AF4 Encodes a Ubiquitous Protein That in Both Native and MLL-AF4 Fusion Types Localizes to Subnuclear Compartments

By Quanzhi Li, Joy L. Frestedt, and John H. Kersey

Acute leukemia with t(4;11)(q21,q23) translocation results from the in-frame fusion of the MLL to the AF4/FEL gene. In previous studies, we and others demonstrated that AF4 transcripts are present in a variety of hematopoietic and nonhematopoietic human cells. To further study the wild-type and leukemia fusion AF4, we used glutathione S-transferase (GST)-fusion proteins as immunogens to produce rabbit polyclonal antibodies that were specific for normal and chimeric AF4 proteins. Using Western blotting, we demonstrated that the AF4 gene encodes proteins with apparent molecular weight of 125 and 145 kD. A 45-kD protein coprecipitated with AF4 protein in immunoprecipitation. Also, the anticipated MLL-AF4-encoded 240-kD protein was detected in all cell lines with t(4;11) translocation; fusion proteins were present in lesser quantity than the wild-type AF4. The proteins recognized by the antibodies are of the predicted sizes of the AF4 and MLL-AF4-encoded proteins based on previous DNA sequencing analysis. The MLL-AF4 fusion protein had a similar subcellular distribution as AF4. Both t(4;11) and non-t(4;11) leukemic cells showed a similar pattern of punctate nuclear staining in all cell lines tested using confocal immunofluorescence microscopy. AF4 antibodies should be useful for further elucidation of the function of AF4 in normal cellular physiology, as well as the function of MLL-AF4 in leukemogenesis. The antibodies should also be helpful for the diagnosis of the MLL-AF4 fusion proteins in t(4;11) leukemias.

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MATERIALS AND METHODS

Cell lines and construction of expression vectors. The RS4;11 cell line was established in our laboratory and is available from ATCC (American Type Culture Collection, Rockville, MD). Two t(4;11) cell lines, B11 and AN4;11, have been described and used in our laboratory for a number of years including Nalm-6, KM3, BLIN-1 (B-cell lines), Raji (B-cell lymphoma), Molt-4, CEM (T-cell lines), M418 (human neuroblastoma), MG-63 (human osteosarcoma), and K562 (myeloid cell line). The cells were grown and maintained in RPMI 1640 tissue culture medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. A 3.3-kb cDNA designated PL12 that contained most of AF4 gene open reading frame was isolated from a human placenta cDNA library. Two regions of the PL12 clone, one from the 5′ end (base pair 612-1110, designated C9) and another from the 3′ end (base pair 494-1100, designated C9) and another from the 3′ end (base pair 612-1110, designated C9) and another from the 3′ end (base pair 612-1110, designated C9).

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sites. Booster injections with the decreasing amounts of antigen complete Freund's adjuvant and injected into two rabbits at multiple preparative electrophoresis were emulsified with an equal volume of (1 mg/mL) of the 44-kD or 36-kD GST-fusion protein obtained from and Burgess.18

Electrode buffer using an electro-elution device. SDS contained in the bands of interest were cut from the gels and eluted into SDS-PAGE stained with Commassie blue staining solution for 2 to 3 minutes. The protein samples were separated in 10% SDS-PAGE gels and surface-proteins were further purified by preparative electrophoresis. The reagent (Pierce, Rockford, IL). After affinity purification, GST-fusion cia). The protein concentration was determined by a protein assay concentrator (Amicon, Bedford, MA) and buffer exchanged to solution containing 25 mmol/L reduced glutathione (Sigma). The polyacrylamide gel electrophoresis (SDS-PAGE). GST and GST-fusion proteins. The presence of the fusion proteins in the cell lysates were sonicated (Branson Ultrasonic Corp, Brandury, CT) to release the proteins. The expected to encode a 44- and a 36-kD protein, respectively (GST, 26 kD; inserts, 18 kD and 10 kD).

Expression and purification of GST-fusion proteins. GST expression vectors with or without the inserts were inserted into DH5 alpha bacteria (GIBCO/BRL, Grand Island, NY) using standard bacterial transformation procedures. The transformed colonies were selected and grown in ampicillin-containing (100 μg/mL) LB medium at 37°C overnight with vigorous shaking. GST-fusion proteins were induced with 0.1 mM IPTG (Isopropyl-B-D-thiogalactopyranoside, Sigma Chemical Co, St Louis, MO) for 3 to 4 hours. The bacteria were harvested by centrifugation, washed and resuspended in PBST-100, pH 7.4 (phosphate-buffered saline, 1% Triton-X100, 1 mM EDTA) and sonicated (Branson Ultrasonic Corp, Brandury, CT) to release the proteins. The presence of the fusion proteins in the cell lysates were determined by analyzing the samples in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). GST and GST-fusion proteins were affinity purified by passing the bacterial lysates through a glutathione sepharose 4B (Pharmacia) column and washed thoroughly with PBST-100. The bound proteins were eluted with 100 mM/mL NaCl solution containing 25 mM/mL reduced glutathione (Sigma). The proteins were concentrated to 2 to 3 mg/mL using a Centricon-10 protein concentrator (Amicon, Bedford, MA) and buffer exchanged to 100 mM/mL HEPES buffer using a PD-10 desalting column (Pharmacia). The protein concentration was determined by a protein assay reagent (Pierce, Rockford, IL). After affinity purification, GST-fusion proteins were further purified by preparative electrophoresis. The protein samples were separated in 10% SDS-PAGE gels and surface-stained with Comassie blue staining solution for 2 to 3 minutes. The bands of interest were cut from the gels and eluted into SDS-PAGE electrode buffer using an electro-elution device. SDS contained in the protein solution was removed by acetone precipitation method of Hager and Burgess.18

Production and purification of antibodies. Two hundred microliters (1 mg/mL) of the 44-kD or 36-kD GST-fusion protein obtained from preparative electrophoresis were emulsified with an equal volume of complete Freund's adjuvant and injected into two rabbits at multiple sites. Booster injections with the decreasing amounts of antigen emulsified with incomplete Freund's adjuvant were performed at a 2-week interval over a period of 84 days. The immune responses of the animals were monitored by indirect enzyme-linked immunosorbent assay (ELISA)19 and Western blotting. The rabbits were killed and the sera collected when the ELISA titers of the immune sera against the immunizing antigen reached 1:5,000 or greater. Our initial attempts to immunize the rabbits with glutathione sepharose 4B purified GST-fusion proteins in denatured or native forms failed to generate useful antibodies. This initial problem led us to further purify the proteins by preparative electrophoresis to obtain a higher proportion of full-length fusion protein for immunizations. Rabbits immunized with these highly purified and denatured antigens stimulated strong antibody responses as determined by immunoblotting and ELISA using native and denatured proteins as coating antigens. Specific antibodies in the sera were purified by affinity chromatography. Briefly, 2 mg of purified GST, GST-C9, or GST-C15 fusion proteins were conjugated to 1 mL Affigel-10 (Bio-Rad, Hercules, CA) following manufacturer's recommendations. Protein-conjugated Affigel-10 was packed into a minicolumn and equilibrated with PBS. The antisera diluted fourfold with PBST (PBS containing 0.1% Tween-20) was passed through the column containing Affigel-10 conjugated GST to remove antibodies against GST. The eluent was then passed several times through the column containing conjugated GST-C9 or GST-C15 fusion protein, washed extensively with PBST, and eluted with 50 mM/mL glycine buffer (pH 2.8). The eluted antibody was neutralized by 1.89 mol/L Tris buffer (pH 8.9) in the collection tubes. Purified antibodies were designated anti-C-9 and anti-C15 and stored at 4°C in a solution containing 0.5% BSA, 0.4 mol/L L-arginine, and 0.02% sodium azide. IgG fractions of preimmune sera were purified by a protein A column (Pierce).

Western blotting. Cultured cell lines in log phase were harvested by low speed centrifugation (1,500g), washed twice with PBS, and subjected to sonication at maximum intensity for 10 seconds. The cell lysates were solubilized in SDS-PAGE sample buffer, heated in boiling water for 2 minutes, and separated on SDS-PAGE gels under reducing-conditions. After electrophoresis, the proteins in the gels were electro-phoretically transferred onto hydrated nitrocellulose membranes for 2 hours. The membranes were blocked with 5% nonfat milk in PBS for 2 hours at 37°C or at 4°C overnight. The membranes were probed with diluted primary antibodies in blocking buffer at room temperature for 1 hour. For detection of AF4 and MLL-AF4-encoded proteins, affinity purified anti-C15 and anti-C9 antibodies (50 to 100 μg/mL) were diluted 1:100 to 1:200 and used in Western blotting. Mouse antihuman β tubulin monoclonal antibody (Sigma) used as an internal control in some experiments was diluted according to supplier's recommenda-tions. After incubating with primary antibodies, the blots were washed either under high or low stringent conditions. Unless specified, all Western blottings were performed under high stringent washing conditions. Under high stringent conditions, the blots were washed three times (15 to 20 minutes each) with PBS containing 0.05% Tween-20. Low stringent wash consisted of three washes (5 to 8 minutes each) with PBS containing 0.01% Tween-20. After washing, the blots were incubated with goat antirabbit or goat antinimouse IgG conjugated to horseradish peroxidase (HRP, Amersham, Alington, IL) for 45 to 60 minutes. The membranes were incubated with horseradish peroxidase (HPR, Amersham, Alington, IL) for 45 to 60 minutes. The membranes were incubated with horseradish peroxidase (HPR, Amersham, Alington, IL) for 45 to 60 minutes.

![Fig 1. Schematic representation of predicted AF4 gene structure.](from www.bloodjournal.org by guest on October 27, 2017. For personal use only.)
minutes at room temperature. The blots were washed as before and incubated with enhanced chemiluminescence (ECL) reagents (Amer-sham) for 1 minute. The blots were blotted dry with filter papers, enclosed in transparent plastic sheets, and exposed to autoradiographic film. Other antibodies used in this study were rabbit anti-LAF4 (A gift from Dr Louis M. Staudt, Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD) and monoclonal anti-MLL antibody (a gift from Dr Lisa H. Butler, John Radcliffe Hospital, Oxford, UK).

Blocking experiment. The specificity of purified anti-C15 and anti-C9 were accessed by blocking with their corresponding immunizing antigens in Western blotting format. Total cell lysate of Km3 was separated in 8% SDS-PAGE (20 to 40 µg/lane) and electrophoretically transferred to nitrocellulose membrane, cut into strips, and incubated with different preparations of primary antibodies. Affinity-purified stock solutions (50 to 100 µg/mL) of anti-C15 and anti-C9 were diluted 1:100 with PBS containing 5% nonfat milk and 80 µg of corresponding immunizing antigens in a final volume of 10 mL. Preimmune IgG and the same amount of antibodies blocked with GST (80 µg/sample) were used as controls. After 30 minutes incubation at room temperature, the nitrocellulose strips were incubated with primary antibodies and the rest of the Western blotting procedures were performed as above.

Immunoprecipitation and pulse chase analysis. A total of 1 × 10^7 cells were harvested by low speed centrifugation and washed twice with cold Hank’s balanced salt solution. The cells were resuspended in 5 mL RPMI medium containing 5% dialized FBS (Gibco) and 0.2 mCi 35 S cold Hank’s balanced salt solution and lysed with 600 µL of lysis buffer (PBS containing 1% Triton X-100, 0.5% SDS, 0.5% deoxycholate, 1% BSA, leupeptin [10 µg/mL] and 0.2 mmol/L phenylmethyl sulfonyl fluoride [PMSF], Sigma). The cell lysate was incubated in ice for 10 minutes and centrifuged in a microcentrifuge at top speed for 10 minutes. The supernatant was incubated with 0.5 to 1 µg anti-C15 or anti-C9 Fab conjugated to fluorescein isothiocyanate (FITC) (Sigma) for 1 hour and washed extensively as described. Stained slides were mounted in glycerol mounting buffer (PBS, 10%; p-phenylenediamine, 10 mg/mL; and Glycerol, 90%) and examined by a Bio-Rad MRC 600 confocal microscope. The images were processed using Adobe photoshop software (Adobe Systems Inc, San Jose, CA).

**RESULTS**

Specificity of anti-C15 and anti-C9 antibodies. The specificity of affinity-purified anti-AF4 antibodies was first evaluated by Western blotting. As shown in a high stringency experiment (see Materials and Methods), affinity-purified anti-C15 recognized a single protein band of about 145 kD (Fig 2A, lane 1) in Western blotting against total Km3 cell lysate. The signal was absent using preimmune serum (lane 2) and was completely blocked by incubating the antibody with immunizing antigen before Western blotting (lane 3). Lane 4 shows lack of blocking with GST. Under low stringent washing conditions, anti-C15 also binds to a 80-kD protein in Western blotting; however, the cross-reactivity could be removed by extensive washing. In contrast to anti-C15, affinity-purified anti-C9 detected four major proteins in Western blotting (Fig 2B, lane 1) with an estimated molecular size of 184, 145, 75, and 50 kD. All four signals were completely blocked by the immunizing antigen.
washed, separated by 10% SDS-PAGE, and autoradiographed.

Because the immunizing antigens of anti-C9 and anti-C15 were
larger protein.

More than one AF4 protein is detected in Western blotting.

As shown in Fig 4A, a side-by-side comparison of anti-C15 and anti-C9 showed that anti-C15 recognized a single
protein band in the cell lysates and anti-C9 bound to several
proteins. One of the proteins detected by anti-C9 (lane A3)
showed the same migration as that detected by anti-C15 (lane
A1). When anti-C9 and anti-C15 were mixed (lane A2) and used
for Western blotting, the AF4 protein signal was stronger than
the signals detected by either antibody alone, while other
protein bands detected by anti-C9 remained comparable. These
results strongly suggest that both antibodies recognized the
same AF4 protein. When the Western blottings were performed
using lower percentage gels (Fig 4B), the AF4 protein migrated
as doublets (145 kD and 125 kD), which were detected by both
anti-C15 (lane B1) and anti-C9 (lane B2). Repeated experiments
showed that anti-C15 generally reacted stronger with the
125-kD band, which is most likely due to the results of alternate
splicing of AF4 mRNA. In previous studies, a 10.5-kb and a
12-kb mRNA transcript of AF4 gene was observed. An
alternate explanation for the doublets is the possibility of
precursor-product relationship of the doublets. To evaluate this
possibility, pulse chase analysis was conducted. Despite re-
peated attempts, we were able to show the doublets in autoradi-
ographs using low percentage gels only after long periods of
incubation (data not shown). A time course study of cell lysates
labeled with 35S and precipitated by anti-C15 indicated that the
doublets were observed in autoradiographs only in cell lysates
that have been labeled for a longer period of time (>3 hours).
The extented labeling time required to show the doublets prevented us from obtaining interpretable results of pulse chase
analysis, which requires a short labeling window of 30 minutes
to 1 hour. Low immunoprecipitation efficiency of anti-C15
may also be in part responsible for the observations.

Anti-C15 and anti-C9 recognize MLL-AF4 and AF4-MLL
reciprocal fusion proteins, respectively. When the cell lysates
were run on low percentage SDS-PAGE gels (4% to 5%), a
protein band with a calculated molecular weight of 240 kD was
detected by anti-C15 in cell lines containing MLL-AF4 fusion
gene (Fig 5). This protein was consistently detected in all cell
lines with known t(4;11) translocations (Fig 5A, lanes 2, 4, and
6), but not in those without the translocations (lanes 1, 3, and 5).
These data indicate that the 240-kD protein is the MLL-AF4–
encoded fusion protein and is consistent with the predicted
protein sizes. A side-by-side comparison of anti-C15 and the
anti-MLL/HRX antibody (HRX107) in Western blotting
showed that a 240-kD protein band was detected by both
antibodies (data not shown). In these low percentage gels,
anti-C15 also generally detected the AF4 doublet in cells used

Fig 4. Comparison of Western blotting patterns of anti-C15 and
anti-C9. (A) Km3 lysate (20 μg/lane) was separated in 8% SDS-PAGE,
transferred to nitrocellulose paper, and probed with anti-C15 (lane 1),
anti-C15 plus anti-C9 (lane 2), and anti-C9 (lane 3). (B) K562 lysate (20
μg/lane) was separated in 4% SDS-PAGE, transferred to nitrocellu-
llose paper, and probed with anti-C15 (lane 1) and anti-C9 (lane 2).
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Fig 5. Western blotting patterns of anti-C15 and anti-C9 in 4% SDS-PAGE. Cell lysates were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and probed with affinity-purified anti-C15 (A) or anti-C9 (B). Lane 1, Nalm-6; lane 2, RS4;11; lane 3, Km3; lane 4, Sem-k2; lane 5, K562; lane 6, B1.

We also evaluated anti-C9 antibody for study of the fusion proteins. Figure 5B shows the immunoblotting patterns of anti-C9 against total cell lysates in 4% gel. In addition to 180 kD and 145 kD proteins, the antibody also recognized a high molecular weight protein (>240 kD) in cells with t(4;11) translocations, RS4;11 and SEM-k2 (lanes 2 and 4, respectively). The B1 cells, which were previously shown to have low to absent levels of 14 kb der4 RNA transcript, were found to have a very weak band as shown in Fig 5B, lane 6. Although the exact size of this high molecular weight protein cannot be determined due to the lack of proper molecular weight markers, by comparing its migration rate with that of the 240 kD MLL-AF4 fusion protein, the size of the protein was estimated in the range of 300 to 350 kD. Because the high molecular weight protein signal is much weaker than that of AF4 protein, the blot in Fig 5B was intentionally overexposed to show the high molecular weight band. Because anti-C9 was raised using N-terminal polypeptide of AF4 as immunogen, it might expect to recognize AF4 and reciprocal AF4-MLL proteins, but not the MLL-AF4 fusion proteins. In view of the observation that the high molecular weight protein detected by anti-C9 was seen only with t(4;11) lines and that the size of the protein falls in the calculated size range of AF4-MLL fusion protein [(430 (MLL) + 140 (AF4) = 240 (MLL-AF4)] = 335 kD (AF4-MLL)], we suspect that anti-C9 recognizes the AF4-MLL reciprocal fusion protein in all t(4;11) lines tested.

AF4 and MLL-AF4 fusion proteins are localized in the nucleus, but not in the cytoplasm. purified anti-C15 was used to evaluate localization of the AF4 and MLL-AF4-encoded fusion proteins in immunofluorescence studies. All cell lines stained with the antibody showed a strong punctate fluorescence staining in nucleus, but not in cytoplasm (Fig 6). The immunostaining patterns were generally similar in all cells tested and there was no significant difference in the patterns and distribution of the labeling for cell lines with or without t(4;11) translocations. Further study will be required to establish whether the AF4 and MLL-AF4 proteins colocalize in the same subcellular compartment. It is also important to determine whether wild-type AF4 and the fusion proteins function in distinct cellular compartments.

DISCUSSION

In this study, we have produced and characterized specific antibodies to AF4-encoded proteins. We have also provided direct evidence that AF4-encoded proteins are present in a variety of human leukemic and nonleukemic cells, as demonstrated by immunoblotting and immunofluorescence microscopy. In Western blotting, anti-C15 and anti-C9 antibodies consistently recognized a protein of about 145 kD in all cell lines tested. Under high stringency conditions of washing, the anti-C15 antibody recognized a single 145-kD AF4 protein in Western blotting, which migrated as a doublet when lower percentage gels (4%) were used, the antibody also detected a 240-kD protein in all cell lines with t(4;11) translocations. The molecular weight of the 240- and 145-kD proteins are consistent with predicted sizes of MLL-AF4 and AF4-encoded proteins based on previous DNA sequencing and Northern analysis.2,4,22 Previous studies demonstrated the presence of 12 kb and 10.5 kb AF4 mRNA transcripts in cell lines tested. The 12-kb and 10.5-kb RNA transcripts identified in the previous studies are most likely to be the result of alternate splicing of AF4 mRNA, which subsequently encode the 145-kD and 125-kD proteins. The 12.5-kb mRNA transcript previously detected in all cell lines with t(4;11) translocations corresponds to the 240-kD protein detected in this study. Coprecipitation of the 45-kD protein with AF4 protein suggests the possibility that it may exert its biological function through interaction with a second protein.

The anti-C9 that was raised against N-terminal end of AF4 protein detected the protein of about 145 kD. However, it also bound to a 184-kD, a 75-kD, and a 50-kD protein in Western blottings. It is not clear whether these proteins are AF4-related. Thus far, mRNA species corresponding to these proteins have not been identified. It was of interest that anti-C9 reacted to a high molecular weight protein (>240 kD) in RS4;11 and Sem-k2 cells with t(4;11) translocations, while the protein was absent from non-t(4;11) cells. These observations suggest that anti-C9 is able to detect AF4-MLL reciprocal fusion protein if it is present.

Molecular analysis of the MLL and AF4 genes by our group and others3,10,11,12,22 indicates that the N-terminal portion of MLL fuses to C-terminal portion of AF4. The C15 clone used for expression and immunization was chosen to be downstream of all known breakpoints. Thus, anti-C15 recognizes the chimeric MLL-AF4 protein in all cell lines tested (B1, RS4;11, and Sem-k2). The MLL-AF4 chimeric proteins in RS4;11, AN4;11, and B1 cells are calculated to contain 2319, 2276, and 2234 amino acid residues, respectively. Fusion proteins seen in the Western blottings migrated as a single band in 4% SDS-
PAGE gel (Fig 5) with apparent similar molecular weight, which is consistent with the predicted sizes and showing that the size difference of the proteins was too small to be resolved under the experimental conditions used.

Recently, Joh et al\textsuperscript{13} have generated rabbit polyclonal antibodies specific for N-terminal epitopes of MLL. These antibodies recognized a 240-kD protein in Western blotting. Butler et al\textsuperscript{14} produced a monoclonal antibody against a 15 amino acid peptide at N-terminal of MLL gene and the antibody (HRX 107) also recognized the 240-kD MLL-AF4 fusion protein in the Sem-k2 cell. It is of some interest that the quantity of MLL-AF4 fusion protein detected by our Western blotting is significantly less than that of the AF4 protein. The significance of these differences is not clear at present.

A gene previously cloned by Ma and Staudt\textsuperscript{6} from a cDNA library of a Burkitt’s lymphoma is closely related to the AF4 gene. Both genes share a high degree of homology (75% homology at C-terminal and 62% at N-terminal), contained a proline/serine-rich region and NLSs at virtually the same position. The LAF4 gene encodes a major protein of 135 kD. However, the expression of LAF4-encoded protein was shown to be restricted to lymphoid cells. As predicted from DNA sequence analysis, our Western blotting results indicate that LAF4 encoded a smaller protein than that detected by AF4 antibodies (data not shown).

Results of our experiments indicate that anti-C15 detects the AF4 protein in subcellular compartments in immunofluorescence microscopy. The subcellular localization of AF4 with punctate distribution in the nucleus is similar to that previously described for transcription-associated proteins. Other proteins with similar distribution are LAF4, MLL,\textsuperscript{13} and several other MLL partners including ENL/LT6,\textsuperscript{13,23} AF9/LT69,\textsuperscript{13} and ELL.\textsuperscript{24} ELL is of interest because of the demonstration that the ELL gene encodes a RNA polymerase II elongation factor.\textsuperscript{25} Consistent with a role for AF4 and LAF4 in transcriptional regulation is the previous observation by Ma and Staudt that AF4 has domains that activate transcription when fused to GAL4 DNA-binding domain. Of note is the observation that the transactivation domain of AF4 is retained within the MLL-AF4 fusion protein.\textsuperscript{6} Further studies will be necessary to determine the significance of the punctate compartmentalized staining seen with anti-AF4 antibody and other antibodies to transcription-related molecules. Previous studies with another transcription factor have demonstrated that phosphorylation of hepatic nuclear factor 4 is

Fig 6. Indirect immunofluorescence microscopy. Cultured cells in log phase were harvested and adjusted to 1 to 2 × 10\textsuperscript{5}/mL. The cells were attached to microscope slides by cytopsin. The cells were probed with anti-C15 followed by goat antirabbit IgG conjugated to FITC. The image of the stained cells was obtained by a Bio-Rad MR C600 confocal microscope and processed using Adobe photoshop software. The cell lines used are indicated.
required for nuclear compartmentalization. It has been shown that the compartmentalized nuclear staining of the protein of Drosophila polycomb (related to Drosophila trithorax) developed homogenous nuclear staining after the protein was mutated.

Finally, the observation that the anti-C15 antibody was able to detect the MLL-AF4 fusion proteins in leukemia lines studied demonstrates the potential use of this AF4-specific antibody as a diagnostic reagent using Western blotting.

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