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Ikaros represses and activates PU.1 cell-type-specifically through the multifunctional *Sfpi1* URE and a myeloid specific enhancer

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Abstract

Generation of myeloid and lymphoid cells from progenitors involves dynamic changes in transcription factor expression and use, and disruption of hematopoietic transcription factor function and expression can contribute to leukemic transformation. PU.1 and Ikaros are pivotal factors whose expression and utilization are dynamically altered during hematopoietic development. Here, we demonstrate that expression of PU.1, encoded by the *Sfpi1* gene, is divergently regulated by Ikaros in distinct cell-type-specific contexts. ChIP analysis and functional perturbations revealed that Ikaros can directly repress or activate *Sfpi1* transcription via different PU.1 cis-elements, with PU.1 and Ikaros collaborating at myeloid-specific elements but not at other elements. Our results thus shed light on how PU.1 and Ikaros can act as lineage competency factors to facilitate both myeloid and lymphoid developmental programs.

Keywords

cis-regulatory element; hematopoiesis; chromatin immune precipitation; context dependent regulation; RNA polymerase II

Introduction

The proto-oncogene PU.1, upregulated in virally-induced erythroleukemias, is an Ets family transcription factor encoded by the *Sfpi1/Spi-1* gene (1). PU.1 plays critical roles in multiple hematopoietic cell types (2, 3) and has emerged as a key determinant regulating cell-type specification.Higher PU.1 dosage restricts erythrocyte specification and enables generation of committed myeloid progenitors or joint myeloid/lymphoid progenitors, the lymphoid-primed multipotent precursors (LMPP) (2, 4). Expressed at the LMPP stage, PU.1 levels rise in myeloid progenitors, but fall in pre-pro-B-cells (5, 6). Differences in PU.1 level strongly affect specification of myeloid vs. B-cells from LMPPs (7). However, PU.1 deficiency blocks both myeloid and B-cell development and reduced PU.1 dosage promotes AML (8, 9). Thus, precise transcriptional controls of PU.1 expression are required.

The mechanisms controlling PU.1 expression as LMPPs become myeloid or B-cells remain unclear. PU.1 positively autoregulates by binding the *Sfpi1* promoter and URE, a compound enhancer element located ~14 kb upstream of the *Sfpi1* transcriptional start site (10, 11, 12,

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Conflicts of interest
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13). Additional myeloid-specific enhancers have also been found between the URE and the promoter (14, 15). A recently proposed gene regulatory network model for the differential control of PU.1 expression in B-cell versus myeloid specification suggests that attenuation of PU.1 autoregulation by two transcription factors, Gfi-1 and Ikaros, can regulate these cell fate decisions (13).

Many cell types that depend on PU.1 also depend on Ikaros. However, the optimal ratios of these two factors diverge in myeloid and B lymphoid lineages (16), and it is unclear whether Ikaros and PU.1 work collaboratively or antagonistically. Ikaros is a lineage determinant with a critical role in priming the lymphoid developmental program in HSCs and progenitors, and mice lacking Ikaros also develop myeloid hyperproliferation (17, 18). The dominant negative Ikaros isoform 6 has been linked with AML (19). Although PU.1 and Ikaros collaborate to control at least one important target in LMPPs (20, 21),genetic evidence suggests that Ikaros opposes PU.1 to support B-cell development over alternative myeloid programs (13, 22). Indeed, expression of a dominant negative version of Ikaros in erythroid cells increased PU.1 levels (23). Because of the central position of both factors in lymphoid developmental gene networks, and their roles in leukemia, it is crucial to resolve whether Ikaros is indeed a direct repressor of PU.1.

Here, we show that Ikaros binds distinct subsets of *Sfpi1* cis-elements, including the URE, in myeloid and B lineage cells. Whereas Ikaros suppresses URE activity in pre-B-cells, we show that Ikaros is a cell-type-specific URE activator in myeloid cells. Furthermore, we find that Ikaros, PU.1 and RNA polymerase II are co-recruited and collaborate to drive activation through additional myeloid-specific enhancers. These results resolve the bifunctional role of Ikaros in PU.1 regulation through lineage-specific recruitment to distinct cis-elements that direct both Ikaros and PU.1 functional specificity.

Results and Discussion

A ~3 kb DNA fragment, CE5-CE3, is a myeloid enhancer lacking B-cell regulatory function

After PU.1 is expressed in LMPPs, its levels diverge between myeloid and lymphoid cells (5). We recently described a *Sfpi1* cis-regulatory region, CE5-CE3, which had cell-type-specific functions. The CE5 element, on its own or as part of CE5-CE3, was an enhancer in a myeloid cell line but not in a pro-T-cell line (14). To determine whether this function is myeloid specific or common to all PU.1 expressing cells, we tested CE5-CE3 function in B lineage cells.

Reporter constructs were made to test segments of the *Sfpi1* upstream region joined to the *Sfpi1* promoter element, CE1 (Fig 1a). In NFS-25 pre-B-cells the construct containing CE5-3 (L5-3) generated similar luciferase expression as L1, the promoter only construct, but in RAW264.7 myeloid cells L5-3 produced strongly enhanced reporter expression (Fig. 1b). In contrast, the conserved URE elements CE9 and CE8 within the L98 reporter enhanced luciferase expression similarly in pre-B and myeloid cells alike (Fig. 1b). Cell-type-specific CE5-CE3 activity was also demonstrated in a chromatin context in stably transfected cell lines. We tested reporters comprising the URE and CE7/6 either without CE5-CE3 (L9-6) or with it (L9-3). L9-6 generated similar luciferase expression in pre-B and myeloid cells. However, L9-3 gave stronger reporter activity only in the myeloid cells (Fig. 1c), implying that CE5-CE3 enhancer activity is myeloid restricted.

CE5 and CE7/6 are myeloid-restricted PU.1 target elements

To identify potentially important transcription factor binding sites within CE5, we used TRANSFAC to analyze regions of CE5 that were conserved in multigenome alignments. As the CE7/6 region may contribute to myeloid specific PU.1 regulation (15), we also examined

CE7/6. The most frequently predicted sites in both CE5 and CE7/6 are overlapping Ikaros and Ets family sites (Fig. 1d). Ikaros is a bifunctional transcription factor able to activate or repress transcription, and Ikaros can compete with Ets factors for the same sites (24). Ikaros, which is required for B-cell development, might constrain PU.1 expression by blocking PU. 1 autoregulation through the URE (13). However, in our myeloid and B-cell lines the URE drove similar levels of reporter activity. Thus, the myeloid specific enhancer activity of CE5-CE3 and its predicted Ets family sites suggested that myeloid restricted PU.1 autoregulation could contribute to the functional specificity of CE5-CE3.

PU.1 chromatin immune precipitation (ChIP) in fact revealed cell-type-specific patterns of PU.1 occupancy at *Sfpi1* regions. PU.1 bound the promoter region (CE1) in myeloid cells (Fig. 2a, red panel) as previously reported (11), but that binding was weak compared with the strong PU.1 binding at the URE elements CE9 and CE8 in these cells (Fig. 2a, red panel). PU.1 also bound CE8 in pre-B-cells (Fig. 2a, blue panel), as well as known PU.1 target genes, *Mef2c* and *Il7r,* in both PU.1-expressing cell lines (red and blue panels)(25, 26); Adh.2C2 pro-T cells, which lack PU.1, showed no binding (green panel). However, only in the myeloid cells was strong PU.1 binding found at CE5 and CE7/6 (Fig.2a, arrows).

PU.1's failure to bind some *Sfpi1* regions in pre-B-cells could be due to other Ets factors competitively binding in preference to PU.1. However, while three Ets factors expressed in the cell types examined, Elf-1, Ets-1, and GABPα, could bind some PU.1 target sites, only PU.1 bound strongly to CE7-CE5 (Fig. 2b-2d)(27).

The URE can recruit RNA polymerase II (Pol II)-containing complexes in PU.1-expressing cells (28, 29). Pol II association with other *Sfpi1* regions was also cell-type-specific. Pol II occupied CE8 in all cell lines tested, but bound to the promoter (CE1) only in PU.1-expressing cells (Fig. 2e). Significantly, however, Pol II bound CE5 and CE7/6 only in the myeloid cells (Fig. 2e, arrows). These results support a functional role for CE5 in myeloid specific PU.1 regulation as well as CE7/6 (15).

Cell-type-specific binding of Ikaros at *Sfpi1* cis-elements

The presence of numerous predicted Ikaros binding sites across CE5, and the absence of PU. 1 binding in the pre-B-cell line, suggested that Ikaros might compete with PU.1 to block PU. 1 autoregulation. Anti-Ikaros ChIP iindeed revealed cell-type-restricted patterns of Ikaros binding. Ikaros bound CE9 and CE8 in macrophages and pre-B-cells (Fig. 3a). Much lower Ikaros binding was seen in the pro-T-cell line, despite high Ikaros expression (discussed below). Additionally, Ikaros uniquely bound CE10 in pre-B-cells, a region that may participate in *Sfpi1* repression in erythroid cells (30)(Fig. 3a, blue arrow). Surprisingly, Ikaros did bind CE7/6 and CE5 too, but only in myeloid cells (Fig. 3a, red arrows).

The cell-type-specific Ikaros association patterns observed imply distinct, context-specific rules for Ikaros recruitment. PU.1 but not Ikaros bound *Il7r* in RAW264.7 (Fig. 2a), whereas Ikaros occupied *Il7r* in Adh.2C2 cells that lack PU.1 expression (Fig. 3a, green arrow). Thus Ikaros binding strength is determined separately from PU.1 at *Il7r*. In contrast, the unique pattern of Ikaros occupancy of *Sfpi1* sites in the myeloid cells suggested that Ikaros recruitment to CE5 and CE7/6 in RAW264.7 cells might involve co-occupancy with bound PU.1.

PU.1 can recruit Ikaros DNA binding

To test whether PU.1 binds concomitantly with Ikaros at the CE5 sites, a DNA probe was made from the CE5 region (CE5-P2) to span adjacent sites predicted to bind PU.1 and Ikaros (Fig. 1d, box). The CE5-P2 probe nucleated myeloid specific complexes in gel shift experiments (Fig. 3b, lanes 1-6 vs. 7-12; M1, M2, and M3). M1, M2, and M3 contained PU.

1, because anti-PU.1 antibody supershifted those complexes (Fig. 3b, lane 5). Additionally, an anti-Ikaros antibody blocked formation of the PU.1 containing M1 complex (lane 6). Mutation of the overlapping PU.1/Ikaros site (mutated in CE5-P2m1) destroyed the ability to compete for the myeloid complexes (lanes 2, 3). Although mutation of the Ikaros specific site in CE5-P2m2 did not prevent competition for M1, M2, and M3 (lane 4), it did prevent probe CE5-P2m2 from nucleating these complexes itself (lanes 13-18). Thus distinct PU.1 and Ikaros binding sites in CE5 may collaborate to form myeloid-specific PU.1-Ikaros complexes. Similar results were found with a probe spanning predicted Ikaros-PU.1-Ikaros sites in CE7/6 (data not shown).

Ikaros could be recruited to CE5-P2 using in vitro translated PU.1 generated in a reticulocyte lysate transcription/translation system. Lysate containing PU.1 could form complex M1 while lysate lacking PU.1 could not (Fig. 3c, lanes 2, 3; arrow). In this system, which contains endogenous Ikaros (31), the M1 complex was sensitive to both anti-PU.1 and anti-Ikaros antibodies (lanes 8, 9). Furthermore, formation of the M1 complex by PU.1 was also blocked by addition of in vitro translated “Plastic”, a dominant negative point mutant of Ikaros that heterodimerizes with wildtype Ikaros family members to inhibit DNA binding (32)(lane 6). Thus PU.1 and Ikaros binding to CE5 can be coordinate, not competitive.

Effect of PU.1 dose on recruitment of PU.1 and Ikaros to *Sfpi1* sites in vivo

The CE5 site where PU.1 and Ikaros co-bind in myeloid cells is not bound by the myeloid driver C/EBPα (15). We therefore tested whether high dose PU.1 alone is sufficient to bind and recruit Ikaros to this site. Adh.2C2 pro-T-cells, which normally express no PU.1 but ≥2x more Ikaros protein than RAW264.7 cells (Fig. 3d), were retrovirally transduced with PU.1 (~60% of cells transduced, data not shown), then analyzed by ChIP to track occupancy patterns of endogenous Ikaros and the exogenous PU.1. Transduced Adh.2C2 cells expressed >2x more PU.1 RNA than RAW264.7 cells (Fig. 3e), and the ectopic PU.1 strongly bound to *Mef2c, Il7r* and both URE elements (Fig. 3f). The presence of PU.1 in PU.1-transduced Adh.2C2 cells now enabled Ikaros to be recruited to CE9 (Fig. 3g), in contrast to untransduced cells (Fig. 3a). Ikaros also bound *Il7r* more strongly (Fig. 3g). Additionally, Pol II now also bound CE9 and *Il7r* in the Adh.2C2+PU.1 cells (Fig. 3h).

However, despite the high dose, PU.1 did not bind CE5 and could only weakly bind CE7/6, similar to NFS-25 cells. Similarly, the myeloid-specific elements where PU.1 and Ikaros bind together were not occupied efficiently by either factor in the PU.1-overexpressing lymphoid cells. Thus, high-level PU.1 is not the sole determinant involved in cell-type-specific CE5 and CE7/6 activity.

PU.1 and Ikaros binding sites are required for CE5 enhancer activity

Gel shift analysis resolved potential PU.1 and Ikaros target sites within CE5 that might be required for CE5 enhancer activity. We tested the functional contribution of those sites by constructing additional L5-3 reporters harboring mutations in these CE5 sites (Fig. 4a). When the m1 and m2 sites, required for the PU.1- and Ikaros-containing M1 complex (Fig. 3b), were mutated in L5-3m1-2, enhancer activity in myeloid cells was abolished (Fig. 4a). Additionally, mutation of another overlapping PU.1/Ikaros site in CE5 also abolished CE5-3 enhancer activity (L5-3m4). In contrast, mutation of a predicted C/EBP family site (L5-3m3) did not affect reporter activity. These results indicate that joint PU.1 and Ikaros sites are required at CE5 for its myeloid cell-specific enhancer function.

PU.1 mediates CE7/6-CE5 enhancer activity

To establish whether the myeloid-specific PU.1 binding is functional, we compared the effects of PU.1 knockdown on the activities of L98 and a reporter that contains both CE7/6

and CE5 joined to the PU.1 promoter (L7-5). Antisense morpholinos (E1 and E2) that target different PU.1 exon/intron boundaries eliminated detectable PU.1 protein expression in myeloid cells as compared with a nonspecific control morpholino (Fig. 4b). PU.1 knockdown not only reduced L98 activity ~40% (Fig. 4c), as expected, but also completely abolished the enhancer function of L7-5 (Fig. 4c). Thus while the URE is PU.1 responsive but partially PU.1 independent, CE7-CE5 strictly requires PU.1 for enhancer activity in these myeloid cells.

Lineage-specific effects of Ikaros on *Sfpi1* cis-elements: positive regulation in myeloid cells but inhibitory activity in pre-B-cells

To determine why Ikaros, a suspected inhibitor of PU.1 expression, should be specifically corecruited to CE7-CE5 under conditions where these elements act as enhancers, we tested the effect of neutralizing Ikaros in distinct cellular contexts, by transfecting *Sfpi1* cis-element driven luciferase reporters into B and myeloid cells with empty vector or the "Plastic" dominant negative mutant of Ikaros. Figure 4d shows that indeed, cotransfection of B-lineage cells with Plastic to neutralize endogenous Ikaros enhances reporter expression driven by the URE (L98), consistent with a repressive role for Ikaros in these cells. In contrast, however, cotransfection with Plastic strongly reduced the activity of the L98 and L7-5 reporters in myeloid cells (Fig. 4e). Thus, via co-occupancy of CE5 and CE7/6 with PU.1, Ikaros is a positive regulator of *Sfpi1* in macrophages.

Concluding remarks

These results show that the PU.1 and Ikaros factors can act both as collaborators and as antagonists in different cellular contexts, based on their cell-type-specific recruitment to distinct subsets of their potential DNA target sites (Fig. 4f). The sites examined here for lineage-specific differential recruitment of PU.1 and Ikaros are all conventionally recognizable based on established position weight matrices, but engagement by these factors is highly context dependent. At the myeloid-specific cis-regulatory elements of *Sfpi1*, neither PU.1 nor Ikaros is sufficient to establish binding in the NFS-25 pre-B or Adh.2C2 pro-T cell lines, yet these elements can mediate potent positive regulation in myeloid cells dependent on both PU.1 and Ikaros. Crucially, the ability of Ikaros to bind with PU.1 at the CE8-CE9 elements themselves is associated with opposite functional consequences in B and myeloid cells. Thus, not only can lineage determinants affect when and where Ikaros will bind DNA, but these distinct patterns of recruitment confer an additional layer of cell-type-specific regulatory interaction which allows Ikaros' functional contributions to be reversed in distinct developmental contexts.

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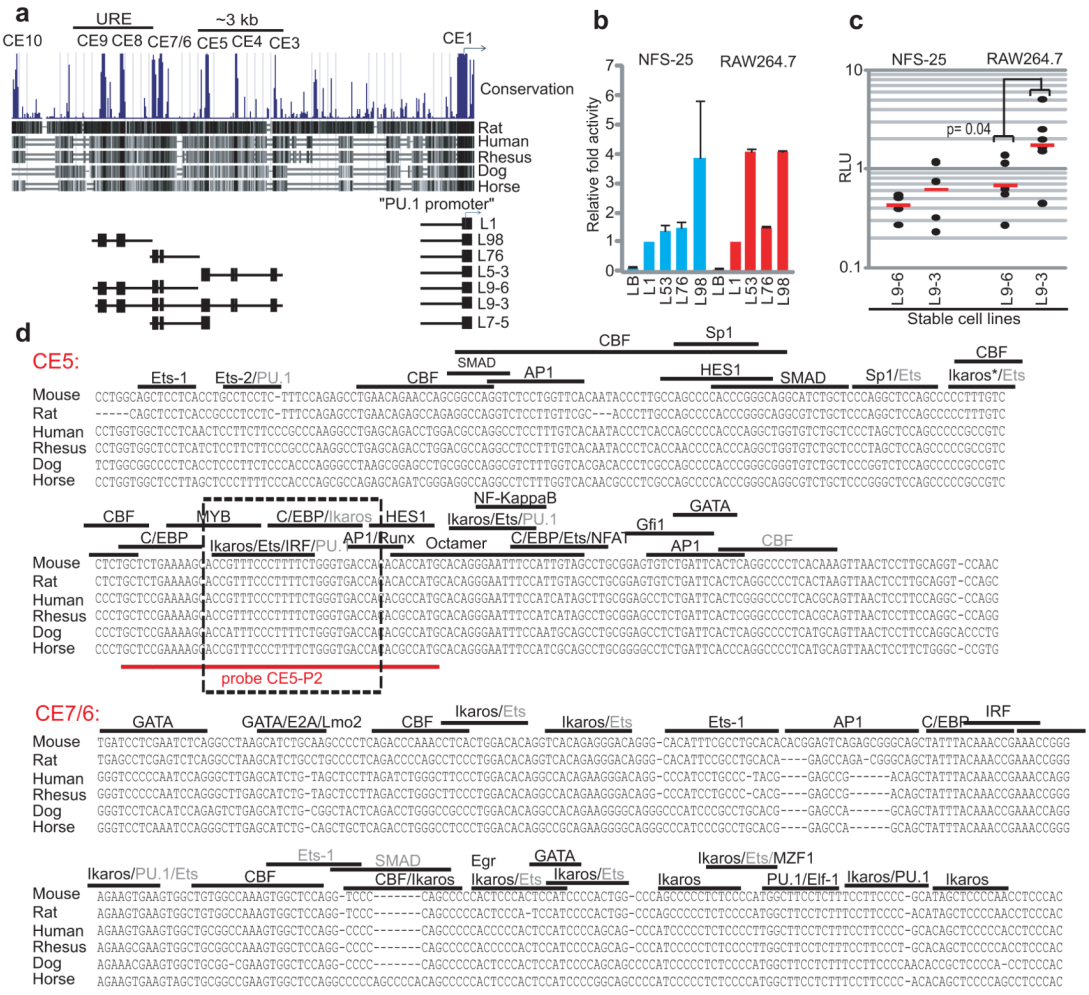

Figure 1.

The *Sfpi1*fragment CE5-CE3 is a myeloid restricted enhancer. (a) A schematic showing the UCSC mammalian conservation track with genome alignment of the ~18 kb upstream region of the *Sfpi1* gene is shown. Regions used in reporter constructs are depicted. Reporter constructs were designed as described previously (14). (b) Transient transfection assays showing *Sfpi1* reporter activity in NFS-25 pre-B-cells and RAW264.7 macrophages. Data represent the average fold difference relative to L1. NFS-25 data are from five independent experiments. RAW264.7 data shown are from a single representative experiment performed in triplicate. Error bars represent standard deviations. Cells were transfected using FuGENE at a 3:1 DNA:FuGENE ratio. NFS-25 cells were grown in RPMI media with 10% fetal bovine serum, penicillin/streptomycin/glutamine, non essential amino acids, sodium pyruvate, and 50 μM 2-mercaptoethanol. RAW264.7 cells were grown in DMEM media with 10% fetal bovine serum and penicillin/streptomycin/glutamine. Transfected cells were harvested 30-48 hours post transfection. Cells were cotransfected with pRL-CMV and lysates were analyzed using Promega's Dual Luciferase system. (c) The CE5-CE3 fragment is a myeloid specific enhancer when integrated into chromatin. Cell lines were stably transfected with the L9-6 and L9-3 constructs depicted in Fig 1A. Bars show the geometric mean of six independent mixed pools (dots) of stably transfected cells 30 days post transfection. Data are reported as relative light units (RLU). P-value is from student's T-test of $\log_{10}$ transformed data. FuGENE:DNA complexes were formed then the same complexes

were aliquot to 6 well plates containing either NFS-25 or RAW264.7 cells. For stable transfections, *Sfpi1* reporters were linearized with Not I prior to transfection. The renilla luciferase was cloned into Invitrogen's pTracer EF/Bsd A and the construct was linearized with Fsp I for cotransfection with *Sfpi1* reporters. Cells were selected with 5-15 μg/ml of Blasticidin for their duration in culture, beginning one day post transfection. (d) Multigenome alignments and transcription factor target site prediction analysis of the CE5 and CE7/6 regions are shown. TRANSFAC analysis was performed through the Biobase TRANSFAC suite's MATCH tool (https://portal.biobase-international.com/cgi-bin/portal/login.cgi). Predicted hematopoietic transcription factors with matrix similarity matches above 0.9 are shown (black). Some matches below 0.9 are also shown (gray). CBF = Core Binding Factor sites for the Runx family. "Ets" labeled sites are general Ets family sites that potentially bind to multiple Ets family factors. Asterisks mark conserved sites present in only 4/6 aligned sequences. All other sites are present in all six sequences. The boxed sequence shows a region of CE5 with a PU.1 target site adjacent to a specifically predicted Ikaros target site. The red bar represents the DNA probe CE5-P2 designed for gel shift assays (Fig. 3).

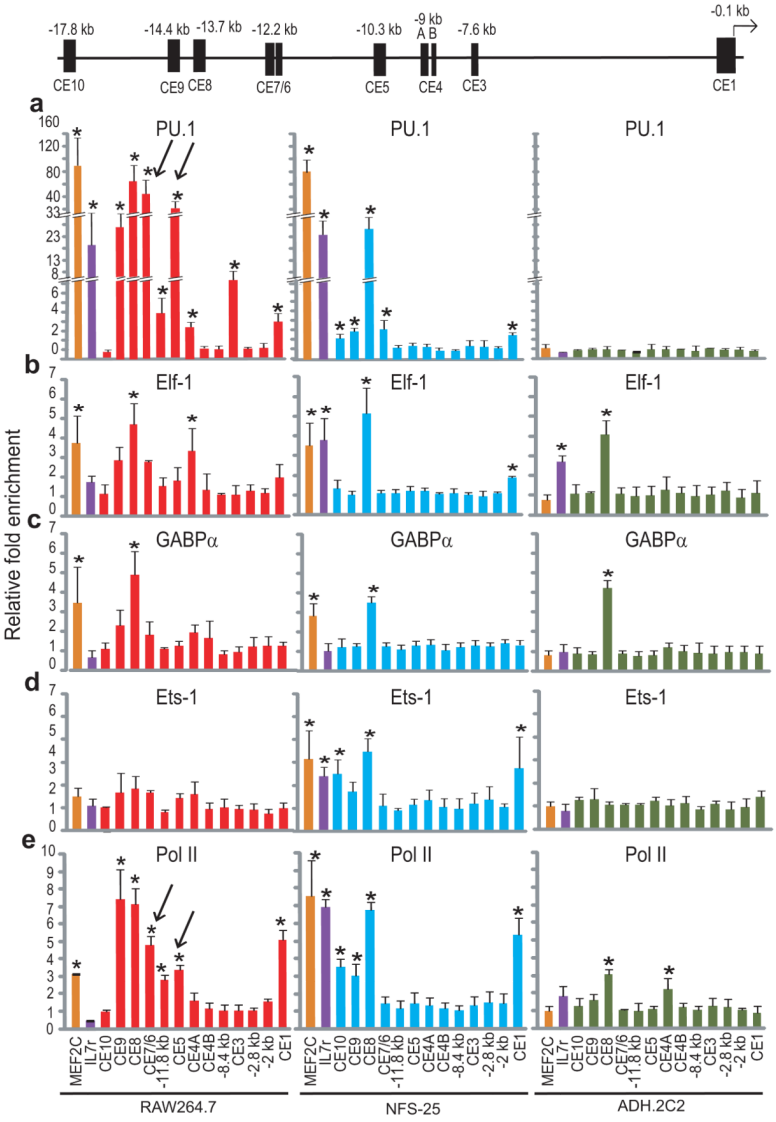

Figure 2.

ChIP analysis reveals restricted and cell-type-specific PU.1 and Pol II binding. A schematic of the regions examined in the ChIP assays is shown. The Conserved Elements are labeled below and approximate locations of forward primers used for QPCR analysis are labeled above. (a), Anti-PU.1 ChIP analyses are shown. ChIP assays were performed using anti-PU. 1 antibody (sc-352 from Santa Cruz). ChIP assays were performed based on Upstate's protocol exactly as described previously (14). Data shown in all ChIP panels are from three or more independent experiments. Orange and purple bars in each panel show data for positive control regions of reported PU.1 target genes *Mef2c* and *Il7r*. Panels with red bars are from myeloid cells (RAW264.7). Panels with blue bars are from pre-B-cells (NFS-25). Panels with green bars represent data from pro-T-cells (Adh.2C2). Primer pairs used for each region are labeled on the x-axis and the primer sequences are reported in (14). ChIP enriched DNA was analyzed by QPCR in triplicate for each experiment. Individual ChIP experiments were normalized first to input DNA then normalized against a region that lacked enrichment to provide relative fold enrichment. Independent experiments were then averaged. Error bars show standard deviations. Iterative one-way ANOVA was used to

analyze ChIP data. ANOVA was initially performed with all regions. If ANOVA generated a p-value < 0.01, data for "regions of interest" were removed from the initial ANOVA test group and ANOVA was repeated on data from the remaining regions until these generated p>0.1. The regions remaining in the test group that lacked a statistically significant enrichment difference were then used as a control group against which to compare the removed "regions of interest" individually by T-tests. The resulting p-values were adjusted using the Bonferroni correction method and regions with $p < 0.0005$ were marked by asterisks in ChIP data figures. (b) Elf-1 ChIP assays using anti-Elf-1 antibody sc-631 are shown as described. (c) GABPα ChIP assays using anti-GABPα antibody sc-22810 are shown as described. (d) Ets-1 ChIP assays using anti-Ets-1 antibodies sc-22802 and sc-111are shown as described. (e) RNA polymerase II ChIP analyses are shown as described using anti-RNAPoll II antibody ab5408 from AbCam.

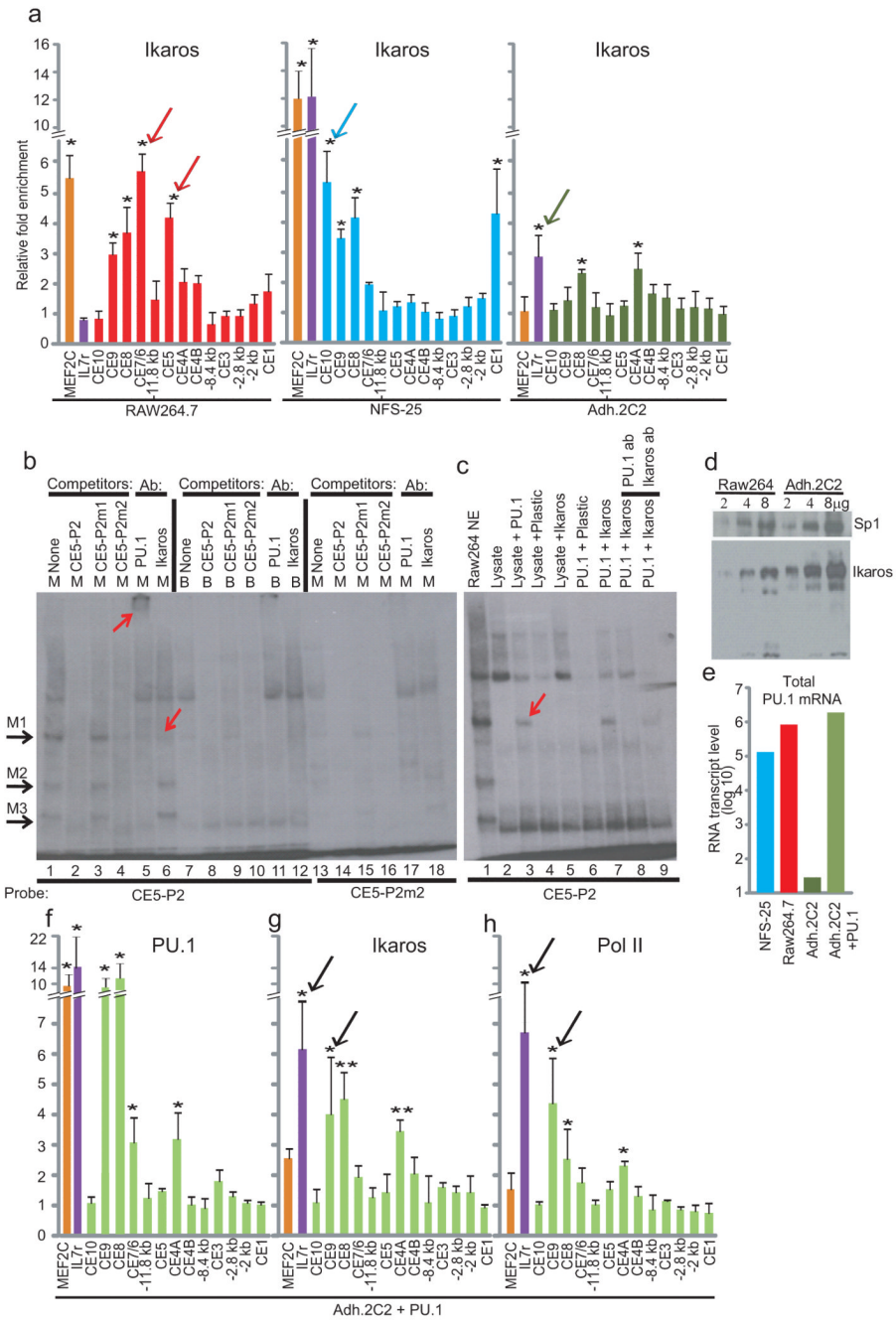

Figure 3.

Cell type specific binding of Ikaros with PU.1 at *Sfpi1* cis-regulatory elements. (a) Ikaros binds the URE region in RAW264.7 and NFS-25 cells, but binds other regions with cell-type-specificity. Ikaros ChIP assays were performed using antibodies Santa Cruz sc-13039 and Active Motif #39291. Data are presented as in Fig. 2. (b,c) The CE5 region nucleates cell-type-specific myeloid complexes in vitro that contain PU.1 and Ikaros proteins. (b) Probe CE5-P2 (see Fig. 1d) and CE5-P2m2 (Ikaros site mutated; see Fig. 4a for mutated sequence) were radiolabeled and incubated with nuclear extracts from RAW264.7 cells (M) or NFS-25 cells (B). Preparation of nuclear extracts and binding conditions were described in (14). Complexes were resolved by 6% SDS PAGE, dried, and then exposed to film.

Myeloid specific complexes are labeled M1, M2, and M3. Anti-PU.1 and anti-Ikaros antibodies used in gel shift experiments are sc-352 and Active Motif #39291, respectively. The overlapping PU.1/Ikaros binding site was mutated in probe CE5-P2m1. The exact sequences mutated for m1 and m2 are shown in red in the schematic in Fig. 4a. Competitor DNA probes were used at 250 fold molar excess; 4 μg of antibodies were used as labeled. (c) PU.1 can direct formation of a PU.1 and Ikaros containing complex on probe CE5-P2. TnT Couple Quick Transcription/Translation system (Promega # L1171 and L2081) was used to generate PU.1, Ikaros, or Plastic proteins. RAW264.7 nuclear extract was included (lane 1) for comparison. All other lanes contained equal volumes of reticulocyte lysate (treated or untreated). For treated lysates, 1 μg of plasmid DNA was incubated with lysates to generate proteins following manufacturer's protocol. Ten μg of total lysate were run per lane. (d) Western blot of Ikaros and Sp1 protein. Nuclear extracts (2-8 μg) from Adh.2C2 and Raw264.7 cells were separated on an 8% SDS-PAGE gel. This blot had been previously probed for Sp1 and was shown in (14). The blot was stripped and reprobed here with anti-Ikaros antibody (sc-13039). (e) Adh.2C2 cells transduced with PU.1 retroviral vector express high levels of PU.1 mRNA. Retroviral vector supernatants were prepared by transfection of pMX-PU.1-IRES-hCD8 plasmid into Phoenix packaging cells with FUGENE 6 reagent. Virus particle-containing medium was collected at 48-72 hours post transfection. Adh.2C2 cells were subsequently transduced to express PU.1 using the TAKARA RetroNectin method. RNA was prepared using Trizol and manufacturer's protocol. cDNA were prepared from RNA using the Superscript III system (Invitrogen #18080-400), then analyzed for PU.1 sequence by quantitative real-time PCR. PU.1 levels were normalized against GAPDH. Data are plotted on a $\log_{10}$ scale. (f) Forced expression of PU.1 in immature T-cells permits site-restricted PU.1 binding. PU.1 ChIP experiments are shown as described previously. Data shown are from five independent transduction and ChIP experiments. (g and h) Ectopic PU.1 expression facilitates restricted recruitment of Ikaros (g) and Pol II (h) to the PU.1 target regions CE9 and *IL7r* (black arrows). Data shown are from two independent experiments. *$p<0.0005$. **$p<0.002$.

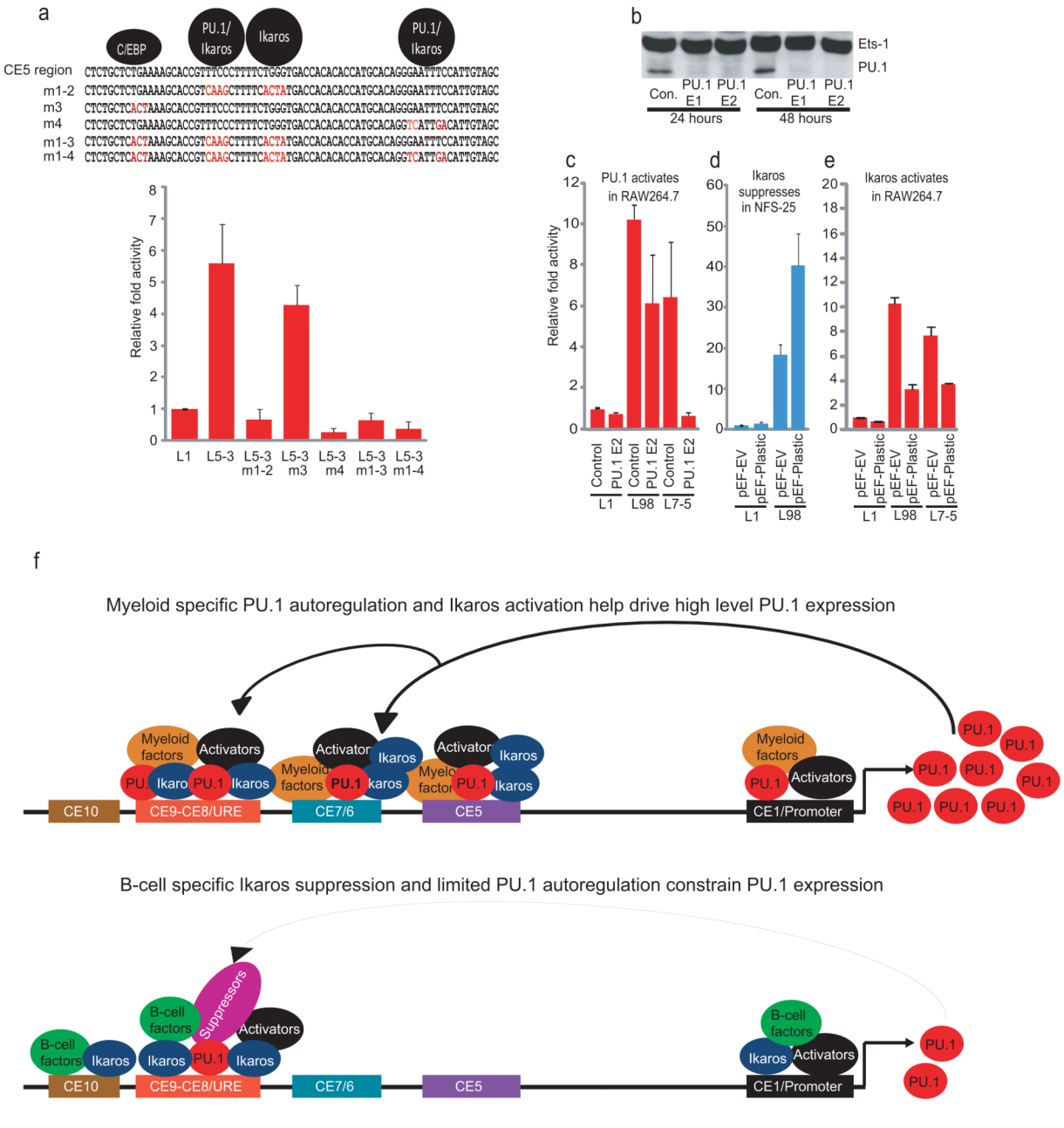

Figure 4.

PU.1 and Ikaros activate *Sfpi1* enhancers in myeloid cells, but Ikaros suppresses the URE enhancer in pre-B-cells. (a) PU.1 and Ikaros target sites in CE5 are required for enhancer activity in RAW264.7 myeloid cells. Schematic shows the CE5 region spanning the CE5-P2 probe and flanking sequence. Nucleotides mutated in the CE5 region of the L5-3 *Sfpi1* reporters are shown in red. Data show the average fold increases in activity of L5-3 and mutated reporters relative to L1, with error bars representing standard deviations. Data are from three independent experiments each performed in duplicate. Reporter plasmids (4 μg) were cotransfected with 0.25 μg of control plasmid pCMV-RL into 5-6 million cells. For transfection conditions see below. Cells were harvested ~24 hours post transfection for analysis. (b) Anti-PU.1 morpholino antisense oligonucleotides can knock down PU.1 protein

expression. 32Dcl5 myeloid precursor cells were used to test the morpholinos and were grown in RPMI medium supplemented with IL-3. Morpholinos E1, overlaps exon1/intron1 boundary; and E2, overlaps exon2/intron2 boundary were ordered from Gene Tools, Inc.: anti-PU.1 (E2) GAGGACCAGGTACTCACCGCTATG; anti-PU.1 (E1) GTAGTGAAGCCCCAGTACTCACAGG; Standard Control oligo CCTCTTACCTCAGTTACAATTTATA. 32Dcl5 cells were nucleofected with 2 picomoles of morpholinos using Solution-V kits and program E-32. Cell samples were harvested for analysis at 24 and 48 hours. The Western blot was probed using anti-PU.1 antibody (sc-5948) and anti-Ets-1 (sc-350) as a control. (c) PU.1 knockdown abolishes L7-5 reporter activity. (d) Ikaros contributes to myeloid enhancer activity in RAW264.7 cells. (e) Ikaros suppresses URE enhancer activity in NFS-25 pre-B-cells. Raw264.7 and NFS-25 cells were cotransfected with *Sfpi1* reporters and pEF empty vector (EV) or pEF-Plastic (Ikaros dominant negative). Transfection data in each panel (c-e) are from three independent experiments performed in duplicate. Error bars show standard deviations. Data are shown as fold difference relative to L1+Control (c) or L1+pEF (d,e). RAW264.7 cells were transfected with *Sfpi1* reporters and 2 picomoles of standard control morpholino or anti-PU.1 morpholino. Transfection and cotransfection were performed by Nucleofection (Lonza/Amaxa) using Solution-V kits and program D-32 for RAW264.7 cells or program A-33 for NFS-25 cells. (f) Schematic depicts cell-type-specific patterns of PU.1 and Ikaros binding to *Sfpi1* cis-elements and the differences in their functional consequences for transcriptional control.