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MLL fusion partners AF4 and AF9 interact at subnuclear foci

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The *MLL* gene is involved in translocations associated with both acute lymphoblastic and acute myelogenous leukemia. These translocations fuse *MLL* with one of over 30 partner genes. Collectively, the *MLL* partner genes do not share a common structural motif or biochemical function. We have identified a protein interaction between the two most common *MLL* fusion partners AF4 and AF9. This interaction is restricted to discrete nuclear foci we have named 'AF4 bodies'. The AF4 body is non-nucleolar and is not coincident with any known nuclear structures we have examined. The AF4–AF9 interaction is maintained by the MLL–AF4 fusion protein, and expression of the MLL–AF4 fusion can alter the subnuclear localization of AF9. In view of other research indicating that other MLL fusion partners also interact with one another, these results suggest that MLL fusion partners may participate in a web of protein interactions with a common functional goal. The disruption of this web of interactions by fusion with MLL may be important to leukemogenesis.
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Introduction

The *MLL* gene (*Hrx, ALL1*) at 11q23 is involved in *de novo* and therapy-related acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL). *MLL* has been implicated in approximately 7–10% of all ALL and 5–6% of all AML.[1,2] MLL's involvement is particularly common in infant leukemia, where *MLL* rearrangement is present in 80% of ALL and 60% of AML.[3] Although internal duplications and deletions of *MLL* have been described in patients with acute leukemia, the vast majority of *MLL* leukemias involve the fusion of MLL to one of over 30 partner genes. There is a strong correlation between the *MLL*-partner gene and the phenotype of the leukemia. Rearrangement of *MLL* is generally considered an adverse prognostic factor. In ALL, rearrangement of *MLL* has been associated with poor prognosis.[4,5] Similarly, infants with ALL and 11q23 abnormalities have a worse prognosis[6] than infants with ALL lacking 11q23 abnormalities. However, recent data from infants with ALL indicate that the t(4;11) that generates the *MLL-AF4* fusion gene, but not other 11q23 abnormalities, is an adverse prognostic indicator.[7] Interestingly, both children and adults with AML and the t(9;11) (which generates the *MLL–AF9* fusion) have a more favorable prognosis than other patients with AML and 11q23 abnormalities or other patients with AML overall.[8,9] Thus, the *MLL* fusion partner can be of clinical significance.

The MLL protein is a 3969 amino acid, 431 kDa[10] human homologue of the *Drosophila trithorax* gene. Trithorax, along with the Polycomb group proteins, act to control homeobox gene expression during *Drosophila* development.[11] MLL generates a leukemic phenotype when fused in-frame to one of over 30 known fusion partner genes.[12] MLL fusion partners do not contain common structure motifs. They can be loosely grouped into gene products which appear to be nuclear or cell surface proteins. There are subgroups of the fusion partners that come from common gene families such as *AF4/AF5q31/LAF4*,[13,14] *ENL/AF9*,[15] and *AF10/AF17*.[16] Owing to their lack of obvious biochemical similarity, the importance of the partner genes to leukemogenesis is unclear. Further questions about the significance of the fusion partner are raised by the discovery of internal duplications and deletions in *MLL* in some patients with acute leukemia[17] and a murine model in which the fusion of a sequence encoding β-gal to *Mll* is leukemogenic.[18] However, in all cases studied, the fusion transcript maintains an open-reading frame well into sequences encoded by the partner gene;[19] there is a strong correlation between the fusion partner and the leukemia's phenotype;[20] mutagenesis in murine model systems indicate that amino acids from the fusion partner are required for efficient leukemogenesis.[18,21]

The *AF4* (or *FEL*) gene fuses with *MLL* in the t(4;11)(q21;q23), and is the most common *MLL* fusion partner.[22] In all, 95% of *MLL-AF4*-associated leukemia is classified as ALL, but shows some lineage infidelity, typically expressing a $CD19^+$ $CD10^-$ $CD15^+$ phenotype.[23] AF4 is a serine/proline-rich nuclear protein with transcriptional activation domains.[24,25] Gene knockout studies indicate *AF4* plays an important role in B and T lymphopoiesis.[26] *AF4* is a member of a gene family that includes two other ALL-associated MLL fusion partners, LAF-4[27,28] and AF5q31,[14] a putative Fragile X mental retardation gene FMR-2,[29] and the *Drosophila melanogaster* pair-rule gene *lilliputian*.[30] Genetic evidence suggests that *lilliputian* performs a partially redundant role in the Ras/MapK differentiation pathway.[31]

AF9 also belongs to a growing family of homologous genes and, like *AF4*, encodes a serine/proline-rich nuclear protein with the ability to activate gene transcription.[15,32] *ENL* at 19p13.3 is a homologue of *AF9*, and is also one of the more common *MLL* partner genes seen in both ALL and AML.[20] Within their carboxy termini, AF9 and ENL share a region of high sequence conservation that includes the terminal 90 amino acids that make up the minimal transactivation domain.[32,33] Also reported within the carboxy termini of both AF9 and ENL are binding domains for the Polycomb 3 protein.[34,35] Polycomb is an antagonist of MLL, whose function is to silence the expression of the genes that MLL activates.[11]

The lack of obvious functional similarity between the MLL fusion partners led us to study the subcellular localization of the most common MLL fusion partner AF4. In addition, we have sought to isolate proteins that interact with AF4 and identify proteins that colocalize with AF4 in the nucleus. We hypothesized that an understanding of the subcellular localization of AF4 and its potential intracellular interactions would provide important insights into partner gene function. We further hypothesized that existence in a common macromolecular

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complex may be the function shared by a significant number of the gene products of *MLL* fusion partners. We have identified a region of AF4 that both controls its subcellular localization and interaction with the AF9 protein. AF4 and AF9 do not colocalize with any of the known nuclear proteins we studied, and AF9's intranuclear localization is dysregulated by expression of the MLL–AF4 fusion protein.

Materials and methods

Cell culture

HeLa cells were grown in RPMI supplemented with 1% penicillin/streptomycin, 1% glutamine, and 10% fetal calf serum. Cos-1 cells and murine embryo fibroblasts (MEFs) were grown in Dulbecco's MEM with the same supplements. K562 cells were grown in RPMI 1640 supplemented with 2.5 mg/ml glucose, 1 mM HEPES, 0.01 mM sodium pyruvate, and 2 mg/ml sodium bicarbonate. Cells were all maintained in a humidified incubator at 37°C and 5% CO_2.

Plasmid construction

GFP-fusion gene expression vectors were made using the pEGFP C1, C2, and pDsRED vectors (Clontech, Palo Alto, CA, USA) with an amino-terminal fluorescent protein and a carboxy-terminal insert. GST fusion proteins were made with the pGEX vector system (Amersham Bioscience, Piscataway, NJ, USA). The FLAG-MLL-AF4 construct was a kind gift of Masao Seto. Both the FLAG-MLL and FLAG-MLL-AF4 constructs were previously used by Hess *et al*.[36] Inserts were generated either by PCR with high-fidelity Pfu polymerase, or by enzymatic excision from other plasmids. PCR inserts were sequenced to ensure proper orientation and correct sequence.

Cell transfection

Cells were transfected with either the ECM 600 Electroporator (BTX Genetronics, San Diego, CA, USA) in the case of Cos-1 and K562 cells, or the T820 ElectroSquarePorator (BTX Genetronics, San Diego, CA, USA) in the case of HeLa cells. Transfections followed the protocols available from the manufacturer. Adherent cells were plated onto glass coverslips following transfection. Cells were incubated for 36 h before visualization of GFP and DsRED. K562 cells were cytospun onto glass slides after the 36-h incubation.

Antibodies

Rabbit anti-Af4 polyclonal antibody 21588 was raised to a peptide comprised of the terminal 15 amino acids of murine AF4 sequence, TRQGLQRLKQSPKG. The 21588 serum, derived from a 10-week terminal bleed, was affinity purified with the peptide using an Affigel matrix column (Biorad, Hercules, CA, USA), and was used at a 1–15 dilution. The chicken anti-AF9 polyconal antibody was raised to the peptide EVKSPIKQSKSDK-QIK (450–465 aa of AF9). The peptide was coupled to a multiple antigenic peptide (MAP) carrier. Antibodies were collected from the eggs of a chicken immunized with the AF9 peptide–MAP conjugate. Antibodies were purified by binding to an AF9 peptide affinity matrix, followed by acid elution. Antibody production was performed by Research Genetics. Monoclonal antinucleolin (Research Diagnostics Inc., Flanders, NJ, USA) was used at 2 μg/ml. Monoclonal anti-PCNA (Oncogene Research, San Diego, CA, USA) was used at 5 μg/ml. Monoclonal anti-fibrillarin (Cytoskeleton, Denver CO, USA) was used at 1 μg/ml. The anti-PML antibody, monoclonal clone 5E10, was a generous gift of the Van Driel Lab,[31] and was used at a 1–10 dilution. The anti-RNA Polymerase II antibody, monoclonal clone 8WG16 (Covance, Princeton, NJ, USA), was used at a 1–50 dilution. The anti-Brdu antibody, monoclonal clone BU33 (Sigma Chemicals, St Louis, MO, USA) is known to crossreact with 5-Fluorouridine (5-FU), and was used at a 1–1000 dilution. Cells were washed in ice-cold PBS, fixed in 4% paraformaldehyde for 30 min on ice, fixed in 0.2% Triton X-100, washed again and blocked in ice-cold PBS containing 10% goat serum and 10% horse serum. Cells were washed again and exposed to the proper dilution of antibody. Cells were washed and exposed to a secondary antibody, with either fluorescent tag, Alexa Fluor 594 or Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). Cells were washed a final time and mounted with PBS only, onto glass slides and subjected to microscopy.

Visualization of RNA transcripts

5-FU was incorporated into nascent RNA transcripts and visualized with the crossreactive antibody to 5-Bromouridine.[37] Cultured HeLa cells were incubated under normal growth conditions with 1 mM 5-FU. The time course for the incubation ranged from 10 s to 60 min, in order to inspect the range of transcripts from immediate/early to late. Cells were quickly rinsed twice and fixed in 4% paraformaldehyde on ice for 30 min. Immunofluorescent staining was performed as described above with anti-BrdU antibody.

Microscopy

Fluorescent microscopy was performed on an Axioplan Fluorescent Microscope (Zeiss, Thornwood, NJ, USA). Immunofluorescent confocal microscopy was performed on a LSM 510 laser-scanning microscope (Zeiss, Thornwood, NJ, USA). EGFP and Alexa Fluor 488 were excited at 488 nm with an argon laser, and emission was visualized with a 505–550 nm band pass filter. Alexafluor 594 was excited at 633 nm with a helium–neon laser and emission was visualized with a 650 nm band pass filter. DsRed was excited at 543 nm with a helium–neon laser and emission was visualized with a 550–615 nm band pass filter. Multitrack processing was used to limit the possibility of fluorochrome bleed though. In the cases where two fluorochromes were used, whenever possible, images in which only one of the two fluorochromes was present were examined in both spectra to insure that bleedthrough was not an issue.

Yeast two-hybrid assay

Yeast cells were grown and transformed with plasmid DNA using standard conditions and protocols. A yeast two-hybrid selection was performed with cDNA encoding 475–569 aa of mouse AF9 as 'bait'. The cDNA was cloned in the yeast expression vector pGBT9. A mouse 11-day embryo cDNA library cloned in vector pGAD10 (Clontech, Palo Alto, CA, USA) was selected in yeast strain PJ69-4A. Following incubation on synthetic medium lacking adenine, leucine, and tryptophan,

Ade$^+$ colonies were isolated and tested for histidine prototrophy on synthetic medium containing 1 mM 3-aminotriazole and lacking histidine, leucine, and tryptophan. Library DNA was isolated from Ade$^+$ His$^+$ colonies, and the phenotype was confirmed by transforming yeast cells with the bait and the selected library 'prey' plasmid DNA. The specificity of the two-hybrid interaction was tested using a lamin bait vector.

GST pull-downs

GST-AF4 chimeric proteins were produced by cloning cDNA fragments encoding amino acids 749–775 into pGEX-5X-1 (Amersham-Pharmacia, Piscataway, NJ, USA). Recombinant protein was isolated from *E. coli* strain BL21 following induction with 0.1 mM IPTG for 4 h at 30°C. Biotinylated AF9 protein was synthesized by coupled *in vitro* transcription/translation, employing T7 polymerase and rabbit reticulocyte lysates according to the manufacturer's protocol (TNT Quick-Promega, Madison, WI, USA). Human AF9 cDNA cloned into pcDNA3 (Invitrogen, Carlsbad, CA, USA) was used as a template for the reaction. Binding assays were performed by mixing 100 μl bacterial lysate to 50 μl GSH-agarose (Amersham-Pharmacia, Piscataway, NJ, USA) at 4°C for 60 min. The affinity matrix was washed five times with cold binding buffer (PBS pH = 7.4, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 0.5% NP-40, 1 mg/ml BSA). After washing, the matrix was resuspended in 200 μl binding buffer. A volume of 10 μl of the biotinylated protein mixture was added and the matrix was incubated at 4°C for an additional 60 min, followed by six washes with cold binding buffer. In all, 300 μl of protein loading buffer was added to the matrix, and the mixture was boiled for 5 min. A volume of 20 μl of the supernatant was separated by SDS-12% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After probing the membrane with alkaline phosphatase–streptavidin conjugate, protein was detected by a colorimetric reaction with Western Blue substrate (Promega, Madison, WI, USA).

Coimmunoprecipitations

NIH 3T3 cells were grown in 100 mm plates with DMEM containing 10% calf serum. Cells were transfected using Lipofectamine 2000 (Invitrogen) and 25 μg of each plasmid vector (p3xFLAG-AF9, pGFP-AF4R13A$^+$, pEGFPC2, p3xFLAG-CMV). After 48 h, cells were washed and harvested in cold lysis buffer (PBS + 0.25% NP-40 + protease inhibitor cocktail (Sigma)). Cells were sonicated for 10 s × 2 and the cell lysates were cleared by centrifugation at 4500 rpm for 10 min at 4°C in a microcentrifuge. A volume of 500 μl of each cell lysate was preincubated with 50 μl (packed volume) Protein-G agarose for 3 h at 4°C. Supernatants were transferred to clean tubes and 5 μl anti-GFP antibody ab290 (Abcam) was added. Lysates were incubated with the antibody for 1 h at 4°C. Protein-G agarose (50 μl) was then added to each tube and incubated for an additional 4 h. Immune complexes were collected by centrifugation and washed × 5 with cold PBS. Immune complexes were then boiled for 5 min in sample buffer, and proteins were resolved by SDS-8% PAGE. Proteins were transferred to a nitrocellulose membrane. Western blot was performed by first blocking the membrane in PBS containing 10% nonfat dry milk. The primary antibody was 10 μg/ml anti-FLAG antibody M2 (Sigma) and the secondary antibody was a 1:2000 dilution peroxidase-conjugated goat anti-mouse antibody (Roche). Signals were detected by ECL-Plus (Amersham).

Results

Control of AF4 subcellular localization

Proteins larger than 50 kDa are generally excluded from the nucleus[38] and their entry into the nucleus is usually dependant on the presence of short amino-acid sequences that direct the binding of chaperone proteins that shuttle these nuclear targeted proteins through the nuclear pores, into the nucleus.[39] Analysis of the deduced amino-acid sequence of *AF4* revealed several putative nuclear localization signals (NLS) (Figure 1a). In all, six classic nuclear localization signals were found clustered in the third quarter of the murine AF4 sequence. Proximal to the most carboxy-terminal classic NLS are three amino acids, two of which are prolines, qualifying that NLS to be a heptamer NLS. A spacer of nine amino acids separates the middle two of the six classic NLS. This specific arrangement of two basic clusters completes the consensus for a bipartite NLS. The proximal basic cluster of the bipartite NLS is separated by 15 amino acids from an upstream pair of lysines. This fits the very precise requirements for the consensus NLS from DNA Helicase-Q1,[22] a homologue of the *E. coli* RecQ Helicase.[40] The NLS for DNA Helicase Q1 has its own chaperone protein, the QIP1 protein, homologous to the importin-alpha NLS receptor. The QIP-1 protein, unlike the two other classes of NLS receptor proteins represented by the proteins Rch1 and NPI-1, binds very discriminately to the specific sequence described.[22]

To test the function of the putative NLS in the murine AF4 sequence, a series of constructs were engineered with the pEGFP plasmid vector (Figure 1b). The largest fragment tested, which consists of full-length murine AF4, lacking the first six amino acids, and the terminal 40 amino acids, displayed a punctate pattern of nuclear expression (Figure 2a). Foci were always within the nuclear space, as defined by a DAPI counter stain (Figure 2d), and numbered from 20 to hundreds per nucleus. This pattern has been reported by others using anti-AF4 antibodies.[36] This murine AF4 expression was always limited to the nucleus and was never cytoplasmic. This was in contrast to EGFP expression, which was seen in both the cytoplasm and nucleus, and appeared diffuse (Figure 2b). The murine AF4 cDNA was divided into four approximately equal portions by restriction endonuclease digestion. The RI-3 fragment, encoding amino acids 647–975, (Figure 1b), contains all the NLS consensus sequences, and proved to be the portion of the protein able to direct murine AF4 subnuclear expression (Figure 2d). The remaining quarters all showed a diffuse whole-cell expression pattern, with some cells exhibiting slight nuclear inclusion or exclusion (for example pattern see Figure 2c). The RI-3 fragment was further divided, based on the inclusion or exclusion of the bipartite NLS (Figure 1b). Of these fragments, only the RI-3A+ fragment, which includes the bipartite NLS and all proximal RI-3 sequence, was able to direct murine AF4 subnuclear expression (Figure 2e). The RI-3A− fragment without the bipartite NLS demonstrated a diffuse whole-cell expression (not shown), indicating that the two amino-terminally situated NLS are not sufficient to direct nuclear localization. The bipartite NLS, present in RI-3A+ but absent in RI-3A−, is sufficient to direct nuclear expression. The two RI-3B fragments, containing the sequence distal to the bipartite NLS (Figure 1b), both showed a diffuse nuclear pattern of expression, indicating that at least one of the two carboxy-most NLS are functional (not shown). However, these results also indicate that the two carboxy-most NLS motifs, while able to direct nuclear targeting, are not enough to direct murine AF4 subnuclear expression. A further division of the RI-3A+

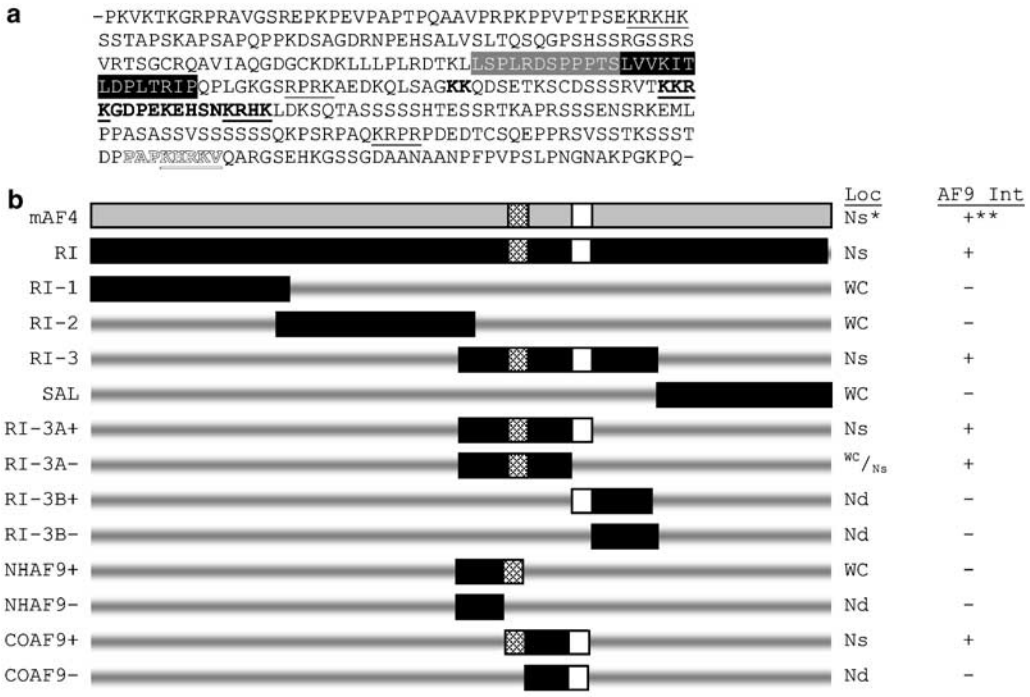

Figure 1 The RI-3 fragment of mAF4 contains both the sequence responsible for AF4 subnuclear localization, and AF9 interaction. (a) The derived amino-acid sequence of the RI-3 fragment. All six putative classic NLS are underlined. The heptamer NLS, with leading prolines, is presented in outline text. The two overlapping bipartite NLS are in bold text. The amino most bipartite NLS is the QIP-1 consensus-binding site. The carboxy most bipartite NLS is the conserved standard bipartite NLS. The two share the central KKRK basic cluster. The yeast AF9 interaction domain is boxed. The black portion of the box is the minimal domain, as determined by point mutation analysis. (b) Schematic of EGFP constructs and summary of results for mAF4 cellular localization and AF9 interaction experiments. Black boxes represent the sequence included in each construct. The hatched box represents the minimal AF9 interaction domain, as determined by experiments in the yeast system. The white box represents the conserved bipartite NLS. Results for subcellular localization are reported as whole cell (WC), nuclear stippled (Ns), and nuclear diffuse (Nd). RI-3A− is reported as having both WC (transfected alone), and Ns (coexpressed with AF9) subcellular localization.

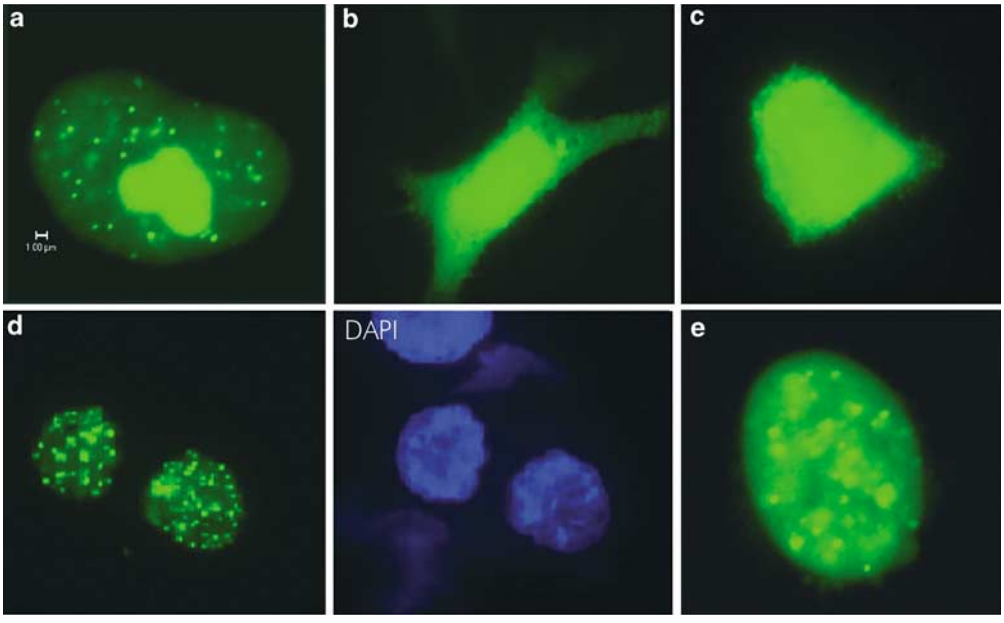

Figure 2 AF4 is localized to small subnuclear foci by sequences in the RI, RI-3, and RI-3A+ fragments. Confocal laser microscopy of HeLa cells transfected with EGFP constructs. (a) The RI fragment (6–1177 aa). (b) Control, EGFP vector without insert. (c) The amino teminal SAL fragment (952–1217 aa). (d) The RI-3 fragment (647–975 aa) containing all mAF4's putative nuclear targeting sequences & DAPI counter-stain of the same field. (e) The RI-3A+ fragment (647–875 aa) containing the bipartite NLS and upstream sequence.

fragment provided the smallest fragment able to achieve subnuclear targeting. This 68 amino-acid fragment, RI-3CO-, contains both the AF9 interaction domain (see below) and the bipartite NLS. This fragment was able to direct murine AF4 subnuclear expression, indicating that the amino-terminal 137 amino acids of the RI-3 fragment are not important in subnuclear targeting (not shown).

AF4 does not colocalize with known nuclear proteins

The intranuclear architecture contains a variety of subnuclear bodies and macromolecular complexes of functional significance. Nucleolin is a phosphoprotein implicated in ribosomal RNA processing, and is a major component of the nucleolus.[41] Using an antinucleolin antibody, we stained nucleoli in HeLa cells transfected with the RI-3A+ fragment of AF4 fused to EGFP. Confocal microscopy with three-dimensional reconstruction demonstrates that the AF4 subnuclear expression pattern is not coincidental with nucleoli (Figure 3a).

The Cajal bodies, also called coiled bodies due to their fibrous appearance under light microscopes,[42] are postulated to be involved in histone assembly.[43] Cajal bodies have also been found localized around the gene clusters for the snRNAs, U1, U2, and U3.[44] We detected Cajal bodies using an antibody to fibrillarin, which is reactive to Cajal bodies and also stains the nucleolus with a dense fibrous pattern. Staining of Cajal bodies, distinguishable from nucleoli by their morphology, in cells expressing the EGFP RI-3A+ fusion demonstrated that Cajal bodies are not coincidental with murine AF4 (Figure 3b). Interestingly, in HeLa cell nuclei, Cajal bodies had one or more AF4 bodies juxtaposed. However, because foci of AF4 were much more numerous, most were found without an associated Cajal body.

The PML body, also called PML Oncogenes Domain (POD) and Kr body, is a small subnuclear body thought to be involved in various aspects of the regulation of transcription and active DNA replication during middle–late S-phase.[45] (The PML fusion is also found fused to the retinoic acid receptor alpha in the t(15;17)(q22;q21) acute promyelocytic leukemia.[46,47] In HeLa cells expressing the RI-3A+ EGFP fusion and stained with anti-PML antibody, it is clear that PODs and AF4 foci are not coincidental (Figure 3c).

Aside from the subnuclear bodies with clean margins and defined shapes, there are nuclear zones of known function that can be visualized. These zones are often associated with RNA and DNA metabolism. Proliferating cell nuclear antigen (PCNA) is involved in both DNA replication[48] and DNA repair.[49] Using an antibody to PCNA, it was determined that the murine AF4 does not colocalize with PCNA in the nucleus (Figure 3d). RNA Polymerase II is a component of the transcription machinery[50] and also colocalizes with pre-mRNA splicing factors.[51] In HeLa cells expressing the EGFP RI-3A+ fusion, RNA Polymerase II did not colocalize with murine AF4 (Figure 3e).

Proteins involved in the post-translational processing and splicing of RNA are identified by their colocalization with the products of RNA transcription. Nascent RNA transcripts were visualized by incorporation of 5-FU into transcripts. The 5-FU was detected by a monoclonal antibody to 5-BrDU. The 5-FU was added directly to the cell culture media. The experiment was done in a pulse chase style, treating cells at different time points to identify early (2 min), mid (10 min), and late (30 min) RNA transcripts. Foci of EGFP AF4 expression showed a pattern of nascent RNA exclusion. Nascent RNA appears as an amorphous cloud inside the nucleus. There are small patches

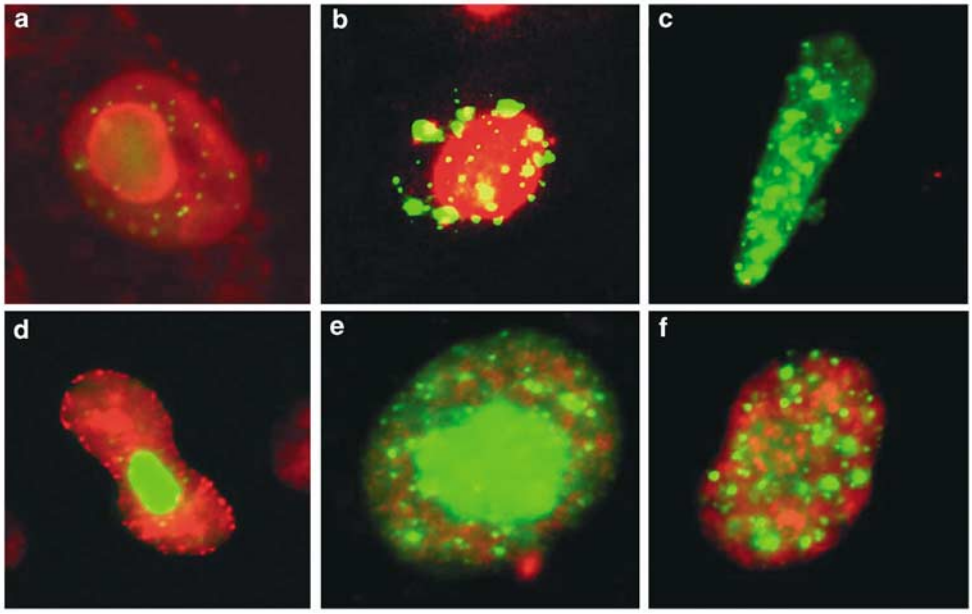

Figure 3 The AAF4 Body does not colocalize with a number of other subnuclear domains and organelles. (a) Confocal laser microscopy of HeLa cells transfected with either an EGFP RI, EGFP RI-3 or EGFP RI-3 A+ construct (green), and counterstained with antibody to a subnuclear protein (red). (a) Monoclonal antinucleolin stains the nucleolus. (b) Monoclonal antifibrillarin stains both the nucleolus (large central) and the Cajal bodies (small foci). This picture was over contrasted to maximize visualization of the Cajal bodies. (c) Monoclonal anti-PML (5E10) stains the PML bodies (PODS). (d) Monoclonal anti-PCNA stains areas of active DNA replication and repair. (e) Monoclonal anti-RNA polymerase II stains areas of active RNA transcription. (f) Nascent RNA transcripts are visualized by staining incorporated 5-FU with a crossreactive anti-BrDU monoclonal antibody.

within the nucleus where the intensity of 5-FU staining is markedly diminished, and AF4 is found within these patches (Figure 3f). This reciprocal staining pattern was confirmed using LSM (Zeiss, Thornwood, NJ, USA) software analysis of the images (not shown).

Proteins encoded by MLL fusion partners AF4 and AF9 interact

We hypothesized that important insights into the biochemical function of the proteins encoded by the MLL fusion partners AF4 and AF9 could be obtained by identifying proteins with which they interact. In addition, we hypothesized that a shared function of MLL fusion partners is coexistence in a multiprotein complex. Therefore, we undertook a series of yeast two-hybrid screens using portions of AF4 and AF9. When fused to MLL, the carboxy-terminal 84 amino acids of the AF9 homolog ENL have been shown to be necessary and sufficient to transform mouse myeloid cells,[52] and the 'knockin' mouse model indicates that the terminal 90 amino acids of AF9 are sufficient, when fused to MLL, to cause myeloid leukemia in chimeric mice.[21,53] With the importance of this region in mind, the carboxy-terminal 93 amino acids of AF9 were used as bait in a yeast two-hybrid screen of a mouse 11-day embryo cDNA library. Of 1.0×10^6 transformed yeast cells, three gave rise to colonies with cDNA molecules encoding proteins that interacted specifically with AF9 in two-hybrid assays. Two of the three clones contained cDNA sequences encoding amino acids 728–814 of the mouse FMR2 protein. FMR2 is an AF4 homologue's therefore we tested and confirmed that the corresponding region of AF4 also specifically interacts with the carboxy-terminus of AF9 in yeast two-hybrid assays. Finally, we performed a deletion mutation analysis to narrow the region of AF9 binding within AF4 to 14 amino acids (Figure 1a).

To confirm the interaction between AF4 and AF9, both GST pull-downs and coimmunoprecipitations were performed. For the pull-downs, cDNA encoding 749–775 aa of AF4 (Figure 1a) was cloned into pGEX-5X1 vector to create a protein with an amino-terminal GST fusion. The GST-AF4 fusion was able to bind biotinylated AF9. Sepharose-bound GST protein alone did not bind to the biotinylated AF9 (Figure 4b). For the coimmunoprecipitations, a FLAG-tagged AF9 protein co-precipitated with a GFP-AF4 protein, but not with GFP alone (Figure 4c). Likewise, an expressed FLAG tag without AF9 sequence showed no interaction with GFP–AF4 protein.

Antisera to both murine AF4 and AF9 were raised in different species (see Materials and methods above). The antisera were affinity purified and used to stain MEF cells. Immunofluorescent microscopy demonstrates colocalization of AF4 and AF9 in these cells (Figure 4a). This colocalization was confirmed in NIH3T3 cells (not shown).

Using the previously described EGFP-AF4 fusion constructs, the localization of the AF4 truncations was compared with a full-length AF9 protein visible by DsRed fusion. DsRed AF9, by itself in cells, directed expression to subnuclear foci similar to AF4 (Figure 5b). In cells cotransfected with EGFP AF4, DsRed AF9 showed perfect colocalization in mathematical overlays (Figure 5a). When the previously described EGFP constructs were used to map the interaction domain in AF4, we saw that the same fusions that were previously able to direct AF4's subnuclear expression (RI, RI-3, RI-3A+ and RI-3CO+),

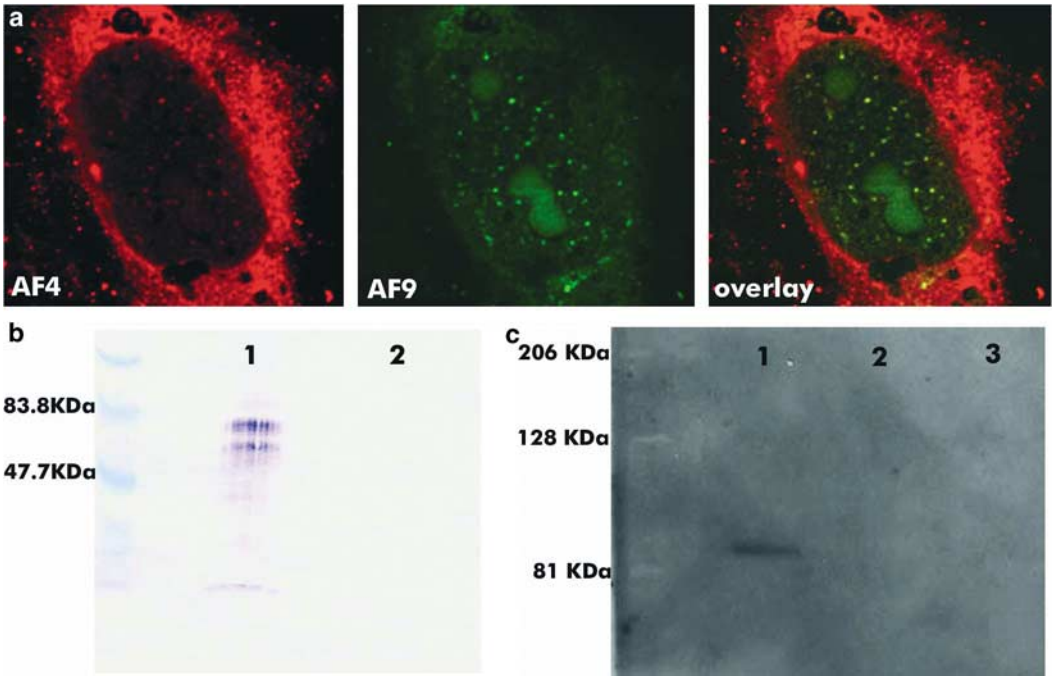

Figure 4 AF4 interacts with AF9. (a) Endogenous AF4 and AF9 colocalize to subnuclear foci. Murine MEF cells were stained with polyclonal antibodies to mAF4 (red) and AF9 (green). The two proteins colocalize, as demonstrated by a mathematical two-color overlay. AF4 antibodies show some addition cytoplasmic staining. (b) GST-RI(AF4) fusion protein can bind to biotynylated AF9. Molecular weight marker is at the left; lane 1, elution from GST-RI 3A+ sepharose shows AF9 (top band, ~63 kDa); lane 2 (no visible bands), elution from GST sepharose. The Western was developed with avadin-HRP. (c) FLAG AF9 coimmunoprecipitates with EGFP-RI3A+. Molecular weight marker is at the left; lane 1, lysate from cells transfected with EGFP-RI3A+ and pFLAG-AF9; lane 2, lysate from cells transfected with EGFP-RI3A+ and pFLAG vector; lane 3, lysate from cells transfected with EGFP-C2 vector and pFLAG AF9. All lysates were immunoprecipitated with anti-GFP antibody and the Western was performed with anti-FLAG antibody.

demonstrated AF9 colocalization (not shown). The same 68 amino-acid region, previously shown to be the smallest portion of murine AF4 able to direct subnuclear expression, was also the smallest fragment able to colocalize with AF9. When coexpressed with AF9, the RI-3A− fragment that previously demonstrated a diffuse cellular expression pattern was retained in the nucleus and demonstrated the murine AF4 stippled pattern (Figure 5c). This indicated that neither the bipartite, nor the QIP-1 NLS, is necessary to direct subnuclear expression of murine AF4. Two new EGFP constructs, RI-3NH+ and RI-3NH−, were designed to include the putative AF9 interaction domain, as determined by yeast two-hybrid studies. Both of these constructs, even the one containing the 14 amino acids of the AF9 interaction domain suggested by yeast two-hybrid studies, showed a whole-cell localization (not shown). This was seen even in the presence of overexpressed AF9. Collectively, the data indicate that more than the 14 amino-acid domain is needed to direct the interaction of AF4 and AF9. The data ultimately place the minimal domain required for nuclear localization and AF9 interaction in a region of 54 amino acids (Figure 1a).

In AF9, the region implicated in AF4 interaction is separate from the region containing the putative NLS. The nuclear localization signals are in the amino-portion of AF9 and are not included in MLL-AF9 fusion. In the amino terminus, there are two classic NLS. The second of the two is a near fit for the QIP-1 recognition sequence (separated by 14 amino acids rather than a perfect fit of 15 amino acids) and has both halves of the bipartite NLS.[40] The carboxy-terminal 100 amino acids of AF9, implicated in murine AF4 interaction and containing the region required for murine knockin leukemogenesis, were fused to the DsRed fluorescent protein tag. In five independent electroporations, the DsRed AF9-100 could not be detected when transfected alone. However, when DsRed AF9-100 was cotransfected with the portions of AF4 that could direct AF4's subnuclear expression, we saw expression of the DsRed Af9-100 fusion (Figure 5d). Expression of DsRed AF9-100 in these cells was able to disrupt the normal subnuclear expression of the AF4 fusions. These data suggest toxicity or silencing of this portion of AF9, except in the presence of overexpressed AF4.

MLL–AF4 fusion protein alters the subnuclear localization of AF9

Both the MLL and AF4 portions of the MLL–AF4 fusion include sequences capable of directing the subnuclear localization of the native proteins. It has previously been shown that the amino terminal 409 amino acids direct the targeted expression of MLL.[36] Endogenous MLL colocalizes with an MLL construct expressing only the first 409 amino acids of MLL.[36] We generated two reporter gene fusions with the first 672 amino acids of MLL. The two constructs EGFP MLL-2Kb and DsRed MLL-2Kb were tranfected into HeLa cells. These constructs give rise to three different expression patterns. The first is a light, diffuse nuclear signal with brighter expression in the nucleolus. This pattern has not been previously reported for MLL. The second expression pattern is similar to the first, with the addition of very bright foci within the nucleolus. The last is the stippled pattern that is most often reported for MLL. When cotransfected with AF4 constructs EGFP RI or EGFP RI-3A+, murine AF4 does not colocalize with any of the MLL expression patterns (Figure 6a).

A FLAG epitope-tagged MLL–AF4 construct was used to study MLL–AF4/AF4 localization. Transfected alone, the MLL–AF4 construct shows two expression patterns similar to that observed for the MLL-2Kb construct – nucleolar and whole nuclear stipples (Figure 6b). When cotransfected with AF4, those portions of AF4 that direct normal AF4 localization do not colocalize with MLL–AF4 (Figure 6c). Therefore, expression of MLL–AF4 does not alter the localization of AF4, and these data suggest that the amino terminus of MLL and not the carboxy terminus of AF4 directs the subcellular localization the MLL–AF4 fusion protein. However, coexpression of FLAG-MLL-AF4

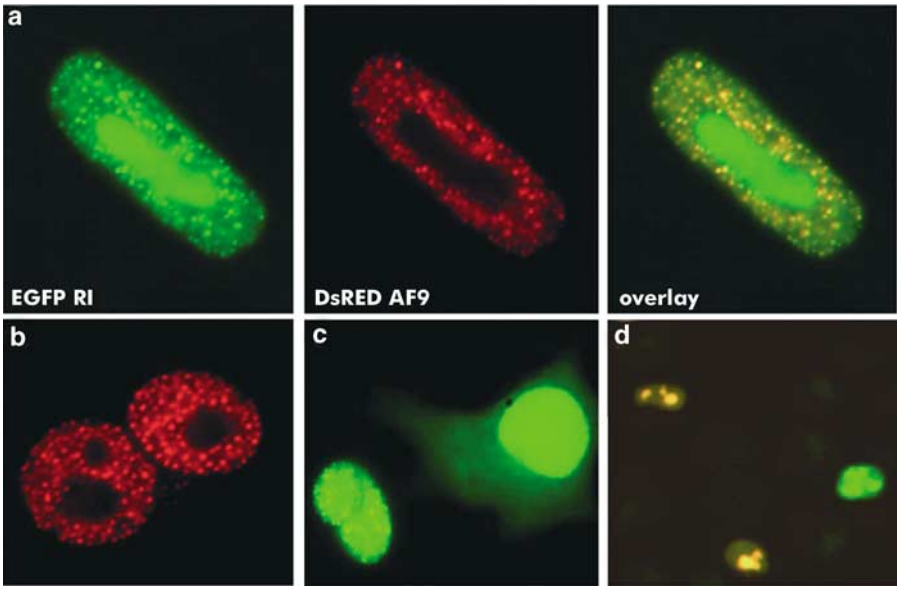

Figure 5 The AF4:AF9 interaction helps direct AF4's subnuclear expression. (a) HeLa cells cotransfected with EGFP-RI and DsRED AF9. A two-color overlay shows colocalization at the AF4 bodies. (b) HeLa cells transfected with DsRed AF9. (c) Two-color overlay of Cos cells cotransfected with EFGP RI-3A− and DsRed AF9. Two cells are visible, on the right a cell transfected with only the EGFP RI-3A− construct shows diffuse whole-cell expression. On the left, a cell coexpressing both constructs shows EGFP RI-3A− expression at the AF4 body. (d) HeLa cells trasfected with EGFP RI-3 and DsRed AF9-100, showing disrupted AF4 expression.

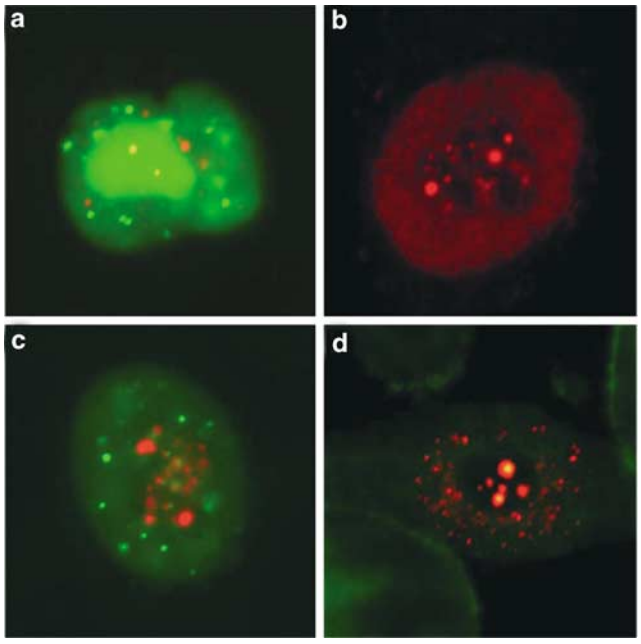

Figure 6 The MLL portion of MLL-AF4 directs MLL-AF4 expression, but retains the ability to bind to AF9. Confocal laser microscopy on HeLa cells. (a) EGFP RI (green) and DsRed MLL 2kb (red) can express in a similar looking pattern, but do not colocalize. (b) FLAG-tagged MLL-AF4 expressing in nucleolar foci. (c) EGFP RI-3 (green) and FLAG-tagged NLL-AF4 (red). (d) DsRed AF9 (red) and FLAG-tagged MLL-AF4 (green) show AF9 expression directed to the nucleolus by MLL-AF4 expression.

with DsRed-AF9 results in colocalization of MLL-AF4 and AF9 in large, nucleolar-like foci (Figure 6d). This pattern of AF9 localization was not observed in the absence of MLL–AF4 expression.

Discussion

In this study, we identify a physical interaction between the two most common MLL fusion partners AF4 and AF9. This interaction occurs within distinct nuclear foci, which we have named 'AF4 bodies'. Importantly, the AF4 body is always restricted to the nuclear space and is excluded from the nucleolus. The AF4 body is not coincidental with PML PODS, Cajal bodies, or other subnucelar structures we examined. Expression of AF4 is not found to be associated with the common nuclear functions of DNA replication, DNA repair, RNA transcription or the post-translational processing of nascent RNAs.

AF4, as well as the MLL-AF4 fusion, has been shown by antibody studies to be distributed in a stippled, nuclear pattern.[54] This is consistent with the AF4 body localization we report. Analysis of the derived amino-acid sequence of murine AF4 reveals a number of nuclear localization signals. The most abundant are the classic, four amino-acid NLS, a number of which prove nonfunctional in our assays. Two overlapping bipartite NLS appear in both human and murine AF4. One is a standard bipartite NLS, which is the only NLS conserved among all of the mammalian AF4 family members. The second is the binding site for QIP-1, an α-importin family shuttle protein.[40] The consensus for QIP-1 binding is much more rigid than for any other NLS. Interestingly, in the small number of proteins that contain a near match for this consensus, we find AF9. Although this may be important to the mutual function of AF4 and AF9, it is not likely to hold importance in the leukemogenic potential of these proteins, as the QIP-1 consensus lies in a portion of AF9 not found in the MLL–AF9 fusion, and it is not vital to AF4's localization. Ultimately, AF4's localization to the AF4 body is not directed by any of the known NLS, but is rather directed by the same sequence that modulates AF4's interaction with AF9. In addition, it appears that some downstream sequences are also required to realize AF4 targeting to the AF4 body.

Mapping of the interaction domains within AF4 and AF9 reveals two important points. First, the domains are located within regions of AF4 and AF9 that are highly conserved within the protein families to which each belongs.[13,15] This suggests that the MLL partner genes ENL (AF9 family) and AF5q31 and LAF4 (both AF4 family) may also interact at the AF4 body. Second, the interaction domains of both AF4 and AF9 are retained in the MLL–AF4 and MLL–AF9 fusions, respectively.[25,55] A recently discovered MLL–AF4 fusion, cloned in a single case of infant lymphoblastic leukemia, does not contain the AF4 transactivation domain, found in all the previously cloned MLL–AF4 fusions.[56] This fusion does, however, contain the AF9 interaction domain we have identified. These findings imply that the interaction that we have characterized plays an important role in MLL leukemogenesis. Furthermore, we show that MLL–AF4 does interact with AF9 and has the ability to misdirect AF9 expression from the AF4 body to alternative foci within the nucleolus.

The AF4–AF9 interaction is not unique among MLL fusion partner proteins. A number of MLL fusion partners have recently been reported to interact either with each other or with a common third protein. ENL has been shown to interact with the MLL partner at 10p11.2, ABI1.[57] ABI1, along with EEN (19p13) and Eps15, the murine homologue of AF1p, all interact with the proteins synaptojanin and dynamin.[58] ELL (19q13.1), another of the more common MLL fusion partners, has recently been shown to interact with the protein EAF1, a small protein with a region of homology to AF4.[59] Many of the indirect interactions between MLL partners are modulated through proteins found in the SWI/SNF complex. The SWI/SNF complex activates transcription by ATP-dependant modulation of chromatin structure.[60] Interestingly, the AF9 homologue ENL, has been identified in a novel human SWI/SNF complex implicated in the activation of the HoxA7 gene.[61] In addition, the MLL partner AF10 interacts with the AF9 family member GAS41 which in turn interacts with INI1, also called SNF5.[62] In yeast, SNF5 also interacts with the AF9 family member TGF3.[63] CBP interacts with a newly discovered member of the SWI/SNF complex, SRCAP.[64] AF10 has been shown to interact with the synovial sarcoma protein SYT,[65] which in turn interacts with the SWI/SNF complex.[66] The interactions are not limited to the nucleus. In the cytoplasm, AF6 interacts with Ras,[67] which is the pathway in which the AF4 fly homologue Lilliputian is thought to play a roll.[68] MLL, present in all the fusions and involved in a leukemogenic internal duplication event,[17] is brought into this web by two interactions. MLL has been shown to interact with CBP,[69] and this interaction is important to the function of both proteins. MLL also interacts with GADD34, which in turn interacts with the SWI/SNF complex through the SNF5 protein.[70] Viewed together, a network of interactions involving MLL and its fusion partners begins to emerge (Figure 7). Given that no one common function or domain has been identified in all the MLL fusion partners in nearly 10 years of study, we propose a new hypothesis based on the interactions we and others have discovered to explain the fusion partner's role in

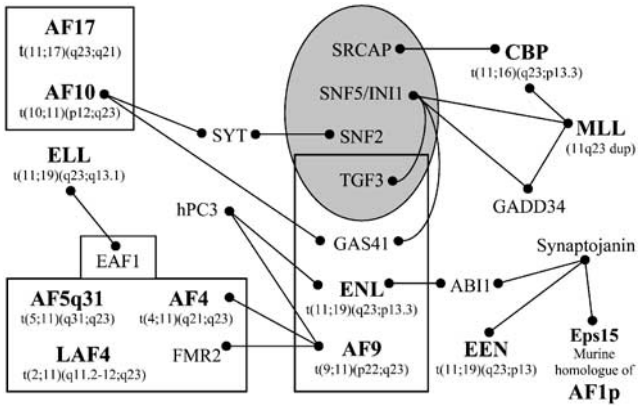

Figure 7 The AMLL Web model of MLL leukemogensis. Genes directly involved in MLL fusions are in black text. Lines indicate a reported interaction between two genes. Boxes contain genes considered to be part of gene families: AF4, AF10/AF17, and AF9/ENL. The gray circle contains proteins known to be part of the SWI/SNF complex.

leukemogenesis. The 'MLL Web hypothesis' considers that no common function or domain exists among the partner genes, but rather, postulates that the partners exist as part of a larger complex that represents a cellular process, somehow perturbed by the MLL fusion. This hypothesis compensates for some of the shortcomings of previous theories of MLL leukemogenesis. Unlike the theories that dismiss the partner genes' importance, it places the partner in a context that allows it to exert some power over the process of leukemogenesis, and might explain the ability of the partner to modulate the phenotype of the blast cell. Unlike the theories that attempt to categorize the partners as transcription factor-like proteins, our hypothesis does not demand that the partners all lend a specific function to the fusion. This explains the partners that do not appear to be transcription factors, such as ELL and AF10. The cytoplasmic partners AF1p and AF6 can also be accommodated by our hypothesis. This process, like many signaling cascades, might begin outside the nucleus, in the cytoplasm where AF6 and AF1p are found. It may then move into the nucleus, and then to subnuclear structures like the AF4 body. The MLL Web currently comprises interactions involving 12 of the known MLL fusions that account for almost 95% of MLL-related leukemia.[20]

The MLL Web hypothesis suggests that the collective role of the fusion partner complexes likely involves chromatin restructuring, many of the interactions being modulated through SWI/SNF complex. In addition, both AF9 and ENL have been shown to interact with hPc3, the human homologue of a Polycomb group protein.[34,35] In the fly, where the MLL homologue trithorax acts to positively regulate the expression of the HOX genes, the Polycomb proteins act to negatively regulate expression of those same genes.[11] The HOX genes, whose spatial and timed expression regulate segment and limb development during embryogenesis, have also been shown to play a pivotal role in hematopoietic lineage determination.[71,72] This makes HOX gene dysregulation a provocative lead in the study of MLL-related leukemia. Polycomb has also been shown to be part of a protein complex called the 'Polycomb repressive complex', which is thought to inactivate gene transcription through chromatin interaction, and is known to exclude the known members of the SWI/SNF complex.[73] It is possible that the AF4 body exists as part of this complex, or perhaps represents a subcellular domain for the assembly of the factors in this complex.

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