The tetramer structure of the Nervy homology two domain, NHR2, is critical for AML1/ETO’s activity

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Summary

AML1/ETO is the chimeric protein resulting from the t(8;21) in acute myeloid leukemia. The Nervy homology 2 (NHR2) domain in ETO mediates oligomerization and AML1/ETO’s interactions with ETO, MTGR1, and MTG16, and with the corepressor molecules mSin3A and HDAC1 and HDAC3. We solved the NHR2 domain structure and found it to be an α-helical tetramer. We show that oligomerization contributes to AML1/ETO’s inhibition of granulocyte differentiation, is essential for its ability to enhance the clonogenic potential of primary mouse bone marrow cells, and affects AML1/ETO’s activity on several endogenous genes. Oligomerization is also required for AML1/ETO’s interactions with ETO, MTGR1, and MTG16, but not with other corepressor molecules.

Introduction

AML1/ETO is the fusion protein resulting from the t(8;21) found in acute myeloid leukemia (AML) of the M2 subtype (Miyoshi et al., 1993). AML1/ETO contains the N-terminal 177 amino acids of RUNX1 fused in frame with most (575 aa) of ETO. RUNX1 (AML1) is the sequence-specific DNA binding subunit of a core binding factor that is required at multiple stages of hematopoiesis (Gronwey et al., 2005; Ichikawa et al., 2004; Okuda et al., 1996; Wang et al., 1996). The Runt domain of RUNX1, which is retained in AML1/ETO, mediates DNA binding as well as heterodimerization with the core binding factor β (CBFβ) subunit. ETO (eight twenty-one) was originally identified as the RUNX1 fusion partner in t(8;21), and many of its biochemical properties have been characterized in light of its role in leukemogenesis. The function of ETO itself is less well understood, although its Drosophila homolog, Nervy, was recently shown to interact directly with the transcription factor daughterless, and to repress the activity of enhancers normally activated by the achaete-scute complex in the sensory organ precursor cell (Wildonger and Mann, 2005). Homozygous disruption of ETO in mice results in a gastrointestinal defect, but no hematopoietic deficiencies were reported (Calabi et al., 2001). A second ETO family member, MTG16, was purified as part of a complex of transcription factors by virtue of their association with the hematopoietic-specific SCL protein (Schuh et al., 2005). MTGR1, the third member of the mammalian ETO family, is required for the maintenance of secretory epithelial cells in the small intestine (Ammann et al., 2005). MTG16 is also translocated to RUNX1 in human leukemia (Gamou et al., 1998), potentially underscoring the importance of sequences that are conserved between the ETO proteins for their oncogenic properties.

ETO shares four regions of homology with Nervy (NHR1-4), all four of which are retained in AML1/ETO (Davis et al., 2003; Hug and Lazar, 2004). NHR1 is homologous to several TATA binding protein-associated factors (TAFs) and interacts with E proteins (Zhang et al., 2004). NHR2 is often referred to as the hydrophobic heptad repeat (HHR) because its amino acid sequence is

SIGNIFICANCE

Chromosomal rearrangements often result in the production of chimeric proteins with altered function. In a number of cases, aberrant oligomerization of kinases or transcription factors through the addition of oligomerization domains contributes substantially to the oncogenic potential of these chimeric proteins. Here, we present the three-dimensional structure of the oligomerization domain (NHR2) from ETO, which becomes fused to the AML1 protein as a result of the 8:21 translocation. We show that NHR2 is a tetramer. We also rigorously demonstrate that oligomerization through the NHR2 domain is essential for AML1/ETO’s effects on the differentiation and enhanced clonogenic potential of primary bone marrow cells and discuss why it will be difficult to develop small molecule inhibitors of tetramer formation for treating t(8:21) leukemia.
indicative of an amphipathic helical structure (Lutterbach et al., 1998a). NHR2 contributes many of the most well-studied biochemical properties of ETO, including oligomerization and protein-protein interactions (Amann et al., 2001; Hildebrand et al., 2001; Lutterbach et al., 1998a; McGhee et al., 2003; Zhang et al., 2001). NHR3 is a predominantly \( \alpha \)-helical domain (Yang et al., 2004) that interacts with the regulatory subunit of type II cyclic AMP-dependent protein kinase (PKA RII\( \alpha \)) (Fukuyama et al., 2001); however, its contribution to AML1/ETO function may be cooperative with adjacent domains (Hildebrand et al., 2001). NHR4, also known as the myeloid-Nervy-DEAF-1 (MYND) domain, has two putative, non-DNA binding zinc fingers. Like NHR2, NHR4 mediates protein-protein interactions thought to be important for leukemogenesis (Gelmetti et al., 1998; Lutterbach et al., 1998a; Wang et al., 1998).

The NHR2 domain mediates interactions with ETO family members, oligomerization of AML1/ETO, and interactions with mSin3A, Gfi-1, BCL6, and HDAC1 and HDAC3 (Hiebert et al., 2001; Hug and Lazar, 2004). The generation of oligomeric transcription factors by translocation partner sequences is emerging as a common transforming mechanism in many hematological malignancies (So and Cleary, 2004); however, its contribution to AML1/ETO function may be cooperative with adjacent domains (Hildebrand et al., 2001). NHR4, also known as the myeloid-Nervy-DEAF-1 (MYND) domain, has two putative, non-DNA binding zinc fingers. Like NHR2, NHR4 mediates protein-protein interactions thought to be important for leukemogenesis (Gelmetti et al., 1998; Lutterbach et al., 1998a; Wang et al., 1998).

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Results

Structure determination

We crystallized the SeMet substituted NHR2 domain (residues 480–551, AML1/ETO numbering), collected diffraction data from a single crystal at three wavelengths (0.97915 Å [peak], 0.97946 Å [inflection], and 0.96422 Å [remote]), and solved the structure at 2.0 Å resolution by multiple wavelength anomalous dispersion (MAD). We traced residues 490–548 in both protein chains but observed no continuous electron density that could be used for model building for residues 480–489 and 549–551. Details of data collection and structure determination are summarized in Table 1. The coordinates and structure factors are deposited in the Protein Data Bank with accession code 1WQ6.

The asymmetric unit contains two molecules (Figure 1A) related by an “approximate” 2-fold noncrystallographic symmetry (backbone r.m.s.d.: 0.97 Å). Each polypeptide chain consists of one long curved \( \alpha \) helix (Asp495–Ala547) with a kink at Ile512. The homotetrameric model is established by rotating the asymmetric unit around the 2-fold axis of the \( \text{P2}_1\text{2}_1\text{2}_1 \) space group (Figure 1A). The two antiparallel molecules in the asymmetric unit are aligned at 173.6º in a head-to-tail fashion and lie on top of the other antiparallel pair (Figure 1B: C3 and C4) at a 21º angle to form a left-handed supercoil. Indeed, the NHR2 primary sequence displays the 7-residue repeat characteristic of left-handed coiled coils (Antonin et al., 2002; Harbury et al., 1993) (Figure 2A). The monomer is amphipathic and interacts with the other three monomers through hydrophobic interactions that bury a total accessible surface area (ASA) of 10,645 Å\(^2\).
The tetramer forms an interaction motif through hydrophobic heptad repeats and ionic/polar interactions

The NHR2 tetramer contains an N-terminal tetrameric region with ten symmetrical interacting layers (−5 to −1 and 1 to 5) and two symmetrical C-terminal dimeric regions with five interacting layers (ε′–a′ and a–ε) (Figure 2A). This unusual interaction pattern occurs as a result of the indented alignment of the anti-parallel molecules and the relatively small intersection angle (21°) between the two dimers. To the best of our knowledge, such an interaction motif has not been reported previously. Most of the layers consist of hydrophobic residues at the “a” and “d” positions of the heptad helical wheel (Figures 2A and 3B). The two molecules in the asymmetric unit (C1 and C2, or C3 and C4) display some asymmetry not only in conformation but also in contact surface (Figures 1B and 2D). All the “a” and “d” residues in the tetrameric region of both molecules are more than 90% buried (Figure 2D), and “a” and “d” residues in the dimeric region are more than 65% buried, except for Ala547, which is at the very end of the helix. This is similar to what is observed for the dimeric leucine zipper domain of GCN4 (Harbury et al., 1993). The “ε” and “γ” residues in the tetrameric region are greater than 60% buried (Figure 2D), consistent with what has been reported for a tetrameric mutant of GCN4: p-LI (Harbury et al., 1993). The hydrophobic core is packed between the four molecules in a sandwich-like fashion (Figure 2C).

A number of polar and charged residues make important stabilizing interactions that are not typically seen in leucine zippers. For example, the Thr519 side chains are buried in layers +2 and −2, and Gln530 is packed in layers +5 and −5 (Figure 2A). The hydroxyl group of Thr519 forms an intramolecular hydrogen bond with the carbonyl group of Met515 (Thr519 Oγ — Met515 CO = 2.8 Å) (data not shown). This type of hydrogen bonding between Thr i and X i-4 has also been observed in the endosomal SNARE complex (Antonin et al., 2002; Fasshauer et al., 1998). In layers +5 and −5, Gln530 of C1 forms a specific intermolecular hydrogen bond with Trp502 of C2 (Gln530 Nε — Trp502 Nε=1 = 2.96 Å), while asymmetrically, Gln530 of C4 in the same layer displays an intermolecular hydrogen bond with Leu526 of C1 (Gln530 Ne2 — Leu526 O = 3.11 Å) (Figure 2E).

Two symmetrical groups of intermolecular salt bridge interactions are observed in the tetrameric complex (Figure 2F). One consists of intermolecular interactions between Arg520 and Asp506 that form solvent-exposed salt bridges (Figure 2F). This type of surface ionic interaction was shown to be important for stability in the GCN4 dimer (Spek et al., 1998). The other symmetrical group occurs at layers a and a′ and involves residues at the “a” and “d” positions of the heptad helical wheel. In leucine zipper oligomers, there are buried hydrophobic residues at these locations that contribute to oligomerization, but the NHR2 tetramer instead contains charged residues that form buried salt bridges with good geometry. For example, Asp533 of C2 forms salt bridges with Arg534 of C3 (Figure 2F). Both Asp533 and Arg534 are almost completely buried (Figure 2D), and the highly favorable electrostatic interaction energy offsets the desolvation penalty for burying the charges.

Oligomerization contributes to AML1/ETO’s activity

We assessed the importance of oligomerization for AML1/ETO function by introducing amino acid substitutions into NHR2 that disrupt tetramer formation. We sought to achieve three goals when designing the mutations: (1) to disrupt the oligomerization interface, (2) to confer favorable solubility properties to the mutant domain, and (3) to preserve the α-helical secondary structure as much as possible. The Leu residues at the interface contribute substantially to the stability of the tetramer, and

(average of 2661 Å² per monomer). The large contact area is consistent with oligomerization rather than crystal contacts. The tetrameric structure was predicted by PITA (protein interaction and assemblies) (Ponstingl et al., 2000) and confirmed in solution by analytical ultracentrifugation (Table 2).
therefore we targeted them for mutagenesis. We tried several different substitutions (see Table S1 in the Supplemental Data available with this article online), only two of which significantly disrupted tetramer formation, and which are reported here. The m7 mutant contained Arg and Glu substitutions for the seven Leu residues, with the charged residues placed appropriately for favorable intrahelical electrostatic interactions (Figure 3B). The placement of charge on the interface was designed to disrupt oligomerization and provide favorable solubility properties to the monomeric domain. In addition, both Arg and Glu have a high α-helical propensity, so it was hoped that the monomeric domain would retain some of its α-helical character. The m4 mutant had four Leu residues replaced with Lys, Glu, or Gln (Figure 3B). The three Leu residues at the reported interface for mSin3A (Amann et al., 2001) were not altered in m4. Finally, we substituted two solvent exposed residues (m2, Figure 3B) that were required for mSin3A binding to the isolated NHR2 domain (Amann et al., 2001).

We characterized the solution oligomerization state and secondary structure of the wild-type and mutant NHR2 domains by sedimentation velocity and circular dichroism (CD) spectroscopy, respectively. Figure 3C shows that the continuous...
Table 2. Analysis of NHR2 oligomerization by sedimentation velocity

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass (kDa)</th>
<th>s (svedbergs)</th>
<th>% Contribution</th>
<th>r.m.s.</th>
</tr>
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<tr>
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<td>36.1</td>
<td>2.68</td>
<td>91</td>
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</tr>
<tr>
<td>m7</td>
<td>7.6</td>
<td>1.09</td>
<td>92</td>
<td>0.0117</td>
</tr>
<tr>
<td>m4</td>
<td>33.3</td>
<td>2.85</td>
<td>84</td>
<td>0.0109</td>
</tr>
<tr>
<td>m2</td>
<td>34.2</td>
<td>2.71</td>
<td>91</td>
<td>0.0053</td>
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* Sedimentation coefficient distribution for NHR2 contains a major peak near s = 2.7 S, corresponding to 91% of the material. The molecular mass of this predominant species is 36.1 kDa (Table 2) which corresponds closely to the predicted tetramer mass of 35.8 kDa. Thus, the NHR2 domain is clearly a tetramer in solution. Hydrodynamic modeling using the crystal structure predicts a sedimentation coefficient of 2.87 S, which agrees well with the experimental value of 2.68 S.

The sedimentation coefficient of m7 was ~1.1 S and the mass 7.6 kDa, indicating that m7 is a soluble monomer (Figure 3C and Table 2). CD spectroscopy indicates that the percentage of residues with helical secondary structure was reduced from 60% in NHR2 to 17% in the m7 mutant (Figure 3D). The m4 mutant was tetrameric at 20°C by analytical ultracentrifugation (Table 2), but CD spectroscopy revealed that it was only 41% helical, suggesting that the m4 tetramer is significantly destabilized. We characterized the stability of m4 more extensively by thermal denaturation and renaturation experiments monitored by CD spectroscopy. The NHR2 domain unfolds with a sharp transition at a melting temperature >90°C (Figure S1), indicative of the extremely high stability of the tetramer. In contrast, m4 unfolding occurs with a broad transition and a midpoint around 50°C; therefore, the m4 tetrameric structure should be destabilized at physiological temperatures. The m2 domain is tetrameric and has a similar sedimentation coefficient (Table 2) and CD spectrum (Figure 3D), and therefore similar structure as the NHR2 domain.

We deleted the NHR2 (Figure 3A) and introduced the m7, m4, and m2 mutations into full-length AML1/ETO and assessed their effect on oligomerization by size exclusion chromatography (SEC) (Figure 3E). As shown by others (Minucci et al., 2000; Zhang et al., 2001), full-length AML1/ETO (84 kDa) migrates with an apparent molecular mass greater than 400 kDa. Deletion of the NHR2 domain (Zhang et al., 2001), the m7 mutation, and the m4 mutation shifted the migration of AML1/ETO to that of lower-molecular weight species (Figure 3E). Consistent with the sedimentation velocity and CD measurements, the m4 version of AML1-ETO shows an elution volume intermediate between m7 and AML1-ETO. The m2 mutation did not affect the SEC elution profile (data not shown).

We used retroviruses to introduce AML1/ETO and its mutated derivatives into lineage-depleted (CD5+, B220+, Mac-1−, Gr-1−, Ter119+) mouse bone marrow cells (Lin−BM) in order to assess the importance of oligomerization for AML1/ETO function. The retroviruses also expressed the green fluorescent protein (GFP) from an internal ribosome entry site, allowing us to monitor successfully transduced cells. We cultured the transduced cells for 2 days in the presence of IL-3, IL-6, and SCF, and for 7 additional days with the same cytokines plus G-CSF to induce granulocyte differentiation, and analyzed GFP+ cells for cell surface Gr-1 and Mac-1 expression (Figure 4A). Approximately 20% of Lin− BM cells expressing GFP alone were Gr-1+ Mac-1−, whereas only 3% of cells expressing AML1/ETO differentiated into Gr-1+Mac-1+ cells. As shown by Hug et al. (2002), deletion of the NHR2 domain partially impaired AML1/ETO’s repression of granulocyte differentiation, resulting in a 3-fold increase in the percentage of Gr-1+Mac-1+ cells. The m7 mutation increased the percentage of Gr-1+Mac-1+ cells to the same extent as the NHR2 deletion. The m4 mutation impaired AML1/ETO’s activity, but significantly less so than the NHR2 deletion. The m2 mutation had no effect on AML1/ETO’s ability to repress granulocyte differentiation.

An AML1/ETO protein truncated at aa 542, C-terminal to the NHR2 domain (NHR2x; Figure 3A) partially inhibits the differentiation of Gr-1+Mac-1+ cells, and the m7 mutation in the context of this truncated NHR2x protein (NHR2x m7) completely abrogates AML1/ETO’s activity (Figure 4A). All of the mutated proteins accumulated to similar steady-state levels in retrovirus-infected NIH 3T3 cells despite the fact that the m4 and m7 mutations partially destabilized NHR2’s helical structure (Figure 4B).

Disruption of oligomerization abrogates AML1-ETO’s ability to promote self-renewal

We assessed the importance of tetramer formation for AML1/ETO’s ability to confer increased self-renewal capacity on hematopoietic progenitors in vitro (Higuchi et al., 2002; Hug et al., 2002; Mulloy et al., 2002). Lin− BM cells were infected with retroviruses expressing AML1/ETO and its mutated derivatives and cultured in IL-3, IL-6, and SCF. Cells expressing AML1/ETO could be propagated for at least 3 weeks in culture and yielded primarily immature myeloid lineage cells (Figures 4C and 4D) and a smaller percentage of differentiated macrophages (data not shown). In contrast to the results of Hug et al. (2002), we found that deletion of NHR2 completely abolished AML1/ETO’s ability to sustain clonogenic activity in vitro, as did the m7 and m4 mutations (Figure 4C). Mutation of the mSin3A binding site in NHR2 (m2), however, had no effect on AML1/ETO’s ability to confer enhanced clonogenic potential (Figure 4C). Truncation of AML1/ETO at the C terminus of NHR2 (NHR2x) eliminated AML1/ETO’s clonogenic activity, despite the observation that a similar construct retaining 9 additional amino acids and a terminal four-residue insertion was shown to augment AML1/ETO’s leukemogenic activity in mice (Yan et al., 2004).

Disruption of AML1/ETO oligomerization alters endogenous gene expression

We transduced Lin− BM cells with retroviruses expressing GFP alone, AML1/ETO, or m7 and assessed the expression levels of candidate target genes 2 days posttransduction by real-time...
PCR (Figures 5A–5D). Genes that were analyzed were either previously implicated as Runx1 or AML1/ETO targets (Elagib et al., 2003; Otto et al., 2003; Peterson and Zhang, 2004) or identified based on hybridization to pathway-specific microarrays (data not shown). Other labs have reported that AML1/ETO expression activates some genes and represses others (Shimada et al., 2000, 2002), and we likewise observed several different patterns resulting from AML1/ETO and m7 expression. Some genes previously shown to be activated by Runx1, including Art1, Csf1r (MCSFR), Eli2 (neutrophil elastase [NE]), and Sfp1 (PU.1) (Otto et al., 2003; Peterson and Zhang, 2004) were repressed in Lin− BM cells transduced with AML1/ETO, and the m7 mutation restored their expression to the levels observed in cells expressing GFP alone (Figure 5A). The cell cycle gene E2f2 and the Jak2 kinase gene also fell into this category. A second pattern seen with Fos (c-fos), the Cd53 tetraspanin gene, and Cdkn1a (p21) was activation by AML1/ETO, with blunting of that activation by the m7 mutation (Figure 5C). Finally, expression of either the putative AML1-ETO target gene Bcl2, nor Bmi1 was significantly altered in the presence of either AML1/ETO or m7 (Figure 5D).

AML1/ETO’s interaction with members of the ETO family of proteins, but not with other corepressors, requires oligomerization

AML1/ETO has been shown to interact directly or indirectly with a number of other proteins, including HDAC1, HDAC2, and HDAC3, N-CoR, SMRT, mSin3A, Gfi-1, BCL6, PLZF, PKA RIÎ±, HEB, and other ETO family members (Hiebert et al., 2001; Hug and Lazar, 2004). We examined whether oligomerization through the NHR2 domain affects the direct or indirect association of AML1/ETO-interacting proteins in vivo. FLAG- or HA-tagged versions of interacting proteins were coexpressed with either HA-tagged or untagged versions of AML1/ETO or

Figure 3. Mutations in the NHR2 domain and their effect on tetramer formation

A: Schematic diagram of AML1/ETO. The Runx1 (AML1) portion is green, and ETO sequences are blue. Truncations and internal deletions used in the analyses are shown below. Parentheses in ΔNHR2 delineate the extent of the deletion. RD, Runt domain.

B: Sequence of the NHR2 domain (Leu in bold, the two amino acids required for mSin3A binding [Amann et al., 2001] in green) and the mutations designed to disrupt tetramer formation (m7 and m4) and mSin3A binding (m2). Diagrammed below are the m7 and m4 mutations in NHR2 in a helical wheel representation. Substituted amino acids are red.

C: Sedimentation velocity analysis of NHR2 and the m7 mutant. Absorbance data at 280 nm (NHR2) and 230 nm (m7) were collected at a rotor speed of 50,000 rpm at 20°C in aluminum-filled Epon double sector cells at a scan interval of approximately 260 s.

D: Circular dichroism spectroscopy of NHR2 domains (recorded at 25°C). The reduction in ellipticity at 222 nm for m7 and m4 is indicative of a loss of helical secondary structure.

E: Size exclusion chromatography (SEC) demonstrating that deletion of the NHR2 domain and the m7 and m4 mutations reduces the apparent molecular weight of full-length AML1/ETO. Proteins were produced by in vitro transcription/translation and fractionated by SEC on a Superdex 200 column, and fractions were analyzed by Western blot using an anti-Runt domain antibody. Fraction numbers are indicated above lanes, and molecular weight markers are on the bottom. (−), reticulocyte lysate incubated with the pcDNA vector; L, load.
AML1/ETO function is impaired by mutations that disrupt tetramer formation, but not by mutations in the mSin3A binding site.

**A:** Representative flow of Lin^-^ BM cells infected with MigR1 retroviruses expressing AML1/ETO and mutated derivatives, following 7 days of culture in the presence of IL-3, IL-6, SCF, and G-CSF. Cells in the R1 gate were analyzed for GFP expression, and GFP-positive cells (R2) were examined for Mac-1 and Gr-1 expression. The experiments were performed at least twice with triplicate samples. The average percentages of Gr-1^+^ Mac-1^+^ cells (± standard deviation) in the illustrated experiments were as follows. Experiment #1: MigR1 (GFP alone), 19.3% (1.1); AML1/ETO, 3.6% (0.2); ΔNHR2, 10.8% (1.0); m7, 8.9% (1.0); m4, 7.5% (0.3). The difference between m7 and m4 versus AML1/ETO was significant (p < 0.01). m7 was not significantly different from ΔNHR2 or m4. m4 was significantly different from ΔNHR2 (p < 0.05). Experiment #2: MigR1, 15.6% (2.1); AML1/ETO, 1.5% (0.1); m2, 1.8% (0.1). m2 and AML1/ETO were not significantly different. Experiment #3: MigR1, 22.4% (5.0); AML1/ETO, 3.1% (0.7); NHR2x, 9.2% (1.1); NHR2x m7, 19.1% (1.5). The difference between MigR1 and NHR2x m7 was not significant.

**B:** Western blot probed with an antibody to the Runt domain, demonstrating expression of AML1/ETO and its mutated derivatives in MigR1-transduced NIH3T3 cells.

**C:** Serial replating of bone marrow cells. Graphs represent the average number of colonies from each round of replating in the presence of IL-3, IL-6, and SCF. Plating number 1 represents colony numbers per 10^3^ cells plated, and plating numbers 2 and 3 are from 10^4^ plated cells. Numbers are averaged from two experiments, each containing triplicate samples. The average numbers of cells (± SD) at week three of replating are as follows: AML1/ETO, 117 (9.5); m2, 141 (26). The other cultures had no cells at 3 weeks.

**D:** Cytospin preparation of cells expressing AML1/ETO from the third replating. The majority of cells (~85%) were immature myelocytes or monocytes, and the remainder were primarily macrophages (data not shown). Only a few mature granulocytes were visible (data not shown).
its mutated derivatives in Cos7 cells, and their interactions were monitored by coimmunoprecipitation (Figure 5E). The interaction of AML1/ETO with endogenous mSin3A was also examined. We found that deletion of the NHR2 domain weakened full-length AML1/ETO’s interactions with mSin3A, to some extent with HDAC2, and dramatically affected interactions with all of the ETO family proteins (ETO, MTG16, and MTGR1). However, when AML1/ETO oligomerization was specifically disrupted by the m7 mutation, only the interactions with ETO, MTG16, and MTGR1 were markedly affected. The m7 mutation in the context of an AML1/ETO protein truncated at the

Figure 5. Oligomerization influences AML1/ETO’s regulation of gene expression, but not its association with interacting proteins, with the exception of ETO, MTG16, and MTGR1

A–D: Real-time PCR of putative Runx1 or AML1/ETO target genes in Lin− BM cells to assess the role of oligomerization in gene expression. Gene expression data are presented as the fold change between control EGFP-transduced cells (normalized to a value of 1, dashed line) and AML1/ETO or m7-transduced cells and represent averages from three independent experiments. Error bars indicate 95% confidence intervals. A: Genes whose expression is repressed by the presence of AML1/ETO and m7 restores expression to that of EGFP alone. B: Genes activated by m7 but not AML1/ETO. C: Genes activated by AML1/ETO and less so by m7. D: Examples of genes whose expression is not affected by either AML1/ETO or m7. The 2-fold reduction in EGFP expression, which was equivalent for AML1/ETO and m7, was most likely due to its translation from bicistronic (AML1/ETO and m7) versus monocistronic (EGFP alone) mRNAs.

E: Cos7 cells were cotransfected with HA-tagged (AML1/ETO [A/E] and m7) or nontagged (ΔNHR2, m4, NHR3x, NHR2x m7, NHR2x m2) AML1/ETO proteins and FLAG-tagged HDAC1, HDAC2, HDAC3, N-CoR, SMRT, PKA RIIα, or HEB. For experiments involving ETO homologs, cells were transfected with HATagged ETO, MTG16, and MTGR1 and untagged AML1/ETO. Top panels: cell lysates immunoprecipitated (IP) with anti-FLAG or anti-HA antibody and blotted with the Runt domain (RD) antibody. Middle panels: 1% of input lysate, blotted with anti-RD to detect AML1/ETO proteins. Bottom panels: membranes from the top panel were reprobed with anti-FLAG, anti-HA, or anti-mSin3A antibodies. The percentages of input AML1/ETO and m7 proteins in the immunoprecipitates were determined by titration analyses and were as follows: HDAC1, 1.3 and 1.4 [AML1/ETO and m7, respectively]; HDAC2, 0.7 and 0.7; HDAC3, 1.3 and 1.3; mSin3A, 0.6 and 0.3; N-CoR, 0.8 and 0.7; SMRT, 0.9 and 0.9; PKA RIIα, 1 and 1; PLZF, 0.8 and 0.7; HEB, 1 and 0.4. ETO homolog immunoprecipitates were not quantified.
Oligomerization is important for the oncogenic potential of a number of leukemia-associated chimeric proteins. This phenomenon was originally documented for the BCR/ABL kinase (McWhirter et al., 1993), and in transcription factors was first observed for fusion proteins involving the retinoic acid receptor \( \alpha \) (RAR\( \alpha \)) (Lin and Evans, 2000; Minucci et al., 2000). Since these initial observations, an in vivo role for oligomerization has been documented for chimeric transcription factors containing RUNX1 (AML1/ETO, AML1/MTG16, TEL/AML1), CBFB\( \beta \) (CBFB\( \beta \)/SMMHC), RAR\( \alpha \) (NPM/RAR\( \alpha \), PML/RAR\( \alpha \), PLZF/RAR\( \alpha \), NuMA/RAR\( \alpha \), Stat5b/RAR\( \alpha \)), and MLL (MLL/GAS7, MLL/AF1p, MLL/GEPRIN), which together account for a high percentage of leukemia cases (Eguchi et al., 2004; So and Cleary, 2004). The paradigm for the effects of aberrant oligomerization on transcription factor function is the fusion of RAR\( \alpha \) to PML and PLZF in acute promyelocytic leukemia, which affects RAR\( \alpha \)’s in vivo function and its binding to the corepressors N-CoR and SMRT (Melnick and Licht, 1999).

The NHR2 domain of ETO is also an oligomerization domain, and it is important for AML1/ETO’s activity. Our structural results show that NHR2 forms a homotetrameric \( \alpha \)-helical bundle with a left-handed supercoil resembling the structure found in the heterotrimeric SNARE complexes (Sutton et al., 1998). We used the NHR2 structure to design mutations that disrupted oligomerization but conferred favorable solubility properties to the domain and preserved a significant proportion of its secondary structure. Unlike a deletion, the mutations we employed do not create artificial restrictions on the relative positioning of other domains and may retain some of the protein-protein interactions mediated by the NHR2 domain. Mutations designed to disrupt oligomerization were introduced into full-length AML1/ETO to determine which biological and biochemical properties previously ascribed to oligomerization through deletion studies can actually be attributed to oligomerization per se. Our results confirm the importance of oligomerization for the activity of AML1/ETO in hematopoietic differentiation, proliferation, and gene expression assays (Hug et al., 2002; Shimada et al., 2000; Zhang et al., 2001). Disrupting oligomerization partially impaired AML1/ETO’s inhibition of granulocyte differentiation, completely ablated its ability to enhance the clonogenic capacity of primary hematopoietic progenitors, and affected its ability to influence the expression of endogenous genes in primary bone marrow stem and progenitor cells.

We also assessed the outcome of disrupting oligomerization on previously described protein-protein interactions. Eliminating oligomerization impaired AML1/ETO’s ability to interact, presumably through tetramerization, with ETO and its homologs MTG16 and MTGR1, as shown previously with an NHR2 deletion (Kitabayashi et al., 1998). MTG16 is found in complexes in hematopoietic cells containing other transcription factors such as SCL, E12/E47, E2.2, Lbd-1, SSDP2, GATA-1, Gfi-1, and LMO2 (Schuh et al., 2005). If AML1/ETO’s activity in leukemia is exerted, in part, through complexes containing MTG16, disrupting its ability to form mixed tetramers with MTG16 could potentially interfere with its activity, a hypothesis previously advanced by others (Amann et al., 2001).

Oligomerization was not required for HDAC1, HDAC2, HDAC3, N-CoR, SMRT, PKA RII\( \alpha \), PLZF, mSin3A, or HEB to coimmunoprecipitate AML1/ETO from cell extracts. Hiebert and colleagues reported that an NHR2 deletion reduced, but did not eliminate AML1/ETO (or ETO) binding to mSin3A, N-CoR, or the HDACs (Amann et al., 2001; Lutterbach et al., 1998a, 1998b). With a more precise oligomerization mutant, we did not see a reduction in AML1/ETO binding to these proteins, except for mSin3A and N-CoR in the context of a truncated AML1/ETO protein. This is not to say that interaction with corepressors is not important for AML1/ETO’s activity, only that oligomerization does not appear to be contributing to AML1/ETO’s activity by increasing its affinity for these proteins. Oligomerization could, however, increase the number of corepressors recruited to the DNA without altering affinity. A tetramer of AML1/ETO (or a mixed tetramer consisting of AML1/ETO and other ETO homologs) would have the potential to recruit 4-fold more repressor molecules to the DNA than monomeric AML1/ETO, thereby increasing the local concentration of corepressors on target genes. Another possible mechanism by which oligomerization might contribute to AML1/ETO’s activity is by augmenting DNA binding, particularly to enhancers and promoters that contain multiple Runx binding sites, allowing it to effectively compete with the normal core binding factors for occupancy. A model for this is the enhanced occupancy of Hox a9 by dimerized forms of MLL (Martin et al., 2003). Establishing cell lines expressing equivalent amounts of AML1/ETO and m7 will be necessary to test these various models by chromatin immunoprecipitation assays.

Disruption of oligomerization in AML1-ETO resulted in clear changes in the expression of a number of endogenous genes that have been implicated in the development of leukemia or myeloproliferative disorders. Some genes were positively and others negatively regulated by AML1/ETO, as has been observed by others (Shimada et al., 2000, 2002). Some genes may be direct and others indirect AML1/ETO targets, which could account for these seemingly opposite activities. The most intriguing differentially regulated target is the \( Sfpi1 \) gene that encodes PU.1, a transcription factor critical for normal myeloid development (McKercher et al., 1996; Scott et al., 1994). Reductions in PU.1 dosage were recently shown to cause an accumulation of c-kit\( ^+ \) hematopoietic precursors in bone marrow, and predisposed mice to acute myeloid leukemia and T cell lymphoma (Rosenbauer et al., 2004, 2006). AML1/ETO is also thought to inhibit the transcriptional activity of the PU.1 protein, and expression of PU.1 in t(8;21) containing Kasumi-1 cells promoted their differentiation (Vangala et al., 2003). AML1/ETO’s ability to reduce both PU.1 dosage and function could be critical for establishing the preleukemic state. Since disrupting tetramer formation ameliorates some of AML1/ETO’s deleterious in vivo effects, an obvious question is whether the tetramer interface is a good target for small molecules or peptides that inhibit oligomerization. We predict that the extremely stable nature of the tetramer, reflected in both its thermal stability and the extensive mutagenesis necessary to disrupt its formation, would make developing small molecule inhibitors of this interaction quite challenging. A more promising approach might be to disrupt the interaction between AML1/ETO and the coregulatory molecules important for its activity.
Identifying which of these molecules is critical for oligomeric AML1/ETO’s oncogenic activity is an important area of investigation.

Experimental procedures

Cloning, expression, and protein purification
Cloning, expression, and protein purification were carried out according to standard procedures (details provided in Supplemental Data). FLAG-tagged mammalian expression constructs for HDAC1–HDAC3, N-CoR, and SMRT, and HA-tagged ETO, MTG16, and MTRG1, were generous gifts from Scott Hiebert. N-terminal FLAG tags were added to PKA RII, HEB, and PLZF using PCR, and the resulting constructs were cloned into pcDNA3.1(+).

Crystallization
The Se-Met labeled NHR2 domain (AML1/ETO residues 480–551) was dia- lyzed into 25 mM Tris (pH 7.5), 150 mM NaCl, and 1 mM DTT and concentrated to 10 mg/ml. The protein was mixed 1:1 with well solution (100 mM NaCit [pH 5.6], 100 mM MgCl2, 40% MPD [2-methyl-2,4-pentanediol]) and crystallized by sitting drop vapor diffusion. Crystals reached their full size in 3–4 days. Crystals were removed from the mother liquor and frozen in liquid nitrogen prior to data collection.

Data collection and structure determination
Data were collected from a Se-Met substituted crystal at the beamline X9B at the National Synchrotron Light Source, Brookhaven National Laboratory. Three data sets were collected (0.9791 Å [peak], 0.97946 Å [inlecnluc], and 0.96422 Å [remote]), reduced, and scaled with HKL2000 (Otwinowski and Minor, 1997). Details of data collection are summarized in Table 1. The structure was solved with the MAD method. A Se-Met substructure was found from peak data with SHELXD (Schneider and Sheldrick, 2002). An initial map was obtained from a single wavelength with SHARP. Due to low solvent content, a better map was obtained with the use of three wave- lengths with SHARP (de La Fortelle and Bricogne, 1997) and subsequently SOLVE/RESOLVE (Terwilliger, 2002). We extended the initial model built by RESOLVE by manual building with O (Jones et al., 1991). We followed the refinement with CNS (Brunger et al., 1998) using the simulated annealing pro- tocol (Brunger et al., 1990) with manual rebuilding taking into account the 2- fold noncrystallographic symmetry (NCS). We used a subset of reflections (5%) for Rfree calculations (Brunger, 1992). Final steps of the refinement were carried out with REFMAC (Murshudov et al., 1997) without NCS re- straints. The refinement statistics are summarized in Table 1. Data collected at 0.97915 Å (peak) were used during refinement. The PROCHECK and MOLPROBITY programs were used for structure validation. The final model contains two protein chains and 59 water molecules.

Sedimentation velocity measurements
Samples were equilibrated by dialysis into a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, and either 1 mM DTT (NHR2 and m4) or 0.5 mM TCEP (m7) (pH 7.5). Extinction coefficients, molecular masses, and partial specific volumes were determined using the SEDNTERP program (Laue et al., 1992). Sedimentation velocity analysis ultracentrifugation was performed with a Beckman Coulter XL-I instrument in aluminum-filled double sector cells at 20°C and 50,000 RPM using a scan interval of ~260 s. The sedimentation velocity concentration profiles were fit to finite element solutions of the Lamm equation using a model of a continuous distribution of discrete, noninteract- ing species with the program SEDFIT (Schuck, 2000). The molecular mass and sedimentation coefficient of the predominant species were determined using a hybrid discrete-continuous model where the major peak was fit as a single discrete species and the material at higher and lower S was accounted for by two continuous distributions. Data obtained at multiple concentrations were globally fit using SEPHAT (Schuck, 2003). Hydrody- namic bead modeling was performed using the crystallographic coordinates for the NHR2 domain using the HYDROPRO package (Garcia De La Torre et al., 2000) with the default effective atomic radii of 3.2 Å.

Western blots
Western blots were performed using standard protocols (details provided in Supplemental Data).

Coimmunoprecipitations
Coimmunoprecipitations were performed using standard protocols (details provided in Supplemental Data).

Retroviral transduction
Phoenix cells were plated in 10 cm dishes in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (FCS, P/S), and transfected after 12–16 hr with 20 μg of MigR1 plasmids by calcium phosphate in the presence of chloroquine, and viral supernatants were harvested at 48 hr. Cells were subjected to 2 hr of spinoculation at 2350 RPM, 25°C in the presence of poly- brene, and then cultured for 48 hr with viral supernatant. We determined transduction efficiencies by comparing the percentage of GFP-positive NIH 3T3 cells with a FACScan flow cytometer (BD Bioscience). Expression of AML1/ETO proteins was assessed by Western blots of nuclear extracts prepared from transduced NIH 3T3 cells.

Granulocyte differentiation
Bone marrow (BM) was harvested from male C57BL/6 x 129Sv (F1) mice and cultured for 2 days in DMEM plus FCS P/S, 10 ng/ml IL-3, 20 ng/ml IL-6, 100 ng/ml SCF (R&D Systems). Cells expressing lineage markers were depleted using a cocktail of antibodies conjugated to magnetic beads (Miltenyi). Lineage-negative (Lin−) cells were infected with retroviruses expressing GFP alone, or GFP in combination with AML1/ETO proteins, and cultured for 7 days in DMEM FCS P/S, 10 ng/ml IL-3, 20 ng/ml IL-6, 100 ng/ml SCF, 60 ng/ml G-CSF (R&D Systems). Cells were harvested and stained for surface expression of Mac1 and Gr-1 (Ly-6c) with antibodies conjugated to PE or APC, respectively (BD Bioscience) using a FACScalibur flow cytometer (BD Bioscience), and the data were analyzed using FlowJo software.

Serial replating
Immediately following retroviral transduction, 103 cells were plated in M3434 complete methylcellulose media (Stem Cell Technologies) and cultured for 7 days. After colonies were enumerated, the cultures were diluted and resuspended, and 104 cells were replated in M3434 media for an additional 7 days, at which point the process was iteratively repeated as indicated. Cyto- spins of cells resulting from replatings were generated using a Shandon cyto- spin and stained with Wright Giemsa.

Real-time PCR
Lin− BM cells were isolated and transduced with MigR1 expressing GFP, AML1/ETO, or m7 as described above. After 48 hr of culture in IL-3, IL-6, and SCF, cells were isolated using a FACSAria Cell Sorting System (BD Bio- science) based on GFP expression and propidium iodide exclusion. Indepen- dent FACS analysis of GFP-positive cells stained with a panel of anti-lineage marker antibodies (Miltenyi, Earhardt, CA) revealed a consistent 95%–98% lineage negativity in all samples (data not shown). Total RNA was extracted from sorted cells with RNeasy spin columns and was Dnase treated (Qiagen Inc, Valencia, CA), RNA integrity was verified by agarose gel electrophoresis and quantified with NanoDrop1000 (Nano-Drop, Wilmington, DE). Total RNA was subjected to two rounds of T7-based linear amplification using the RiboAmp RNA Amplification kit (Artcuric Inc, Mountain View, CA). Amplified RNA (aRNA) from each sample was used to generate three independent preparations of double-stranded cDNA (dsDNA) for quantitative analysis of gene expression. An aliquot of this material was subjected to a third round of amplification in the presence of biotinylated dUTP using the Bioarray High Yield RNA Transcript Labeling Kit (ENZO, Farmingdale, NY). Biotinylated RNA was used to interrogate pathway-specific oligonucleotide arrays (He- matopoietic Stem Cell and Hematopoiesis, and Cell Cycle; SuperArray, Fred- erick, MD) as a primary screen to identify differentially expressed genes. Real-time quantitative PCR of candidate AML1/ETO target genes was then performed in triplicate for each sample using SYBR-Green (Applied Biosys- tems, Foster City, CA) on Applied Biosytem’s 7500 Real-Time PCR Sys- tem. Linear regression analysis of unknown samples was performed using the relative standard curve method.

Supplemental data
The Supplemental Data include Supplemental experimental procedures, one supplemental figure, and one supplemental table and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/4/ 249/DC1.
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Accession numbers

The coordinates and structure factors are deposited in the Protein Data Bank with accession code 1WQ6.