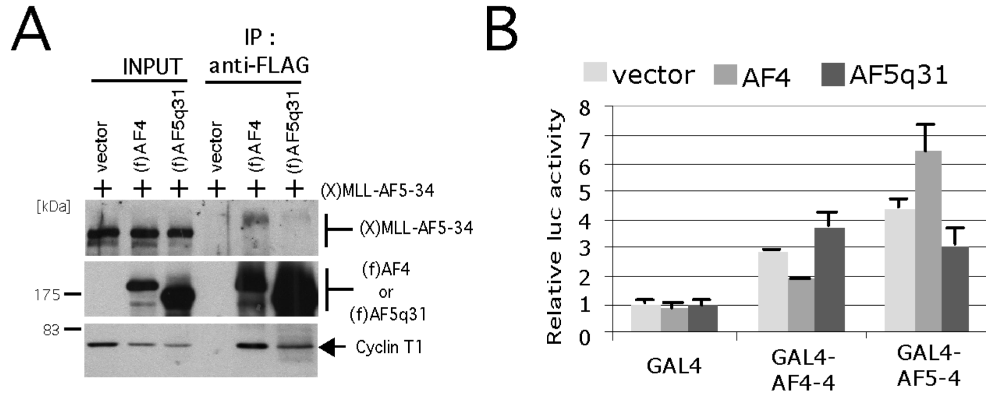

Figure S3, related to Figure 3. Supporting evidence for preferential hetero association of AF4 and AF5q31.

(A) Hetero-dimerization of AF4 and MLL-AF5q31 was assessed by IP western blot analysis. Xpress-tagged MLL-AF5-34 [(X)MLL-AF5-34] and FLAG-tagged AF4 or AF5q31 [(f)AF4 or (f)AF5q31] were co-expressed in 293T cells. IP was performed with anti-FLAG antibody and the precipitates were immunoblotted with anti-Xpress, anti-FLAG or anti-cyclin T1 antibodies. Co-IP of endogenous cyclin T1 served as a positive control. The mobility of MLL fusion proteins was disturbed presumably by excess amount of protein in the sample (e.g. IgG).

(B) (f)GAL4-AF4-4 and AF5-4 were tested for their transactivation activity with or without AF4(m) or AF5q31(m) co-expression. The subregion AF4-4 retains weak transactivation activity that is presumably derived from AF4 family proteins (Figure 3C). Because AF4 preferentially forms a heterodimer with AF5q31, AF4 co-expression depletes endogenous AF5q31 for GAL4-AF4-4 to associate with in order to transactivate. A similar result was obtained for GAL4-AF5-4. Error bars represent standard deviations of triplicate analyses.

Supplemental Figure 4. Yokoyama et al.

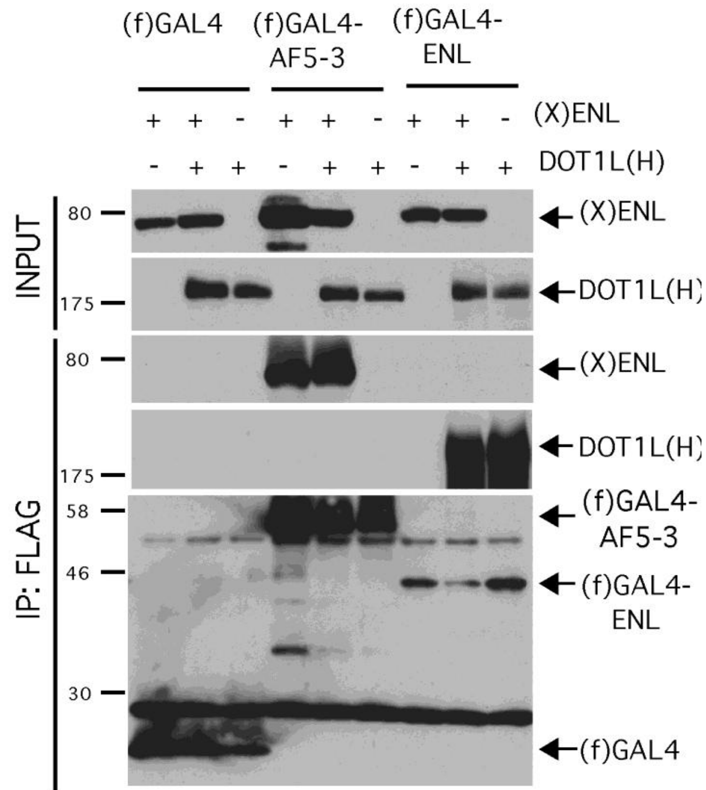

Figure S4, related to Figure 5. Supporting evidence for the mutually exclusive association of ENL with DOT1L and AF5.

Various GAL4 fusion proteins, (X)ENL and DOT1L(H) were expressed in 293T cells and analyzed by IP western blotting, which reveals that GAL4-AF5-3 co precipitates ENL but not DOT1L under conditions where GAL4-ENL pulls down DOT1L

Supplemental Figure 5. Yokoyama et al.

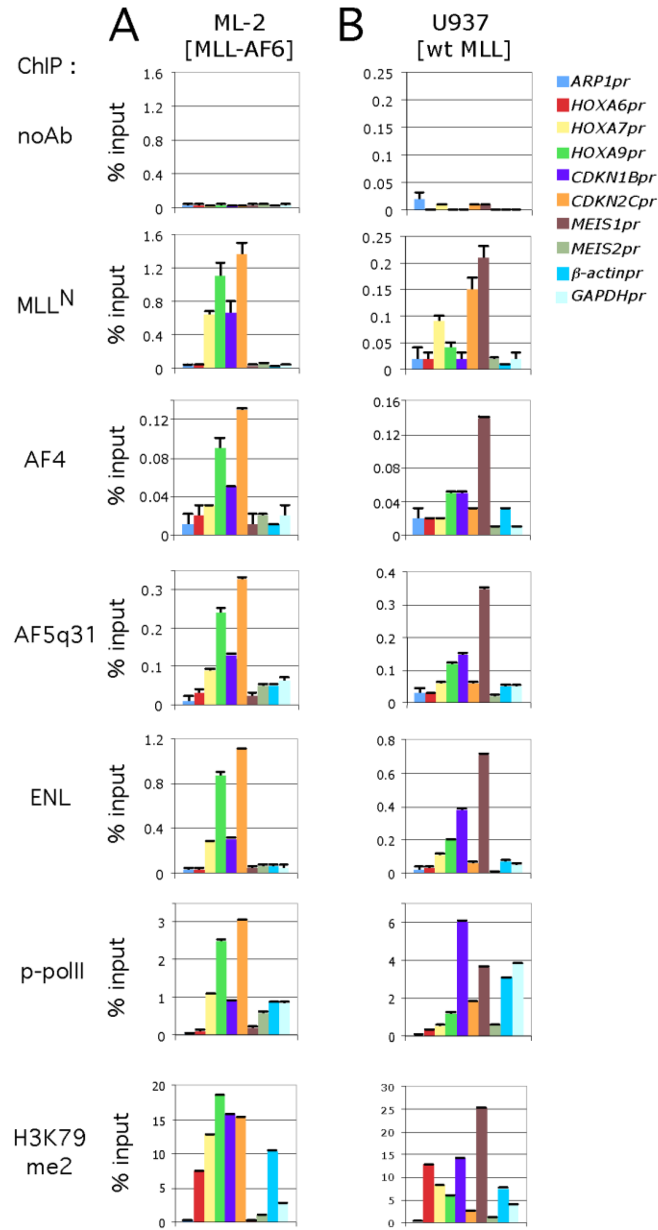

Figure S5, related to Figure 6. Supporting evidence for the indirect recruitment of AEP to MLL-AF6-occupied or wt MLL-occupied loci.

(A & B) Unprocessed chromatin IP data as shown for ML-2 (A) and U937 (B) cells. IP signals relative to input are shown for the promoter adjacent regions indicated by the color key (right). Error bars represent standard deviations of triplicate PCRs.

Supplemental Experimental Procedures

Purification of the AEP complex

Nuclear extracts were prepared by a modified Dignam procedure (Dignam et al. 1983). All purification steps were carried out in buffer A (20 mM Hepes, pH 7.5, 0.5 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 2 µg/ml each of leupeptin, aprotinin, and pepstatin A) supplemented with various amounts of NaCl. K562 nuclear extract containing 400 mg total protein was loaded onto a 75 ml SP sepharose column in buffer A with 150 mM NaCl. The column was washed sequentially with buffer A containing 150 mM (SP150), 250 mM (SP250), 400 mM (SP400), and 1M (SP1M) NaCl. AF4 protein eluted exclusively in the SP400 fraction, which was diluted with buffer A to reduce NaCl concentration to 200 mM before being loaded onto a 5 ml HiTrap heparin column (Amersham). The heparin column was washed sequentially with buffer A containing 200 mM (Hep200), 500 mM (Hep500), 600 mM (Hep600), and 1M (Hep1M) NaCl. For size fractionation, 5 ml of K562 nuclear extract was loaded onto an XK16/70 (Amersham) superose 6 column and eluted with buffer A containing 300 mM NaCl in 5 ml fractions. Monoclonal antibodies were crosslinked to protein G-sepharose beads (Amersham) at 2 mg/ml. The Hep600 fraction, which exclusively contained AF4 and AF5q31 proteins, was diluted with buffer A to reduce NaCl concentration to 400 mM. The fraction was then divided into 2 equal aliquots, each of which was mixed with 200 µl of anti-GST-protein G or anti-AF4 (or anti-AF5q31)-protein G beads, respectively. Complex binding was carried out at 4°C for 4 hours on a rotation wheel. The beads were then washed extensively with buffer A containing 400 mM NaCl and 0.1% NP-40. Bound proteins were eluted with 100 mM glycine (pH 3.0), resolved on 4-15% gradient SDS-PAGE (Bio-Rad), and visualized by Coomassie blue staining. Bands were excised for mass spectrometry analysis and protein identification performed by the Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford University.

Antibodies used in this study

Antibodies used for IP western blotting or ChIP.			
Antigen	**Antibody type**	**ID**	**Source/reference**
MLL^{N}	Mouse monoclonal	mmN4.4	Yokoyama et al., 2002
MLL^{N}	Rabbit polyclonal	rpN1	Yokoyama et al., 2002
AF4	Mouse monoclonal	C2-1	This study
AF5q31	Mouse monoclonal	1.3	This study
ENL	Mouse monoclonal	3.1	This study
Menin	Goat polyclonal	C19	Santa Cruz Biotechnology, Inc.
Menin	Rabbit polyclonal	BL342	Bethyl Laboratories, Inc.
Cyclin T1	Goat polyclonal	T-18	Santa Cruz Biotechnology, Inc.
CDK9	Rabbit polyclonal	C-20	Santa Cruz Biotechnology, Inc.
RNA pol II	Rabbit polyclonal	N-20	Santa Cruz Biotechnology, Inc.
GAL4	Rabbit polyclonal	DBD	Santa Cruz Biotechnology, Inc.
Xpress epitope	Mouse monoclonal	D-8	Santa Cruz Biotechnology, Inc.
Myc tag	Mouse monoclonal	4A6	Upstate Biotechnology, Inc.
Phospho RNA pol II	Mouse monoclonal	CTD4H8	Upstate Biotechnology, Inc.
FLAG tag	Rabbit polyclonal	F-7425	Sigma
FLAG tag	Mouse monoclonal	M2	Sigma
Di-methyl histone H3K79	Rabbit polyclonal	ab3594	Abcam
Tri-methyl histone H3K4	Rabbit polyclonal	ab3580	Abcam
Acetyl histone H3K9	Rabbit polyclonal	ab4441	Abcam
Di-methyl histone H3K9	Rabbit polyclonal	ab7312	Abcam
Histone H3	Rabbit polyclonal	ab1791	Abcam

Primers used in this study

Primers used for RT-PCR.		
Gene	**Probe ID/sequence**	**Source**
Murine genes		
Enl	Mm00452080_m1	Applied Biosystems, Inc.
Gapdh	Mm99999915_g1	Applied Biosystems, Inc.
Hoxa9	Mm00439364_m1	Applied Biosystems, Inc.
Hoxc8	Mm00439369_m1	Applied Biosystems, Inc.
β-Actin	Mm00607939_m1	Applied Biosystems, Inc.
Human genes		
ARP1	Hs00165626_m1	Applied Biosystems, Inc.
HOXA6	Hs00430615_m1	Applied Biosystems, Inc.
HOXA7	Hs00600844_m1	Applied Biosystems, Inc.
HOXA9	Hs00365956_m1	Applied Biosystems, Inc.
CDKN1B	Hs00153277_m1	Applied Biosystems, Inc.
CDKN2C	Hs00176227_m1	Applied Biosystems, Inc.
MEIS1	Hs00180020_m1	Applied Biosystems, Inc.
MEIS2	Hs00542638_m1	Applied Biosystems, Inc.
β-ACTIN	Hs99999903_m1	Applied Biosystems, Inc.
GAPDH	Hs99999905_m1	Applied Biosystems, Inc.
AF5q31	Hs00232683_m1	Applied Biosystems, Inc.
MLL (exon 9-10)	Fwd: GCCCAAGTATCCCTGTAAAACAAAA Rev: CCTGCATTCTCCTGCTTATTGAC Probe: CAAAAGAAAAGGAAAAACC	Custom made

Primers used for chromatin immunoprecipitation assays.			
Probes	**Forward Primer Seq.**	**Reverse Primer Seq.**	**Reporter 1 Sequence**
hARP1pr	CGGTGTCCCAGCTAAGCT	CGAACGAGCTAGGCTTGTCA	CAGCGTCCCGGCCTT
hACTBpr	AGGGCAGTTGCTCTGAAGTC	CTGCAGAAGGAGCTCTTGGA	ACTGCCTGGCCACTCC
hGAPDHpr	CCCCTCCTAGGCCTTTGC	GCTGAGAGGCGGGAAAGTT	ACTACCGCAGAGCCTC
hMEIS1pr	CGGCGTTGATTCCCAATTTATTTCA	CACACAAACGCAGGCAGTAG	CCGCCAGCTTTATTTT
hMEIS2pr	GGCCGCGCCACAAG	CACTACGAGCCCGGAGTTG	TTGCCGCCCAGCAGC
hCDKN2Cpr	CTCCACAACCGTCTTAAATAACAAACC	GCGGGCTTGAGTCTGTGA	CAGCTGCCCCAATTC
hCDKN1Bpr	GTCCCGAGGGTCCCTTC	GTGTGCCTACCTCATCTCATACG	CAGCTGTCACATTCTG
hHOXA6pr	AACTCGCACCCACGAATAGG	GCAGTGACAAAGGTGGCTTT	CCTGCCGGCCTGCACT
hHOXA7pr	GCCTTCCCCGTCTGGAT	ACTCTGCCCAAGTCTTCTCTCA	CAGGCCGGACTTAGAC
hHOXA9pr	TGGCTGCTTTTTTATGGCTTCAATT	CCGCGTGCGAGTGC	CCCCTCACATAAAATT
hHOXA6-7center	GCCTATATGGCCGGGAAATCT	GTCCAGCTCCGGTCAGG	CCCCGCCTCGGCTAC
hHOXA7downstream	CCAGAGCTGATTCCGGATTCG	CCTCGCCTGGTCTGCA	CCCTGAACCCAGCCCC
hHOXA7intron	TGCCAGGGTCCATTTCAAGATG	CCCTCATCCCCAGGACCTT	CTCTGTCCTCATTCCC
hHOXA7-9center	CTAGGGATCACTCACTCACTGGAT	AGGCAAATTGCTTCCTAAAGGTTTC	CACCCTTGCCTCTCTC
hHOXA9intron	AGTGGCGGCGTAAATCCT	TGATCACGTCTGTGGCTTATTTGAA	CCCGCAGCCTCATC
hHOXA9downstream	GGATATTCCACCAAAGCCCTTTCAT	GGCAATGCCAATAAAAGAGGTGTTT	CTGCCGCCGATAAAG
hMEIS1in	TCTCAGCGCCTCCAAATCTTG	TTTGTGTGTGTGAAATTTAGCTATTTAGGTTTT	CCAGGCAGTTATTTTC
mhoxc8pr	GTCTACTCTTTTGCACCCTTCAGT	GTCTAGCCCAACTCAAGAGATTTCA	CCAGCTGTAATCTCC

Supplemental References

Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11, 1475-1489.