Eya2 is critical for the *E2A-HLF*-mediated immortalization of mouse hematopoietic stem/progenitor cells

Running title: *E2A-HLF*-MEDIATED LEUKEMOGENESIS THROUGH EYA2

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ABSTRACT

The *E2A-HLF* oncogenic fusion gene, generated by t(17;19)(q22;p13) translocation in childhood B-cell acute lymphoblastic leukemia with a very poor prognosis, encodes a chimeric transcription factor in which the transactivation domains of immunoglobulin enhancer-binding factor (E2A) are fused to the DNA-binding and dimerization domain of hepatic leukemia factor (HLF). E2A-HLF was shown to have an anti-apoptotic effect. However, the molecular mechanism underlying *E2A-HLF*-mediated leukemogenesis remains unclear. We identified the transcriptional coactivator Eya2, the forced expression of which is known to immortalize mouse hematopoietic stem/progenitor cells (HSPCs), as a direct target molecule downstream of E2A-HLF. *E2A-HLF*-immortalized mouse HSPCs expressed *Eya2* at a high level in the aberrant self-renewal program. Chromatin immunoprecipitation-quantitative polymerase chain reaction and a reporter assay revealed that E2A-HLF enhanced the *Eya2* expression by binding to the promoter region containing the E2A-HLF-binding consensus sequence. *Eya2* knockdown in *E2A-HLF*-immortalized cells resulted in reduced colony-forming efficiency. These results suggest a critical role of Eya2 in *E2A-HLF*-mediated leukemogenesis.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most frequent childhood malignancy. Chromosomal translocations are the hallmark of pediatric ALL that generate fusion genes encoding chimeric transcription factors. The translocation t(17;19)(q22;p13) that results in the fusion gene *E2A-HLF* (1,2) defines a rare subtype of ALL that accounts for approximately 1% of pediatric B-cell precursor ALLs and is associated with a very poor prognosis (3,4).

The *E2A* gene encodes two proteins, E12 and E47, which are members of the basic helix-loop-helix (bHLH) family of transcriptions factors, and is required for proper B-cell development (5-7). *HLF* encodes a transcription factor of the basic leucine zipper (bZIP) family containing a proline- and acidic amino acid-rich (PAR) domain, which enables it to form either homodimers or heterodimers with other PAR protein family members (1,8,9). In E2A-HLF fusion protein, the two transactivation domains AD1 and AD2 of E2A are fused to the bZIP DNA-binding and dimerization domain of HLF (2). The expression of the *E2A-HLF* fusion gene results in transcriptional reprogramming with dedifferentiation in pre-leukemic cells (10). E2A-HLF promotes the anchorage-independent growth of murine fibroblasts (11). Human leukemic cells expressing E2A-HLF rapidly undergo apoptosis when programmed to express a dominant-negative mutant of E2A-HLF. In addition, the conditional expression of E2A-HLF prevents apoptosis induced by cytokine withdrawal in IL-3-dependent mouse Ba/F3 cells (12). However, B-cell progenitor-specific conditional *E2A-HLF* knock-in mice showed hyposplenia and lymphopenia, whereas hematopoietic stem/progenitor cell (HSPC)-specific ones were embryonically lethal (13). The E2A-HLF fusion likely requires additional events to cause leukemia because immunoglobulin enhancer and promoter-driven *E2A-HLF* transgenic and knock-in mice showed maturation arrest and apoptosis in cells expressing *E2A-HLF* (13-15). Several molecules downstream of E2A-HLF or cooperative with E2A-HLF have since been identified, including transcription factor LMO2, which is involved in T-cell ALL (16,17); transcriptional repressor SLUG (18); E4BP4 (19); Groucho-related genes (20); ANNEXIN VIII and sushi-repeat protein upregulated in leukemia (SRPUL), which have paraneoplastic roles in E2A-HLF-expressing leukemia (21); Zfp521 (22); SURVIVIN (23); and death receptors DR4/DR5 (24).

Drosophila eyes absent homologue 2 (eya2) protein belongs to the eyes absent (eya) family of proteins and acts as a transcriptional co-activator. Eya proteins play a critical role in fly eye development and are also involved in many processes, such as organ development, innate immunity, and DNA damage repair. Eya protein has threonine phosphatase and transactivation activities in the N-terminal domain and tyrosine phosphatase and protein-interacting activities in the C-terminal domain. Eya is located in the cytoplasm and translocated into the nucleus after binding to Six protein for transactivation (25-27). Human *EYA2* is located in chromosome 20q13 (28). *EYA2* was shown to be upregulated in ovarian and breast cancers and astrocytoma (29-31). Eya2 was also reported to promote metastasis of breast cancer cells (30) and to promote the proliferation and invasion of human astrocytoma cells (31). In contrast, silencing of *EYA2* promoted tumor growth in pancreatic adenocarcinomas, implying a tumor suppressive function of EYA2 (32). *Eya2* is differentially expressed in mouse long-term hematopoietic stem cells (33) and confers an aberrant self-renewal capacity in HSPCs (34). In addition, Eya2 is critically involved in leukemogenesis by *PLZF-RARA* resulting from t(11;17)(q23;q21) in patients with acute promyelocytic leukemia (34).

To identify effective therapeutic targets in leukemia with *E2A-HLF*, we must clarify the molecular mechanism underlying E2A-HLF-mediated leukemogenesis. We herein report the upregulation of *Eya2* by E2A-HLF through promotor binding. We also show that Eya2 has a crucial role in the aberrant self-renewal capacity conferred by E2A-HLF. *Eya2* knockdown experiments via retrovirally expressed shRNA revealed the critical involvement of Eya2 in immortalizing HSPCs. Our findings therefore identify Eya2 as one of the key players downstream of oncogenic E2A-HLF.

MATERIALS AND METHODS

Mice

All animal studies were approved by the Animal Care Committees of Mie University.

Reagents

G418 (Invitrogen, Carlsbad, CA, USA) and puromycin (Sigma-Aldrich, St. Louis, MO, USA) were used at final concentrations of 1 mg/ml and 1 μg/ml, respectively for drug selection.

Construction of the plasmids and retroviral vectors

The retroviral vectors used in this study pMYs-IRES-Neomycin^r (pMYs-IN) and pMXsU6-KO (Kusabira Orange) were previously described (35, 36). The pMYs-E2A-HLF-IN was also described (35). To produce *E2A-pre-B-cell leukemia transcription factor 1* (*E2A-PBX1*) (37) fusion fragment, a portion of *PBX1* encoding amino acid residues 89-430 (38) generated by polymerase chain reaction (PCR) was inserted into pMYs-E2A-HLF-IN to replace that of *HLF*. *E2A-HLF* mutants were prepared as previously reported (39). Mutants lacking the AD1 domain, AD2 domain, bZIP domain, and a part of the basic region, respectively, were generated by site-directed mutagenesis using PCR with the wild-type *E2A-HLF* construct as a template, followed by cloning in a series of pMYs retroviral vectors (36). The *E2A-HLF* mutants are as follows: (i) ΔAD1, which lacks 426 bp (Met-1 to Gly-142); (ii) ΔAD2, which lacks 405 bp PvuII-NaeI restriction fragment (Leu-278 to Ala-412) in the E2A transactivation region; (iii) ΔbZIP, which lacks 132 bp (Try-508 to Ala-551) in the bZIP domain of HLF; and (iv) Δ509-518, which lacks 30 bp (Ala-509 to Ala-518) in the basic region of HLF. For the chromatin immunoprecipitation (ChIP) analysis, E2A-HLF was fused with the FLAG epitope tag at the N-terminus in pMYs-E2A-HLF-IN. PCR for construction was performed with Phusion High-Fidelity DNA Polymerase (New England Biolabs Inc., Ipswich, MA, USA) according to the manufacturer's protocol. Each insert fragment in the plasmid was validated by DNA sequence analysis. For the reporter assay, *E2A-HLF* and *E2A-PBX1* cDNAs were subcloned into the pcDNA3.1+ (Invitrogen) expression vector.

Purification of mouse HSPCs

Mouse HSPCs were purified as described (35). In brief, bone marrow mononuclear cells (BMMNCs) were prepared from 8- to 12- week-old C57/BL6 mice. Using a MACS cell separation system (Miltenyi Biotec, Auburn, CA, USA), lineage (Lin)-depleted cells were isolated from BMMNCs, and c-Kit+Sca-1+Lin- (KSL) cells were purified from Lin-depleted cells using a FACSAria (BD Biosciences, Franklin Lakes, NJ, USA).

Retrovirus production and transduction

Plat-E packaging cells (40) were plated at the density of 4.5×10^5/mL and transfected next day with retroviral constructs using the Polyethylenimine "Max" (Polysciences, Inc., Warrington, PA, USA) according to the manufacturer's recommendations. Retroviral supernatants of transfected Plat-E cells were harvested 48 h post-transfection after two times of medium changes. KSL and immortalized cells were transduced with retroviruses using RetroNectin (Takara Bio Inc., Otsu, Japan) for 48-72 h at 37 ℃ as previously described (35).

Myeloid immortalization assay

Myeloid immortalization assays by serial replating were performed as previously described (35). In brief, every 5 to 7 days, colonies were enumerated, followed by replating of the harvested cells (1×10^4 cells/dish) in the methylcellulose culture medium supplemented with 25 ng/ml mouse stem cell factor (SCF), 10 ng/ml each of mouse IL-3, human IL-6, and mouse granulocyte macrophage-colony stimulating factor (GM-CSF) (Miltenyi Biotec).

To evaluate the effects of knockdown of *Eya2* in the immortalized cells, the Eya2-depleted cells were plated in the same methylcellulose culture medium as that used in immortalization assays. Relative colony-forming units (CFUs) were calculated as the percentage of the colony numbers compared with the corresponding controls (normalized to 100%) in each experiment after culture for 5-7 days.

Fluorescence-activated cell sorting (FACS) analysis

An immunophenotypical analysis was performed using a FACS Calibur (BD Biosicences) as described (41).

Gene silencing by RNA interference

The target sequences against the *Eya2* and *luciferase* genes were 5′-GTGTTTCAGAGACAATCAT-3′ (shE09) and 5′-GCCTTATGCCGCCATCTTG-3′ (shE12), and 5'-GGCTATGAAGAGATACGCC-3' (shLuc), respectively. The shE09 and shE12 are two out of twelve sequences (shE01-shE12) designed for *Eya2* knockdown, which showed most efficient effects (34). To design a short hairpin structure, a loop sequence (5′-CTTCAAGAGAG-

3′) was used (34). Short hairpin RNA (shRNA) sequence(s) against *Eya2* or *luciferase* was cloned into the retroviral vector pMXsU6-KO (35). Each 9 μg DNA of pMXsU6-KO derivative was transfected into Plat-E cells (40) in a 10 cm dish to produce retroviruses as described above. The filtered culture supernatant of Plat-E cells containing retroviruses 48 h after transfection was used for transduction of *E2A-HLF*-immortalized mouse KSL cells (2.5×10^5) for 48 h at 37 ℃ using RetroNectin (Takara Bio Inc.) in liquid culture containing SCF, IL-3, IL-6, and GM-CSF. The shRNA-transduced cells were then subjected to sorting by Kusabira Orange (KO) expression on the FACSAria. The sorted KO^+ cells were cultured for 5-7 days for colony-forming assay as previously described (35).

ChIP

ChIP was performed as described (35). In brief, the chromatin prepared from FLAG-tagged *E2A-HLF*-immortalized cells was precipitated using Dynabeads anti-Mouse IgG (Invitrogen) preincubated with a mouse monoclonal anti-FLAG (M2; Sigma-Aldrich), a mouse monoclonal anti-RNA polymerase II (CTD4H8; Millipore, Temecula, CA, USA), or a mouse IgG1 antibody (BioLegend, San Diego, CA, USA). The purified DNA in precipitates was subjected for quantitative PCR (qPCR).

PCR

Total RNA was extracted using TRI Reagent LS (Molecular Research Center Inc., Cincinnati, OH, USA). Reverse transcription (RT) was performed with random hexamers by using SuperScript II reverse transcriptase (Invitrogen) as described (35). The qPCR analyses were performed using the KOD SYBR qPCR Mix (for ChIP products; TOYOBO, Osaka, Japan) or PowerSYBR® Green PCR Master Mix (for cDNA) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) (35). For the RT-qPCR analysis, PCR was performed for 40 cycles (94°C for 15 sec, 57°C for 15 sec, and 72°C for 34 sec) in a total volume of 12 μl containing appropriately diluted cDNA. After quantifying the expression of samples using the threshold cycle ($2^{-\Delta\Delta CT}$) method and performing normalization relative to *B2m*, the relative expression was calculated. The sequences of the primers used (*Eya1, Eya2, Eya3, Eya4,* and *B2m*) have been described (34,35). For the ChIP-qPCR analysis, PCR was performed for 40 cycles (98°C for 10 sec, 60°C for 10 sec, and 68°C for 34 sec) in a total volume of 20 μl. After the CT values of ChIP products were measured and normalized with those of corresponding input samples using the $2^{-\Delta\Delta CT}$ method, the percentages of samples relative to the input were calculated. Primer sets ($Eya2_{c\text{-}4}$) for ChIP-qPCR

have been described elsewhere (34). The promoter region of *Hbb-b1* was used as a negative control. To detect the transcripts of *E2A-HLF*, PCR amplification of cDNA was performed for 30 cycles (98°C for 10 sec and 68°C for 40 sec) using LA Taq (Takara Bio Inc.). The primers specific for E2A-HLF were as follows: E2A S4, 5'-GATAGAAGACCACCTGGACGAG-3'; E2A S2, 5'-GTGAGGACTACGGCAGGGAT-3'; HLF AS1, 5'-CCAGCTCCTTCCTCAAGTCAG-3'; and HLF ASx, 5'-gaaagaattcaCAGGGGCCCGTGCCTGG-3' (small letters, non-related sequence). To detect transcripts of *B2m*, PCR amplification of cDNA was performed for 26 cycles (94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec) using Quick TaqR HS DyeMix (TOYOBO). The primers specific for *B2m* were as previously described (42).

Western blot analyses

The expression of transgenes in transfected Plat-E cells was examined by a Western blot analysis as described (43). In brief, the transfected cells were harvested with lysis buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich) and 2 mM phenylmethylsulfonylfluoride. The lysates were mixed with an equal volume of 2×SDS sample buffer and boiled for 5 minutes. Western blot analyses of the samples obtained from the cells transfected with pMYs-IN, pMYs-E2A-HLF-IN, pMYs-E2A-PBX1-IN, pMYs-ΔAD1-IN, pMYs-ΔAD2-IN, pMYs-ΔbZIP-IN, and pMYs-Δ509-518-IN were performed using mouse monoclonal anti-E2A (Yae) (sc-416; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-α-tubulin (Sigma-Aldrich) antibodies.

Luciferase reporter constructs and assays

To generate pGL3-mP, a minimal promotor (mP) sequence (derived from pGL 4.23; Promega, Madison, WI, USA) was inserted between NheI and Hind III sites in pGL3-Basic Vector (Promega). The putative promotor region of *Eya2* (-2219 to -226, relative to the ATG of translation initiation site) was inserted upstream of the mP sequence in pGL3-mP to generate pGL3-E2pro3. pGL3-E2pro3CSmut with the sequence CT*ATT*CTAGT instead of CTTACCTAGT, a putative DNA-binding sequence of E2A-HLF that is similar to the consensus sequence (CS) GTTACGTAAT (9), was generated by PCR-mediated mutagenesis. All constructs for appropriate insertions were confirmed by DNA sequence analyses. Human leukemic K562 (2.5×10^6) cells were resuspended in 400 μl of K-PBS (NaCl 30.8mM, KCl 120.7mM, Na_2HPO_4 8.1mM, KH_2PO_4

1.46mM) containing reporter (6.6 μg of pGL3-E2pro3 or pGL3-E2pro3CSmut), equimolar amounts of effecter (pcDNA-E2A-HLF, pcDNA-E2A-PBX1, or empty vector), and 0.5 μg of internal control (phTK-RL; Promega) plasmid DNAs. The reporter-to-effecter molar ratio of the DNA was 2:1. These cells were electroporated at 170 V and 950 μF in a 4 mm cuvette using Gene Pulser Xcell (Bio-Rad, Hercules, CA, USA), and were incubated in the culture medium for 48 h. Cells were harvested after incubation and lysed in 100 μl of lysis buffer (Promega). Activities of firefly and *Renilla* luciferases in each lysate were measured sequentially using a Dual Luciferase Assay System (Promega) on a luminometer (TD-20/20; Turner Biosystems, Sunnyvale, CA, USA) according to the manufacturer's instructions.

Statistical Analyses

Comparisons of bar charts between two groups were performed by a *t*-test. As for the statistical comparisons of more than two groups, first the assumption of homogeneity of variances was tested by a Bartlett's test. In a case that the variances were not equal at the 0.05 level, statistical differences were analyzed using a Friedman test as a non-parametric analysis of variance, followed by a Steel's test as post hoc multiple comparison.

RESULTS

Eya2 upregulation in E2A-HLF-immortalized cells.

KSL cells were sorted from Lin-depleted bone marrow cells, retrovirally transduced with *E2A-HLF* fusion oncogene, and subjected to myeloid immortalization assay. *E2A-HLF* transduction immortalized KSL cells (Fig. 1A-C). Since we recently revealed that Eya2 is important for inducing aberrant self-renewal activity in HSPCs (34), we analyzed the expression of the *Eya* family genes *Eya1*, *Eya2*, *Eya3*, and *Eya4* in *E2A-HLF*-transduced colony-forming cells. An RT-qPCR analysis revealed that *Eya2* was exclusively highly expressed among these genes after serial replating (Fig. 1D). Although the *Eya1* expression was higher than that of *Eya2* in the first-round colonies, the *Eya1* expression was much lower than that of *Eya2* in the third-round colonies. *Eya3* and *Eya4* were not highly expressed in either the first- or third-round colonies. These results

suggest that an E2A-HLF fusion product induced upregulation of *Eya2* in HSPCs, which might be associated with aberrant self-renewal activity in *E2A-HLF*-immortalized HSPCs.

E2A-HLF upregulates the Eya2 expression in the aberrant self-renewal program.

To investigate the molecular mechanism underlying the upregulation of *Eya2* by E2A-HLF in HSPCs, we retrovirally transduced mouse KSL cells with *E2A-HLF* and its mutants (Fig. 2A) and subjected them to myeloid immortalization assays (Fig. 4A). The immortalized cells exhibited an immunophenotype of myeloid lineage with the high expression of c-kit (Fig. 2B). Initial plating showed similar colony morphologies and reflected the transduction efficiency. In serial replating, *E2A-HLF*-transduced cells yielded and maintained increased numbers of dense colonies, whereas *E2A-HLF* mutant-transduced cells formed loose colonies that were smaller in size than those with *E2A-HLF* at the third round (Figs. 2C, 2D, and 3A) and failed to be immortalized (data not shown). A Western blot analysis showed a similar expression of each mutant protein to that of wild-type E2A-HLF (Fig. 3B). The expression of the chimeric transcripts of *E2A-HLF* and *E2A-HLF* mutants was detected by RT-PCR of total RNA extracted from the transduced colonies at the first- and third-round plating, respectively. RT-PCR using primers E2A S4 and HLF AS1 in E2A-HLF, ΔAD1, and Δ509-518 generated 497, 497, and 467 bp products, respectively, while that using primers E2A S4 and HLF ASx in ΔbZIP generated a 426 bp product, and that using primers E2A S2 and HLF AS1 in ΔAD2 generated a 696 bp product (Fig. 3C).

ΔAD1 and ΔAD2 mutants lack each transactivation domain of E2A to abolish the transactivation ability but retain DNA-binding and dimerization abilities, while ΔbZIP lacks the basic region and the leucine zipper domain of HLF to abolish DNA-binding and dimerization abilities, and Δ509-518 lacks a part of the basic region of HLF to abolish DNA-binding ability (Fig. 2A). Wild type *E2A-HLF* transduction immortalized KSL cells in serial colony replating (Figs. 1C, 4A, and 4B). Consistent with a previous report demonstrating that both transactivation domains of E2A and leucine zipper dimerization domain of HLF are essential for the transforming potential of the E2A-HLF in NIH3T3 cells (11), ΔAD2- and ΔbZIP-transduced cells failed to form colonies in serial replating. In addition, Δ509-518-transduced cells failed to form colonies (Fig. 4B). ΔAD1-

transduced cells formed some colonies at the third round, but lost the ability at the fifth round (data not shown). Transduction of *E2A-PBX1*, a fusion oncogene generated by t(1;19)(q23;p13) (37), also induced immortalization of KSL cells (Fig. 4B). RT-qPCR analyses showed that *Eya2* was highly expressed in the transduced cells with wild-type *E2A-HLF* or *E2A-PBX1*. The mutant ΔAD1- and ΔAD2-transduced cells showed an elevated *Eya2* expression, albeit to a lesser extent than the wild-type. However, ΔbZIP- or Δ509-518-transduced cells showed no induction of *Eya2* expression (Fig. 4C). It should be noted that the colony-forming ability by E2A-HLF and its mutants roughly correlates with the level of *Eya2* expression. These results suggest that both AD domains of E2A are required for the full activity of *Eya2* transactivation, while the DNA-binding and dimerization abilities of HLF are indispensable for *Eya2* transactivation in KSL-derived cells.

Eya2 is critical for E2A-HLF-mediated myeloid immortalization of HSPCs

To corroborate the involvement of Eya2 in myeloid immortalization by E2A-HLF, *Eya2* was depleted in *E2A-HLF*-immortalized cells using shRNA expression (Fig. 5A-D). The expression of *E2A-HLF* chimeric transcripts was not affected after the shRNA transduction in RT-PCR of total RNA extracted from shRNA-transduced cells (Fig. 5B). Both shE09 and shE12 showed effective depletion of endogenous *Eya2* expression in the *E2A-HLF*-immortalized cells (Fig. 5C), and *Eya2* knockdown by shE09 and shE12 reduced the clonogenicity to about 40% and 70% of that in the control at the first round of plating, respectively (Fig. 5D). We were unable to perform rescue experiments on *Eya2*-knocked down HSPCs that were immortalized with *E2A-HLF* by forced expression of *Eya2* without the shRNA target sequence, likely due to the genotoxic stress induced by *Eya2* overexpression, as the forced expression of *Eya2* resulted in dramatically reduced colony-forming efficiency in *E2A-HLF*-immortalized cells (data not shown).

E2A-HLF upregulates the Eya2 expression by binding to the promoter region.

We previously found that, in *promyelocytic leukemia zinc finger* (*Plzf*)-mediated immortalization, plzf binds to the putative *Eya2* promoter region around exon 1c of *Eya2* (34), in agreement with

public data of ChIP sequencing. Therefore, a ChIP-qPCR analysis of FLAG-tagged *E2A-HLF*-immortalized HSPCs with the primers around exon 1c was performed. The analyses showed the increased binding of E2A-HLF to the *Eya2* promoter region in the immortalized cells, accompanied by RNA pol II binding signals (Fig. 6A). These results suggest that E2A-HLF drives the aberrant expression of *Eya2* in HSPCs by promoter binding. We next confirmed the effect of E2A-HLF expression on *Eya2* promoter activity using a reporter assay. The luciferase assay in K562 cells showed that E2A-HLF and E2A-PBX1 activated the reporter gene expression through the *Eya2* promoter region (Fig. 6C), and transactivation through the *Eya2* promoter region was significantly reduced by the mutation of the putative E2A-HLF binding consensus sequence (Fig. 6B and D). Taken together, these findings suggest that E2A-HLF upregulates *Eya2* transcription through the E2A-HLF binding consensus sequence.

DISCUSSION

To clarify the mechanism underlying E2A-HLF-mediatred leukemogenesis, we examined the mechanism whereby E2A-HLF leads to transformation of HSPCs and explored therapeutic targets for novel treatments.

Since E2A-HLF alone reportedly cannot immortalize mouse hematopoietic cells *in vitro* under the lymphoid condition on irradiated stromal cells unless *BCL-2* is forcedly co-expressed (44), we employed a myeloid condition to assess the transforming potential of E2A-HLF on mouse HSPCs *in vitro*, where *E2A-HLF* expression alone is sufficient to immortalize mouse HSPCs. We found that E2A-HLF binds to the promotor of *Eya2* to elevate the *Eya2* expression in HSPCs where endogenous *Eya2* is preferentially expressed (33). We also identified the *cis*-acting element that resembles the previously reported DNA-binding consensus sequence (9) within the *Eya2* promoter region in transactivation by E2A-HLF. We recently found that the forced expression of *Eya2* using retroviral vector induces aberrant self-renewal in mouse HSPCs (34). The suppressive effect induced by *Eya2* depletion on clonogenicity of *E2A-HLF*-immortalized mouse HSPCs in the present study suggests the involvement of Eya2 in the aberrant self-renewal capacity of *E2A-HLF*-immortalized HSPCs.

Previous studies revealed the anti-apoptotic activity of E2A-HLF in IL-3-dependent murine pro-B cells through the AD1 and AD2 transactivation domains of E2A, but not the bZIP domain (12,18, 39). However, the molecular mechanism underlying the function of E2A-HLF on the self-renewal and/or cell proliferation of hematopoietic cells is poorly understood. E2A-HLF was previously shown to transactivate the *LMO2* oncogene through the AD1 and AD2 domains of E2A and the basic region of HLF (16). Similar to this finding, we revealed that both transactivation domains of E2A and the basic region of HLF are essential for the E2A-HLF-mediated transformation of mouse HSPCs. In the present study, we focused on Eya2 as one of the key molecules downstream of E2A-HLF. Eya2 as an oncogenic molecule has been shown to be involved in solid tumors with a poor prognosis (29-31). We recently found that Eya2 is critical for leukemogenesis by *PLZF-RARA* (34). The present study further showed that Eya2 is also involved in *E2A-HLF*-mediated leukemogenesis. The same might be true for E2A-PBX1, which also activated the *Eya2* expression in the present study. Therefore, Eya2 may be involved more generally in leukemogenesis, and further studies using clinical samples will be helpful to prove this hypothesis. Of note, *Eya2* knockout mice (45) seemed to have no severe abnormalities, suggesting that Eya2 may be a potential target of molecular therapy without major adverse effects for certain subtypes of leukemia.

In conclusion, this study demonstrated the critical role of Eya2 in the *E2A-HLF*-mediated transformation of mouse HSPCs *in vitro*.

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RO and TN designed the research. BDM and RO performed most and some of the experiments, respectively. BDM, RO, and TN analyzed the results. BDM and TN wrote the manuscript.

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Figure Legends

Figure 1. **Myeloid immortalization of KSL cells by E2A-HLF in association with the *Eya2* expression.** (A) Lin-depleted (Lin-) bone marrow (BM) cells are shown with the sorting gate for KSL cells. (B) The experimental strategy for myeloid immortalization assays of KSL cells with retroviral transduction of *E2A-HLF*. pMYs-IRES-Neomycin^r (pMYs-IN) was used as the backbone vector (empty) in (C, D). (C) Myeloid immortalization assays in KSL cells after retroviral transduction. The transduced cells were harvested 5-7 days after the transduction, and followed by replating of 1×10^4 cells. (D) The expression of *Eya* family genes by RT-qPCR normalized to *B2m* in the myeloid immortalization assays. *E2A-HLF*-transduced cells were harvested at the end of the first round (1R) and the third round (3R) of plating in (B). The *Eya4* expression at 3R in *E2A-HLF*-transduced cells was below the detection level. The relative induction of each *Eya* family gene expression after transduction of *E2A-HLF* was calculated in comparison with transduction of the empty vector. Bar graphs show the mean±SD of three independent experiments.

Figure 2. **A schematic representation of the chimeric proteins E2A-HLF, E2A-HLF mutants, and E2A-PBX1 used in this study and the characterization of the cells immortalized by *E2A-HLF*.** (A) The chimeric oncoproteins E2A-HLF and E2A-PBX1 produced by the t(17;19) and t(1;19) translocations, respectively, retain the N-terminal transactivation domains of E2A. The E2A C-terminus including the bHLH DNA-binding and protein dimerization domain is replaced with the bZIP DNA-binding and dimerization domain of the HLF protein and a region of PBX1 containing a homeobox DNA-binding domain, respectively. AD, activation domain; HD, homeodomain. Bold arrows indicate fusion sites. The proportions of each domain are not accurate in this representation to facilitate comparisons. (B) Immunophenotype of the cells immortalized by retroviral transduction of *E2A-HLF*. (C, D) Typical morphology of the colonies generated by E2A-HLF (C) and Δ509-518 (D) at the end of the third round. Colonies generated by the E2A-HLF mutant are smaller and less dense than those generated by the wild-type. Bars indicate 200 μm.

Figure 3. Colony formation in the methylcellulose assay by E2A-HLF and E2A-HLF mutants and the expression of these proteins. (A) Colonies generated by E2A-HLF and its mutants at the end of the third-round plating. Colonies generated by the mutants show altered morphology. **a**, E2A-HLF; **b**, ΔAD1; **c**, ΔAD2; **d**, ΔbZIP; **e**, Δ509-518. Colonies were viewed with an Olympus CKX41 microscope. Bars indicate 200 μm. (B) The expression of wild-type and mutant E2A-HLF chimeric proteins by a Western blot analysis. Lysates extracted from Plat-E cells (40) transfected with empty vector (pMYs-IN), pMYs-E2A-HLF-IN, E2A-HLF mutants (pMYs-ΔAD1-IN, pMYs-ΔAD2-IN, pMYs-ΔbZIP-IN, and pMYs-Δ509-518-IN), and pMYs-E2A-PBX1-IN were blotted with the anti-E2A antibody (αE2A), followed by reprobing with the anti-α-tubulin antibody (αTub) as an internal control. (C) The expression of *E2A-HLF* by RT-PCR in the cells from the first- and third-round cultures. *B2m* was used as an internal standard. M, 1-kb DNA ladder (New England Biolabs Inc.); lane 1, mock (pMYs-IN); lane 2, *E2A-HlF*; lane 3, Δ*AD1*; lane 4, Δ*AD2*; lane 5, Δ*bZIP*; lane 6, Δ*509-518*; lane 7, negative control (water instead of cDNA mixture); lane 8, *E2A-HlF*; lane 9, Δ*AD1*; lane 10, Δ*AD2*; lane 11, Δ*bZIP*; lane 12, Δ*509-518.*

Figure 4. **The *Eya2* expression in *E2A-HLF*-mediated immortalization of HSPCs.** (A) The experimental strategy for myeloid immortalization assays of KSL cells. The cells were retrovirally

transduced with empty vector, *E2A-HLF*, its mutants, and *E2A-PBX1* in pMYs-IN. (B) Myeloid immortalization assays in KSL cells after retroviral transduction. The transduced cells were harvested 5-7 days after the transduction, and followed by replating of 1×10^4 cells. (C) Expression levels of *Eya2* by RT-qPCR normalized to *B2m* in colony-forming cells at the end of the first (1R)- and third (3R)-round of plating in panel (A). Bar graphs show the mean±SD of three independent experiments.

Figure 5. Suppressive effects by Eya2 depletion on the clonogenicity of *E2A-HLF*-immortalized KSL cells. (A) Experimental strategy for the analysis of *E2A-HLF*-immortalized cells with Eya2 depletion by retroviral transduction of shRNA/Kusabira-Orange (KO) co-expressor in pMXsU6-KO. (B) The expression of *E2A-HLF* by RT-PCR in the sorted cells and colony-forming cells at the end of the first-round of plating. *B2m* was used as an internal standard. M, 1-kb DNA ladder; lane 1, shLuc; lane 2, shE09; lane 3, shE12; lane 4, negative control, lane 5, shLuc; lane 6, shE09; lane 7, shE12. (C) The expression of *Eya2* by RT-qPCR in the cells sorted from shRNA-transduced *E2A-HLF*-immortalized cells and subsequent colony-forming cells at the end of the first-round of plating. (D) Relative CFUs of the cells at the end of the first- and second-round of plating. CFUs: colony-forming units. Bar graphs show the mean±SD of three independent experiments.

Figure 6. E2A-HLF upregulates the *Eya2* expression by binding to the promoter region. (A) A ChIP-qPCR analysis on the *Eya2* promoter region in *E2A-HLF*-immortalized mouse HSPCs. The relative binding activity of E2A-HLF (detected by the anti-Flag antibody) and RNA polymerase II around exon 1c of *Eya2* in KSL cells immortalized by FLAG-tagged E2A-HLF is shown. The promoter region of *Hbb-b1* was examined as a negative control. Statistical analysis was performed using a Friedman test, followed by a Steel's test for post hoc nonparametric multiple comparison. * $p<0.05$. (B) A schematic representation of two reporter constructs for the luciferase assay. Mutations in the consensus sequence (9) are underlined. The position of the 5'end of the protein coding sequence in exon 2 of mouse *Eya2* is numbered +1. The consensus sequence (CS) of E2A-HLF binding is located within the amplified region by ChIP-qPCR in panel A. (C) The effects of E2A-HLF and E2A-PBX1 on the *Eya2* promoter activity in the

luciferase assay in K562 cells. (D) A luciferase assay using a wild-type *Eya2* and a mutated reporter construct containing a putative E2A-HLF-binding CS. Statistical analysis was performed by a *t*-test. * $p<0.05$. Bar graphs show the mean±SD of three independent experiments.

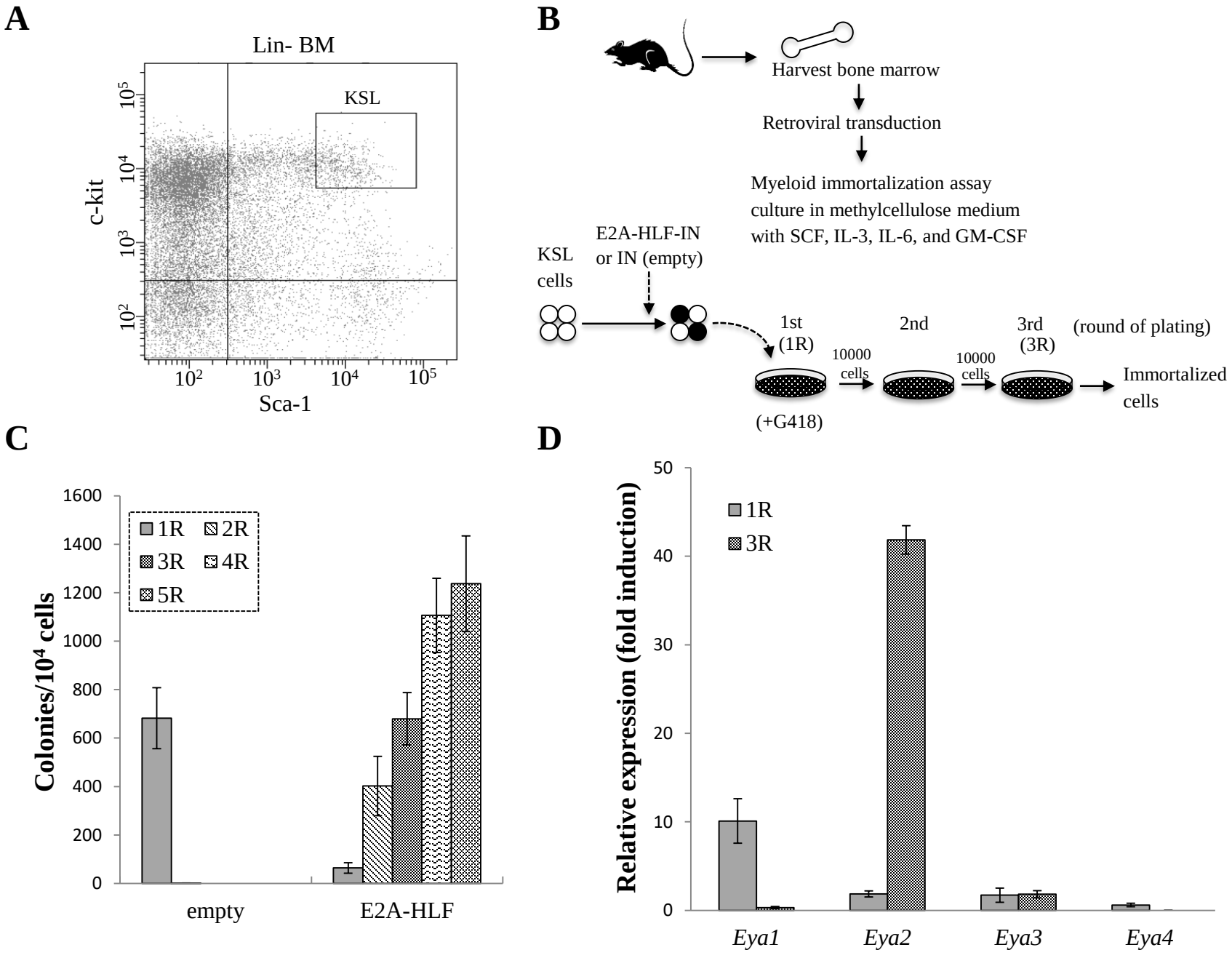
A
Lin- BM
KSL
c-kit
Sca-1
B
Harvest bone marrow
Retroviral transduction
Myeloid immortalization assay
culture in methylcellulose medium
with SCF, IL-3, IL-6, and GM-CSF
KSL
cells
E2A-HLF-IN
or IN (empty)
1st
(1R)
2nd
3rd
(3R)
(round of plating)
10000
cells
10000
cells
Immortalized
cells
(+G418)
C
Colonies/10⁴ cells
1R
2R
3R
4R
5R
empty
E2A-HLF
D
Relative expression (fold induction)
1R
3R
Eya1
Eya2
Eya3
Eya4

Figure 1

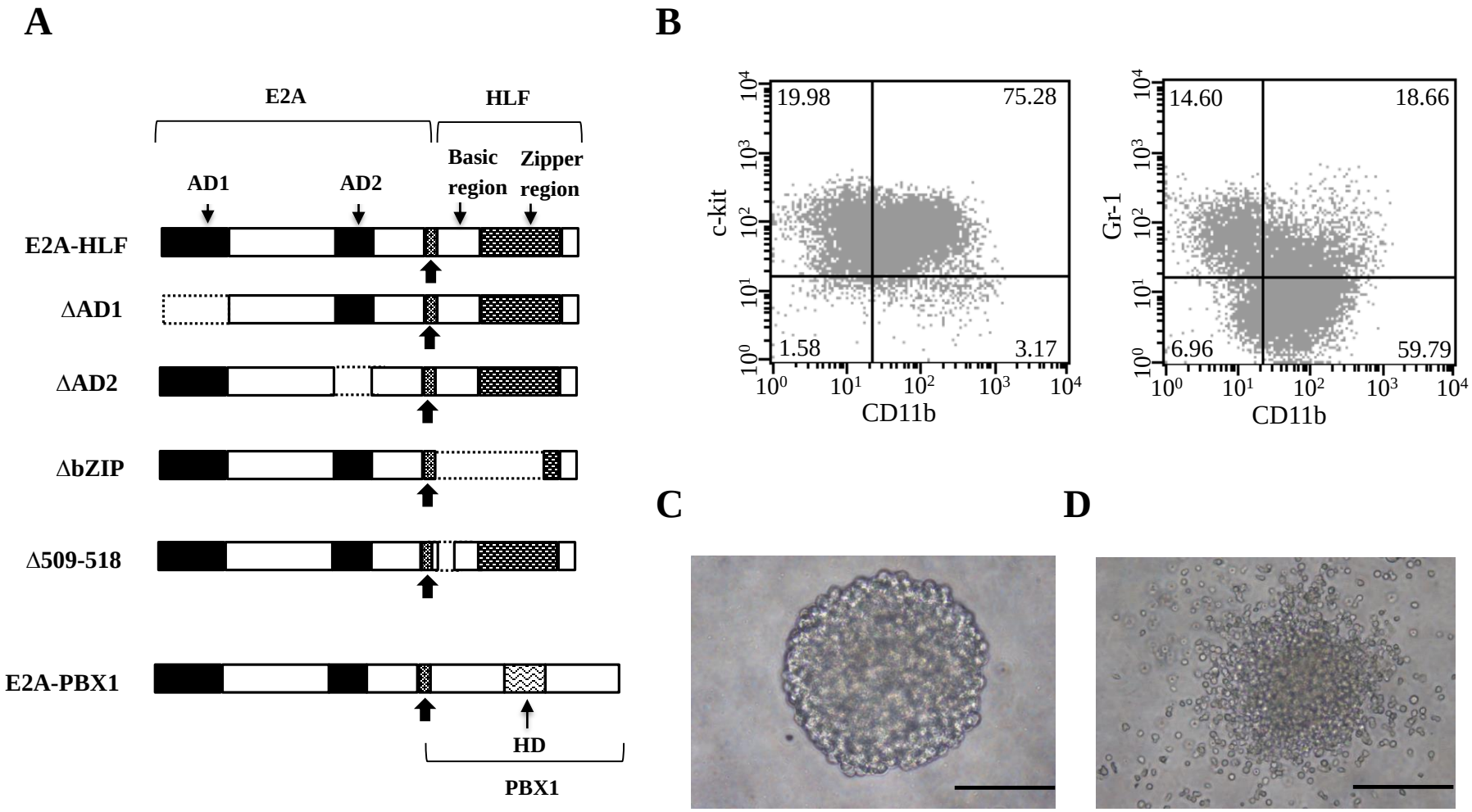
A
E2A
HLF
AD1
AD2
Basic region
Zipper region
E2A-HLF
ΔAD1
ΔAD2
ΔbZIP
Δ509-518
E2A-PBX1
HD
PBX1
B
c-kit
CD11b
19.98
75.28
1.58
3.17
Gr-1
CD11b
14.60
18.66
6.96
59.79
C
D

Figure 2

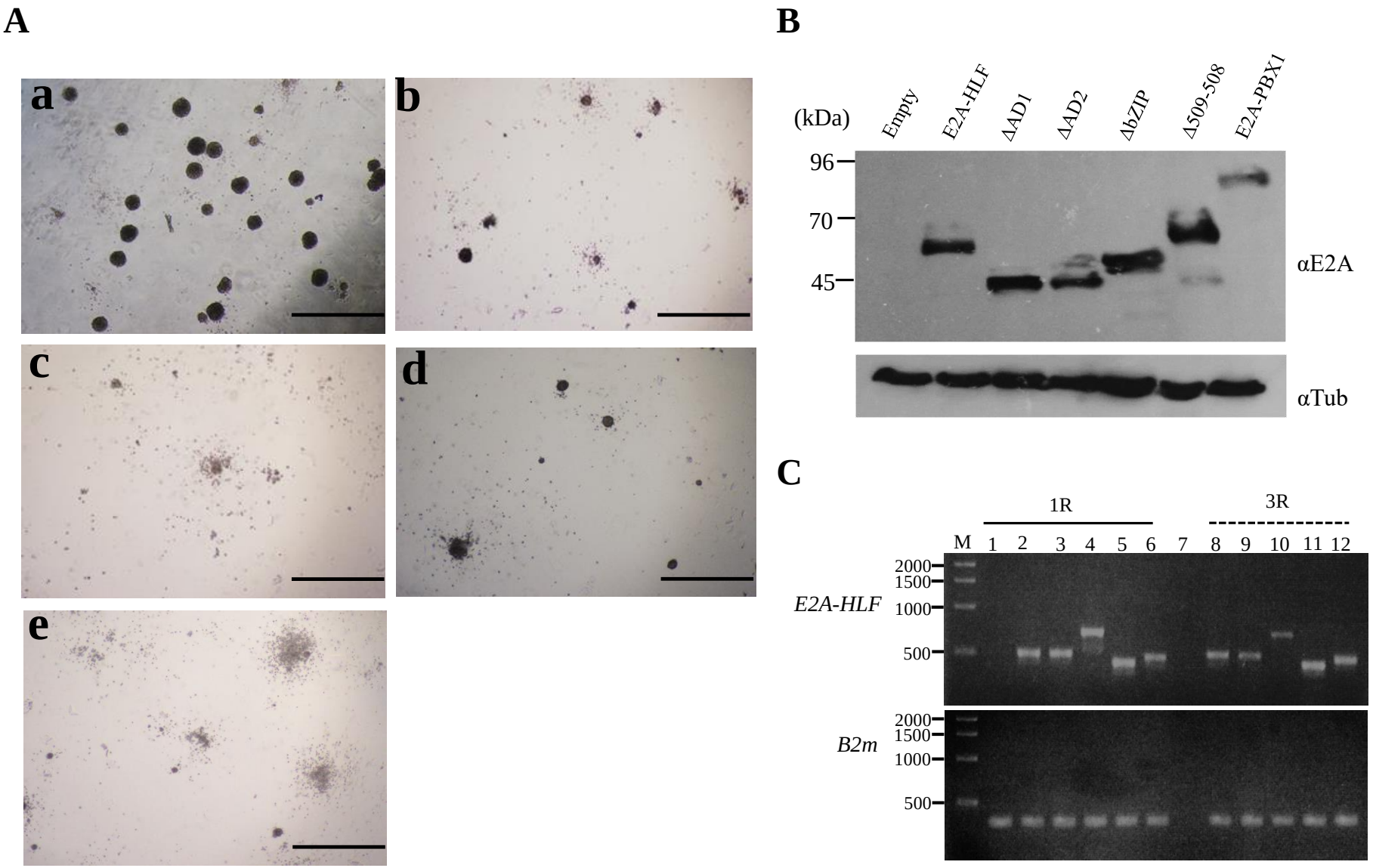
A
a
b
c
d
e
B
(kDa)
Empty
E2A-HLF
ΔAD1
ΔAD2
ΔbZIP
Δ509-508
E2A-PBX1
96
70
45
αE2A
αTub
C
1R
3R
M 1 2 3 4 5 6 7 8 9 10 11 12
2000
1500
1000
500
E2A-HLF
B2m

Figure 3

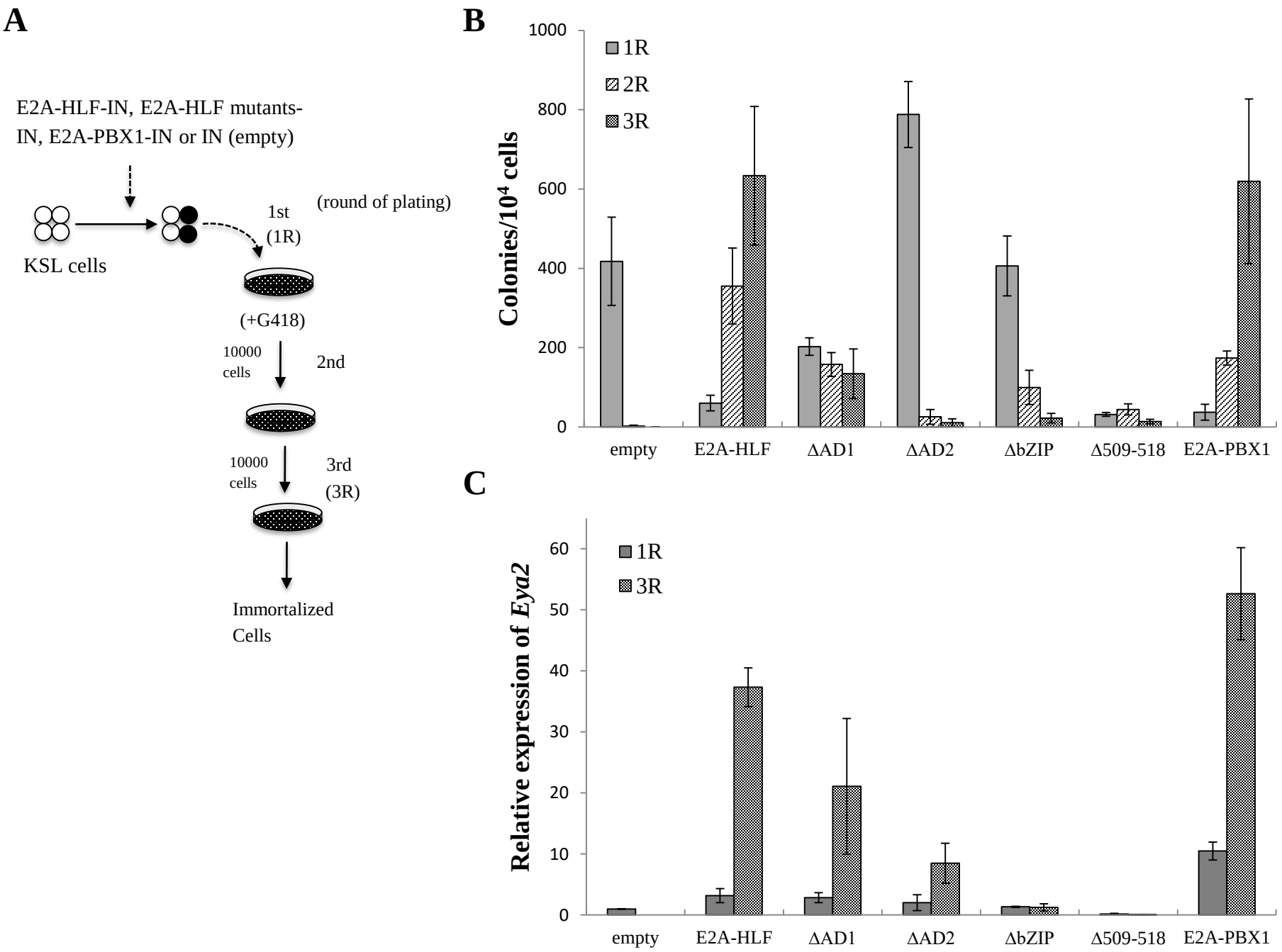
A
E2A-HLF-IN, E2A-HLF mutants-IN, E2A-PBX1-IN or IN (empty)
KSL cells
1st (1R)
(round of plating)
(+G418)
10000 cells
2nd
10000 cells
3rd (3R)
Immortalized Cells
B
Colonies/10⁴ cells
1R
2R
3R
1000
800
600
400
200
0
empty
E2A-HLF
ΔAD1
ΔAD2
ΔbZIP
Δ509-518
E2A-PBX1
C
Relative expression of Eya2
1R
3R
60
50
40
30
20
10
0
empty
E2A-HLF
ΔAD1
ΔAD2
ΔbZIP
Δ509-518
E2A-PBX1

Figure 4

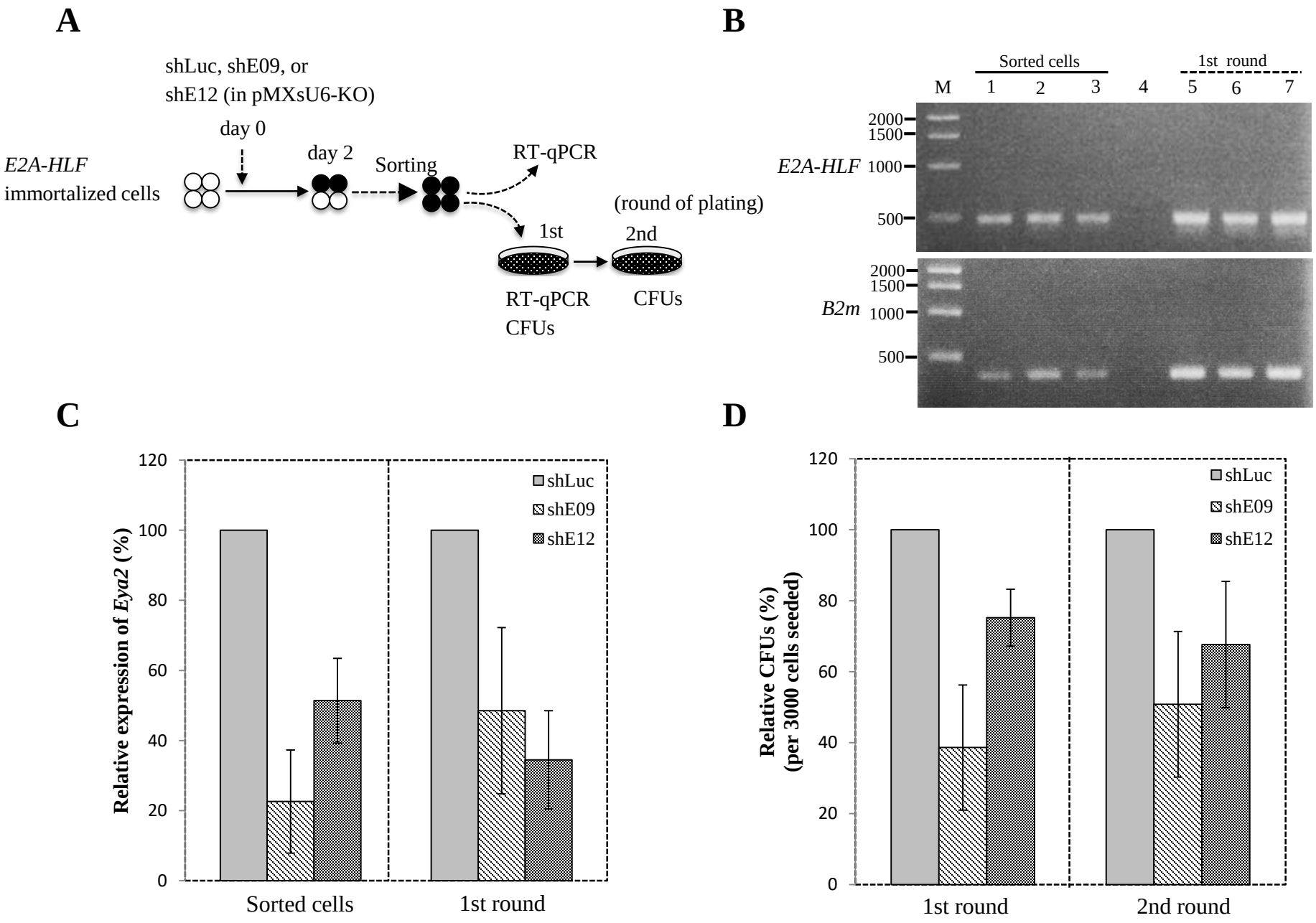
A
shLuc, shE09, or
shE12 (in pMXsU6-KO)
day 0
day 2
Sorting
RT-qPCR
E2A-HLF
immortalized cells
(round of plating)
1st
2nd
RT-qPCR
CFUs
CFUs
B
Sorted cells
1st round
M
1
2
3
4
5
6
7
2000
1500
1000
500
E2A-HLF
B2m
C
Relative expression of Eya2 (%)
120
100
80
60
40
20
0
shLuc
shE09
shE12
Sorted cells
1st round
D
Relative CFUs (%)
(per 3000 cells seeded)
1st round
2nd round

Figure 5

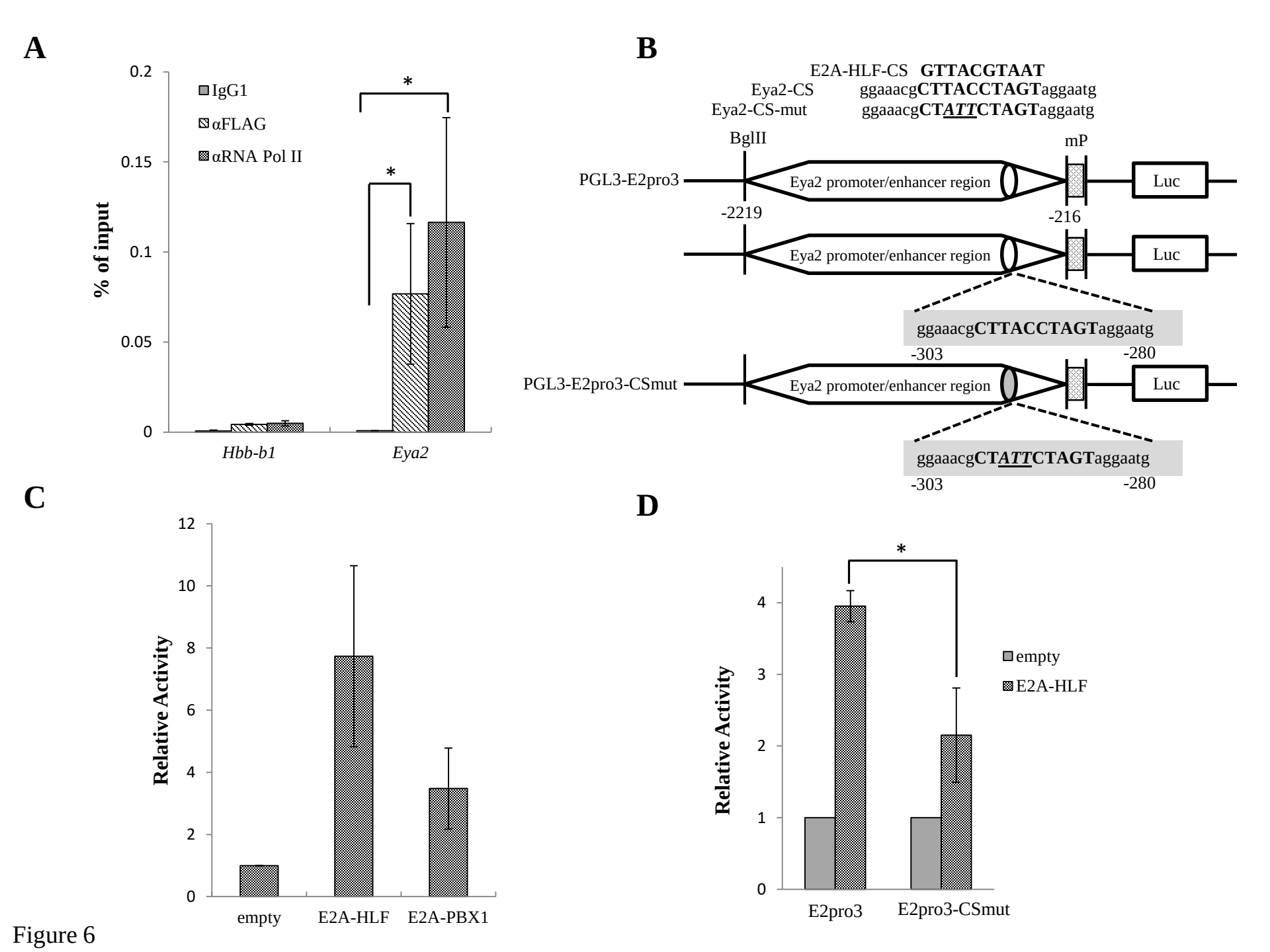
A
0.2
0.15
0.1
0.05
0
% of input
IgG1
αFLAG
αRNA Pol II
*
*
Hbb-b1
Eya2
B
E2A-HLF-CS GTTACGTAAT
Eya2-CS ggaaacgCTTACCTAGTaggaatg
Eya2-CS-mut ggaaacgCTATTCTAGTaggaatg
BglII
mP
PGL3-E2pro3
Eya2 promoter/enhancer region
Luc
-2219
-216
Eya2 promoter/enhancer region
Luc
ggaaacgCTTACCTAGTaggaatg
-303
-280
PGL3-E2pro3-CSmut
Eya2 promoter/enhancer region
Luc
ggaaacgCTATTCTAGTaggaatg
-303
-280
C
12
10
8
6
4
2
0
Relative Activity
empty
E2A-HLF
E2A-PBX1
D
*
4
3
2
1
0
Relative Activity
empty
E2A-HLF
E2pro3
E2pro3-CSmut

Figure 6