別紙３

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

がん抑制因子 p53 を標的とした新しいがん治療法の開発に関する研究

分担研究者　江成　政人　　国立がんセンター研究所　生物学部　室長

研究要旨　　がん抑制タンパク質 p53 の活性化を阻害する Negative regulators（COP1、Pirh2、ARF-BP1、Synoviolin、Mdm2、Mdmx）の様々な欠失変異体をグルタチオン-S-トランスフェラーゼ（GST）との融合タンパク質として作製し、p53 との結合実験および p53 との結合量を定量する実験系を確立した。そして、190 種類の低分子化合物をスクリーニングしたところ、p53 と Mdmx との相互作用を阻害する化合物を同定した。その低分子化合物の薬効試験において、その低分子化合物をがん細胞に添加すると、p53 の活性化を伴い、p53 依存的にがん細胞が死滅させることがわかった。また、様々ながん細胞を用いて、その低分子化合物の細胞増殖抑制活性を調べたところ、野生型 p53 を持つがん細胞は、変異型 p53 を持つがん細胞に比べ、その低分子化合物による細胞増殖抑制効果が高い傾向にあった。ここでの発見は、将来、副作用の少ない抗癌剤の開発に繋がるものと期待できる。

A．研究目的

がん抑制遺伝子 p53 は約半数のヒト癌で変異が認められており、がん発生の抑制に重要な役割を担っている。しかしながら、残りの半数の癌では p53 が野生型であり、多くの場合、p53 の Negative regulators が過剰発現していることが報告されている。がん抑制因子 p53 の Negative regulators である E3 ユビキチンリガーゼ Mdm2 と Mdm2 のような E3 ユビキチンリガーゼ活性を持たないが Mdm2 の機能を高める活性を持つ Mdmx が p53 タンパク質と結合し、p53 の分解を促進することで、p53 のがん抑制機能を阻害している。Mdm2 を標的とした抗癌剤の開発が進行している一方で、近年、Mdm2-Mdmx 系と同様、p53 に結合し、p53 の分解を促進する E3 ユビキチンリガーゼが 4 種類同定された。Mdm2-Mdmx 系に加え、これらのユビキチンリガーゼは野生型 p53 を持つヒト癌で過剰発現している場合が多く、野生型 p53 を持つ癌細胞に対する新規抗癌剤開発の分子標的となることが考えられる。すなわち、p53 とそれら Negative regulators との相互作用を阻害する低分子化合物が開発されれば有用な抗癌剤になると考えられる。そこで、p53 とそれら Negative regulators との相互作用をモニターできる簡便な方法を確立、そして、その相互作用を阻害する低分子化合物を探索し、p53 経路を標的とした新たな抗癌剤の開発を目指す。

B．研究方法

p53 とその Negative regulators の相互作用を阻害する低分子化合物を探索するために、以下の実験手順でその定量法（改良型 ELISA 法）を確立した。まず、大腸菌大量発現系を用いて、6 種類の Negative regulators の全長および欠失変異体をグルタチオン-S-トランスフェラーゼ（GST）との融合タンパク質として調製し、p53 との結合領域の同定を試みた。同定した p53 結合部位を含む GST 融合タンパク質をグルタチオンセファロースで精製し、0.05%Tween20 を含むリン酸緩衝液で透析した後、グルタチンコートしたプレートに固相化した。そして、大腸菌大量発現系を用いて、FLAG エピトープ標識した p53 を調製し、固相化した GST 融合タンパク質と反応させた。反応後、horseradish peroxidase 標識した抗FLAG 抗体および発色基質を用いて、p53-ユビキチンリガーゼの結合量を定量した。また、低分子化合物のスクリーニングには文部科学省がん特定領域・化学療法基盤情報支援班より分与された 190 種類からなる低分子化合物ライブラリーを用いた。また、低分子化合物の薬効試験には、野生型 p53 を持つがん細胞（MCF7 細胞、A427 細胞、HT1080 細胞）および変異型 p53 を持つがん細胞（A431 細胞、MDA-MB-468 細胞）を用いて検討し、細胞増殖抑制活性はクリスタル・バイオレット法を用いて定量した。

C．研究結果

p53 とその Negative regulators の相互作用を阻害する低分子化合物を探索するために、まず様々な Negative regulators の欠失変異体を作製、そして、p53 への結合部位を同定し、p53 結合部位を含む GST 融合タンパク質をグルタチンコートしたプレートに固相化した。そして、FLAG エピトープ標識した p53 と固相化した GST 融合タンパク質と

を反応させた。その方法を用いて p53 と Negative regulators との結合量を定量したところ、加えた p53 の容量に依存してその結合量が増加することがわかった。この系を用いて、p53 と Negative regulators 間相互作用を阻害する低分子化合物のスクリーニングを行ったところ、p53 と Mdmx との相互作用を阻害する低分子化合物が得られた。実際、野生型 p53 遺伝子を持つがん細胞に得られた低分子化合物を添加すると、p53 の活性化を伴い、その低分子化合物の用量依存的にがん細胞を死滅させることがわかった。また、様々ながん細胞を用いて、その低分子化合物の細胞増殖抑制活性を調べたところ、野生型 p53 を持つがん細胞は、変異型 p53 を持つがん細胞に比べ、その低分子化合物による細胞増殖抑制効果が高い傾向にあった。

D．考察

本研究において、p53 を負に調節するタンパク質と p53 との結合を簡便にモニターすることのできるアッセイ系を確立し、p53 と Mdmx との相互作用を阻害する低分子化合物が得られた。そして、その低分子化合物は p53 を発現しているがん細胞特異的に細胞増殖抑制効果を示すことから、今後、この発見によって、Mdmx の過剰発現が原因で p53 の不活化が起こり、それに伴って悪性化しているがんの新たな治療法の開発に発展していくことが期待される。特にヒト網膜芽腫や神経芽腫の多くで Mdmx の過剰発現が認められることから、これらのがんに対する治療戦略に非常に有用であり、将来的に p53 経路を標的とした副作用の少ない抗癌剤の開発へと進展していくことが期待される。

E．結論

p53 の Negative regulators である Mdmx と p53 との結合を阻害する低分子化合物を同定した。その低分子化合物は細胞増殖抑制活性を有し、p53 依存的な経路を活性化することでがん細胞の増殖を阻害していることがわかった。

G．研究発表

1．論文発表

Masami Kodama, Chihiro Otsubo, Toru Hirota, Jun Yokota, Masato Enari*, and Yoichi Taya: Requirement of ATM for Rapid p53 Phosphorylation at Ser46 without Ser/Thr-Gln Sequences. Mol. Cell. Biol., 30, 1620-1633, 2010. *Corresponding author

Hirokazu Ohata, Nobuyuki Ota, Mikako Shirouzu, Shigeyuki Yokoyama, Jun Yokota, Yoichi Taya and Masato Enari*: Identification of a function-specific mutation of clathrin heavy chain (CHC) required for p53 transactivation. J. Mol. Biol., 394, 460-471, 2009. *Corresponding author

2．学会発表

チロシンキナーゼ ALK による p53 を介した転写の抑制機構：江成政人、吉田祐輔、横田淳、田矢洋一　第 68 回日本癌学会学術総会、横浜、2009 年 10 月 2 日

Nuclear mitotic apparatus (NuMA)タンパク質による p53 を介した転写選択制御機構：江成政人、大畑広和、田矢洋一　第 82 回日本生化学会大会、神戸、2009 年 10 月 23 日

p53 による細胞運動制御機構の解析：大坪千裕、大友亮、横田淳、江成政人　第 32 回日本分子生物学会年会、横浜、2009 年 12 月 9 日

H．知的所有権の出願・登録状況（予定を含む。）

1．特許取得

発明の名称：「ｐ５３－ｍｄｍｘ相互作用を阻害する低分子抗がん剤」
発明者：江成政人、上里新一
出願日：2010 年 2 月 26 日
出願番号：特願 2010-43548
出願人：財団法人ヒューマンサイエンス振興財団、
　　学校法人　関西大学

2．実用新案登録

なし

3．その他

なし

別紙4

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Aikawa Y, Katsumoto K, Zhang P, Shima H, Shino M, Terui K, Ito E, Ohno H, Stanley ER, Singh H, Tenen DG, Kitabayashi I.	PU.1-mediated upregulation of M-CSFR is critical for leukemia stem cell potential induced by MOZ-TIF2.	Nat Med	16	580-585	2010
Yokoyama A, Lin M, Naresh A, Kitabayashi I, Cleary ML.	A Higher-Order Complex Containing AF4 and ENL Family Proteins with P-TEFb Facilitates Oncogenic and Physiologic MLL-Dependent Transcription.	Cancer Cell	17	198-212	2010
Yamagata K, Kitabayashi I.	Sirt1 physically interacts with Tip60 and negatively regulates Tip60-mediated acetylation of H2AX.	Biochem Biophys Res Commun	390	1355-1360	2009
Kitoh A, Ono M, Naoe Y, Ohkura N, Yamaguchi T, Yaguchi H, Kitabayashi I, Tsukada T, Nomura T, Miyachi Y, Taniuchi I, Sakaguchi S.	Indispensable role of the Runx1-Cbfbeta transcription complex for in vivo-suppressive function of FoxP3+ regulatory T cells.	Immunity	31	609-620	2009
Hibiya K, Katsumoto T, Kondo T, Kitabayashi I, Kudo A.	Brpf1, a subunit of the MOZ histone acetyl transferase complex, maintains expression of anterior and posterior Hox genes for proper patterning of craniofacial and caudal skeletons.	Dev Biol..	329	176-190	2009
Rokudai S, Aikawa Y, Tagata Y, Tsuchida N, Taya Y, Kitabayashi I.	MOZ interacts with p53 to induce p21 expression and cell-cycle arrest.	J Biol Chem.	284	237-244	2009
Miyazawa,Y, Uekita,T, Hiraoka, N, Fujii, S, Kosuge, T, Kanai, Y, Nojima, Y, Sakai, R	CDCP1, a prognostic factor for human pancreatic cancers, promotes cell migration and extracellular matrix degradation.	Cancer.Res..		In press	2010
Yamaguchi, H, Yoshida, S, Muroi E, Kawamura, M, Kouchi, Z, Nakamura, Y, Sakai, R, Fukami, K	Phosphatidylinositol 4,5-bisphosphate and PIP5-kinase Iα are required for invadopodia formation in human breast cancer cells.	Cancer Sci.		In press	2010
Tanaka, M, Kamata, R, Yanagihara, K, Sakai, R	Suppression of gastric cancer dissemination by ephrin-B1-derived peptide.	Cancer Sci.	101	87-93	2010
Futami, H, Sakai, R	RET protein promotes non-adherent growth of NB-39-nu neuroblastoma cell line.	Cancer Sci.	100	1034-9	2009

Azuma, K, Urano, T, Horie-Inoue, K, Hayashi, S, Sakai, R, Ouchi, Y, Inoue, S	RUNX1 permits E4orf6-directed nuclear localization of the adenovirus E1B-55K protein and associates with centers of viral DNA and RNA synthesis.	Cancer.Res.	69	2935-40	2009
Ikeda, J, Oda, T, Inoue, M, Uekita, T, Sakai, R, Okumura, M, Aozasa, K, Morii, E	Acetylation of PML is involved in histone deacetylase Inhibitor-mediated apoptosis.	Cancer Sci.	100	429-33	2009
Miyake, I, Ohira, M, Nakagawara, A, Sakai, R	Phosphorylation of Runx1 at Ser249, Ser266, and Ser276 is dispensable for bonemarrow hematopoiesis and thymocyte differentiation.	Oncogene	28	662-73	2009
Tanaka, M, Sasaki, K, Kamata, R, Hoshino, Y, Yanagihara, K, Sakai, R	Phosphorylation of PML is essential for activation of C/EBPe and PU.1 to accelerate granulocytic differentiation.	Mol. Cell. Biol	29	402-13	2009
Cui H, Kamino H, Nakamura Y, Kitamura N, Miyamoto T, Shinogi D, Goda O, Arakawa H, Futamura M.	Regulation of apoptosis by p53-inducible transmembrane protein containing sushi domain.	Oncol Rep		In press	2010
Ohnishi S, Futamura M, Kamino H, Nakamura Y, Kitamura N, Miyamoto Y, Miyamoto T, Shinogi D, Goda O, Arakawa H.	Identification of NEEP21, encoding neuron-enriched endosomal protein of 21 kDa, as a transcriptional target of tumor suppressor p53.	Int J Oncol		In press	2010
Miyamoto Y, Futamura M, Kitamura N, Nakamura Y, Baba H, Arakawa H.	Identification of UNC5A as a novel transcriptional target of tumor suppressor p53 and a regulator of apoptosis.	Int J Oncol	36	1253-1260	2010
Tanikawa C, Furukawa Y, Yoshida N, Arakawa H, Nakamura Y, Matsuda K.	XEDAR as a putative colorectal tumor suppressor that mediates p53-regulated anoikis pathway.	Oncogene	28	3081-3092	2009
Maida Y, Yasukawa M, Furuuchi M, Lassmann T, Possemato R, Okamoto N, Kasim V, Hayashizaki Y, Hahn WC, Masutomi K	An RNA dependent RNA polymerase formed by TERT and the RMRP RNA	Nature	461	230-235	2009
Masami Kodama, Chihiro Otsubo, Toru Hirota, Jun Yokota, Masato Enari*, and Yoichi Taya *Corresponding author	: Requirement of ATM for Rapid p53 Phosphorylation at Ser46 without Ser/Thr-Gln Sequences.	Mol. Cell. Biol.	30	1620-1633	2010
Hirokazu Ohata, Nobuyuki Ota, Mikako Shirouzu, Shigeyuki Yokoyama, Jun Yokota, Yoichi Taya and Masato Enari* *Corresponding author	Identification of a function-specific mutation of clathrin heavy chain (CHC) required for p53 transactivation.	J. Mol. Biol.	394	460-471	2009

朝日新聞
2010年5月11日朝刊

急性骨髄性白血病 幹細胞を狙い治療

国立がん研究センター

国立がん研究センターなど日米の研究チームが、急性骨髄性白血病の発症にかかわる細胞に特徴的な受容体を見つけた。この受容体を目印にして細胞を取り除くことで発症を抑えることに、マウスの実験で成功した。再発を防ぐ治療法の開発につながる可能性がある。

同センターの北林一生・分子腫瘍学部長らは、ヒトの白血病の原因遺伝子を組み込んだマウスを使い、白血病が発症する仕組みを調べた。

その結果、M－CSFというたんぱく質の受容体がある細胞を遺伝子操作で取り除くと、白血病の原因遺伝子があっても発症しないことを発見。この受容体を持つ細胞の中に、白血病を発症させる「がん幹細胞」が含まれていると結論づけた。

さらに、この受容体の働きを抑える薬剤を白血病のマウスに投与したところ、発症が抑えられ、生存日数も2倍ほどに延びたという。

米医学誌ネイチャー・メディシン（電子版）に発表した。（福島慎吾）

日本経済新聞
2009年9月28日朝刊

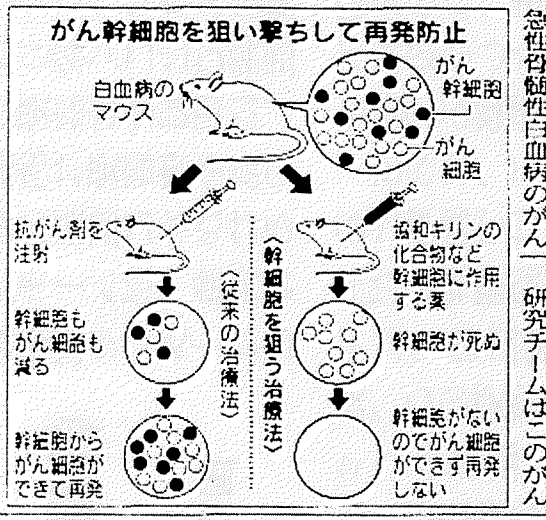
がん、幹細胞狙い再発防ぐ

「元凶」の研究相次ぎ成果

「がん幹細胞」と呼ぶ細胞の研究で成果が相次いでいる。国立がんセンターと協和発酵キリンは白血病のがん幹細胞の構造を解明し、マウスの実験ではこの細胞を特殊な化合物で死滅させることに成功した。東京医科歯科大学も胃がんのがん幹細胞を見つけた。がん幹細胞はがんを引き起こす元凶と考えられ、研究成果はいずれもがんの根治薬の開発につながりそうだ。

がんセンターと協和発酵キリン 「白血病」で死滅

東京医科歯科大 胃がんでも発見



国立がんセンター研究所の北林一生部長らが発見し構造を解明したのは急性骨髄性白血病のがん幹細胞。急性骨髄性白血病は治療しても再発する場合がある。

研究チームはこのがん幹細胞を白血病のマウスから取り除き、がん細胞も死滅させることに成功した。がん幹細胞を標的とした治療で一定の効果が確認できたのは初めてという。

このがん幹細胞は表面に「M-CSFR」というたんぱく質がある。正常なマウスでも、このたんぱく質ができた細胞が体内で増えたマウスはすべて白血病になった。

研究チームは協和発酵キリンが開発したM-CSFRの働きを妨げる化合物を白血病のマウスに注射した。がん幹細胞が死滅した結果、がん細胞もなくなった。治療に使われている抗がん剤を与えたマウスに比べ生存期間は3倍に延びた。

国立がんセンターの北林部長は「がん幹細胞をたたくことで再発を防ぎ根治につながる」とみている。今後、化合物を改良して治療薬の実現を目指す。

東京医科歯科大学の深町博史講師らは、胃がんにもがん幹細胞があることを確認した。患者から採取した腫瘍（しゅよう）の一部を免疫機能のほとんどないマウスに移植。マウスにできた腫瘍を取り出し分析した。構造の解明を急ぐ。

横浜市立大学の谷口英樹教授らは大腸がんのがん幹細胞と考えられる細胞の特徴の研究で成果を上げた。すでに分かっている細胞の目印（たんぱく質）のほかに、正常な腸の上皮細胞の表面にあるたんぱく質も多いことを突き止めた。研究チームは一正常な幹細胞からがん幹細胞が生まれる一とみており、新しい治療法につなげるため、がん幹細胞の働きを抑える方法の検討を始めた。

成果はいずれも10月1日から横浜市で始まる日本癌学会学術総会で発表する。

▼がん幹細胞　遺伝子の変異によって生じるがんの最初の細胞。自己複製して無限に増える。がん細胞はがん幹細胞が分裂してできたもので、何度か分裂すると死ぬがが、がん幹細胞はなかなか死なず、がん再発の要因とみられる。

科学

がん幹細胞は1997年に白血病で見つかり、乳がんや悪性脳腫瘍にも存在することが分かった。現在は肝臓がんや胃がんなどその他のがんでも研究が進む。がん幹細胞を標的にできれば、根治療が実現すると考えられている。

nature medicine

PU.1-mediated upregulation of *CSF1R* is crucial for leukemia stem cell potential induced by MOZ-TIF2

Yukiko Aikawa[1], Takuo Katsumoto[1], Pu Zhang[2], Haruko Shima[1], Mika Shino[1], Kiminori Terui[3], Etsuro Ito[3], Hiroaki Ohno[4], E Richard Stanley[5], Harinder Singh[6], Daniel G Tenen[2,7] & Issay Kitabayashi[1]

Leukemias and other cancers possess self-renewing stem cells that help to maintain the cancer[1,2]. Cancer stem cell eradication is thought to be crucial for successful anticancer therapy. Using an acute myeloid leukemia (AML) model induced by the leukemia-associated monocytic leukemia zinc finger (MOZ)-TIF2 fusion protein, we show here that AML can be cured by the ablation of leukemia stem cells. The MOZ fusion proteins MOZ-TIF2 and MOZ-CBP interacted with the transcription factor PU.1 to stimulate the expression of macrophage colony–stimulating factor receptor (CSF1R, also known as M-CSFR, c-FMS or CD115). Studies using PU.1-deficient mice showed that PU.1 is essential for the ability of MOZ-TIF2 to establish and maintain AML stem cells. Cells expressing high amounts of CSF1R (CSF1R^{high} cells), but not those expressing low amounts of CSF1R (CSF1R^{low} cells), showed potent leukemia-initiating activity. Using transgenic mice expressing a drug-inducible suicide gene controlled by the *CSF1R* promoter, we cured AML by ablation of CSF1R^{high} cells. Moreover, induction of AML was suppressed in CSF1R-deficient mice and CSF1R inhibitors slowed the progression of MOZ-TIF2–induced leukemia. Thus, in this subtype of AML, leukemia stem cells are contained within the CSF1R^{high} cell population, and we suggest that targeting of PU.1-mediated upregulation of CSF1R expression might be a useful therapeutic approach.

Chromosomal translocations that involve the *MOZ* gene[3] (official gene symbol *Myst3*) are typically associated with acute myelomonocytic leukemia and predict a poor prognosis[4]. Whereas MOZ is essential for the self-renewal of hematopoietic stem cells[5,6], MOZ fusion proteins enable the transformation of non–self-renewing myeloid progenitors into leukemia stem cells[7]. We previously generated a mouse model for AML by introducing c-Kit$^+$ mouse myeloid stem/progenitor cells infected with a retrovirus encoding MOZ-TIF2 and EGFP into lethally irradiated mice[8].

To identify leukemia-initiating cells (LICs), we investigated the bone marrow cells of these mice for various cell surface markers by FACS analysis. CSF1R^{high} and CSF1R^{low} cells were present in the bone marrow (**Fig. 1a**) and expressed equivalent amounts of MOZ-TIF2 protein (**Fig. 1b**). To determine the LIC activity of these cell populations, we isolated CSF1R^{high} and CSF1R^{low} cells by cell sorting and transplanted limited numbers (10 to 1×10^4 cells) into irradiated mice. One hundred CSF1R^{high} cells were sufficient to induce AML in all transplanted mice (**Fig. 1c**). Conversely, no mice developed AML after 1×10^5 CSF1R^{low} cells were transplanted per mouse, and only half of the mice developed AML with delayed onset when 1×10^4 CSF1R^{low} cells were transplanted (**Fig. 1d**). Thus, the CSF1R^{high} cells showed a >100-fold stronger LIC activity than CSF1R^{low} cells.

FACS analysis indicated that the CSF1R^{high} cell population had the phenotype of both granulocyte-macrophage progenitors (GMPs, Kit$^+$Sca-1$^-$CD16/CD32$^+$) and differentiated monocytes (Mac-1^{low}Gr-1$^+$) (**Supplementary Fig. 1a**). Comparison of the CSF1R^{high} and CSF1R^{low} cell populations indicated that Mac-1 expression was lower in CSF1R^{high} than in CSF1R^{low} cells (**Fig. 1e**). However, we did not observe significant differences between the CSF1R^{high} and CSF1R^{low} cell populations with respect to their cell morphology (**Fig. 1f**), colony-forming ability in methylcellulose medium (**Fig. 1g**), cell cycle distribution (**Supplementary Fig. 1b**) or homeobox A9 (HoxA9) expression (**Supplementary Fig. 1c**). To investigate whether downstream pathways of CSF1R signaling were activated, we measured phosphorylation levels of signal transducer and activator of traranscription-5 (STAT5) and extracellular signal–regulated kinase (ERK) in CSF1R^{high} and CSF1R^{low} cells. STAT5 was highly phosphorylated in the CSF1R^{high} cell population but not in the CSF1R^{low} population, whereas ERK was equivalently phosphorylated in the two cell populations (**Fig. 1h**).

Side population cells, which are present in some types of normal and malignant stem cell populations, were present in the bone marrow of MOZ-TIF2–induced AML mice (**Supplementary Fig. 2a**). Whereas most side population cells were CSF1R^{high}, the non–side population fraction contained both CSFR1^{high} and CSF1R^{low} cells (**Supplementary Fig. 2b**). LICs were approximately tenfold more enriched in the side population fraction than in the non–side population fraction (**Supplementary Fig. 2c,d**). Because the side population fraction was very small (~0.12% of total bone marrow cells), the fraction of LICs in the side population fraction was also small (~1% of all LICs), and most LICs were present in the non–side population fraction (~99%).

[1]Molecular Oncology Division, National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo, Japan. [2]Harvard Stem Cell Institute, Boston, Massachusetts, USA. [3]Department of Pediatrics, Hirosaki University School of Medicine, Hirosaki, Japan. [4]Pharmacological Research Laboratories, Research Division, Kyowa Hakko Kirin, Gunma, Japan. [5]Albert Einstein College of Medicine, Bronx, New York, USA. [6]Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois, USA. [7]Cancer Science Institute, National University of Singapore, Singapore. Correspondence should be addressed to I.K. (ikitabay@ncc.go.jp).

Received 11 January; accepted 18 February; published online 25 April 2010; doi:10.1038/nm.2122

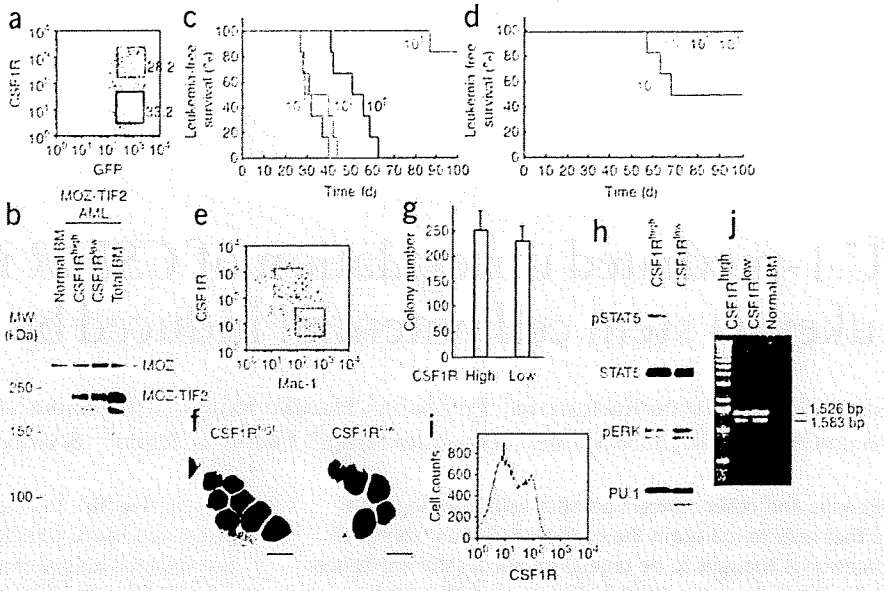


Figure 1 CSF1R^{high} cells show potent leukemia-initiating activity. (**a**) FACS analysis of bone marrow cells from mice with MOZ-TIF2–induced AML for expression of GFP and CSF1R. The red and black boxes signify CSF1R^{high} and CSF1R^{low} cell fractions, respectively. (**b**) Immunoblot analysis of MOZ-TIF2 expression in CSF1R^{high} and CSF1R^{low} cell populations (sorted by flow cytometry) with a MOZ-specific antibody. MW, molecular weight; BM, bone marrow. (**c**,**d**) Leukemia-free survival after the indicated numbers of flow-sorted CSF1R^{high} (**c**) and CSF1R^{low} (**d**) cells were transplanted into sublethally irradiated mice. $n = 6$. $P = 0.0001$ (1×10^6, 1×10^5 and 1×10^4) and 0.3173 (1×10^3) (CSF1R^{high} versus CSF1R^{low} cells). (**e**) FACS analysis of Mac-1 and CSF1R expression in bone marrow cells from mice with MOZ-TIF2–induced AML. The red and blue boxes signify CSF1R^{high} and CSF1R^{low} cell fractions, respectively. (**f**–**h**) CSF1R^{high} and CSF1R^{low} cells were sorted and analyzed for morphology by staining with May-Giemsa (**f**), colony-forming activity in methylcellulose medium (**g**) and levels of total and phosphorylated STAT5, phosphorylated ERK and PU.1 (**h**). Scale bars represent 10 μm in **f**. The error bars represent s.d. in **g**. (**i**) FACS analysis of CSF1R expression in bone marrow cells from an individual with AML with a t(8;16) translocation; the cells were cultured for 3 d in 10 ng ml^{-1} human M-CSF. (**j**) RT-PCR analysis of MOZ-CBP transcripts in CSF1R^{high} and CSF1R^{low} cells of the individual with t(8;16) AML. The results are representative of 25 (**a**,**e**), four (**b**), three (**c**,**d**,**f**–**h**) and two (**i**,**j**) independent experiments.

To determine whether a high level of CSF1R expression also occurs in human AML cells with *MOZ* translocations, we investigated CSF1R expression in bone marrow cells from a subject with AML harboring a t(8;16) translocation, yielding a MOZ-CREB–binding protein (CBP, encoded by the *Creblp* gene) fusion[9]. FACS analysis indicated that both CSF1R^{high} and CSF1R^{low} cells were present among the bone marrow cells with this translocation (**Fig. 1i**). We detected *MOZ-CBP* fusion transcripts in both the CSF1R^{high} and CSF1R^{low} cell populations (**Fig. 1j**).

These results suggest that leukemia stem cells in this subtype of AML express a high amount of CSF1R, indicating that leukemia might be cured by inducing apoptosis of CSF1R^{high} cells. To test this idea, we used transgenic mice expressing a drug-inducible FK506-binding protein (FKBP)-Fas suicide gene and EGFP under the control of the *CSF1R* promoter[10] (**Fig. 2a**). The suicide gene products are inactive monomers under normal conditions but can be activated by injection of the AP20187 dimerizer, inducing apoptosis of cells expressing high amounts of CSF1R[10]. We infected c-Kit$^+$ bone marrow cells of transgenic mice with the MOZ-TIF2 retrovirus and transplanted them into lethally irradiated wild-type mice. These mice developed AML ~2 months after transplantation. In the bone marrow of these mice, we observed morphologically indistinguishable CSF1R^{high} and CSF1R^{low} cells. As expected, endogenous CSF1R expression was proportional to EGFP and FKBP-Fas expression (**Fig. 2b** and **Supplementary Fig. 3a**).

Next, we transplanted the bone marrow cells of these AML mice (1×10^5 cells per mouse) into secondary sublethally irradiated recipient mice. Seven days after transplantation, we injected the mice with

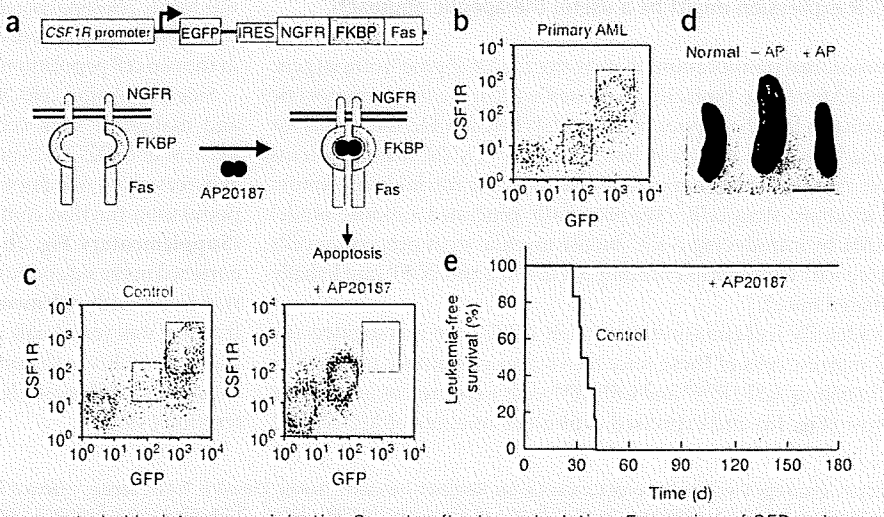


Figure 2 Cure of AML by ablation of CSF1R^{high} cells. (**a**) Top, structure of the *CSF1R* promoter-EGFP-NGFR-FKBP-Fas suicide construct. Bottom, schematic showing the activation of the NGFR-FKBP-Fas fusion protein: in transgenic mice carrying this suicide construct, ablation of cells expressing high levels of CSF1R can be induced by exposure to the AP20187 dimerizer. (**b**) FACS analysis of GFP and CSF1R expression in bone marrow cells of mice with AML 2 months after the transplantation of MSCV-MOZ-TIF2-IRES-GFP–transfected bone marrow cells derived from transgenic mice into lethally irradiated C57BL/6 mice. The red boxes signify CSF1R^{high} and CSF1R^{low} cell fractions. (**c**–**e**) Bone marrow cells (1×10^5) of primary transplanted mice with AML, generated as in **b**, were transplanted into sublethally irradiated C57BL/6 mice. Administration of AP20187 or solvent (control) to the secondary transplanted mice was started by intravenous injection 3 weeks after transplantation. Expression of GFP and CSF1R in bone marrow cells (**c**) and spleen sizes (**d**) were analyzed 4 weeks after transplantation. Scale bars, 1 cm. (**e**) Leukemia-free survival of the untreated ($n = 6$) and AP20187-treated ($n = 6$) secondary transplanted mice. $P < 0.0001$. The results are representative of five (**b**), four (**c**) and three (**d**,**e**) independent experiments.

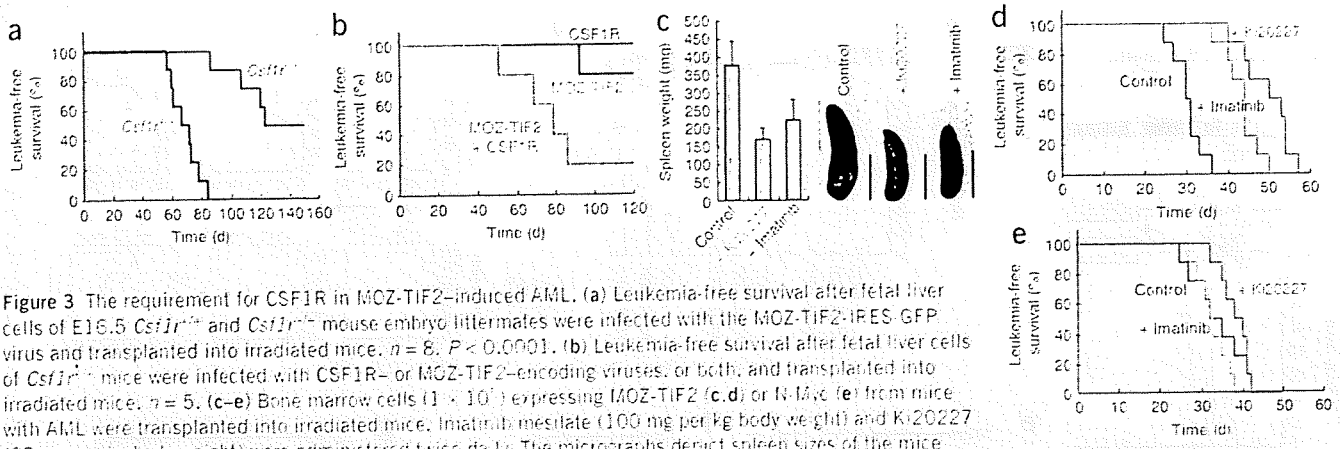


Figure 3 The requirement for CSF1R in MOZ-TIF2–induced AML. (**a**) Leukemia-free survival after fetal liver cells of E16.5 *Csf1r*$^{+/+}$ and *Csf1r*$^{-/-}$ mouse embryo littermates were infected with the MOZ-TIF2-IRES-GFP virus and transplanted into irradiated mice. $n = 8$. $P < 0.0001$. (**b**) Leukemia-free survival after fetal liver cells of *Csf1r*$^{-/-}$ mice were infected with CSF1R– or MOZ-TIF2–encoding viruses, or both, and transplanted into irradiated mice. $n = 5$. (**c–e**) Bone marrow cells (1×10^6) expressing MOZ-TIF2 (**c**,**d**) or N-Myc (**e**) from mice with AML were transplanted into irradiated mice. Imatinib mesilate (100 mg per kg body weight) and Ki20227 (20 mg per kg body weight) were administered twice daily. The micrographs depict spleen sizes of the mice transplanted with MOZ-TIF2–expressing cells, analyzed three weeks after transplantation (**c**). Scale bars, 1 cm. (**d**,**e**) Leukemia-free survival of the control and drug-treated mice was analyzed. In **d**, $n = 8$. $P < 0.0001$ (control versus + Ki20227 and control versus + imatinib). In **e**, $n = 8$. $P = 0.3825$ (control v.s. + Ki20227) and 0.4051 (control versus + imatinib).

AP20187 or a control solvent, as previously described[10]. We observed an increase in the number of CSF1R^{high} cells (**Fig. 2c**) and splenomegaly (**Fig. 2d**) in the control-treated mice 3 weeks after transplantation. However, we detected neither CSF1R^{high} cells nor splenomegaly in the AP20187-treated mice after a 1-week course of treatment (**Fig. 2c,d**). Although we observed CSF1R^{low} cells in the bone marrow and peripheral blood after the 1-week treatment course, we did not detect these cells after three months of treatment (**Fig. 2c** and **Supplementary Fig. 3b**). All control-treated mice developed AML 4–6 weeks after transplantation, but none of the AP20187-treated mice died of AML within 6 months of transplantation (**Fig. 2e**). These results indicate that ablation of the CSF1R^{high} cells was sufficient to cure MOZ-TIF2–induced AML, and that a high level of CSF1R expression is a key contributor to leukemia stem cell potential.

As it has been reported that N-Myc overexpression rapidly causes AML in mice[11], we next tested the specificity of the requirement for CSF1R^{high} cells in AML progression. We transfected the bone marrow cells of suicide gene–expressing transgenic mice with a retrovirus encoding N-Myc and EGFP, and transplanted the cells into lethally irradiated recipient mice, which developed AML. In these mice, GFP$^+$ leukemia cells were Mac1$^+$Gr1$^+$CSF1R$^-$ blast cells (**Supplementary Fig. 4a,b**), and treatment with AP20187 did not affect AML induction (**Supplementary Fig. 4c**). These results indicate a specific role of CSFR expression in MOZ-TIF2–induced AML.

To investigate the role of CSF1R in the development of MOZ-TIF2–induced AML, we infected wild-type and *Csf1r*$^{-/-}$ (ref. 12) mouse fetal liver cells of embryonic day 16.5 (E16.5) littermate embryos with the MOZ-TIF2 virus and transplanted them into lethally irradiated mice. All mice transplanted with wild-type cells developed AML within 3 months (**Fig. 3a**). In contrast, AML induction was initially suppressed in mice transplanted with *Csf1r*$^{-/-}$ cells, but half of the mice developed AML after a longer latency period (**Fig. 3a**). The suppression of AML was rescued by co-infection with the retrovirus encoding CSF1R (**Fig. 3b**). STAT5, which was highly phosphorylated in CSF1R^{high} cells but not in CSF1R^{low} cells (**Fig. 1h**), was phosphorylated in the bone marrow of recipient mice transplanted with *Csf1r*$^{+/+}$ cells but not with *Csf1r*$^{-/-}$ cells (**Supplementary Fig. 5**). To test the specificity of the requirement of CSF1R for AML induction by MOZ-TIF2, we transfected *Csf1r*$^{+/+}$ and *Csf1r*$^{-/-}$ fetal liver cells with the retrovirus encoding N-Myc and transplanted them into irradiated recipient mice. All of the mice transplanted with either *Csf1r*$^{+/-}$ or *Csf1r*$^{-/-}$ cells expressing N-Myc developed AML (**Supplementary Fig. 4d**). These results indicate that CSF1R has a key role in AML induction by MOZ-TIF2, but not by N-Myc.

The above results suggest that signaling through CSF1R might be a therapeutic target for kinase inhibitors in leukemogenesis induced by MOZ fusions. To test this, we used the CSF1R-specific inhibitor Ki20227 (ref. 13) and the tyrosine kinase inhibitor imatinib mesylate (ST1571), which inhibits CSF1R[14–16]. Oral administration of Ki20227 or imatinib inhibited MOZ-TIF2–induced splenomegaly (**Fig. 3c**) and slowed MOZ-TIF2–induced AML onset (**Fig. 3d**). However, the drugs did not affect the progress of N-Myc–induced AML (**Fig. 3e**).

Next, we investigated the molecular mechanism of CSF1R expression in the leukemia cells. Monocyte-specific expression of CSF1R is reportedly regulated by transcription factors such as AML1, PU.1 and CCAAT/enhancer-binding proteins (C/EBPs)[17]. We previously found that MOZ interacts with AML1 and PU.1, but not with C/EBPα or C/EBPε, to stimulate transcription of their target genes[5,18]. Deletion analysis indicated that PU.1 interacted with the N-terminal and central regions of MOZ (**Fig. 4a** and **Supplementary Fig. 6**), and that the acidic amino acid–rich region (DE region) of PU.1 was required for its high-affinity interaction with MOZ (**Fig. 4a** and **Supplementary Fig. 7a–d**). Although binding of PU.1 to N-terminal MOZ (amino acids 1–513) was inhibited by several deletions in the PU.1 protein (**Supplementary Fig. 7c**), binding to full-length MOZ was not completely inhibited by these deletions (**Supplementary Fig. 7b**), suggesting that there may be other PU.1-binding sites in MOZ, its associated proteins or both. A pull-down assay with *Escherichia coli*–produced GST-PU.1 or GST-AML1 and *in vitro*–produced N-terminal MOZ indicated a direct interaction between both PU.1 and MOZ and between AML1 and MOZ (**Supplementary Fig. 8**). However, we cannot rule out a possibility that other factors may facilitate interactions between PU.1 or AML1 and MOZ *in vivo*.

To investigate transcriptional regulation of CSF1R, we performed reporter analysis with a *CSF1R* promoter–luciferase construct and found that MOZ, MOZ-TIF2 and MOZ-CBP could all activate the *CSF1R* promoter in the presence of PU.1 but not in the presence of AML1 (**Fig. 4b**). Moreover, MOZ, MOZ-TIF2 and MOZ-CBP did not activate a *CSF1R* promoter mutant lacking PU.1-binding sites (**Fig. 4c**). These results suggest that MOZ and MOZ fusion

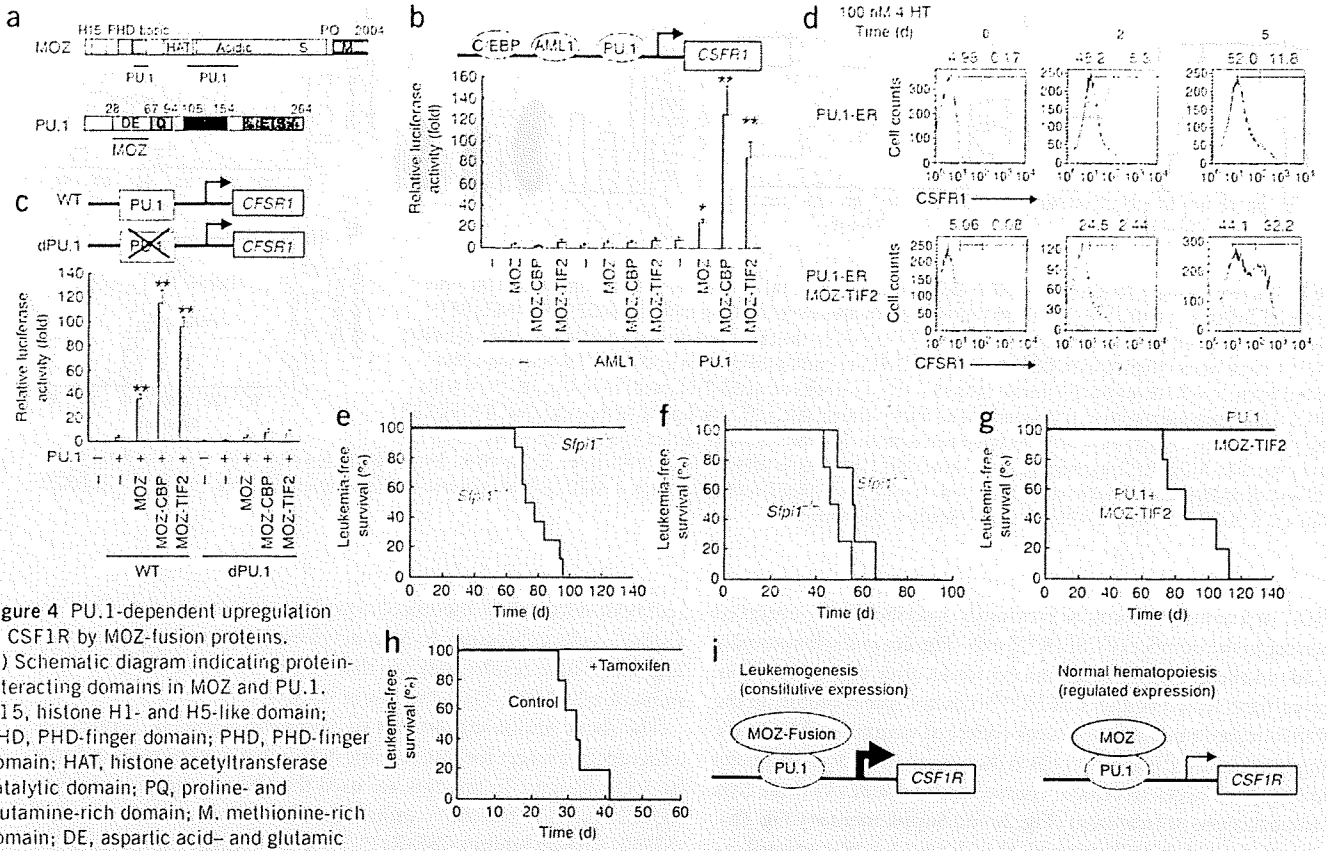


Figure 4 PU.1-dependent upregulation of CSF1R by MOZ-fusion proteins. (**a**) Schematic diagram indicating protein-interacting domains in MOZ and PU.1. H15, histone H1- and H5-like domain; PHD, PHD-finger domain; PHD, PHD-finger domain; HAT, histone acetyltransferase catalytic domain; PQ, proline- and glutamine-rich domain; M, methionine-rich domain; DE, aspartic acid– and glutamic acid–rich domain; Q, glutamine-rich domain; PEST, proline-, glutamic acid–, serine- and threonine-rich domain; ETS, Ets DNA-binding domain. (**b**) Effects of MOZ, MOZ-CBP and MOZ-TIF2 on AML1- and PU.1-mediated transcription of the *CSF1R* promoter. Osteocarcinoma SaOS2 cells were transfected with the CSF1R-luciferase construct and the indicated effector constructs encoding AML1 or PU.1 together with MOZ, MOZ-CBP or MOZ-TIF2. Luciferase activity was analyzed 24 h after transfection. Error bars represent s.d. $^{*}P < 0.01$ and $^{**}P < 0.005$ (comparison to PU.1 only). The results are representative of six independent experiments in which three samples were tested for each group in each experiment. (**c**) PU.1-dependent activation of CSF1R promoter. SaOS2 cells were transfected with the wild-type (WT) *CSF1R*-luciferase construct or its mutant lacking the PU.1-binding site (dPU.1), together with the indicated effectors. Error bars represent s.d. $^{*}P < 0.01$ and $^{**}P < 0.005$ (comparison to PU.1 only). The results are representative of three independent experiments in which three samples were tested for each group in each experiment. (**d**) FACS analysis of CSF1R expression in PUER cells infected with MSCV-GFP (top) or MSCV-MOZ-TIF2-IRES-GFP (bottom) retroviruses and exposed to 100 nM 4-HT for 0, 2 or 5 d. Population (%) of $CSF1R^{high}$ and $CSF1R^{low}$ cells were indicated. The results are representative of three independent experiments. The horizontal lines and the numbers above the graphs indicate $CSF1R^{high}$ (right) and $CSF1R^{low}$ (left) cell fractions and their populations (%), respectively. (**e**,**f**) Leukemia-free survival after fetal liver cells of E12.5 $Sfpi1^{+/+}$ and $Sfpi1^{-/-}$ mouse embryo littermates were infected with either MOZ-TIF2– (**e**) or N-Myc– (**f**) encoding viruses and transplanted into irradiated mice. (**g**) Leukemia-free survival after fetal liver cells of $Sfpi1^{-/-}$ mice were infected with PU.1– or MOZ-TIF2–encoding viruses, or both, and transplanted into irradiated mice. In **e**, $n = 8$, $P < 0.0001$; in **f**, $n = 4$, $P = 0.0943$; in **g**, $n = 5$, $P = 0.0001$ (PU.1 + MOZ-TIF2 versus either PU.1 or MOZ-TIF2). (**h**) Fetal liver cells of E14.5 $Sfpi1^{flox/flox}$ ER-Cre mice were infected with the MOZ-TIF2–encoding virus and transplanted into irradiated mice, which developed AML. The bone marrow cells of these mice were then transplanted into sublethally irradiated wild-type mice. Tamoxifen or solvent (control) was administered to the secondary transplanted mice every 2 d by intravenous injection starting 17 d after transplantation, when GFP^{+} cells were detected in peripheral blood. Leukemia-free survival of the secondary transplanted mice is shown. $n = 5$, $P = 0.0018$. (**i**) Model for transcriptional regulation by normal and fusion MOZ proteins. MOZ fusion proteins stimulate constitutive CSF1R expression to induce leukemia (left). Normal MOZ protein controls CSF1R expression by binding to PU.1 to regulate normal hematopoiesis (right).

proteins activate *CSF1R* transcription in a PU.1-dependent manner. It was recently reported that although chromatin reorganization of *Csf1r* requires prior PU.1 expression together with AML1 binding, stable transcription factor complexes and active chromatin can be maintained at the *Csf1r* locus without AML1 once the full hematopoietic program has been established[19]. This might explain why we found that AML1 was not required for MOZ-TIF2–mediated activation of *Csf1r*. Deletion analysis indicated that the DE-rich, Q-rich and ETS DNA-binding domains of PU.1, as well as the histone H1 and H5-like (H15) and the central PU.1-binding domains of MOZ and MOZ fusion proteins, are required for the activation of *CSF1R* transcription (**Supplementary Figs. 7e** and **9**). A truncated version of MOZ (1–1518) lacking the C-terminal region failed to activate transcription, indicating that the transcriptional activity of MOZ-TIF2 and MOZ-CBP, which do not contain that C-terminal region, requires the TIF2 or CBP portion of the fusion protein.

To test the requirement of PU.1 for the expression of endogenous *CSF1R*, we used PU.1-deficient ($Sfpi1^{-/-}$) myeloid progenitors expressing the PU.1–estrogen receptor fusion protein (PUER). Upon restoration of PU.1 activity by exposure to 4-hydroxytamoxifen (4-HT), PUER cells can differentiate into macrophages[20]. We infected PUER cells with the MOZ-TIF2 retrovirus or control retrovirus, sorted them for GFP expression and cultured the GFP^{+} cells in the presence of 4-HT. The results of FACS (**Fig. 4d**) and quantitative RT-PCR (**Supplementary Fig. 10**) analyses indicated that CSF1R expression was induced after exposure to 4-HT, and that MOZ-TIF2 enhanced

the PU.1-induced upregulation of CSF1R. Notably, 5 d after exposure to 4-HT, we detected $CSF1R^{high}$ and $CSF1R^{low}$ cells in the population of PUER cells expressing MOZ-TIF2, but only $CSF1R^{low}$ cells were in the control PUER cell population (**Fig. 4d**). We did not detect CSF1R expression before addition of 4-HT, even in PUER cells expressing MOZ-TIF2 (**Fig. 4d**), indicating that functional PU.1 is required for MOZ-TIF2–induced CSF1R expression. Chromatin immunoprecipitation (ChIP) analysis indicated that PU.1, MOZ-TIF2 and possibly endogenous MOZ were recruited to the *Csf1r* promoter in the bone marrow cells of mice with MOZ-TIF2–induced AML (**Supplementary Fig. 11a**). In PUER cells expressing MOZ-TIF2, recruitment of MOZ-TIF2 and MOZ to the *Csf1r* promoter was detected after 4-HT treatment, but not before the treatment (**Supplementary Fig. 11b**), suggesting that the recruitment of MOZ-TIF2 and MOZ is dependent upon functional PU.1.

To determine whether PU.1 is essential for the development of MOZ-TIF2–induced AML, we infected wild-type and $Sfpi1^{-/-}$ fetal liver cells of E12.5 littermates with retroviruses encoding MOZ-TIF2 or N-Myc and transplanted them into irradiated mice. Although mice transplanted with $Sfpi1^{+/+}$ cells expressing MOZ-TIF2 developed AML 8–14 weeks after transplantation, mice transplanted with $Sfpi1^{-/-}$ cells were healthy for at least 6 months (**Fig. 4e**). In contrast, all mice transplanted with either wild-type or $Sfpi1^{-/-}$ cells expressing N-Myc developed AML 6–10 weeks after transplantation (**Fig. 4f**). When both PU.1 and MOZ-TIF2 were introduced into PU.1-deficient fetal liver cells, the transplanted mice developed leukemia (**Fig. 4g**). However, introduction of either PU.1 or MOZ-TIF2 alone was not sufficient for AML induction. Thus, we conclude that PU.1 is required for the initiation of MOZ-TIF2–induced AML.

To determine whether PU.1 is also required for the maintenance of MOZ-TIF2–induced AML, we infected fetal liver cells of PU.1 conditional knockout mice ($Sfpi1^{flox/flox}$ and expressing estrogen receptor (ER)-Cre) with MOZ-TIF2 and transplanted them into irradiated recipient mice, which developed AML. We next transplanted bone marrow cells of these mice into irradiated secondary recipients and then treated half of the mice with tamoxifen to induce PU.1 deletion. All of the control mice died of AML within 6 weeks, but none of the tamoxifen-treated mice developed AML for at least for 6 months (**Fig. 4h**). These results indicate that PU.1 is also required for the maintenance of MOZ-TIF2–induced AML stem cells.

Taken together, our results indicate that MOZ and its leukemia-associated fusion proteins activate PU.1-mediated transcription of the monocyte-specific gene *Csf1r*. MOZ fusion proteins might constitutively stimulate high *Csf1r* expression to induce AML (**Fig. 4i**). In contrast, we previously found that MOZ fusion proteins inhibit AML1-mediated activation of granulocyte-specific *Mpo* gene transcription[18]. Because MOZ fusion proteins are associated with monocytic leukemia, commitment to the monocytic lineage may be determined by differential regulation of target genes by MOZ fusion proteins (that is, upregulation of monocyte-specific genes such as *Csf1r* and downregulation of granulocyte-specific genes such as that encoding myeloperoxidase). It is also likely that the normal MOZ protein modulates *Csf1r* expression to an appropriate level to regulate normal hematopoiesis (**Fig. 4i**), as *Csf1r* expression was impaired in $MOZ^{-/-}$ fetal liver cells (**Supplementary Fig. 12**).

Although AML induction was suppressed in mice transplanted with $Csf1r^{-/-}$ cells, half of these mice developed AML, albeit at a longer latency. Thus, MOZ-TIF2 can provoke either a rapid induction of AML in a CSF1R-dependent manner or a slower induction in a CSF1R-independent manner. There are several possibilities to explain this CSF1R independence. First, we observed increased HoxA9 expression in both $CSF1R^{high}$ and $CSF1R^{low}$ cells. HoxA9 overexpression is reportedly not sufficient to induce AML and additional mutations or oncogene activation is required for AML induction in this context[21,22]. Thus, MOZ-TIF2–transfected $Csf1r^{-/-}$ cells might require additional mutations to induce leukemia. Second, because we used a retrovirus vector to introduce MOZ-TIF2, it is possible that oncogene activation by retroviral integration might mediate AML pathogenesis.

In conclusion, our results indicate that PU.1-mediated upregulation of *Csf1r* is crucial for leukemia stem cell potential induced by MOZ-TIF2. Our findings add to previous work associating CSF1R with AML. CSF1R upregulation has been reported in human[23–25] and mouse[26] AML. CSF1R is also known as the oncoprotein c-Fms, and transplantation of bone marrow cells expressing the v-fms oncoprotein induces multilineage hematopoietic disorders[27]. A chromosomal translocation resulting in expression of a fusion protein in which RNA-binding motif protein-6 (RBM6) is fused to CSF1R has recently been reported to be associated with AML[28]. CSF1R may thus be crucial for not only leukemia induced by MOZ fusions but also a wider subset of AML.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We would like to thank D.E. Zhang for the CSF1R promoter mutant lacking PU.1-binding sites, Y. Kamei and A. Iwama for MOZ-TIF2 cDNA, H. Ichikawa for N-MYC cDNA, T. Taya for SaOS2 cells (National Cancer Center Research Institute) and A. Kuchiba for help with statistical analyses. This work was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Health, Labor and Welfare and from the Japanese Ministry of Education, Culture, Sports, Science and Technology (I.K.), by the Program for Promotion of Fundamental Studies from the National Institute of Biomedical Innovation of Japan (I.K.), and by US National Institutes of Health grants R01-CA41456 (D.G.T.), CA32551 and 5P30-CA13330 (E.R.S.).

AUTHOR CONTRIBUTIONS

Y.A., I.K., T.K. and M.S. conducted experiments in AML mice. Y.A., H. Shima and I.K. performed western blotting, immunoprecipitation, GST pull down, ChIP and reporter assays. P.Z. and D.G.T. conducted experiments in PU.1-deficient mice. E.R.S. designed and performed experiments in CSF1R-deficient mice. K.T. and E.I. analyzed expression of CSF1R in human AML cells. H. Singh designed and performed experiments in PUER cells. H.O. prepared Ki20227. I.K. and Y.A. analyzed data and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturemedicine/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Bonnet, D. & Dick, J.E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* **3**, 730–737 (1997).
2. Reya, T., Morrison, S.J., Clarke, M.F. & Weissman, I.L. Stem cells, cancer and cancer stem cells. *Nature* **414**, 105–111 (2001).
3. Borrow, J. *et al.* The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. *Nat. Genet.* **14**, 33–41 (1996).
4. Katsumoto, T., Yoshida, N. & Kitabayashi, I. Roles of the histone acetyltransferase monocytic leukemia zinc finger protein in normal and malignant hematopoiesis. *Cancer Sci.* **99**, 1523–1527 (2008).
5. Katsumoto, T. *et al.* MOZ is essential for maintenance of hematopoietic stem cells. *Genes Dev.* **20**, 1321–1330 (2006).

6. Thomas, T. *et al.* Monocytic leukemia zinc finger protein is essential for the development of long-term reconstituting hematopoietic stem cells. *Genes Dev.* **20**, 1175–1186 (2006).
7. Huntly, B.J. *et al.* MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell* **6**, 587–596 (2004).
8. Deguchi, K. *et al.* MOZ-TIF2–induced acute myeloid leukemia requires the MOZ nucleosome binding motif and TIF2-mediated recruitment of CBP. *Cancer Cell* **3**, 259–271 (2003).
9. Terui, K. *et al.* Two novel variants of MOZ-CBP fusion transcripts in spontaneously remitted infant leukemia with t(1;16;8)(p13;p13;p11), a new variant of t(8;16)(p11;p13). *Haematologica* **93**, 1591–1593 (2008).
10. Burnett, S.H. *et al.* Conditional macrophage ablation in transgenic mice expressing a Fas-based suicide gene. *J. Leukoc. Biol.* **75**, 612–623 (2004).
11. Kawagoe, H., Kandilci, A., Kranenburg, T.A. & Grosveld, G.C. Overexpression of N-Myc rapidly causes acute myeloid leukemia in mice. *Cancer Res.* **67**, 10677–10685 (2007).
12. Dai, X.M. *et al.* Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies and reproductive defects. *Blood* **99**, 111–120 (2002).
13. Ohno, H. *et al.* A c-fms tyrosine kinase inhibitor, Ki20227, suppresses osteoclast differentiation and osteolytic bone destruction in a bone metastasis model. *Mol. Cancer Ther.* **5**, 2634–2643 (2006).
14. Taylor, J.R., Brownlow, N., Domin, J. & Dibb, N.J. FMS receptor for M-CSF (CSF-1) is sensitive to the kinase inhibitor imatinib and mutation of Asp-802 to Val confers resistance. *Oncogene* **25**, 147–151 (2006).
15. Dewar, A.L., Zannettino, A.C., Hughes, T.P. & Lyons, A.B. Inhibition of c-fms by imatinib: expanding the spectrum of treatment. *Cell Cycle* **4**, 851–853 (2005).
16. Dewar, A.L. *et al.* Macrophage colony-stimulating factor receptor c-fms is a novel target of imatinib. *Blood* **105**, 3127–3132 (2005).
17. Zhang, D.E. *et al.* CCAAT enhancer-binding protein (C/EBP) and AML1 (CBF alpha2) synergistically activate the macrophage colony-stimulating factor receptor promoter. *Mol. Cell. Biol.* **16**, 1231–1240 (1996).
18. Kitabayashi, I., Aikawa, Y., Nguyen, L.A., Yokoyama, A. & Ohki, M. Activation of AML1-mediated transcription by MOZ and inhibition by the MOZ-CBP fusion protein. *EMBO J.* **20**, 7184–7196 (2001).
19. Hoogenkamp, M. *et al.* Early chromatin unfolding by RUNX1: a molecular explanation for differential requirements during specification versus maintenance of the hematopoietic gene expression program. *Blood* **114**, 299–309 (2009).
20. Walsh, J.C. *et al.* Cooperative and antagonistic interplay between PU.1 and GATA-2 in the specification of myeloid cell fates. *Immunity* **17**, 665–676 (2002).
21. Kroon, E. *et al.* Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. *EMBO J.* **17**, 3714–3725 (1998).
22. Jin, G. *et al.* Trib1 and Evi1 cooperate with Hoxa and Meis1 in myeloid leukemogenesis. *Blood* **109**, 3998–4005 (2007).
23. Wang, C. *et al.* Expression of the CSF-1 gene in the blast cells of acute myeloblastic leukemia: association with reduced growth capacity. *J. Cell. Physiol.* **135**, 133–138 (1988).
24. Rambaldi, A. *et al.* Expression of the macrophage colony-stimulating factor and c-fms genes in human acute myeloblastic leukemia cells. *J. Clin. Invest.* **81**, 1030–1035 (1988).
25. Preisler, H.D., Kinniburgh, A.J., Wei-Dong, G. & Khan, S. Expression of the protooncogenes c-myc, c-fos and c-fms in acute myelocytic leukemia at diagnosis and in remission. *Cancer Res.* **47**, 874–880 (1987).
26. Gisselbrecht, S. *et al.* Frequent c-fms activation by proviral insertion in mouse myeloblastic leukaemias. *Nature* **329**, 259–261 (1987).
27. Heard, J.M., Roussel, M.F., Rettenmier, C.W. & Sherr, C.J. Multilineage hematopoietic disorders induced by transplantation of bone marrow cells expressing the v-fms oncogene. *Cell* **51**, 663–673 (1987).
28. Gu, T.L. *et al.* A novel fusion of RBM6 to CSF1R in acute megakaryoblastic leukemia. *Blood* **110**, 323–333 (2007).

ONLINE METHODS

Human subjects, mice and cells. The study involving human samples was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent. C57BL/6 mice were purchased from CREA Japan. NGF-FKBP-Fas transgenic mice[10] (Jackson Laboratories), *Csf1r*-deficient mice[12] (provided by E.R.S.), PU.1-null (*Sfpi1*$^{-/-}$) and PU.1 conditionally deficient (*Sfpi1* floxed) mice[29] (provided by D.G.T.), CreERT2 knock-in mice (TaconicArtemis GmbH)[30] and MOZ-deficient mice[5] were backcrossed to C57BL/6 mice at least five times. Mouse experiments were performed in a specific pathogen–free environment at the Japan National Cancer Center animal facility according to institutional guidelines and with approval of the Japan National Cancer Center Animal Ethics Committee. PUER cells[20] were provided by H. Singh.

Generation of acute myeloid leukemia mouse models. MSCV-MOZ-TIF2-IRES-EGFP, MSCV-N-Myc-IRES-EGFP, MSCV-CSF1R-pgk-pac and MSCV-PU.1-pgk-pac constructs were generated by inserting cDNAs encoding MOZ-TIF2, N-Myc, CSF1R or PU.1 into the appropriate vector. The constructs were transfected into Plat-E cells[31] cells using the FuGENE 6 reagent (Roche Diagnostics) and supernatants containing retrovirus were collected 48 h after transfection. c-Kit$^+$ cells (1×10^5 cells) were selected from bone marrow or fetal liver cells using CD117-specific MicroBeads (Miltenyi Biotec); the cells were then incubated with retroviruses using RetroNectin (Takara Bio) for 24 h in StemPro-34 serum-free medium (Invitrogen) containing cytokines (20 ng ml^{-1} stem cell factor (PeproTech), 10 ng ml^{-1} interleukin-6 (PeproTech), 10 ng ml^{-1} interleukin-3 (a gift from Kirin Pharmaceuticals)). The infected cells were then transplanted together with bone marrow cells (2×10^5) into lethally irradiated (9 Gy) 6- to 8-week-old C57BL/6 mice by intravenous injection. Secondary transplants were performed by intravenous injection of bone marrow cells from primary AML mice into sublethally irradiated (6 Gy) C57BL/6 mice.

Administration of AP20187, imatinib or Ki20227. AP20187 (a gift from Ariad Pharmaceuticals; 10 mg per kg body weight) was administered daily by intravenous injection for 5 d, and then 1 mg per kg body weight AP20187 was administered every 3 d thereafter as described previously[10]. Mice were orally administered imatinib mesylate (Novartis Pharmaceuticals; 100 mg per kg body weight), Ki20227 (ref. 13) (a gift from Kirin Pharmaceuticals; 20 mg per kg body weight) or solvent twice daily from 7 d after transplantation.

Immunofluorescent staining, detection of side population cells, flow cytometric analysis and cell sorting. Bone marrow cells from mice with AML were preincubated with rat IgG and then incubated on ice with the following staining reagents: antibody to CD115 (AFS98) conjugated to phycoerythrin (PE) (eBioscience), antibody to Mac-1 (M1/70) conjugated to PE-Cy7 (eBioscience), antibody to Gr-1(RB6-8C5) conjugated to allophycocyanin (APC) (BD Pharmingen) and antibody to c-Kit (2B8) conjugated to APC (BD Pharmingen). For the detection of side population cells, bone marrow cells were stained with 5 μg ml^{-1} Hoechst 33342 in the presence or absence of 50 μM verapamil at 37 °C for 60 min. Flow cytometric analysis and cell sorting were performed using the JSAN cell sorter (Baybioscience) and the results were analyzed with FlowJo software (Tree Star).

Reporter analysis. *CSF1R*-luciferase constructs were generated by insertion of *CSF1R* promoter constructs, either wild type or lacking the PU.1-binding site[32], into pGL4.10 (luc2) (Promega). SaOS2 cells (a gift from T. Taya) were transfected with *CSF1R*-luciferase constructs and pGL4.75 (hRL-CMV) (Promega) together with various expression constructs (pLNCX-AML1 (ref. 18), pLNCX-PU.1 (ref. 33), pLNCX-MOZ[18], pLNCX-MOZ-TIF2 (ref. 18) and pLNCX-MOZ-CBP[18]) in 24-well plates, and luciferase activity was assayed 24 h after transfection using the microplate luminometer GLOMAX (Promega). The results shown for the reporter assays represent average values for relative luciferase activity generated from at least three independent experiments; relative values were obtained by normalizing to the luciferase activity of phRL-CMV, which served as an internal control.

Immunoprecipitation and immunoblotting. For Flag tag immunoprecipitation experiments, cells were lysed in a lysis buffer containing 250 mM NaCl, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate, 10 mM NaF, 0.1% NP-40, 5 mM dithiothreitol, 1 mM phenylmethanesulfonylfluoride and Complete protease inhibitor (Roche). Cell lysates were incubated with Flag-specific antibody–conjugated agarose beads (Sigma) and rotated at 10 r.p.m. (TAITEC RT-50) at 4 °C overnight. The adsorbed beads were washed three times with lysis buffer. Precipitated proteins were eluted from the beads by Flag peptide and dissolved with the same volume of 2× SDS sample buffer. When immunoprecipitation was not performed, total protein lysates were prepared in 2× SDS sample buffer. Antibodies were detected by chemiluminescence with ECL plus Detection Reagents (Amersham Biosciences). The primary antibodies used in this study were Flag-specific antibody (M2) (Sigma), hemagglutinin-specific antibody (3F10) (Roche) and MOZ-specific antibody[18], which was generated by immunizing rabbit with peptides corresponding residue 441–460 of human MOZ.

GST pull-down assay. The HindIII-ClaI fragment corresponding to the N-terminal region (1–664) of MOZ was cloned into the pSP64polyA vector. [^{35}S]-MOZ (1–664) was produced by incubating pSP64polyA-MOZ with [^{35}S]-methionine using the TNT Coupled Rabbit Reticulocyte Lysate System (Promega). pGEX-6P-PU.1 and pGEX-6P-AML1 were generated by subcloning full-length human PU.1 and AML1 cDNAs into pGEX-6P (GE Healthcare). GST, GST-PU.1 and GST-AML1 were produced in *Escherichia coli* BL21 containing pGEX-6P, pGEX-6P-PU.1 and pGEX-6P-AML1, respectively. The [^{35}S]-MOZ (1-664) protein was incubated with GST-, GST-PU.1– or GST-AML1–conjugated glutathione-agarose at 4 °C for 60 min in lysis buffer, washed three times with lysis buffer, analyzed by SDS-PAGE and detected by autoradiography.

Statistical analyses. We performed unpaired two-tailed Student's *t* tests for comparisons and a log-rank test for survival data with JMP8 software (SAS Institute).

29. Iwasaki, H. *et al.* Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* **106**, 1590–1600 (2005).
30. Seibler, J. *et al.* Rapid generation of inducible mouse mutants. *Nucleic Acids Res.* **31**, e12 (2003).
31. Morita, S., Kojima, T. & Kitamura, T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* **7**, 1063–1066 (2000).
32. Zhang, D.E., Hetherington, C.J., Chen, H.M. & Tenen, D.G. The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor. *Mol. Cell. Biol.* **14**, 373–381 (1994).
33. Yoshida, H. *et al.* PML-retinoic acid receptor α inhibits PML IV enhancement of PU.1-induced C/EBPε expression in myeloid differentiation. *Mol. Cell. Biol.* **27**, 5819–5834 (2007).

doi:10.1038/nm.2122

Cancer Cell

A Higher-Order Complex Containing AF4 and ENL Family Proteins with P-TEFb Facilitates Oncogenic and Physiologic MLL-Dependent Transcription

Akihiko Yokoyama,[1,*] Min Lin,[2] Alpana Naresh,[2] Issay Kitabayashi,[1] and Michael L. Cleary[2,*]
[1]Molecular Oncology Division, National Cancer Center Research Institute, Tokyo 104-0045, Japan
[2]Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA
*Correspondence: ayokoyam@ncc.go.jp (A.Y.), mcleary@stanford.edu (M.L.C.)
DOI 10.1016/j.ccr.2009.12.040

SUMMARY

AF4 and ENL family proteins are frequently fused with MLL, and they comprise a higher order complex (designated AEP) containing the P-TEFb transcription elongation factor. Here, we show that AEP is normally recruited to MLL-target chromatin to facilitate transcription. In contrast, MLL oncoproteins fused with AEP components constitutively form MLL/AEP hybrid complexes to cause sustained target gene expression, which leads to transformation of hematopoietic progenitors. Furthermore, MLL-AF6, an MLL fusion with a cytoplasmic protein, does not form such hybrid complexes, but nevertheless constitutively recruits AEP to target chromatin via unknown alternative mechanisms. Thus, AEP recruitment is an integral part of both physiological and pathological MLL-dependent transcriptional pathways. Bypass of its normal recruitment mechanisms is the strategy most frequently used by MLL oncoproteins.

INTRODUCTION

Leukemia is a heterogeneous disease with distinctive biological and clinical properties that are conferred by a variety of acquired genetic mutations (Gilliland, 2002). Chromosomal translocations of the *MLL* gene account for 5%–10% of acute leukemias and are generally associated with poor prognosis (Daser and Rabbitts, 2004; Krivtsov and Armstrong, 2007; Pui et al., 2004). *MLL* gene rearrangements create fusion genes that contain the 5′ portion of *MLL* and the 3′ portion of its fusion partner, whose products cause sustained expression of MLL target genes and consequent enhanced proliferation of hematopoietic progenitors (Ayton and Cleary, 2003; Lavau et al., 1997; Cozzio et al., 2003). The amino-terminal portion of MLL serves as a targeting unit to direct MLL oncoprotein complexes to their target loci through DNA binding (Ayton et al., 2004; Slany et al., 1998) and association with menin and LEDGF (Yokoyama et al., 2005; Yokoyama and Cleary, 2008), whereas the fusion partner portion serves as an effecter unit that causes sustained transactivation (Cheung et al., 2007; Lavau et al., 2000; DiMartino et al., 2000; 2002; Slany et al., 1998; So and Cleary, 2002; 2003). To date, approximately 50 different fusion partners have been reported to form chimeric MLL oncoproteins (Huret et al., 2001). However, the mechanisms underlying this molecular diversity have not been revealed.

The AF4 and ENL protein families are the most frequent MLL fusion partners, accounting for two-thirds of *MLL*-associated leukemia incidence (Huret et al., 2001). The AF4 family comprises four paralogous proteins, including AF4, AF5q31, LAF4, and FMR2. The ENL family includes ENL and AF9 and has structural homology to the yeast Anc1 protein. The members of both protein families possess transactivation domains and therefore are thought to be involved in transcriptional regulation (Prasad et al., 1995; Ma and Staudt, 1996; Morrissey et al., 1997; Slany et al., 1998). All but *FMR2* have been reported to form fusion genes with *MLL* in leukemia (Domer et al., 1993; Taki

Significance

MLL is fused by chromosomal translocations in 5%–10% of acute leukemias to a variety of partner proteins (>50) of diverse molecular composition and function. Recent studies show that several of the more common MLL fusion partners (e.g., AF4, ENL, and AF9) associate in a higher-order complex containing transcription elongation factors. Here we show that this complex is biochemically distinct from the MLL histone methyltransferase complex, but nevertheless normally present at MLL target genes during physiologic gene expression. In acute leukemias, the complex is constitutively recruited to target chromatin by covalent fusion of MLL with one of several complex components or noncovalent mechanisms used by other MLL fusion proteins, thereby representing a unifying mechanism for MLL-mediated leukemogenesis that can be targeted by molecular therapy.

et al., 1999; von Bergh et al., 2002; Iida et al., 1993; Nakamura et al., 1993; Tkachuk et al., 1992). AF4 family proteins associate with ENL family proteins and P-TEFb (Positive Transcription Elongation Factor b) (Erfurth et al., 2004; Zeisig et al., 2005; Bitoun et al., 2007; Mueller et al., 2007). P-TEFb is composed of CDK9 and cyclin T1 (or cyclin T2) and is capable of phosphorylating the carboxy-terminal domain (CTD) of RNA polymerase II (RNAPII) and DSIF to facilitate transcriptional elongation (Saunders et al., 2006; Peterlin and Price, 2006). AF4 functions as a positive regulator of P-TEFb kinase (Bitoun et al., 2007), which, in turn, controls the transactivation activity or stability of AF4 and ENL family proteins. ENL family proteins also associate with DOT1L (Bitoun et al., 2007; Mueller et al., 2007; Zhang et al., 2006), the major histone methyltransferase responsible for the H3K79 methylation mark (Jones et al., 2008), which is predominantly associated with actively transcribed genes (Steger et al., 2008). It has been reported that DOT1L also associates with MLL-AF10 and plays a critical role in its oncogenic transformation (Okada et al., 2005). However, the molecular roles of these components in MLL-dependent leukemogenesis have not been clearly defined.

In this study, we investigated the contributions of a higher order complex containing AF4 and ENL family proteins with P-TEFb in physiologic and pathologic MLL-dependent transcription.

RESULTS

AF4 Forms a Higher Order Complex with AF5q31, ENL, and P-TEFb in Hematopoietic Cells

To identify AF4-associated proteins in vivo, we biochemically purified AF4 complexes from K562 cells using column chromatography followed by immuno-affinity purification with a highly specific anti-AF4 monoclonal antibody (Figure 1A). Mass spectrometry identified AF5q31, ENL, CDK9, and cyclin T1 in the purified materials (Figure 1B). Reciprocal immunoprecipitation (IP) further confirmed that all five proteins compose an endogenous bona fide complex (Figure 1C) consistent with previous observations (Erfurth et al., 2004, Zeisig et al., 2005; Bitoun et al., 2007; Mueller et al., 2007). In gel filtration analysis, the AF4 complex components codistributed in fractions that eluted at an average mass of ~0.8 MDa (Figure 1D). A similar complex was obtained using a monoclonal antibody specific for AF5q31 in the immunoaffinity step (see Figure S1A available with this article online). However, neither purification process yielded other proteins previously reported to interact with ENL (e.g., DOT1L and AF10) (Zeisig et al., 2005; Bitoun et al., 2007; Mueller et al., 2007). These data demonstrate that AF4, AF5q31, and ENL associate in an endogenous higher-order complex (hereafter referred to as "AEP" for the AF4 family/ENL family/P-TEFb complex) containing P-TEFb in hematopoietic lineage cells.

Leukemogenic Fusion Proteins Inappropriately Tether AEP Components with MLL

Co-IP analyses were performed to determine whether MLL chimeric oncoproteins participate in higher-order associations that recapitulate the composition of AEP. Reciprocal IP using human leukemia cell lines that express MLL-ENL, MLL-AF4, or MLL-AF5q31 showed that the respective fusion proteins form similar AEP-like complexes (Figure 1E and Figure S1B). Conversely, MLL-AF6, an MLL fusion with a cytoplasmic protein that was not copurified with AF4 or AF5q31, did not coprecipitate any of the AEP components in ML-2 cells (Figure 1E). Similarly, wild-type (WT) MLL did not pull down AEP components in K562 cells while coprecipitating menin, a component of the MLL complex (Yokoyama et al., 2004) (Figure 1C). Therefore, the MLL and AEP complexes are separate biochemical entities that are inappropriately tethered to form MLL/AEP hybrid complexes by a subset of covalent fusions of MLL in human leukemia cells.

MLL-ENL and MLL-AF4 Consistently Recruit AEP Components to MLL Target Genes

Genomic localizations of MLL chimeric proteins and AEP components were analyzed by chromatin immunoprecipitation (ChIP) in human leukemia cell lines. Histone marks indicative of open chromatin states (tri-methyl H3K4 and acetyl H3K9) (Li et al., 2007) were associated with transcriptionally active loci, whereas histone marks indicative of closed chromatin (di-methyl H3K9 and high levels of histone H3) were associated with transcriptionally inactive loci (Figures 2A–2C), verifying the integrity of ChIP assays. In HB1119 cells, MLL-ENL specifically colocalized with AF4 and AF5q31 at promoter-adjacent regions of the *HOXA9* and *MEIS1* genes, which are known to serve critical roles in MLL-associated leukemogenesis (Ayton and Cleary, 2003; Nakamura et al., 1996; Wong et al., 2007), whereas the presence of AEP at non-MLL target loci such as *β-ACTIN* and *GAPDH* was minimal or negligible (Figure 2B and Figure S2A). Similarly, ChIP analysis showed that AF5q31 and ENL colocalized with endogenous MLL-AF4 on the *HOXA9* and *MEIS1* promoters in MV4-11 cells (Figure 2C and Figure S2B). Colocalization of AEP components with MLL oncoproteins was also observed on other MLL target genes, such as *CDKN1B* and *CDKN2C* (Milne et al., 2005), and the transcribed regions of *HOXA9* and *MEIS1* (Figures 2B and 2C), suggesting that MLL/AEP hybrid complexes may function in transcriptional elongation. Therefore, a subset of MLL oncoproteins results in consistent recruitment of AEP components at MLL target chromatin in leukemia cells.

Formation of a Higher Order MLL-AF5q31/AEP Hybrid Complex Is Required for Sustained Transcription of Target Genes and Transformation

AF4 and AF5q31 share extensive sequence similarity that resides in four subregions of the respective proteins (Figure 3A). A structure/function analysis (Figures 3B and 3C) revealed that: (1) P-TEFb interacts with AF4 and AF5q31 via subregion 1, which contains the N-terminal homology domain (NHD) (Nilson et al., 1997); (2) strong transactivation activity is conferred by subregion 2, consistent with previous observations (Prasad et al., 1995; Ma and Staudt, 1996; Morrissey et al., 1997); (3) ENL interacts with AF4 and AF5q31 through subregion 3 that encompasses the AF9 interaction domain (Srinivasan et al., 2004; Zeisig et al., 2005); and (4) the C-terminal homology domain (CHD) within subregion 4 mediates hetero-association of AF4 and AF5q31, which appears to be highly preferred over their respective homo-dimerization (Figure 3B). Preferential hetero-dimerization was also observed in co-IP experiments of endogenous or transfected MLL-AF5q31 (Figure 1E and

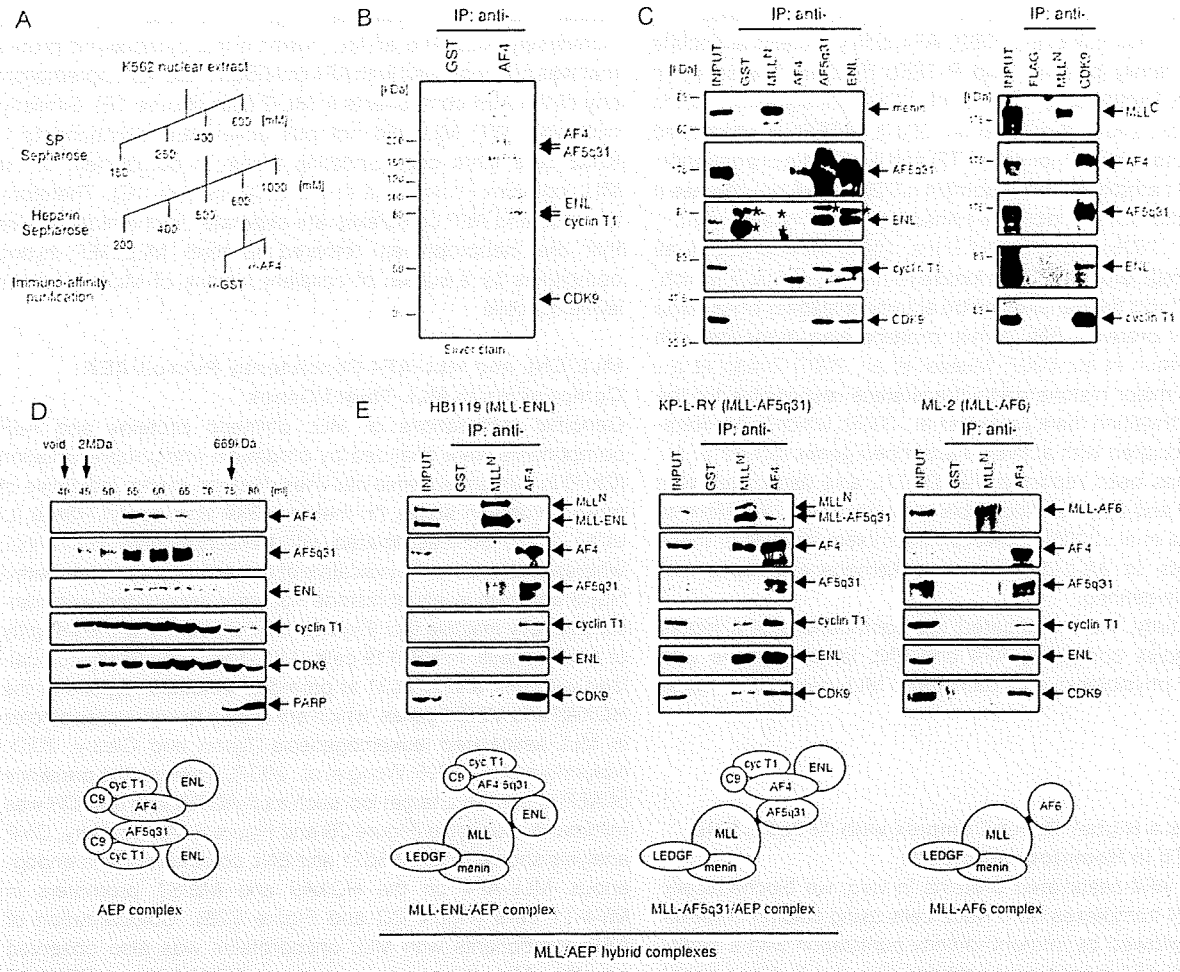


Figure 1. Heterologous Associations of Wild-Type and Oncogenic AF4 and ENL Family Proteins

(A) The scheme used for purification of the AF4 complex.

(B) A silver-stained image shows the proteins immuno-purified using anti-AF4 antibody and subsequently identified by mass spectrometry, as indicated by arrows on the right. Anti-GST antibody served as a negative control.

(C) K562 nuclear extracts were analyzed by IP western blotting. IP was performed with the antibodies indicated on the top, and the precipitates were immunoblotted with the antibodies indicated on the right. Anti-GST and anti-FLAG antibodies served as negative controls. Asterisks indicate signals from IgG used for IP.

(D) Selected fractions from gel filtration analysis of K562 nuclear extracts were analyzed by western blotting for AF4-associated factors (PARP served as a negative control). Molecular weight standards are shown on the top. A cartoon of a putative AEP complex is depicted. C9, CDK9; cyc T1, cyclin T1.

(E) IP western blot analysis was performed as in (C) on human leukemia cell lines that harbor MLL chromosomal translocations and express MLL chimeric oncoproteins (indicated at tops). Cartoons of putative MLL fusion complexes are depicted below. See also Figure S1.

Figure S3A), as well as an interaction assay based on GAL4-dependent transactivation (Figure S3B).

MLL fusion proteins containing the respective subregions of AF4 or AF5q31 were assessed for their oncogenic potentials in a myeloid progenitor transformation assay (Figure 3D) (Lavau et al., 1997). Only MLL-AF5q31 constructs containing subregion 4 (MLL-AF5-4 and MLL-AF5-34) induced serial replating activity and up-regulation of *Hoxa9* transcription (Figures 3E and 3F). This result indicates that none of the single functions (i.e., P-TEFb recruitment, transactivation, or association with ENL) is sufficient for transformation but rather CHD-mediated association with endogenous AEP is required. The corresponding MLL-AF4-4 and MLL-AF4-34 proteins were not stably expressed and thus unable to be evaluated (Figure 3F). Although recruitment of Enl was not sufficient for MLL-AF5q31-dependent transformation, Enl was required because its knockdown by sh-RNA substantially decreased the clonogenicity and *Hoxa9* expression of MLL-AF5q31-transformed cells (Figures 3G–3J). This phenotype was rescued by exogenous expression of human ENL, thus verifying the target specificity of the sh-RNA. Hence, formation of a higher order MLL/AEP hybrid complex on target genes is necessary for MLL-AF5q31-dependent transformation.

Transforming Properties of MLL-ENL and MLL-AF9 Correlate with Association with AF4 Family Proteins and DOT1L

A similar structure/function analysis of MLL-ENL demonstrated that C-terminal ENL residues (494–559) are required for the interaction with AF5q31 (Figures 4A and 4B). This region, which is evolutionally conserved with AF9 and *Saccharomyces cerevisiae*

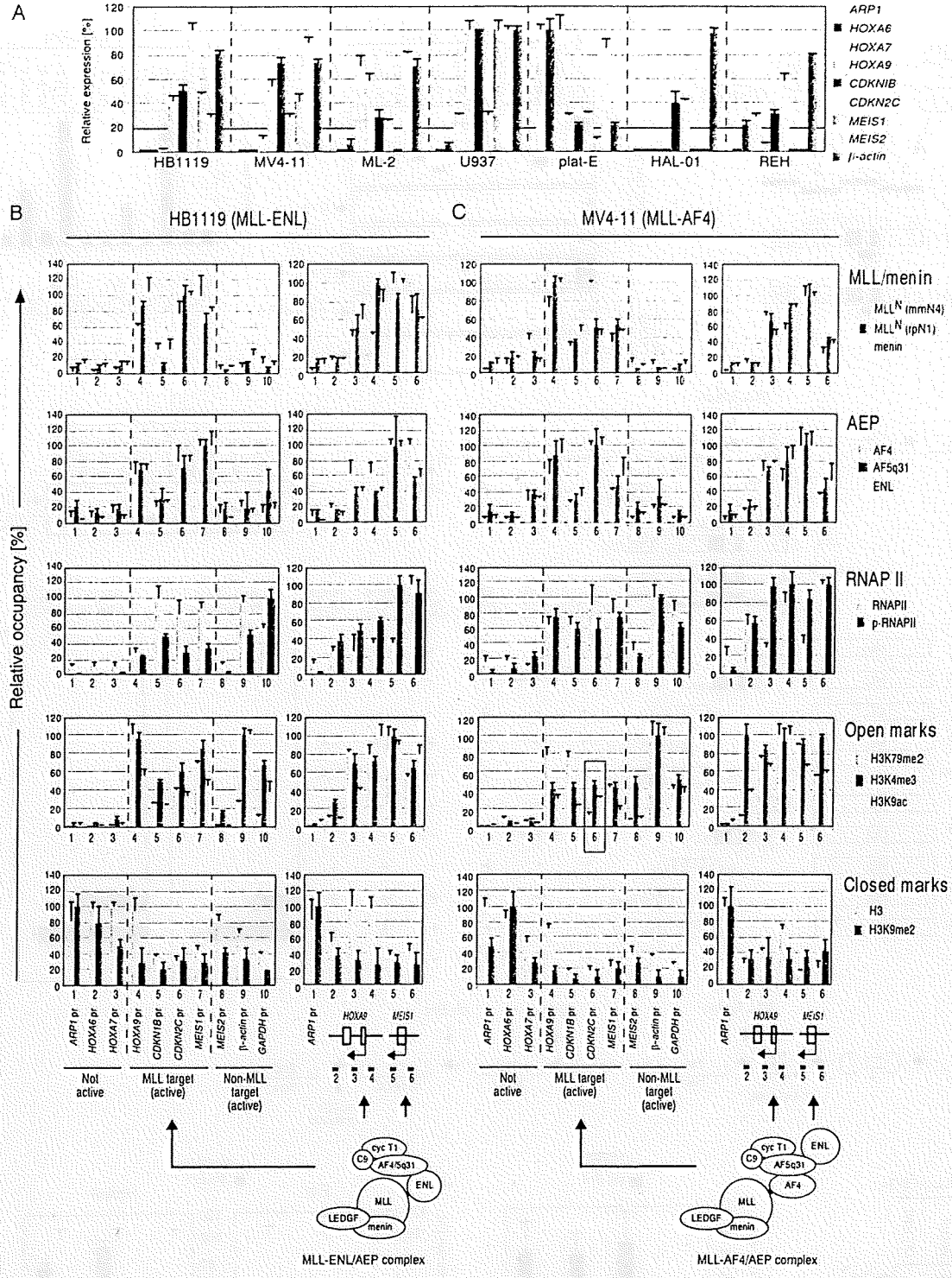
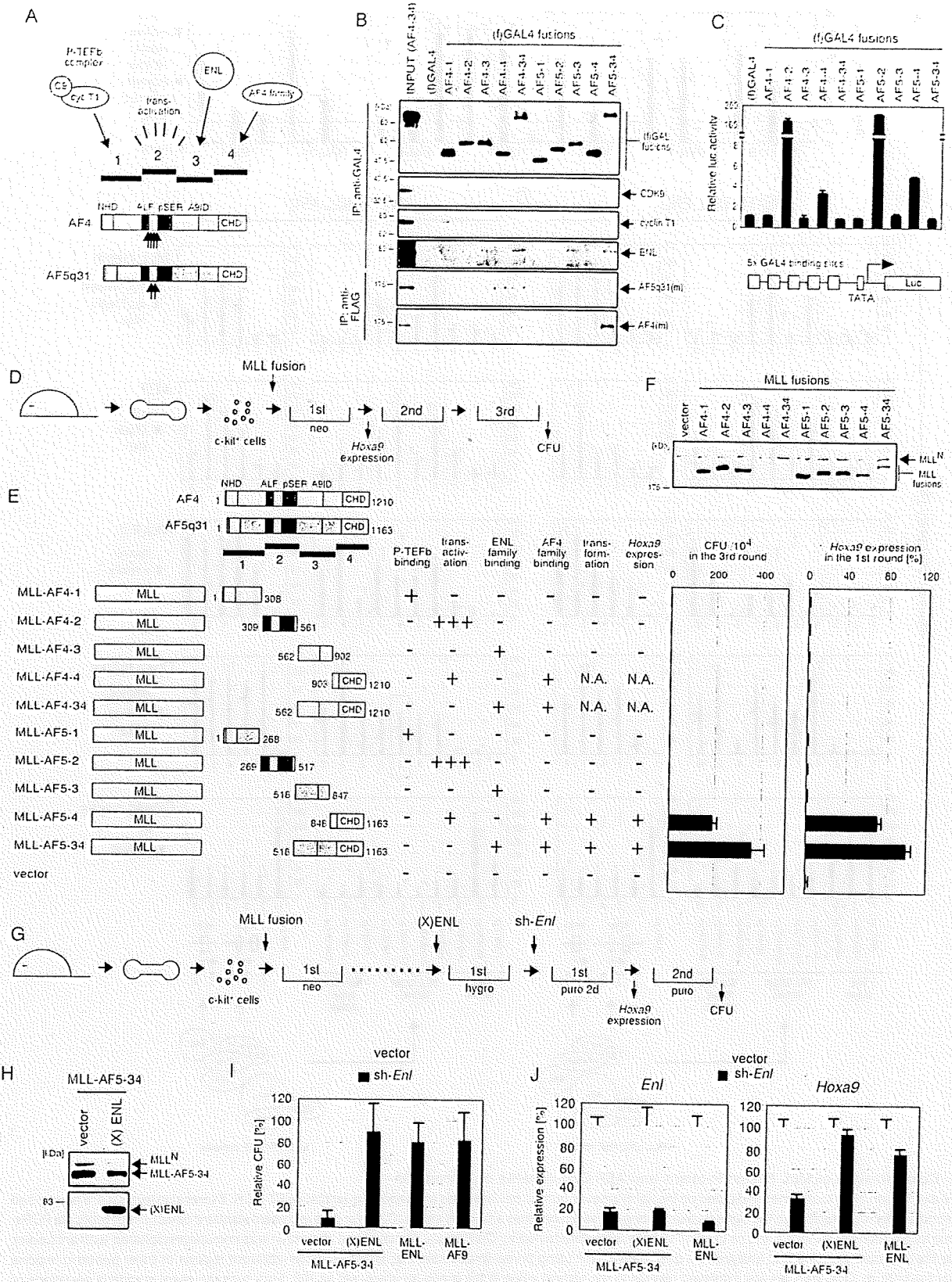


Figure 2. Colocalization of MLL Fusion Proteins and AEP Components on Chromatin

(A) Relative expression of various genes (indicated on the right) in seven human cell lines was analyzed by quantitative RT-PCR. Expression levels were normalized to *GAPDH* and are depicted relative to the highest value among the seven cell lines arbitrarily set as 100. Error bars represent standard deviations of triplicate PCRs.

(B) Genomic localizations of various proteins in HB1119 cells were determined by ChIP assay. Cross-linked chromatin was immunoprecipitated with antibodies specific for the indicated proteins and analyzed by quantitative PCR using primer/probe sets that target promoter-adjacent regions or other genomic regions indicated at the bottom. Occupancies are displayed relative to the highest value in the group arbitrarily set as 100. Error bars represent standard deviations of triplicate PCRs. Genes expressed more than 20% of the highest levels in (A) are defined as active genes.

(C) A comparable analysis as in (B) was performed for MV4-11 cells, which harbor a t(4;11) translocation and express MLL-AF4 proteins. The purple rectangle highlights a locus on which dimethyl H3K79 marks were absent, but the MLL-AF4/AEP complex was present. See also Figure S2.


A
P-TEFb complex
CE
cyc T1
ENL
AF4 family
trans-activation
1
2
3
4
NHD
ALF pSER ASID
CHD
AF4
AF5q31
B
(f)GAL4 fusions
INPUT (AF4-34)
(f)GAL4
AF4-1
AF4-2
AF4-3
AF4-4
AF4-34
AF5-1
AF5-2
AF5-3
AF5-4
AF5-34
IP: anti-GAL4
IP: anti-FLAG
(f)GAL4 fusions
CDK9
cyclin T1
ENL
AF5q31(m)
AF4(m)
C
(f)GAL4 fusions
Relative luc activity
5x GAL4 binding sites
TATA
Luc
D
MLL fusion
c-kit+ cells
1st
neo
2nd
3rd
Hoxa9 expression
CFU
E
AF4
AF5q31
P-TEFb binding
trans-activation
ENL family binding
AF4 family binding
trans-formation
Hoxa9 expression
MLL-AF4-1
MLL-AF4-2
MLL-AF4-3
MLL-AF4-4
MLL-AF4-34
MLL-AF5-1
MLL-AF5-2
MLL-AF5-3
MLL-AF5-4
MLL-AF5-34
vector
N.A.
CFU 10-4 in the 3rd round
Hoxa9 expression in the 1st round [%]
F
MLL fusions
vector
MLLN
MLL fusions
G
MLL fusion
(X)ENL
sh-Enl
c-kit+ cells
1st
neo
1st
hygro
1st
puro 2d
2nd
puro
Hoxa9 expression
CFU
H
MLL-AF5-34
vector
(X) ENL
MLLN
MLL-AF5-34
(X)ENL
I
vector
sh-Enl
Relative CFU [%]
vector
(X)ENL
MLL-ENL
MLL-AF9
MLL-AF5-34
J
vector
sh-Enl
Enl
Hoxa9
Relative expression [%]
vector
(X)ENL
MLL-ENL
MLL-AF5-34

Anc1 (designated AHD: Anc1 homology domain), displayed transactivation potential that correlated with association with AF4 family proteins (Figure 4C). The AHD of ENL also mediated association with DOT1L (Figure 4D), consistent with results of previous studies (Mueller et al., 2007). Mutations of MLL-ENL that abolished AF5q31 and DOT1L interaction (including a single L550E point mutation) resulted in failure to up-regulate *Hoxa9* transcription and transform myeloid progenitors (Figures 4E–4G). Similarly, the portion of AF9 retained in MLL-AF9 oncoproteins, which includes AHD (residues 502–568) (Figure 4A), mediated AF5q31 and DOT1L association and conferred GAL4-dependent transactivation, MLL-dependent *Hoxa9* expression, and myeloid transformation (Figures 4B–4G). Unlike MLL-AF5q31-transformed cells, MLL-ENL- and MLL-AF9-transformed cells did not require WT Enl because their clonogenicities were unaffected by its knockdown (Figures 3I and 3J), consistent with the observation that MLL-ENL did not form a complex with WT ENL in HB1119 cells (Figure 1E). These results suggest that association with AF4 family proteins and/or DOT1L is required for the oncogenic properties of MLL-ENL and MLL-AF9.

Interactions of ENL with DOT1L or AF4 Family Proteins Are Mutually Exclusive

To determine whether ENL can simultaneously coassociate with AF4 family proteins and DOT1L, IP analysis was performed on cells transiently expressing ENL, AF5q31, and DOT1L. Although ENL coprecipitated both AF5q31 and DOT1L, the latter two did not pull down each other (Figure 5A), indicating that the three proteins do not form a trimeric complex. Similarly, GAL4-AF5-3 effectively coprecipitated ENL but not DOT1L under conditions where GAL4-ENL successfully pulled down DOT1L (Figure S4). These data demonstrate that the associations of ENL family proteins with AF4 family proteins or DOT1L are mutually exclusive. Therefore, the ENL/DOT1L complex is a separate entity from AEP (Figure 5B).

Recruitment of AEP, versus DOT1L, Plays a Predominant Role in MLL-Dependent Leukemogenesis

The ability of MLL-ENL to associate with AF4 family proteins or DOT1L raised the issue of which interaction (MLL-ENL/AEP vs. MLL-ENL/DOT1L) is essential for leukemic transformation (Figure 5B). To address this issue, an artificial MLL fusion with DOT1L (MLL-DOT1L) that does not associate with AF4 (Figure 5C) but retains the HMT catalytic domain (thus mimics the MLL-ENL/DOT1L complex) was assessed for its transformation potential. MLL-DOT1L failed to sufficiently activate *Hoxa9* expression to immortalize myeloid progenitors (Figures 5D and 5E), despite the comparable levels of protein expression in packaging cells (Figure 5F) and mRNA expression in first-round colonies (Figure 5E). In the same experimental condition, MLL-AF5q31 successfully transformed myeloid progenitors (Figure 5E) without being able to directly associate with DOT1L (Figure 5C). These results, which contrast with those of previous studies (Okada et al., 2005), indicate that simple recruitment of DOT1L HMT activity alone to MLL target genes is not sufficient for transformation and support a more predominant role for AEP recruitment.

Nevertheless, DOT1L-dependent H3K79 methylation colocalized with the presence of MLL-ENL at all target loci tested in HB1119 cells (Figure 2B), indicating that not only AEP components but also DOT1L is consistently recruited by MLL-ENL. In MV4-11 cells, H3K79 methylation marks also colocalized at most of the MLL-AF4-occupied loci, consistent with previous observations (Krivtsov et al., 2008; Guenther et al., 2008), despite the apparent inability to directly recruit DOT1L (Figures 2C and 5C). However, the signal intensities of H3K79 dimethylation were relatively low at MLL-AF4-target loci, compared with those at MLL-ENL-target loci (compare relative intensities to those of *β-ACTIN* and *GAPDH*, which served as internal standards) (Figures 2B and 2C; Figure S2) and were minimal at the *CDKN2C* promoter in spite of the localization of abundant AEP components (Figure 2C, purple rectangle). Thus, DOT1L-dependent

Figure 3. Formation of an AEP-Like Complex Is Required for MLL-AF5q31-Dependent Myeloid Transformation

(A) The structures of AF4 and AF5q31 are schematically illustrated. Subregions (1–4) of AF4 and AF5q31 are indicated with associated functions. Upward arrows indicate the sites of fusion with MLL in human leukemia oncoproteins (Jansen et al., 2005) (A9ID, AF9 interaction domain; Srinivasan et al., 2004).

(B) The four subregions fused to GAL4 DNA binding domain were expressed in 293T cells (upper four panels) or coexpressed with myc-tagged AF4 or AF5q31 [AF4(m) or AF5q31(m)] (lower two panels) and analyzed by IP western blotting. IP antibodies are indicated on the left and proteins detected by western blotting are indicated on the right. (f)GAL4 fusions and myc-tagged AF4 family proteins were visualized with anti-FLAG and anti-myc antibodies, respectively.

(C) Transactivation activity of respective GAL4 fusions was analyzed using the reporter gene shown below. Error bars represent standard deviations from triplicate analyses.

(D) The experimental scheme of myeloid progenitor transformation assays to evaluate the oncogenic potentials of various MLL mutants shows the time points at which CFU (colony forming unit) activity or *Hoxa9* expression was examined.

(E) The structures of various MLL-AF4/AF5q31 mutants and their associated functions are summarized schematically. *Hoxa9* levels were normalized to *Gapdh* and displayed relative to *MLL-AF5-34*-transduced cells arbitrarily set at 100%. Error bars represent standard deviations of three independent analyses (left) or triplicate PCRs (right). N.A., not applicable because of unstable expression of MLL fusion proteins.

(F) Protein levels of respective MLL mutants in virus-packaging cells were examined by western blotting with anti-MLL^N antibody. MLL-AF4-4 and MLL-AF4-34 proteins were not stably expressed.

(G) The experimental scheme to evaluate the effect of *Enl* knockdown on MLL transformation is shown schematically. (X)ENL, Xpress-tagged human ENL.

(H) Transduced myeloid progenitors were analyzed by western blotting with anti-MLL^N (top) and anti-Xpress (bottom) antibodies to detect exogenous MLL-AF5q31 and human (X)ENL, respectively.

(I) The clonogenic potentials of MLL-AF5-34-transformed cells transduced with or without *(X)ENL* are shown at the second plating after sh-RNA transduction (vector or sh-*Enl*). MLL-ENL- or MLL-AF9-transformed cells were also subjected to sh-RNA transduction for comparison. CFUs are expressed relative to the vector control arbitrarily set as 100. Error bars represent standard deviations of three independent analyses.

(J) Cells from first-round colonies following sh-RNA transduction (vector or sh-*Enl*) were analyzed by RT-PCR for expression of endogenous *Enl* or *Hoxa9*. Expression levels were normalized to *Gapdh* and displayed relative to the vector/vector control cells arbitrarily set at 100. Error bars represent standard deviations of triplicate PCRs. See also Figure S3.

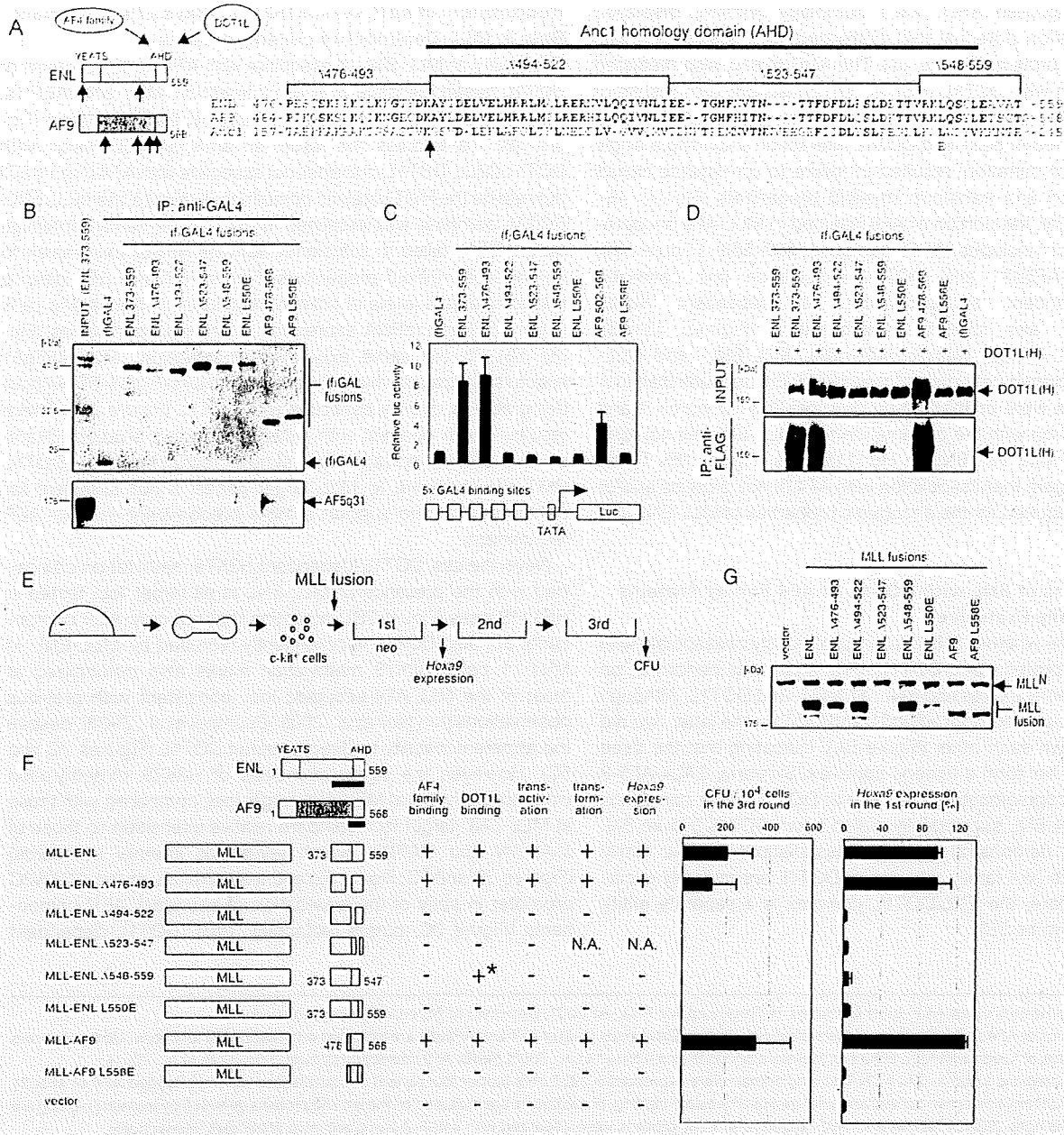


Figure 4. MLL-ENL and MLL-AF9 Transform Myeloid Progenitors via the AHD, which Is Responsible for Association with AF4 Family Proteins and DOT1L

(A) The structures of ENL and AF9 are schematically illustrated with associated functions (Zeisig et al., 2005). Aligned amino acid sequences for the minimum transformation domain are also shown with the positions of deletion or substitution mutations and AHD. Upward arrows indicate the sites of fusion with MLL in human leukemia oncoproteins (Jansen et al., 2005).

(B) Domain mapping of ENL family proteins for association with AF5q31 was performed with FLAG-tagged GAL4 fusion constructs of ENL (372–559 aa) and AF9 (478–568 aa). IP was performed with anti-GAL4 antibody, and the precipitates were immunoblotted with anti-FLAG antibody for (f)GAL4 fusions or anti-AF5q31 antibody for endogenous AF5q31.

(C) Transactivation activity of indicated GAL4 constructs was analyzed by luciferase assay as in Figure 3C.

(D) The same set of GAL4 fusion proteins used in (B) and HA-tagged DOT1L [DOT1L(H)] were coexpressed in 293T cells and analyzed by IP western blotting. IP was performed with anti-FLAG antibody and the precipitates were immunoblotted with anti-HA antibody.

(E) The experimental scheme is shown for myeloid progenitor transformation assays to evaluate the oncogenic potentials of MLL mutants.

(F) The structures of MLL-ENL and MLL-AF9 mutants and their associated functions are summarized with schematic representations. *Hoxa9* expression levels were normalized to *Gapdh* and displayed relative to the *MLL-ENL*-transduced cells arbitrarily set at 100%. Error bars represent standard deviations of three independent analyses (left) or triplicate PCRs (right). N.A., not applicable because of unstable expression of MLL fusion proteins. The asterisk indicates that association of ENL Δ548–559 mutant with DOT1L was detected but reduced substantially, compared with WT ENL.

(G) Protein levels of respective MLL mutants in virus packaging cells were examined by western blotting with anti-MLL^N antibody. MLL-ENL Δ523–547 was not stably expressed.