Investigating the Transcriptional Mechanisms Controlling *Sfpi1*, a Critical Regulatory Node within Multiple Lineage Specifying Subcircuits of the Hematopoietic Gene Regulatory Network

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

2010

Defended December 11, 2009

Acknowledgements

Properly acknowledging people cannot be accomplished in a few sentences, but perhaps I should start this not by acknowledging others, but by first acknowledging my own journey.

In many regards, over the past several years I have been fighting a war against maturity, both intellectual and emotional. No doubt this was done with great consternation felt by those witnessing it. My realization that I was engaged in this covert war was bittersweet. After all, it was a war I was actually winning! Be that as it may, once recognized, I had to seek an accord with the real world after realizing the war was best abandoned. I am still suffering from my wounds, but they will heal and I will be better for it.

So to those who watched from the sidelines as I raged my battles, I thank you for your patience and understanding and your willingness not to abandon hope.

Most especially I have to thank my wife Melanie, without whom I may have fallen in battle. I must also thank my advisor Ellen Rothenberg who frequently and generously attempted to triage my wounds even though I was often too blind to realize it.

Next to Ellen, Shelley Diamond has done more than anyone to help make my servitude productive and always showed me a willingness to help that I will no doubt appreciate and miss for years to come.

Of course I am grateful to my committee, Eric Davidson, Paul Sternberg, and Barbara Wold, for not tossing me out.

To everyone who passed through the Rothenberg lab, I learned from you all.

I have to acknowledge Devin Tesar since he acknowledged me. Really though, Devin opened my world back up to social consciousness and introduced me to people who have greatly increased the quality of my life over the past ~3 years. The first on that list is Dave Seal who has been a joy to interact with. Graeme Fordyce has also contributed to my joy, quite literally by giving me lots of his money through poker. Yeah, I went there. To the rest of the JPLers, Scott, Eric, Ly, Dave T., Charlie, you have all provided me with important outlets. Thank you.

Abstract

The *Sfpi1* gene encodes PU.1, a critical transcription factor in multiple hematopoietic lineages. PU.1 expression is upregulated as hematopoietic stem cells become granulocyte-macrophage progenitors. In contrast, *Sfpi1* must be silenced after progenitors undergo T-lineage specification. If unrestrained in early T-lineage cells, PU.1 can both block developmental progress and induce diversion to a myeloid fate. When PU.1 expression is not sufficiently increased or maintained in myeloid lineage cells, myeloid hyperproliferation and cancer can result. In mouse DN thymocytes, PU.1 mRNA begins at high levels in early T-cell progenitors, but drops about fivefold as cells enter the T-cell program (DN2) and then falls tenfold further as the cells reach T-lineage commitment (DN3). This implies operation of a stage-specific repression mechanism correlated with commitment. Only one major cis-regulatory element has previously been described for *Sfpi1*, which is a compound conserved region around -14 kb that is thought to mediate activation as well as some repression. However, it cannot account for all PU.1 regulation in early T-lineage cells nor in myeloid cells. In particular, that -14 kb element can show strong enhancer activity in an immature T-cell line in which the endogenous *Sfpi1* gene is profoundly repressed. Additionally, absence of the -14 kb element does not abolish PU.1 expression in myeloid lineages. We now present evidence for another complex of conserved noncoding elements that appear to mediate several cell-type-specific regulatory features, including cell-type-specific repression in early T-cells. We describe fine mapping of a T-cell specific bipartite silencer and show that the T lineage specific repressive activity requires Runx1. We also describe additional regulatory complexes that may contribute to lineage specific regulation of PU.1 in early hematopoietic progenitors, including a myeloid specific enhancer. We provide evidence

of lineage restricted occupancy of these additional regulatory elements and show that the novel enhancer elements are additional sites of PU.1 auto regulation.

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Chapter 1:

Introduction

Hematopoietic Stem Cells, a Paradigm of Hierarchical Development

Differential gene regulation provides the foundation of all biology. In embryogenesis differential gene expression is the pivotal means by which the genetic program produces an organism with a predetermined DNA encoded body plan. In a developing mouse embryo, as early as the fifth cleavage, or 16 to 32 cell stage, asymmetric distributions of cytoplasmic material, particularly maternal transcripts within blastomeres, has generated spatial cues within the mutlicellular aggregate of the morula that lead to cellular differences in transcriptomes whose processing by a cell's assemblage of cis regulatory elements directs commitment of the first two discernable cell lineages, cells of the inner cell mass and trophoectoderm, a point at which totipotency begins to be lost (Suwinska et al. 2008). As embryogenesis continues, cell to cell interactions and/or signaling gradients from secreted molecules continue to provide spatial and temporal cues that lead to continued alterations in the regulatory transcriptome of new cells, ultimately giving rise to a programmed body plan. Sometime after embryogenesis many organisms also undergo various types of maturation, such as sexual maturation where an organism generates additional pronounced physical changes to the body plan, often accompanied by changes in cell lineages and gain of novel cellular functions. These changes are typically inevitable but temporally distinct in onset, reflecting another deeper layer of programming below that used in embryogenesis. Additionally, complex organisms must have developmental programs for homeostasis. It is currently accepted that rare stem cells exist in multiple tissues, each able to give rise to a subset of cellular lineages that replace and maintain healthy tissue throughout an animal's lifespan. How the genome encodes multiple, diverse, developmental programs and how these programs are used hierarchically to produce distinctive body plans of

mixed cell types at the correct time and place is perhaps the most fundamental of all biological questions.

We now know that such developmental programs are the product of highly controlled differential gene regulation through networked cis elements whose lineage specific outputs are controlled by the combinatorial interplay of multiple transcription factors. To understand how the regulatory circuitry of networked cis elements process the information represented by the regulatory transcriptome (the combination of all transcription factors of a cell), giving rise to various developmental outcomes, the critical regulatory nodes within the developmental networks must be identified and dissected to uncover the logic of their functional interactions.

Blood development from hematopoietic stem cells (HSCs) has historically been the most accessible mammalian developmental program. The hematopoietic lineage is one of the earliest to arise in mammalian development, being specified from the first set of mesodermal precursors generated by gastrulation (reviewed in Kyba and Daley, 2003). Since first being isolated more than 20 years ago (Spangrude et al. 1988) HSCs and their development into immune cells has served as a paradigm for stem cell biology and the study of gene regulatory networks. The immune system represents a complex developmental system that is particularly amenable to studying hierarchical gene regulatory networks (GRNs). Cells of the immune system can be isolated in relatively pure form with various developmental stages delineated by surface proteins. This accessibility has facilitated perturbation and monitoring of lineage decisions that has led to the identification of numerous factors involved in lineage choice, allowing the elucidation of regulatory subcircuits involved in cell fate decisions within the

hematopoietic compartment. However, many pleiotropic effects and the factors that drive them are still undergoing identification and characterization, with new models of hematopoiesis being reiterated as evidence accumulates.

A Hierarchical Model of Hematopoietic Lineage Development

Recent work has led to a revised model of hematopoiesis (Figure 1) where short- and long-term hematopoietic stem cells (LSK: $Lin^{-}Sca\text{-}1^{Hi}c\text{-}kit^{Hi}$) can be distinguished by their expression of the various surface markers, including CD34. These cells are self renewing and must develop within a developmental gene regulatory network that is designed to enable maintenance of the HSC program while facilitating the ability of some cells to become nonself-renewing committed progenitors that subsequently enter various lineage specifying programs. Short-term $CD34^{+}$ HSCs (ST-HSC) give rise to a mixed population of VCAM-1^{+} multipotent progenitors (MPPs). Various primed and/or lineage restricted progenitors emerge from the MPP population that can be further delineated by relative expression of the receptor tyrosine kinase Flt3, which mediates cell survival and proliferation (Adolfsson et al. 2001 and 2005; Lai et al. 2005; Lai et al. 2006).

The MPP population contains the earliest lineage restricted progenitors, CMPs, GMPs, and LMPPs. The $Flt3^{-/Lo}$ VCAM-1^{+} common myeloid progenitor (CMP) generates either a committed megakaryocyte/erythroid progenitor (MEP) or a committed granulocyte-macrophage progenitor (GMP), but not lymphoid cells (Lai and Kondo, 2006). The MPP population also contains a committed lymphoid primed multipotent progenitor (LMPP) that has lost the capacity to generate erythrocytes or megakaryocytes. This early segregation of lineage potential is marked by high expression of Flt3, with

LMPPs being LSK CD34^{+}Flt3^{Hi} (Adolfsson et al. 2005). The VCAM-1^{-} LMPP fraction of the MPP population has lost most GM potential and preferentially produces early lymphoid progenitors (ELPs) that express Rag1, a factor critical to B and T-cell receptor rearrangement (Igarashi et al. 2002; Lai and Kondo, 2006). ELPs are thought to be precursors to early T-cell progenitors (ETPs) and common lymphoid progenitors (CLPs), the latter gives rise to B-cells and is distinguished by earlier expression of IL-7Rα which mediates cell survival and proliferation (Allman et al. 2003). LMPPs are also precursors to Flt3^{Hi} VCAM-1^{+} GMPs.

To understand how the process of commitment and lineage specification through these progenitor states is canalized to produce terminally differentiated cell types it is critical to identify the transcription factors expressed by the first HSCs and onward down their developmental hierarchy. Understanding how these factors are regulated, what their targets are, and how the combinatorial use of multiple factors might facilitate specification through multiple accessible lineage choices is a massive endeavor, but through identifying and dissecting the cis regulatory modules controlling critical nodes, in conjunction with genetic analysis, the entire hierarchical gene regulatory network can be constructed to advance understanding of lineage commitment from HSCs down through T-cells.

Factors Controlling Emergence and Maintenance of HSCs

Identifying critical nodes and their functions within the hematopoietic gene regulatory network should begin with an understanding of how HSCs themselves first

emerge and form a stable population of self-renewing cells. It was recognized nearly a century ago that hematopoiesis shares origins with vasculogenesis, but what transcription factors and signaling pathways are pivotal to the process of HSC development has only recently been revealed (reviewed in Kyba and Daley, 2003). The further question of how these factors are networked to specify HSC emergence from the precursor of endothelial progenitors and HSCs, the hemangioblast, is still being clarified. Work with xenopus, zebrafish, and mouse embryos has led to construction of a tentative evolutionarily conserved core hemangioblast regulatory circuit (Liu et al. 2008; Pimanda et al. 2007). At the top of this circuit, Fli-1 expression is initiated and becomes autoregulatory, possibly with input from the bone morphogenic pathway (BMP) that functions through activation of the Smad family of transcription factors. Next, the BMP pathway, Notch signals, and Fli-1 drive GATA2 expression, followed by Fli-1 and GATA2 driven expression of the bHLH factor SCL. SCL then feeds back into GATA2 (and Fli-1 in the mouse). In Pimanda and colleagues' murine hemangioblast circuit, SCL and GATA2 are also autoregulatory (Gering et al. 1998; Kobayashi-Osaki et al. 2005). The hemangioblast circuit also requires the function of SCL's cofactor Lmo2, which can be regulated by SCL and Fli-1 and forms a complex with SCL and GATA factors to positively regulate targets (Liu et al. 2008; Landry et al. 2005). It should be noted however, that SCL can bind corepressors like Sin3A and silence target genes, therefore reserving the potential to disrupt the same networks in which it promotes feed forward regulation in earlier developmental stages (Huang and Brandt. 2000). Together, Fli-1, GATA2, and SCL/Lmo2 maintain the transient hemangioblast regulatory state until specification toward endothelium or HSCs is triggered.

Hemangioblast specification to HSCs requires further BMP signaling impingement on the hemangioblast core circuit, resulting in a complex of SCL/ GATA2 /Lmo2, Ets factors, and Smad factors coordinating the initiation of Runx1 expression through binding to Runx1's +23 enhancer element and its distal promoter (Pimanda et al. 2007; Nottingham et al. 2007). A failure to turn on Runx1 results in death due to its essential role in definitive hematopoiesis (Okuda et al. 1996). Notch signals also contribute to Runx expression, however it is unclear if the effect is mediated by Notch regulation of GATA2 (Burns et al. 2005; Robert-Moreno et al. 2005; de Pooter et al. 2006). After initial expression, Runx1 is autoregulatory through Runx1 target sites in its distal promoter, and possibly through additional Runx1 binding to its +23 enhancer (Ghozi et al. 1996; North et al. 1999; Telfer and Rothenberg, 2001; Nottingham et al. 2007).

SCL is absolutely required for the emergence of HSCs and continues to be expressed with critical roles in megakaryopoiesis and erythropoiesis. While SCL is dispensable for HSC maintenance and function an additional bHLH family member, LYL1, compensates for loss of SCL and loss of both impairs HSC function (Mikkola et al. 2003; Souroullas et al. 2009). In contrast to SCL, Fli-1 remains essential for normal hematopoietic maintenance and function, as does Erg, another Ets family member that may facilitate Runx1 activation in cooperation with GATA2, Fli-1, and SCL (Pimanda et al. 2007; Loughran et al. 2008; Kruse et al. 2009). Runx1 was also recently found to be dispensable for HSC maintenance and function in postnatal animals (Chen et al. 2009). In fact, an increase in the number of LSK $CD34^-$ LT-HSCs has been observed with Runx1 excision, indicating that Runx1 may function to limit HSC self renewal in addition to its roles in differentiation (Ichikawa et al. 2004). As $Runx1^{-/-}$ mice die in early

gestation due to a lack of definitive hematopoiesis, Runx1 targets appear critical for HSC maintenance and function (Okada et al. 1998). Therefore a closer examination of defects in Runx1$^{-/-}$ mice could reveal additional critical nodes within the hematopoietic GRN.

Sfpi1, the Gene Encoding PU.1, Is a Critical Runx1 Target and Is First Expressed in Emerging HSCs

Runx1$^{-/-}$ fetal liver lacked expression of Flt3, PU.1, and the pan-hematopoietic Vav, suggesting that one or more of these factors are downstream targets of Runx1 and might have a role in maintenance of HSCs (Okada et al. 1998). While Flt3 is critical to early progenitors, it is not expressed by the LT-HSCs, implying that Vav and/or PU.1 might have effects in LT-HSCs. As Runx1 is dispensable to HSC function once Vav expression is initiated, and PU.1 has been shown to regulate Vav, Flt3, and itself, Runx1's initiation of PU.1 expression may be one of its critical functions in HSCs (Denkinger et al. 2002; DeKoter et al. 2002; Okuno et al. 2005). Consistent with this, targeted deletion of *Sfpi1* in HSCs revealed it contributes to their maintenance and function, (Kim et al. 2004; Iwasaki et al. 2005). Furthermore, forced expression of PU.1 in SCL$^{-/-}$ embryonic stem (ES) cells, followed by in vitro differentiation, partially rescued myeloid lineage development (Tsuneto et al. 2005). Since PU.1$^{-/-}$ mice are deficient in more than myeloid lineages (Scott et al. 1994), failure to rescue additional lineages in the SCL$^{-/-}$ suggests that SCL or its targets, such as Runx1, may be needed by PU.1 for combinatorial regulation of additional lineage programs. Together, these results indentify *Sfpi1* as a critical Runx1 regulated node in emerging HSCs.

Sfpi1 is a Critical Node within the Hematopoietic GRN, with Unique Functions in Multiple Developmental Stages

The *Sfpi1* gene product PU.1, also called Spi-1, is a member of the divergent Spi subfamily of the E26 transformation-specific (Ets) transcription factor family, which also includes Spi-B and Spi-C (Laudet et al. 1999; Bemark et al. 1999). PU.1 was first identified as a critical hematopoietic factor when it was observed that proviral DNA integration into its upstream region resulted in upregulation of PU.1 expression. This *Sfpi1* deregulation led to erythropoietin independent erythroblastic leukemia in mice (Moreau-Gachelin et al. 1988). Fli-1 is also a frequent target of proviral insertion with deregulation contributing to leukemia, underscoring the critical need to maintain proper expression of Ets family members in hematopoietic development (Ben-David et al. 1991).

The Spi family is distinguished from other Ets family members through unique functional domains and their relatively low DNA binding domain homology (40%) with Ets-1. In contrast to lower homology with Ets-1, PU.1, Spi-B, and Spi-C Ets domains are 60% to 73% homologous. While PU.1 and Spi-B are divergent across their N-terminal region, there is some low homology. In contrast, Spi-C has no homology with PU.1 or Spi-B, or any other Ets family member outside of the Ets domain (Rao et al. 1999; Bemark et al. 1999). PU.1 transactivation involves two domains, an acidic domain and a flanking glutamine rich region (Klemsz and Maki, 1996). Spi-B also has an acidic domain, but it is highly divergent from PU.1, and its activation function is primarily thought to depend on its unique proline/serine/threonine rich region (Rao et al. 1999). Both PU.1 and Spi-B have a PEST domain that mediates protein-protein interactions,

most critically with members of the IRF family, but Spi-C does not interact with IRFs (Brass et al. 1999; Carlsson et al. 2003).

Non Spi related Ets family members expressed in hematopoietic cells, including Fli-1, Erg, Ets-1, and GABPα contain a POINTED domain involved in divergent protein-protein interactions and transactivation relative to the Spi subfamily; the POINTED domain may also facilitate Ets-Ets interactions (Slupsky et al. 1998; Anderson et al. 1999). The DNA binding domain of Ets family members also mediates important protein-protein interactions, such as interactions with Runx1 and C/EBPα (Petrovick et al. 1998).

Despite similarities to PU.1, neither Spi-B nor Spi-C is fully redundant to PU.1. Unlike the severe loss of blood cells in $PU.1^{-/-}$ mice, $Spi\text{-}B^{-/-}$ mice are normal with only slight defects in B-cell signaling (Su et al. 1997). $Spi\text{-}C^{-/-}$ mice also have essentially normal hematopoietic development, except for a loss of red pulp macrophages (Kohyama et al. 2009). To investigate Spi family functions in myeloid and B-cell development, Spi factors were ectopically expressed in $PU.1^{-/-}$ fetal liver cells. Ectopic Spi-B could only partially rescue myeloid and B lineage development at ~50% the efficiency of PU.1 restoration. Spi-C was even worse and could only restore 1% and 3% of myeloid and B lineage development compared to PU.1, respectively (Schweitzer et al. 2006). Collectively, these studies imply that PU.1 mediates essential functions through interactions unique amongst the Ets family.

PU.1 Expression Diverges as Early Progenitors Navigate Lineage Specifying Programs

Early investigations demonstrated PU.1 expression to be restricted to hematopoietic cells (Hromas et al. 1993). Initially, PU.1 is expressed at nearly equivalent levels with moderately high expression maintained across many of the earliest differentiation events. However, PU.1 expression diverges (figure 1) when restricted megakaryocyte/erythroid progenitors (MEPs), restricted granulocyte-macrophage progenitors (GMPs), or committed lymphoid progenitors emerge from the multipotent progenitor population (MPPs). PU.1 expression is thought to fall as MEPs are specified and expression is fully eliminated during terminal differentiation of erythrocytes. *Sfpi1* transcriptional output increases in MPPs specified to become GMPs. In contrast, MPPs that enter the lymphoid program maintain relatively stable PU.1 levels until cells commit to B or T lineages. In the case of pro-pre-B-cells, commitment to the B lineage is accompanied by a reduction in *Sfpi1* transcriptional output that is stably maintained. On the other hand, while early T-cells maintain HSC levels of PU.1 expression, during the DN2 to DN3 transition *Sfpi1* is silenced as cells become committed DN3 T-lineage cells (Nutt et al. 2005).

The mechanisms controlling the divergent PU.1 expression levels described above have not been adequately explained yet. So far, studies of *Sfpi1* transcriptional output have focused on the promoter and a compound enhancer element (URE) located at -14 kb upstream of the *Sfpi1* transcriptional start site (TSS). This URE contains important Runx1 target sites thought to mediate the Runx1 dependent *Sfpi1* activation previously discussed (Rosenbauer et al. 2006; Hoogenkamp et al. 2009). The URE also has critical

target sites through which PU.1 may be autoregulatory (Okuno et al. 2005). However, the URE is dispensable for PU.1 expression. The consequences and implications of deleting the URE, as well as discussions of what is known about how the URE functions to regulate *Sfpi1* transcription will be discussed in greatest detail in the introductions to chapters 2 and 3 of this report. Chapter 2 specifically will discuss the URE's relevance and inadequacy in explaining *Sfpi1* silencing during T-cell development and will introduce novel regulatory regions that include a T-lineage silencer. Chapter 3 seeks to advance our understanding of how PU.1 expression might be autoregulated and maintained at a higher expression level in myeloid cells.

The following sections will address some of what is known with respect to *Sfpi1* transcriptional control. Much of what follows will also attempt to expound on the combinatorial roles PU.1 plays in various lineages where *Sfpi1* transcription has diverged from HSCs. Factors involved in *Sfpi1* transcriptional control will be discussed in multiple contexts, and potential controversies and difficulties with respect to interpretations of factors' involvement will be highlighted.

PU.1 Is the Primary Competence Factor Facilitating Lineage Specification of Granulocyte-Macrophage Progenitors

All myeloid lineages can be specified from granulocyte-macrophage progenitors (GMPs). Whether a granulocyte lineage (eosinophil, basophil, mast cell, or neutrophil) or a monocyte lineage (macrophage, dendritic cell, or osteoclast) is the terminal cell fate of a GMP is contingent upon balanced PU.1 functional interactions with a select group of

regulatory proteins. GMPs express PU.1, Ikaros, Gfi-1, Egr, C/EBP family members, MEF2C, c-Jun, IRF8, STAT3 and many cytokine receptors. Each of these factors is an integral cog within the complex GMP lineage specifying machinery. Different factors work together to construct unique gears that operate restrictive lineage programs. In addition to cytokines, Notch1 and Notch2 signals also provide important cell extrinsic cues to promote or restrict differentiation by signaling cells to prevent or induce transcriptional gear shifts (Ohishi et al. 2000). However, the universal feature of the myeloid machinery is the PU.1 cog, without which the myeloid specification, commitment, or terminal differentiation machinery screeches to a halt (Scott et al. 1994; Anderson et al. 1998; Dakic et al. 2005). Therefore closely examining PU.1 function and expression in myeloid cells should foster understanding of how the *Sfpi1* node is built into other developmental programs in the hematopoietic compartment.

Transcription Factors and Cytokine Pathways Associated With GMP Specification and Increased *Sfpi1* Transcription

After megakaryocyte and erythroid (MEP) potential is lost, lymphoid primed multipotent progenitors (LMPPs) can become restricted lymphoid progenitors or restricted myeloid progenitors (GMPs). At least some less-restricted common myeloid progenitors (CMPs) that can give rise to MEPs can also become GMPs. Regardless of which developmental branch GMPs originate from, important transcription factor expression changes correlate with GMP specification. First, and of greatest relevance to this thesis, PU.1 expression rises during the specification of GMPs (Nutt et al. 2005). The increase in PU.1 is preceded by loss of GATA1 which is associated with loss of

MEP potential. Greatly reduced expression of the important HSC factor GATA2, and elevation of C/EBPα expression also occurs (Yoshida et al. 2006).

HSCs lacking C/EBPα have a profound loss of GMPs but still have less-restricted common myeloid progenitors (CMPs), consistent with a requirement for C/EBPα activation of PU.1 during GMP specification. However, targeted deletion of C/EBPα in GMPs does not prevent their subsequent differentiation while deletion of PU.1 does (Zhang et al. 2004). This suggests C/EBPα is needed independent of PU.1 for GMP specification, while PU.1 but not C/EBPα is required for further differentiation. This is a somewhat surprising result as C/EBPα is needed for granulocyte and monocyte specification (discussed below). However, it may be that excision of C/EBPα in the GMP population occurs late and after specification, or other C/EBP family members may be partially redundant after the GMP transition.

Independently and/or collaboratively, PU.1 and C/EBPα regulate the development of myeloid lineages. They do so in part by controlling the expression of several critical myeloid cytokine receptors. These cytokine receptors then facilitate survival, proliferation, and differentiation, and include G-CSFR, CSF1R (M-CSFR), GM-CSFR, and IL-6Rα (Anderson et al. 1998; Zhang et al. 1998; Petrovick et al. 1998). PU.1 also regulates the expression of Flt3, the cytokine receptor that delineates the LMPP population and is highly expressed by GMPs (DeKoter et al. 2002; Adolfsson et al. 2005). With the exception of CSF1R, all of these cytokine receptors are reported to activate STAT3 in the presence of ligand (and other STATs to varying degrees) which drives some but not all of the receptor's effects. In the case of Flt3, STAT1 and STAT5 are also activated. It should be noted that the combination and dosage of cytokines

controls the strength of STAT responses and as activated STATs can form homodimers or heterodimers with other STATs, different cytokine combinations can also alter the stoichiometry of dimer formation to facilitate context dependent functions.

The relevance and interest in STAT3 has risen because it was recently discovered that STAT3 contributes to *Sfpi1* regulation in primary erythroblasts, possibly through binding to conserved target sites located at -8.7 kb (CE4B region described in chapter 2) and -9.6 kb upstream of the *Sfpi1* TSS (Hegde et al. 2009). These sites may also contribute to interferon induced STAT3 activation of PU.1 expression in cells with myeloid and erythroid potential (Panopoulos et al. 2003; Gutirrez et al. 1997).

It turns out STAT3 can enhance C/EBPα expression in some contexts and C/EBPα can regulate cytokine receptors that activate STAT3, like IL-6Rα (Numata et al. 2005; Zhang et al. 1998). STAT3 physically interacts with C/EBPα too, and together they regulate targets in the liver (Kim et al. 2009; Choi et al. 2007; Numata et al. 2005). Moreover, C/EBPα can regulate PU.1 expression through binding to the *Sfpi1* URE and promoter (Yeamanns et al. 2007; Cai et al. 2007).

This potential crosstalk between STAT3 activation pathways, C/EBPα and *Sfpi1* transcriptional control makes interpreting the causes of PU.1 upregulation and GMP specification difficult. In STAT3 deficient mice, PU.1 levels in bone marrow progenitors are normal (Laouar et al. 2003). However, STAT3 deficiency causes severe myeloid hyperproliferation which would be consistent with lower PU.1 expression in the myeloid compartment (Welte et al. 2003; Zhang et al. 2005; Rosenbauer et al. 2004). It should be noted that while STAT3 deficient mice have severe myeloid defects, lymphoid development is essentially intact (Wei et al. 2008). This implies that Flt3/STAT1/STAT5

signaling operates in LMPPs preferring a lymphoid fate while Flt3/STAT3 signaling influences LMPPs (and perhaps CMPs) primed to generate GMPs. The STAT3 knockout results also indicate that STAT3 may be involved in PU.1 upregulation but not basal HSC and MPP PU.1 expression. How then, do LMPPs decide to become GMPs and not lymphoid cells?

The C/EBPα studies described earlier may be critical to unraveling GMP specification and *Sfpi1* transcriptional divergence at the LMPP to GMP transition. ~60% of $Flt3^+$ LMPPs express IL-6Rα, a C/EBPα dependent target gene, and activation of the IL-6/IL-6R pathway leads to Id1 upregulation (Maeda et al. 2009). Id1 is a member of the Inhibitor of DNA (Id) binding family that acts as negative regulators of bHLH factors including E2A. E2A promotes lymphoid development of LMPPs and restricts myeloid fates (Dias et al. 2008). Taken together, a model of GMP specification independent of initial PU.1 dosage may emerge.

GMP specification may involve a regulatory circuit built in part on Flt3/STAT-C/EBPα-IL-6/IL-6R/Id1. In this hypothetical model, C/EBPα is required for the expression of IL-6Rα and through IL-6 triggered Id1 upregulation a myeloid fate can be promoted. Since STAT3 deletion does not prevent myeloid lineage specification, the IL-6/IL-6R/Id1 pathway would be predicted to depend on other STATs. Furthermore, C/EBPα might be regulated by Flt3L/Flt3/STAT3 in normal cells. This could allow IL-6Rα expression to depend on a threshold of C/EBPα-STAT3 activity within the LMPP compartment. Only those LMPPs that have sufficient C/EBPα-STAT3 activity would then turn on IL-6Rα, followed by IL-6 enhancement of Id1. This pathway would also sustain and/or augment STAT3 activation and C/EBPα expression that could then

enhance *Sfpi1* transcription coincident with GMP specification. This is important as previous studies have suggested PU.1 dosage differences dictate myeloid versus lymphoid lineage specification (discussed later).

While the above model is hypothetical, it is evident that potentially complex cross regulation between C/EBPα and STAT3 exists and this confounds understanding of how higher PU.1 expression and/or autoregulation might be stabilized where STAT3 activation and C/EBPα are not present or steadily maintained. In the absence of C/EBPα, It may be that C/EBPβ can compensate beyond the GMP stage, allowing for terminal differentiation of lineages and also maintenance of PU.1 expression. Alternatively, once PU.1 is upregulated during GMP specification, PU.1 may become sufficiently autoregulatory and/or rely on other inputs. These considerations aside, what is not hypothetical or controversial is the critical role PU.1 plays in GMP lineage choice postspecification.

PU.1 versus C/EBPα Activity in the Absence of GATA Factors Determines Neutrophil versus Monocyte Lineage Choice

Neutrophil versus monocyte/macrophage lineage choice is influenced by the relative transcriptional activities of C/EBPα and PU.1 in GMPs (figure 2). A high PU.1 level relative to C/EBPα favors monocyte specification over a neutrophil fate choice (Dahl et al. 2003). While cooperative with PU.1, it has been suggested that C/EBPα can inhibit PU.1 function in some contexts through direct binding to PU.1's important coactivator, c-Jun. This may impair c-Jun autoregulation and activation of PU.1 targets,

thus favoring neutrophil over monocytic cell fates (Rangatia et al. 2002). More recently, it has been shown that C/EBPα needs to interact with c-Jun to promote monocyte development. This was demonstrated by showing that a C/EBPα variant able to form homodimers but unable to zipper with c-Jun could not enhance generation of monocytes (Wang et al. 2006). In addition, C/EBPα can directly inhibit PU.1 through protein-protein interactions at high concentrations, perhaps establishing an upper limit on PU.1 expression in early myeloid progenitors (Reddy et al. 2002). Collectively, these studies indicate that the stoichiometric balance of C/EBPα, c-Jun, and PU.1 is essential to lineage determination.

PU.1 promotes monocyte development in part through regulation of MEF2C (Stehling-Sun et al. 2009). C-Jun is sensitive to the maintenance of PU.1 expression and function as the direct PU.1 regulated transcription factor MEF2C enhances c-Jun expression (Schuler et at., 2008). Consequently, enhanced PU.1 function relative to C/EBPα promotes MEF2C expression which sustains or augments c-Jun expression to prevail over C/EBPα interference. This results in progenitors being pushed toward a monocyte/macrophage lineage choice over a neutrophil fate. Stabilization of the monocyte fate results from persistent strong PU.1 expression and/or function to induce another secondary determinant, Egr. Egr functions to repress the C/EBPα activated Gfi-1. If C/EBPα activity dominates over PU.1, Gfi-1 expression wins out and opposes PU.1 activated factors Egr-1/2, stabilizing a regulatory state permissive to terminal differentiation of neutrophils (Laslo et al. 2006).

Despite competing to specify neutrophil versus monocyte fates, C/EBPα and PU.1 may also work together to restrict other alternative myeloid fates. C/EBPα and

PU.1 coregulate important genes like neutrophil elastase (NE) that is expressed at highest levels in myeloid progenitors (Oelgeschlager et al. 1996). Interestingly, NE may itself be important in restricting lineage choice of GMPs. NE enzymatic activity has been associated with proteolysis and inhibition of Notch2 function (Doan et al. 2004). As a result, progenitor NE levels may affect Notch2 sensitivity thresholds to counteract Notch2 triggered C/EBPα repression and hinder Notch2 induced GATA3 induction (Sakata-Yanagimoto et al. 2008). In this way, NE levels could hamper alternative granulocyte lineage specification (discussed below). NE is later repressed by Gfi-1, but as Notch expression decreases during neutrophil differentiation, reduced NE may be inconsequential after specification away from alternative fates (Person et al. 2003; Ohishi et al. 2000). In summary, sufficient NE levels may restrict lineage choice to either neutrophil or monocyte fates as a function of higher PU.1 and C/EBPα transcriptional competence.

Relative Levels of PU.1, GATA Factors, and C/EBPα Help Dictate Granulocyte Sublineage Choice

The eosinophil, mast cell, and basophil lineages are alternative granulocyte fate choices that require restoration of GATA factor expression after GMP specification. Should a GMP be undecided whether to enter the neutrophil or monocyte lineage program, and during that indecision should it receive sufficient Notch input, the alternative granulocyte lineage pathways become available (figure 3).

Mast cell specification from GMPs depends on the ratio of PU.1 and GATA2. A high level of PU.1 relative to GATA2 represses GATA2 function and expression to promote a monocytic fate while a lower level of PU.1 collaborates with GATA2 to drive a mast cell fate (Walsh et al. 2002). Although GATA2 and PU.1 are cross antagonistic they are not mutually exclusive and both factors remain expressed in terminally differentiated mast cells. However, PU.1 is expressed at a low level in mast cells relative to its expression in GMPs (Arinobu et al. 2005). Moreover, PU.1 and GATA2 advancement of mast cell specification requires an ordered shifting of gears, with reduced expression of C/EBPα in GMPs preceding upregulation of GATA2; or else alternative granulocyte sublineage differentiation into eosinophils results (Iwasaki et al. 2006).

Notch signaling induces the GMP gear shift that allows the developmental machinery to produce mast cells, basophils, or eosinophils. Recently, C/EBPα repression by Hes-1 and GATA3 activation were shown to be triggered by Notch2 signaling, initiating mast cell differentiation (Sakata-Yanagimoto et al. 2008). Which occurs first, loss of C/EBPα or GATA factor activation, may be fully cell intrinsic and stochastic, or it may also depend on cytokines and STAT activity. Regardless, if GATA factor expression rises before C/EBPα is lost, eosinophils are generated. If C/EBPα goes down first, followed by GATA factor activation, PU.1 levels fall and bipotent mast/basophil progenitors are obtained (Arinobu et al. 2005). As GATA2 and GATA1 are activated by GATA3, they may be constraining PU.1 autoregulation (discussed in a following section) and STAT3 activation leading to reduced *Sfpi1* transcription. To what extent GATA3, normally considered a T-cell factor, might also be capable of inhibiting PU.1 is unclear. Interestingly, PU.1 expression stays low in basophils despite return of higher C/EBPα

expression, suggesting the restoration of GATA factor expression has a dominant effect to prevent a reinstatement of GMP levels of PU.1.

Combinatorial Use of PU.1 Decides Additional Terminal Monocyte Lineage Choices

Once the balance of PU.1 and C/EBPα expression and function has led to monocyte specification, the monocyte progenitor still has access to the osteoclast and dendritic cell (DC) fates in addition to becoming mature macrophages (Servet-Delprat et al. 2002; Montesoro et al. 2006). Several factors continue to affect PU.1 transcriptional output to dictate monocytic cell fate choices, including IRF8, MafB, C/EBPβ, the Ikaros family, Gfi-1, STAT3, and Notch signaling (figure 4).

Osteoclast or dendritic cell differentiation from monocytes is mediated by specific PU.1 interactions. PU.1 forms complexes with MITF and NFATc1, and in response to RANK ligand signaling promotes osteoclast differentiation (Matsumoto et al. 2004; Sharma et al. 2007). The Ikaros family member Eos counters some PU.1 and MITF cooperative transactivation by converting PU.1/MITF into a repressive complex that restricts osteoclast related gene expression (Hu et al. 2007). Additionally, Notch signaling blocks macrophage and osteoclast cell fate determination, but permits dendritic cell development, again reflecting the importance of Notch mediated spatiotemporal network steering to navigate lineage determining paths (Yamada et al. 2003; Bai et al. 2008; Ohishi et al. 2001). Moreover, PU.1 interacts with IRF8 to regulate monocyte and DC target genes (Tailor et al. 2008). IRF8 is also important to monocyte versus

neutrophil fate choice as mice deficient in IRF8 have an increase in neutrophils relative to immature myeloid progenitors (Holtschke et al. 1996). On the other hand, IRF8 negatively regulates osteoclast development (Zhao et al. 2009). In summary, IRF8, the Ikaros family member Eos, and Notch signals can act in parallel to promote dendritic cell fate determination by restricting alternative programs, but they do not do so alone as yet more factors are part of the PU.1 interplay controlling monocyte lineage choice.

MafB was recently revealed to play a role in monocyte lineage choice by blocking osteoclast and dendritic cell fate decisions, thereby promoting macrophage specification and terminal differentiation. MafB inhibits osteoclast development through binding to NFATc1 and MITF, competing with PU.1 (Smink et al. 2009). PU.1 and MafB are coexpressed in HSCs and monocytes, but upon PU.1 expression beyond a critical threshold relative to MafB, PU.1 becomes inhibitory to MafB function through protein-protein interactions and also suppresses MafB expression, resulting in a DC fate (Sarrazin et al. 2009; Bakri et al. 2005). The above studies imply *Sfpi1* transcriptional output must either be stabilized while MafB expression drops, or there must be a mechanism to drive further PU.1 expression to prevail over MafB to promote a dendritic cell fate.

The C/EBP family may also affect dendritic cell development. A C/EBP dominant negative can block both macrophage and neutrophil development, switching lineage choice to the dendritic fate (Iwama et al. 2002). C/EBPβ cooperates with PU.1 and c-Jun to drive macrophage target genes (Grondin et al. 2007). In addition, C/EBP□ contributes to terminal macrophage differentiation and restricts other fates in part by regulating MafB expression in monocytes, in spite of MafB's potential to limit activation of targets by interacting with and inhibiting c-Jun (Smink et al. 2009; Metcha-Grigoriou

et al. 2003). Thus C/EBPβ provides itself a window of opportunity to complete macrophage commitment before other factors can impose alternative lineage programs.

The above results raise some questions about *Sfpi1* transcriptional control. As C/EBPα or β can bind the *Sfpi1* URE, the dominant negative (DN) result suggests a few possibilities (Yeamanns et al. 2007). One possibility is that both MafB and PU.1 transcription fall in the presence of the C/EBP DN, but without MafB and C/EBPα opposing a possibly lowered PU.1 expression, dendritic lineage specification still takes place. Another possibility is that *Sfpi1* transcription needs to remain high to drive a dendritic cell fate and is unaffected by a loss of C/EBP function at these stages. In this case, blocking C/EBP function might act to more quickly close the window of developmental opportunity that MafB keeps open. Examining PU.1 expression in a C/EBP DN system that gives rise to DCs could thus prove enlightening with respect to *Sfpi1* transcriptional maintenance.

How factors might work in combination with PU.1 to facilitate dendritic cell development is not well understood beyond PU.1 needing to overcome MafB, nor is it clear at what point the DC fate option is excluded, or what the primary in vivo precursors are. While blood monocytes can differentiate into DCs, many subtypes exist with distinct phenotypes and functions. Recent studies have indicated that most if not all DC subtypes come from a common precursor found amongst the Lin^{-} $Flt3^{+}$ $CSF1R^{+}$ population of the bone marrow, with some precursors then migrating from the bone marrow to seed lymphoid tissues and complete Flt3 ligand dependent development (Karsunky et al. 2003; D'Amico and Wu, 2003; Liu et al. 2009). Four factors have now been linked with the development of most DC subtypes, PU.1, Ikaros, STAT3, and Gfi-1. Mice deficient in

either factor are severely defective in their generation of DCs (Scott et al. 1994; Anderson et al. 2000; Wu et al. 1997; Laouar et al. 2003). The requirement of some of these factors may not be surpring as both PU.1 and Ikaros control Flt3 expression while STAT3 appears to be the primary mediator of Flt3 signals in myeloid cells. Furthermore, Ikaros and Gfi-1 are expressed at high levels in GMPs (Yoshida et al. 2006; Zeng et al. 2004). As PU.1 expression must either rise to overcome MafB, or stay steady while MafB falls, the requirement of Ikaros and Gfi-1 marks these factors as candidate regulators of *Sfpi1* transcriptional output, perhaps working with STAT3 to maintain or augment PU.1 levels through monocyte lineage determining pathways.

Sfpi1 Transcriptional Regulation and PU.1 Function in MEPs

PU.1 is a dual functioning transcription factor able to activate or repress transcription, and through specific combinatorial mechanisms, antagonizes or promotes differing lineage choices. In early progenitors mutual antagonism between PU.1 and GATA1 segregates myeloid from megakaryocytic and erythroid lineage potential (MEP). How GATA1 itself is first activated to generate MEPs is unclear, but once expressed at sufficient levels GATA1 dominates PU.1 function and inhibits its expression to promote MEP differentiation.

At the transcriptional level, GATA1 may negatively regulate *Sfpi1* in MEPs through binding to a recently identified region at -18 kb upstream from the *Sfpi1* TSS (Chou et al. 2009). GATA2 was shown able to bind the -18 kb region and the *Sfpi1* promoter in an MEP-like cell line deficient in GATA1. As knockdown of GATA2 increased PU.1 expression in these cells, this work suggests that GATA2 may constrain

PU.1 levels through the -18 kb region and/or promoter. HSC-like PU.1 expression level is present in the GATA1 deficient MEP-like cells. After restoration of GATA1 expression, GATA1 is found to bind at the -18 kb and promoter target sites, accompanied by reduction in GATA2 occupancy. Consequently, PU.1 expression was reduced approximately 5-fold by day three post GATA1 restoration. This indicates that GATA1 is a more potent repressor of PU.1 expression than GATA2.

While GATA1 and GATA2 could bind the -18 kb region, the element and its GATA factor binding have not yet been directly tested for function. Thus GATA factor regulation of *Sfpi1* may not depend on this region and might primarily repress PU.1 through well known protein-protein interaction mechanisms described below.

To promote erythroid lineage development, GATA1 levels rise to overcome PU.1 so that GATA1 antagonizes PU.1 transcription, possibly by competing with c-Jun, the essential PU.1 coactivator discussed earlier (Zhang et al. 1999). Likewise, PU.1 antagonizes erythroid lineage specification and commitment by concentration dependent interaction with DNA bound GATA1, followed by recruitment of Retinoblastoma (Rb) and repressor complexes (Stopka et al. 2005). PU.1 interaction with Rb is mediated by the N terminus, yet the PU.1 N terminus was found dispensable for direct PU.1 interactions with Sin3A and HDAC1. Thus PU.1 can also mediate Rb independent repression (Kihara-Negishi et al. 2001). GATA1's ability to physically inhibit PU.1 autoregulation, along with its potential to directly repress *Sfpi1* may be enough to culminate in the eventual loss of PU.1 expression to promote terminal erythrocyte differentiation.

However, the recent work uncovering *Sfpi1* transcriptional responses to STAT3 at regions not associated with GATA binding or PU.1 positive feedback cannot be fully accounted for with the above GATA1 repression mechanisms and implies there is more to the GATA story. The PU.1 increase correlated with GATA2 knockdown in the MEP-like cells was observed under cytokine conditions (TPO and GM-CSF) that can activate STAT3 (Chou et al. 2009). Moreover, there was more than a 6-fold increase in C/EBPα in the MEP-like cells, as well as activation of other monocyte related genes (Chou et al. 2009). Since GATA1 and GATA2 can physically interact with STAT3, inhibiting expression of STAT3 target genes like c-myc, it is unclear if their effects on PU.1 expression directly involve the -18 kb cis element, or even the promoter (Ezoe et al. 2005). Therefore an alternative mechanism through which GATA factors could indirectly constrain or block *Sfpi1* transcription may involve another concentration dependent protein-protein interaction, this time with STAT3 and not PU.1. Taken together, it is difficult to attribute changes in PU.1 expression to GATA factor binding to *Sfpi1* cis elements. The potentially complex interplay between C/EBPα, GATA factors, and STAT3 obviates any conclusion about the mechanisms of *Sfpi1* regulation in the work discussed.

MEPs lack expression of the receptor tyrosine kinase Flt3 while CMPs, GMPs, and LMPPs express Flt3. Since signaling through Flt3 can activate STAT3, Flt3 instructive potential was investigated by transducing $Flt3^-$ MEPs with human Flt3 (Onai et al. 2006). Strikingly, signaling through hFlt3 was sufficient to reprogram MEPs into CMPs, increasing STAT3 expression, and restoring PU.1, C/EBPα, and myeloid related cytokine receptor expression (Onai et al. 2006). Interestingly, forced expression of STAT3 or PU.1 in MEPs repressed GATA1 expression and restored mouse Flt3

expression although hFlt3 alone did not do either. These results indicate that the balance between GATA1 and PU.1 mutual antagonism could be tipped by the influence of cytokines acting through STAT3, thus extending our understanding of how lineage choice might be altered by extrinsic signals impinging on stable progenitor regulatory states.

It is worth emphasizing the potential significance of the above results. The ability of hFlt3 to increase STAT3 and the finding that even higher forced STAT3 expression can turn on mFlt3 indicates that the STAT3 pathway is sensitive to positive feedback. PU.1 also activated Flt3 expression. Therefore PU.1 and STAT3 may be able to promote a self sustaining STAT3/PU.1 feedback loop past some threshold. Moreover, the ability of Flt3 to restore and maintain myeloid lineage potential in MEPs highlights the need to constrain Flt3 expression as a mechanism of lineage fidelity control.

Regardless of uncertainty over which possible mechanism might be in use, GATA1 inhibition of PU.1 function is well established and critical to erythroid development. Nevertheless, the general portrayal of GATA1 and PU.1 as antagonistic factors in erythroid development is misleading as PU.1 may have positive benefits in MEPs too. Low levels of PU.1 are present in early erythroid progenitors and experiments have shown it plays a function in self-renewal and proliferation before being fully silenced to allow terminal erythroid differentiation (Back et al. 2004). Furthermore, while not strictly required for megakaryocyte development, PU.1 may promote megakaryocyte over erythroid lineage choice by facilitating Fli-1 expression; and it may do so in collaboration with GATA1 (Starck et al. 1999; Barbeau et al. 1999). Moreover, cytokines used in platelet development, including IL-11, IL-6, and TPO activate STAT3,

which may maintain PU.1 expression and the proliferation of MEPs (Weich et al. 1997; Schulze et al. 2000; Tong and Lodish, 2004). An additional MEP benefit may be PU.1 collaboration with SCL to maintain the expression of MEF2C, which has recently been shown to be a direct SCL and PU.1 target with contributions to megakaryopoiesis (Gekas et al. 2009; Stehling-Sun et al. 2008).

Though PU.1 is antagonistic to GATA1 at high levels, and ultimately expendable to MEP function and development, the above discussion further exemplifies several important lessons. First, PU.1 regulation is very complex and involves the context dependent interplay of transcription factors and cytokine signaling crosstalk. Second, overexpression or deficiency of PU.1 can obscure interpretation of PU.1's normal physiological functions. And finally, erythroid and megakaryocyte development, like other blood lineages discussed, illustrate the ubiquity of PU.1 function contributions to normal hematopoiesis.

Sfpi1 Transcriptional Control Mechanisms in B-lineages Is Essentially Unknown

There is a shortage of information on *Sfpi1* transcriptional mechanisms in B-lineages. As described earlier, *Sfpi1* transcriptional output is reduced as early progenitors commit to the B-lineage. How PU.1 expression is modulated has not been determined, but may involve IRF8 binding to the *Sfpi1* promoter (Wang et al. 2008). However, the *Sfpi1* promoter cannot drive PU.1 expression in a chromatin context on its own (Li et al. 2001). So while several factors have been found to affect B-cell promoter activity, they

cannot adequately explain *Sfpi1* transcriptional output in the B-lineage. DMS footprinting indicates Runx1 and PU.1 sites in the *Sfpi1* -14 kb URE are occupied, but to date, in B-cells no factor has been shown to functionally regulate *Sfpi1* through the URE althouth the URE does contribute to B-cell *Sfpi1* transcriptional control.

PU Is a Context dependent Regulator in B-cells

While knowledge of B-lineage *Sfpi1* transcription control mechanisms may be lacking, it has long been recognized that PU.1 is an important context dependent B-lineage regulator. It has been nearly twenty years since PU.1 was recognized to bind and positively regulate the immunoglobulin kappa enhancer in collaboration with IRF4/NF-EM5 (Pongubala et al. 1992). Identification of important context dependent targets of PU.1 and IRF4 collaboration continues. For example, PU.1 interacts with IRF4 to regulate CD68 expression, but this time PU.1-IRF4 represses transcription. In contrast, PU.1 collaborates with Elf-1 to activate CD68 expression in myeloid cells (O'Reilly et al. 2003). PU.1 is now well known for its interactions with multiple IRF family members. For instance, PU.1 interacts with IRF8 to regulate dendritic target genes, but in early B-cells PU.1 cooperation with IRF4 or IRF8 helps activate the critical B-cell factor, Pax5 (Decker et al. 2009; Tailor et al. 2008).

Although important, IRFs are not the only B-lineage PU.1 collaborators. PU.1 also cooperates with Pax5 to recruit the Grg4 repressor and silence IgH, but in Pax5's absence PU.1 activates IgH in combination with NFKappaB (Linderson et al. 2001 and 2004). PU.1 can also recruit BCL6 to repress target genes in germinal center B-cells (Wei et al. 2009).

These examples of PU.1 context dependent function provide a cautionary tale with important lessons. First, despite very different PU.1 expression levels, myeloid and B lineages can still use PU.1 to regulate overlapping targets like CD68, therefore simple dosage models will sometimes mislead. Second, since PU.1-IRF4 complexes can activate or repress different targets, the amount of contextual information needed to predict function may be greater than often appreciated. Third, the CD68 and IgH examples demonstrate that the directionality of PU.1 transcriptional effects are readily altered through different combinatorial occupancy patterns, further precluding easy prediction of PU.1 functional relevance even when binding is observed on the same target.

Does PU.1 Dosage Affect Myeloid Versus Lymphoid Fate Choices?

Previous sections highlighted the ways in which multidimensional PU.1 levels affect regulatory choices in erythroid and myeloid lineages. It has also been argued that one dimensional PU.1 dosage influences myeloid versus B-cell lineage choice with higher PU.1 concentration promoting myeloid lineage choice at the expense of B-cells from a common precursor (DeKoter and Singh, 2000). The authors noted in their original discussion that there could be two possible interpretations of their data. Either differing amounts of PU.1 specify distinct lineage fates, or commitment may be initiated from nearly equivalent levels of PU.1 with levels consequently changing as a by-product of specification so that committed cells can complete terminal differentiation (DeKoter and Singh, 2000). The authors favored the former argument because it was believed PU.1 was expressed at low levels in multipotent progenitors.

Recent *Sfpi1* reporter mice have established that the original premise for arguing a simple dosage effect is not valid. PU.1 is expressed uniformly in HSCs, CMPs, and CLPs, but rises in GMPs and falls in B and T precursors only after lineage specification (Nutt et al. 2005; Back et al. 2004). Furthermore, mice with reduced functional PU.1 protein levels still undergo myeloid specification but are defective in differentiation. These mice also show defects in B-cell development, indicating a minimal threshold of expression is required to allow B lineage development and subsequent myeloid differentiation postspecification (Rosenbauer et al. 2004; Houston et al. 2007). This does not come as a surprise as the B-cell compartment is not a mixture of lineages constructed by different PU.1 amalgams like the myeloid compartment, thus B-cells can simply change collaborators under conditions of relatively stable PU.1 expression to alter PU.1 mediated transcriptional outputs.

PU.1 Is a Subversive but Essential T-lineage Regulator

Absence of PU.1 results in a T lineage developmental defect with $PU.1^{-/-}$ progenitors arrested at the T lineage specification stage where cells transition from DN1 ($CD44^{+}CD25^{-}$) to DN2 ($CD44^{+}CD25^{+}$) (Spain et al. 1999). Forced PU.1 expression in early thymocytes also induces a developmental arrest at a slightly later stage, indicating normal T lineage development is dependent on maintenance and silencing of *Sfpi1* (Anderson et al. 2002).

Analysis of PU.1 overexpression in pro-T-cells has demonstrated that PU.1 can repress T-lineage associated genes while activating myeloid lineage targets, even diverting some cells to a myeloid-like or dendritic cell fate (Dionne et al. 2005; Franco et

al. 2006; Lefebvre et al. 2005; Laiosa et al. 2006). Additionally, when removed from thymic Notch signaling, PU.1 expressing DN2 thymocytes can generate DCs in the spleen after intravenous transfer (Wu et al. 1996). Deletion of Notch1 in pro-T-cells also increases DC numbers in the thymus (Feyerabend et al. 2009). Collectively, these studies indicate PU.1 provides both T lineage fidelity and instability with Notch signaling able to contextually restrain PU.1 dependent lineage competence functions in the normal thymic environment. While it is clear PU.1 is required for efficient T-cell development, and PU.1 activity must be contextually limited to maintain the T-lineage program, it remains vague what the true functions and targets of PU.1 are at normal physiological expression levels in early T-cells.

At least one known PU.1 target benefits T-cell development, Flt3. Early T progenitors (ETPs) are part of a mixed population of DN1 cells within the thymus. Several multipotent progenitors have now been found circulating in the blood that can seed the thymus to give rise to ETPs, thereby contributing to T-lineage development (Schwarz and Bhandoola, 2004). The most efficient T-cell generators appear to be the $Flt3^{Hi}$ LMPPs and their early progeny. Flt3 is essential to thymic seeding, in part due to its regulation of the thymic homing factor CCR9 (Schwarz et al. 2007; Zlotoff et al. 2008). After ETP seeding, Flt3 signaling continues to contribute to DN1 expansion within the thymus. As already discussed, the Flt3 pathway is also associated with myeloid potential. Additionally, $Flt3^{Hi}$ ETPs retain B lineage potential, in part due to insensitivity to early thymic Notch signaling through expression of Zbtb7a and MINT (Maeda et al. 2007; Tsuji et al. 2007). So while Flt3 benefits the thymic citizenry, it is loss of Flt3 expression that in part marks B-cell restriction and limits myeloid potential (Sambandam et al. 2005).

Increasing Notch Signaling during the DN1 to DN2 Transition Constrains PU.1 Function and Facilitates T-lineage Specification, in Part through Altering Reliance on PU.1 Targets Flt3 and IL-7Rα

Notch signaling potency increases during the DN1 to DN2 transition and facilitates T-lineage specification. Notch target genes Hes1 and GATA3 are already activated in $Flt3^+$ ETPs, suggesting Notch signaling has already begun, perhaps prethymically. However, activation of the Notch target Deltex is not immediate. Instead it coincides with Flt3 repression and CD25 upregulation at the DN1 to DN2 transition, suggesting strengthening Notch signaling with loss of Zbtb7a and MINT is needed to initiate the T-lineage program (Sambandam et al. 2005; Taghon et al. 2006). Furthermore, coculture of sorted $Flt3^+$ ETPs on OP9 stroma lacking the Notch ligand DL1 fail to downregulate Flt3. Collectively, these results demonstrate a requirement for increasing Notch signaling to constrain expression of a PU.1 target and in so doing, block alternative lineage potential.

By downregulating Flt3 expression, Notch alters the cytokine landscape of early T-lineage cells. In fact, Notch regulates the cytokine response pathways involved in multiple hematopoietic lineages. In monocyte progenitors, Notch activation blocks nondendritic cell fates by triggering apoptosis in the presence of M-CSF (Ohishi et al. 2000). DCs overcome potential Notch induced cell death through GM-CSF and/or Flt3L activation of STAT3 and its target gene Survivin (Gu et al. 2007; Zhou et al. 2009). In the absence of Flt3/STAT3 mediated Survivin expression, T-lineage cells shift to survival mechanisms that rely in part on c-Kit and the less myeloid friendly IL-7/IL-7Rα pathway

for survival and proliferation (Yu et al. 2004). Neither IL-7Rα nor c-Kit is known to be a strong activator of STAT3.

The above results may seem inconsistent as Notch silences Flt3 in the thymus, but Notch has been shown to be necessary for some dendritic cell development. This implies that Notch's ability to modulate Flt3 expression may be contextually specific, possibly through quantitative control of Notch signaling potency (Cheng et al. 2003; Dallas et al. 2005; De Smedt et al. 2005). Additionally, recall that STAT3 has the potential for positive feedback to regulate Flt3 expression, therefore GM-CSF and Flt3L together may be enough to sustain Flt3 expression, preventing Notch mediated repression. Consistent with the above, forced expression of GM-CSFR (or IL-2β), a potent STAT3 activator, can quickly convert DN1 or DN2 cells into myeloid cells (King et al. 2002). Taken together, the ability of Notch1 to promote T lineage specification may be incompatible with coexpression of other myeloid/DC drivers. As Flt3 and PU.1 together contribute to developmental instability by allowing alternative fate specification, one factor must give way. Therefore Notch silences Flt3 as PU.1 itself may yet be required for IL-7Rα expression and/or further maintenance of T-lineage fidelity.

Thymocytes Avoid a Notch, PU.1, and GATA3 Induced Mast Cell Fate through Mutual Constraint

One PU.1 dependent myeloid fate is partially restrained by intrathymic Notch repression of Flt3. However, the myeloid fate problem only becomes more paradoxical when considering the relationship of PU.1, GATA3, and Notch in the mast cell fate decision, as immature T lineage cells requires these very same developmental inputs.

As discussed previously, mast lineage specification is dependent upon balanced PU.1 and GATA activity in the presence of little or no C/EBPα. Primary myeloid progenitors exposed to Notch repress C/EBPα via HES1 and strongly activate GATA3 expression, in turn leading to primarily mast cell formation in mixed colony assays (Sakata-Yanagimoto et al. 2008). DN1 and DN2 cells express plentiful HES1 and GATA3 too (Sambandam et al. 2005; Taghon et al. 2005). Moreover, DN2 cells have strongly downregulated C/EBPα expression, which may be related to losing Flt3 (Laiosa et al. 2006; Rothenberg et al. 2008). Since C/EBPα can shut off Notch1 and GATA3 expression when overexpressed, repressing C/EBPα is also an important event in maintaining T-lineage fidelity (Laiosa et al. 2006). With plentiful PU.1 and GATA3, and little C/EBPα, why do DN1 and DN2 thymocytes not generate thymic mast cells en masse? While Notch is a natural candidate for protecting DN1 and DN2 cells from transdifferentiation, another may actually be PU.1.

Although Notch constrains PU.1's subversive behavior, Notch may also be commandeering PU.1 and turning it into a T-lineage guardian. Notch has been observed to boost PU.1 expression in thymocytes (Taghon et al. 2007). This could be due to

HES1's reported ability to cross talk with the JAK/STAT pathway by directly binding STAT3, promoting its phosphorylation and enhancing its transcriptional activity in glial differentiation (Kamakura et al. 2004). Thus Notch signaling may ironically induce a transient activation of PU.1 expression through the very same STAT3 mechanism that silencing of Flt3 would serve to prevent. However, HES1 induced STAT3 activity could be short lived as Notch can also trigger the expression of the STAT3 inhibitor SOCS3, which may serve to limit or block cytokine or HES induced STAT3 activation in the wrong context (Narayana and Balaji, 2008). Notch might not just transiently regulate PU.1 though. In a myeloid progenitor cell line, overexpression of an activated Notch1 triggers PU.1 upregulation and differentiation (Schroeder et al. 2003). Since Notch activates GATA3, but GATA3 fails to trigger GATA2 and GATA1 expression unless forcibly overexpressed, it is conceivable that sustained PU.1 expression is constraining GATA3 just as Notch is constraining PU.1, likely with GATA3 also inhibiting PU.1 (Taghon et al. 2007). However, this is inconsistent with observed PU.1 downregulation observed in mast cells and suggests there is more to consider (Arinobu et al. 2005).

Continuous direct or indirect Notch maintenance of PU.1 in the thymus may be protecting T-lineage fidelity in early development while transient or weaker Notch signaling in myeloid progenitors may be more permissive to mast cell specification. Transient or less potent Notch signaling must be sufficient for initial HES1 and GATA3 activation, as evidenced by their expression in ETPs. Thus if myeloid progenitors within the bone marrow obtain transient or less potent Notch signals, they could still repress C/EBPα. Additionally, GATA3 can sustain Notch-independent HES1 expression while initiating GATA2 expression, which then autoregulates (Taghon et al. 2007). So long as the spatiotemporal Notch signaling in the bone marrow is sufficient to allow GATA2

activation, but too impotent or transient to consequently activate a PU.1 maintenance program, PU.1 expression would be allowed to drop and a new regulatory state could be established. In this way, Notch could prevent legacy *Sfpi1* regulators from working against T lineage specification in the thymus, while coopting PU.1 to be a T lineage guardian able to counterbalance Notch activation of GATA3.

Consistent with the above scenario, overexpression of GATA3 in early T-cells redirects some cells toward a mast cell fate, overcoming any PU.1 imposed constraint (Taghon et al. 2007). Absence of Notch signaling enhances GATA3 induced mast cell diversion, which indicates PU.1 independent Notch regulted constraint of GATA3 is also important. GATA3 rapidly induces GATA1 and GATA2 expression with accompanied suppression of high PU.1 expression, followed by establishment of an expression level similar to bone marrow derived mast cells (Taghon et al. 2007). While GATA3 overexpression could activate a mast cell diversion program even in the presence of Notch, only DN2 thymocytes in the absence of Notch could give rise to mast cells without forced GATA3 overexpression. This is consistent with a hypothesis that DN2 cells may have switched to reliance on a Notch dependent PU.1 maintenance program in the absence of other HSC legacy regulators. Subsequent removal from Notch signaling could then result in a drop in PU.1 expression unless Flt3 or other STAT3 activating cytokine receptor expression is restored first. Without sufficient Notch and PU.1, some cells might then have an opportunity to enter the mast cell program.

Switching Gears, What T-lineage Player Throws the Wrench into the *Sfpi1* Transcriptional Machinery?

The preceding discussions in this chapter have highlighted many factors involved in *Sfpi1* transcriptional control, partly with the hope of better understanding what factors might be relevant in thymocytes, but the main lesson here is that none of the factors discussed, nor any other literature can readily explain PU.1 expression in the T-cell compartment. C/EBPα is expressed, but at relatively low levels and falls in the earliest T-cell precursors, before PU.1 is shut off. Loss of STAT3 does not affect T-cell development and the cytokine receptors that early T-cells rely on are not known to activate STAT3. GATA1 is not present, but GATA2 is expressed in early T precursors. However, its expression decrease before *Sfpi1* silencing, and it might be expected to regulate PU.1 in the opposite direction (Rothenberg et al. 2008). While Notch was discussed as a potential regulator, that hypothesis has yet to be tested. Taken together, *Sfpi1* transcriptional maintenance involves unrecognized players and will need to be further explored.

A more pressing question is what factor disrupts the *Sfpi1* transcriptional machinery at the DN2 to DN3 transition? Examining DN3 cells reveals several interesting shifts in transcription factor family ratios and one that is particularly relevant is Runx1/Runx3 (Taghon et al. 2007; Rothenberg et al. 2008). Runx1 has already been recognized as being an important silencer in the T-lineage. Although it was involved in turning on *Spfi1* in the first place, its peak expression at the stage where most cells are committed to the T-lineage makes it an interesting candidate for *Sfpi1* silencing, and will be the subject of the next chapter.

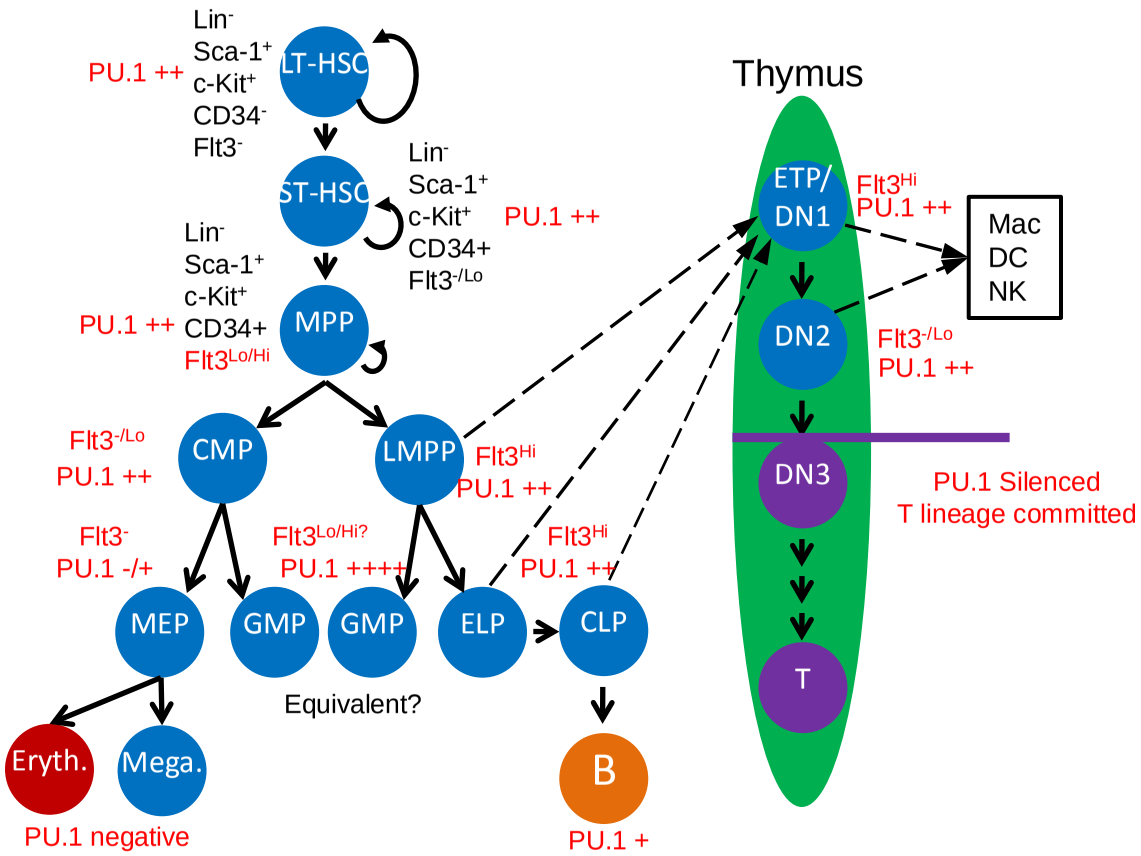

Figure 1. Hierarchical Model of Hematopoiesis.
LT-HSC (Long Term Hematopoietic Stem Cell), ST-HSC (Short Term Hematopoietic Stem Cell), MPP (Multipotent Progenitor), CMP (Common Myeloid Progenitor), LMPP (lymphoid Primed Multipotent Progenitor, GMP (Granulocyte Macrophage Progenitor) , ELP (Early Lymphoid Progenitor), CLP (Common Lymphoid Progenitor), MEP (Megakaryocyte Eruthroid Progenitor), ETP (Early T Progenitor)

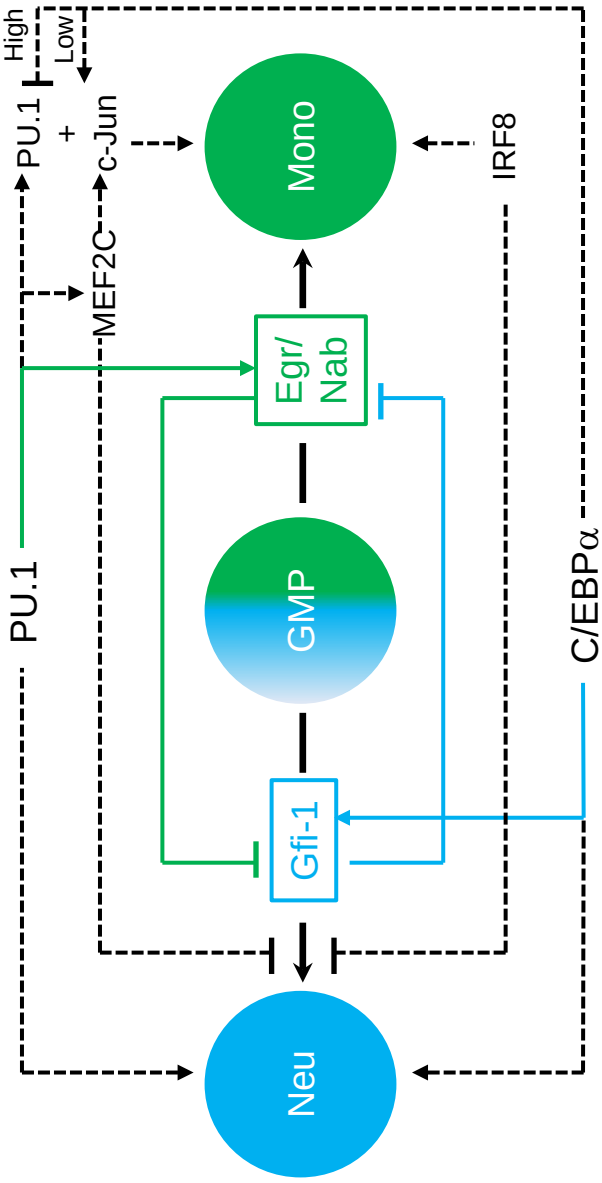

Figure 2. Model of Neutrophil versus monocyte lineage specification.

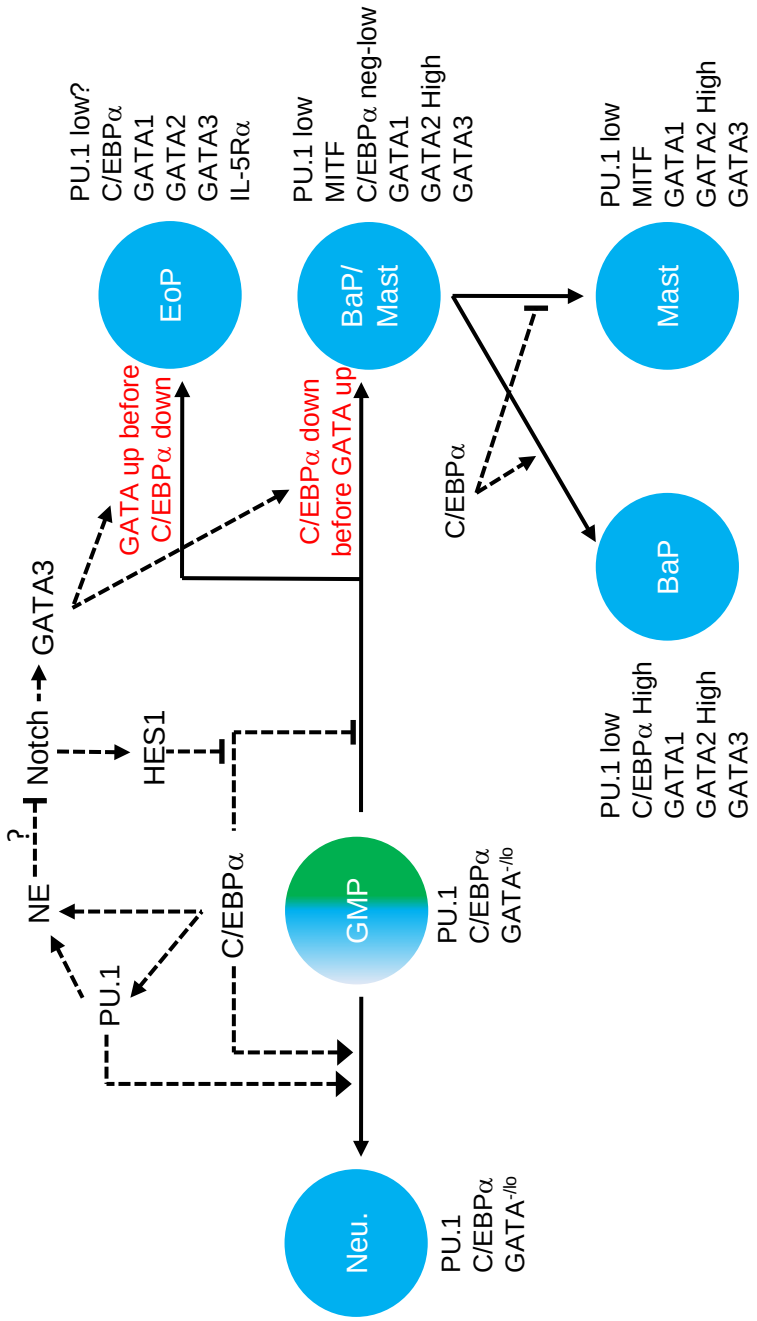

Figure 3. Model of Granulocyte lineage specification.

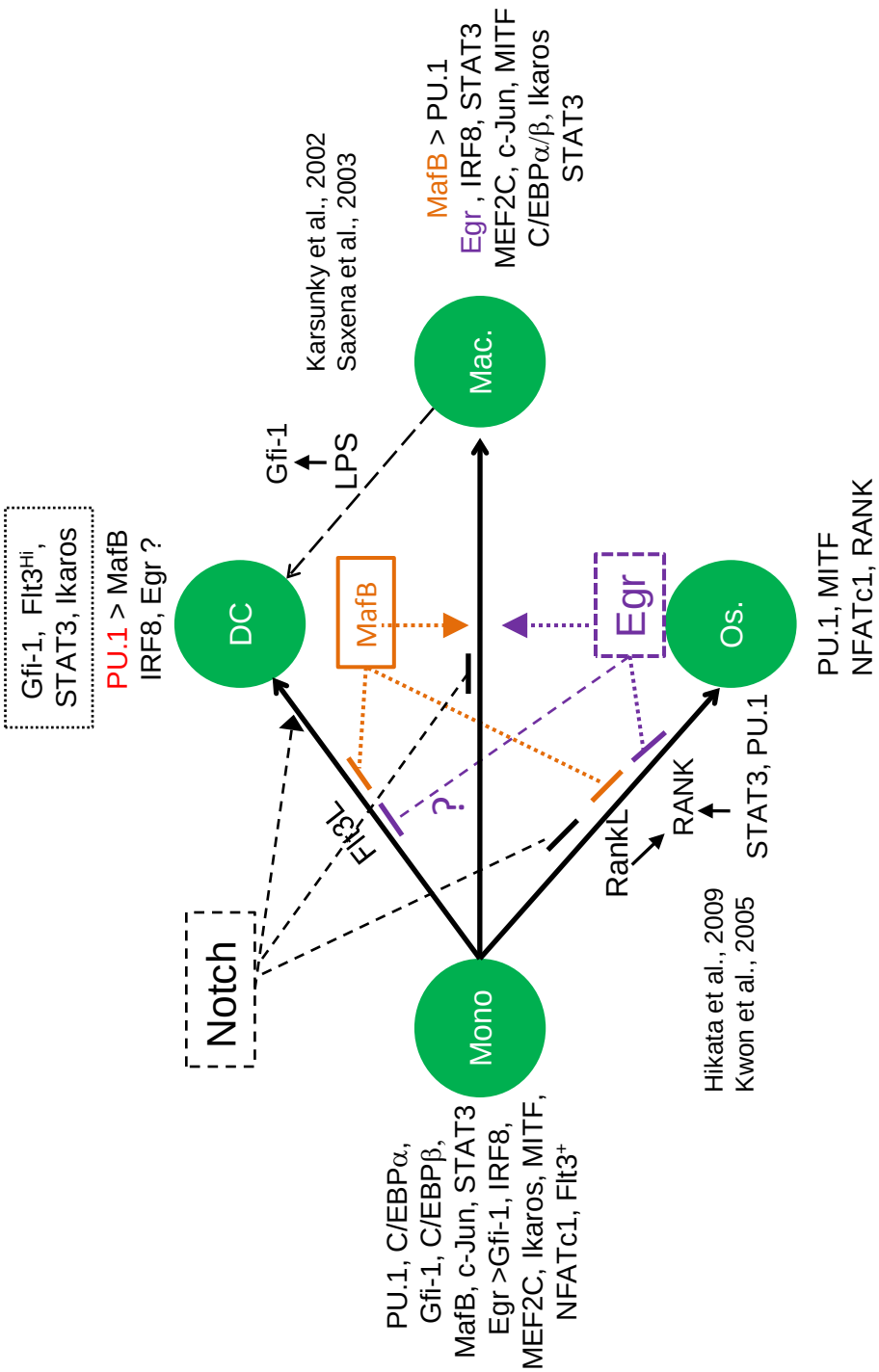

Figure 4. Model of monocyte terminal lineage choice.

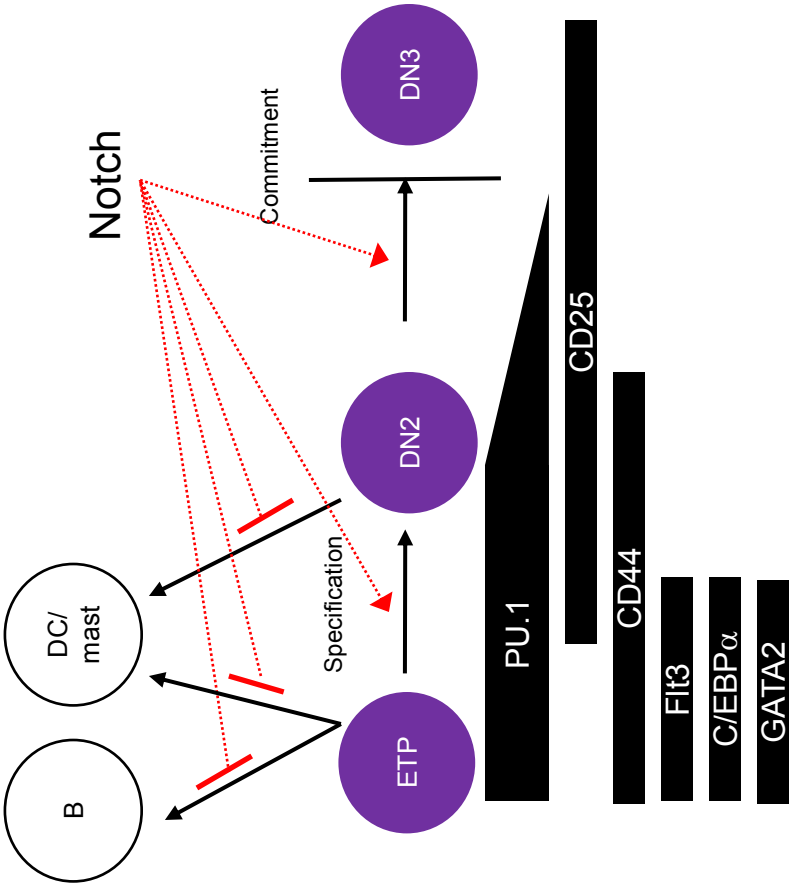

Figure 5. Early T-cell development

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Chapter 2

Cell type-specific cis-regulatory elements for PU.1: repression in pro-T cells through a dedicated, Runx-dependent silencer

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This chapter represents a manuscript describing the Runx1 dependent silencing of PU.1 in immature T-cells. The discovery of multiple novel *Sfpi1* regulatory modules is also reported.

Abstract

PU.1, a transcription factor critical in multiple hematopoietic lineages, is required during T-cell specification but is then silenced before commitment. If uncontrolled, PU.1 can both block T-cell development and induce diversion to a myeloid fate. This implies operation of a stage-specific repression mechanism correlated with commitment. Two major cis-regulatory elements have been described for *Sfpi1*, the gene encoding PU.1, namely the promoter and a compound conserved region around -14 kb that is thought to mediate activation and repression. However, the -14 kb element is dispensable for most *Sfpi1* downregulation in early T-cells, and it can exert enhancer activity in an immature T-cell line in which the endogenous *Sfpi1* gene is repressed. We now present evidence for another complex of conserved noncoding elements that can mediate several discrete, cell type-specific regulatory features of *Sfpi1*, including cell type-specific repression in early T-cells. We describe mapping of the silencer core and show that the T-cell specific repressive activity requires Runx1 acting through multiple nonconsensus sites. These newly characterized sites recruit Runx1 binding in early T-cells in vivo and define a functionally specific scaffold for Runx-dependent repression complexes.

Introduction

Differentiation of hematopoietic stem cells (HSCs) is a highly regulated process whereby sequence specific transcription factors drive differentiation through activation of lineage specific developmental programs and by restricting or repressing alternative fates. Some of these transcription factors are pleiotropic inputs acting as regulators of multiple cell fate decisions. Such functions depend on tight regulation of the factors themselves, distinguishing various cell lineages and differing developmental phases.

One such dynamically regulated factor is PU.1, an Ets family DNA binding protein critical to many hematopoietic cell types. This transcription factor is essential for the development of myeloid and lymphoid lineages (20, 28). PU.1 expression is restricted to hematopoietic cells, and is highly expressed in HSCs. Initiation of PU.1 expression depends on Runx1 which unfolds the PU.1 chromatin structure and primes the gene for expression in HSCs (14, 23). Upon differentiation of HSCs, PU.1 expression diverges in distinct lineages. PU.1 expression is elevated in macrophages, continues at high levels in neutrophils and most types of dendritic cells, and is fixed at lower levels in committed B cells (22).

A more dramatic shift of PU.1 expression occurs in the development of T-cells. Although the earliest intrathymic precursors express PU.1 at HSC-like levels, PU.1 expression is silenced during the transition to the DN3 stage of T-cell development, as the cells undergo lineage commitment (3, 31, 33). This silencing is crucial as forced expression of PU.1 beyond this stage causes a developmental block. PU.1 overexpression in DN3 thymocytes or a DN3 like immature T cell line, Adh.2C2, can also cause the cells to gain myeloid characteristics (9, 17). This suggests that silencing of PU.1 is needed to

block alternative fate choices during commitment of cells to the T-lineage. The mechanism of this essential silencing event is not fully understood.

To date, most aspects of PU.1 regulation have been explained by invoking just two regulatory elements: the promoter and an upstream regulatory element (URE) at ~14 kb upstream of the transcription start site of the *Sfpi1* gene which encodes PU.1. Early studies indicated that potential PU.1 regulatory differences might be mediated through cell type-specific use of different transcription factors at the promoter. The *Sfpi1* promoter contains octamer binding sites affecting B-cell expression (6), and PU.1 can bind its own promoter with Sp1 to regulate itself in myeloid cells (7). *Sfpi1* promoter activity can also be directed in myeloid cells by C/EBPα and AP1 (4). These regulatory inputs to *Sfpi1* may be modulated by cell type-specific DNA methylation (1). The PU.1 promoter has also been shown to be more active in a myeloid cell line than in a mature T cell line, implying that it can mediate cell type specificity (18).

The promoter alone cannot drive reporter expression in a chromatin context, and the search for added regulatory function yielded the conserved URE which acted as a myeloid specific enhancer, enhancing promoter activity in a myeloid cell line but not in a mature T cell line (18). In myeloid cells, the URE binds C/EBPα (5, 34) and PU.1 and may thus contribute to autoregulation as well (24, 29). Data suggest that the URE also mediates silencing function in T cells. To account for its repressive activity, two mechanisms have been offered. A TCF/LEF site in the URE was argued to mediate repression as long as Wnt signals are absent (26). However, this mechanism does not explain continued PU.1 repression later, when T cells must undergo canonical Wnt signaling (10, 34). Also, the proximal URE enhancer has three conserved Runx1 sites able to bind Runx1. This region of the URE is in an open state of accessibility, with the

Runx sites occupied in both myeloid and T lineage cells (13). This Runx input was argued to mediate both activation and silencing (15).

This claim implies that opposite effects on *Sfpi1* regulation can be mediated by the same factor binding to the same sites. Indeed, mice with a deletion either of Runx1 itself or of these URE Runx sites showed a decrease in PU.1 expression in myeloid and B cells. In T-lineage cells, deletion of Runx1 produces a developmental block at the DN2 stage (11, 16) and the surviving cells have higher PU.1 expression, which may add to this arrest (15). The argument is that not only the URE function overall, but also Runx1 binding to the same cis-regulatory sites in the URE is inherently bifunctional.

However, it is not proven that all regulation goes through the URE. Deletion of the URE (UREΔ) neither fully blocked activation of PU.1 expression within hematopoietic cells, nor fully blocked T-cell silencing (26, 27). Thus, while required for normal regulatory output, the URE was dispensable for turning on PU.1 (27). Also, the UREΔ mice still had T-cells in which PU.1 expression was effectively silenced (26). Thus, even though the Runx1 bound to the URE in T-cells might be part of a repressive complex, lack of the URE was less harmful to T-cell development than the severe DN2 developmental block observed with deletion of Runx1 itself.

Here, we have identified a set of conserved, previously uncharacterized cis regulatory regions for *Sfpi1*. We show that at least one is a novel enhancer that can contribute to the myeloid specific expression of PU.1. We also show that in an immature, DN3-like T-cell line, in contrast to a mature T-cell line, the URE remains an active enhancer though endogenous PU.1 is repressed. However, another new element we define is a bipartite silencer that is necessary and sufficient for full silencing within a chromatin context in this immature T-cell line. Using scanning mutagenesis, we mapped

sites required for the core silencer's function and we show that these sites are novel Runx binding sites. These *Sfpi1* silencer Runx sites bind Runx1 in a dose-dependent, cell type specific way, and perturbations of Runx1 protein function block silencer activity mediated through these sites. Thus, this study identifies novel sites for nucleating a distinct set of transcription factor complexes through which Runx1 can influence PU.1 expression. The existence of these lineage-specific, functionally dedicated cis-elements reveals a new set of mechanisms for cell type-specific regulation of PU.1.

Materials and methods

Cell culture:

Adh.2C2 and EL4 cell lines were grown in RPMI media supplemented with 10% fetal bovine serum, penicillin/streptomycin/glutamine, non essential amino acids, sodium pyruvate, and 2-ME. Raw264.7 and NIH-3T3 cells were grown in DMEM media with 10% fetal bovine serum, penicillin/streptomycin/glutamine.

DNase I HS Mapping and Southern blot analysis experiments:

DNase I hypersensitivity assay were carried out as described (21) with slight modifications. Briefly, about 100 million cells were harvested and washed three times with RS buffer (10mM Tris-HCl, pH 7.5, 10mM NaCl, 5 mM $MgCl_2$, and 2 mM $CaCl_2$). Cells were resuspended in 0.5ml RS buffer and mixed with 0.5ml 1% NP-40 and incubated on ice for 10 min. The pelleted nuclei were washed twice with RS buffer and resuspended in 0.2ml RS buffer. Twenty five microliters of nuclei suspension were digested with 25 µl of DNase I (Roche) at varying concentrations ranging from 0 to 40 unit/ml for 5 min at 20°C. Reactions were terminated by adding 450 µl of digestion

buffer (100 mM NaCl, 20 mM EDTA, pH 8.0, 6mM EDTA, 0.5% SDS) containing 100μg of RNase A and incubated for 30 min at 56°C. Proteinase K was then added to 0.1 μg/μl and the mixture was incubated at 56°C overnight. The genomic DNAs were then extracted by phenol-chloroform and precipitated by ethanol, and resuspended in 50μl of H_2O. All obtained DNAs were digested with different restriction enzymes to completion according to the regions analyzed, and fragments were resolved by gel electrophoresis and transferred to a nylon membrane. Probes were amplified by PCR from the mouse genomic DNA using the primer sets in the selected regions. The PCR products were gel purified using the Qiagen gel extraction kit and radiolabeled by random priming. Hybridization was performed at 42°C for 24 hours according to the protocol provided by BD Clontech.

Cloning of reporters and expression constructs:

PU.1 DNA was obtained by PCR using the BAC RP23-20F9 (bacpac.chori.org). Reporter constructs were made by cloning PU.1 sequences into Promega's pGL3-basic vector. Detailed maps of reporters and their construction are available upon request. For scanning mutagenesis analysis, CE4A core silencer fragments with M2, M14, or M5+M9 mutations were synthesized by GenScript and used to construct reporters. All other L98+4A-5 mutants were made using overlap PCR to produce mutated core silencers for reporter construction. L9-3mut-Runx was made by excising the CE4 region and replacing it with a mutated sequence synthesized by GenScript. Runx1 dominant negative and full length Runx1 cDNAs were kind gifts from Dr. Janice Telfer, and were cloned into Invitrogen's pEF1/Myc-His B vector. The Ikaros dominant negative, Plastic, was synthesized by GenScript based on published sequence (Papathanasiou et al. 2003) and

cloned into pEF1. The following sequence with the M5a mutation and predicted Runx sites mutated was synthesized by GenScript and used to replace the wild type sequence in L93 to construct L93-M5-mut-Runx:

AGCTCTTAAGGGACTGAGGACTAAGCAAGATGCTGAGTTCTGGAGACGGGAC

TGTCTTCTTCCCCAGATTGAGATGCCAGGCATGTGTGTCTCACACAGACTCTG

TGCCTACTACCTCAGTTAGCCTTGAGAAATCCCCACCTCCATTCCCAGAGGTA

TCTTCTATTATTGCTCCTATCTGGGGACAAAGAGCCTGAGGTCCCTAGAAGTG

GGTTCCTGGCTCTCAGTTGTGAAGATAATTAGGTATAGGGAGTCACACTGCA

GGTCACAGAAAGCACTGGCAGAAGCCAATGAAAGAGGCACATACTAAGTAG

ACTTTTAGTCTTGGAAACAAGGCTAGGAGGTGATTCTTGTTGATGTCTCTCTG

TAGAGCTGAGCCTAAGTTCTGGAGAGGGGAAGGAACTCAGAAGGCTACATG

GCCAATCCATGGGGGTTGGGGGAGAACCCGTGGAGCTAGAGATGGGATGGT

AGAGGGGGCGCCTTAGAGGAGGTAGGCCTGAGTGGGGAAGCAGCTCTTGTCC

TTGGTGAGCAAGCTGGAGGTGTTCTGCTGCCCGTGGCGAGCAGACGACAGTT

GCTGTTAGTTACGGTTAGTTTGATCTGCAGGAGACTGAGTGATGTTACCAGGA

GGTGAGAGCTCCGCATCTGCAGGCCTGGTCAGCAGGAGACGGGGTTCAGTAA

GATTCAGAGGAGTGTTAGCTGAACTGGAGATTTGTATCTCTCAGTCACCGGCC

CTGGAACACATGGGACCAGGAACCGGAATAGAACAGGAGGAGAAACTGAGG

CAAGGCTTGGGAAAGACAGAGCAAACTGAAAAAAAAAAAAAAAGGGCACTT

AGAGGAGTGTCCAGTAGGGTGTTAAAGACAGTGAGAGCCTGTGTGAGCAAA

GCCTGTTAGAGATTGAGAAAGAGCAGAGCTTCTGGACATGTTGAGTCTTCTT

ACGCATCATGGGGGTAGGGCTAGCTGGACTCCCAGTGTAGGAGGCTCCAGCA

CAGGCCTCCAAGGTATGGGCTCCAGCTCTGGACAGGTAAGAGCTGAGGAAGA

CTTCCAGGTAGGGAGAGACACAAGAAGCCAAGAGGTGAGACAGCTGAAGAA GGCCAGGCCCTAGG.

The M5a mutation in L93-M5-mut-Runx was corrected by PCR mutagenesis to generate L93mut-Runx. The M5 mutation was introduced into L93-M5 by overlap PCR of CE4 which was then used to replace the wild type sequence. Sequences of primers used to construct reporters are available upon request. All reporter constructs were sequence verified.

Transfections and luciferase assays:

Cells were transfected in some experiments with FuGENE 6 reagent, at a FuGENE:DNA ratio of 3:1. Alternatively, cells were transfected by Nucleofection (Lonza/Amaxa). Solution-V kits were used when nucleofecting Adh.2C2 cells with program D-19, or Raw264.7 cells with program D-32 (Lonza/Amaxa). EL4 cells were nucleofected with Solution-L kits and program C-09. Cells were harvested ~48 hours post FuGENE treatment or ~24 hours post nucleofection. Cells were cotransfected with pRL-CMV and lysates were analyzed using Promega's Dual Luciferase system. 3-6 μg *Sfpi1* reporters were used in transfections with 100-200 ng pRL-CMV control. For stable transfections, *Sfpi1* reporters were linearized with Not I prior to transfection. The renilla luciferase was cloned into Invitrogen's pTracer EF/Blasticidin A and the construct was linearized for mixed transfection with the *Sfpi1* reporters. After transfection, cells were aliquot into 6-well plates then selected with 5-15 μg/ml Blasticidin for their duration in culture, beginning one day post transfection. The pTracer-Renilla control was linearized with Fsp I. 10 μg of *Sfpi1* reporters were transfected with 1-2 μg of pTracer-Renilla. For some stable transfections, insert copy number was determined by quantitative PCR with

primers specific to firefly luciferase and the pTracer-Renilla control vector's GFP-Blasticidin sequence: Luc-F ACGATTTTGTGCCAGAGTCC and Luc-R AGGAACCAGGGCGTATCTCT; GFP/Blast1-F GTCAGTGGAGAGGGTGAAGG and GFP/Blast1-R ACGGGAAAAGCATTGAACAC. All antisense morpholino transfections were performed by nucleofection with 2 nanomoles of morpholinos. The following morpholino antisense oligos were order from Gene Tools, Inc.: anti-Runx1 CAGGCAGGAGTACCTTGAAAGCGAT; anti-PU.1 GAGGACCAGGTACTCACCGCTATG; anti-CBFβ CCTCCCCAACCGCCTCACCTCGCAC

Transcription factor binding site predictions:

TRANSFAC analysis was used to predict potential transcription factor binding sites. Biobase's (https://portal.biobase-international.com/cgi-bin/portal/login.cgi) TRANSFAC suite's MATCH tool was used for the analysis. Matrix similarities >0.925 were shown.

Gel Shift Assays:

Nuclear extracts were prepared by hypotonic swelling in buffer A, followed by NP40 lysis, nuclei pelleting, and extraction with buffer C containing protease inhibitors (Roche #11873580001). Buffer A: 10 mM HEPES pH 7.9, 60 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, followed by addition of NP40 to 0.625%. Buffer C: 20 mM HEPES pH 7.9, 0.4 M NaCl, 1mM EDTA, 1mM EGTA, 1 mM DTT. Protein was quantified by the Bradford method. Gel shifts were performed with ~6 μg extract in 30 μl volume containing 1-2 μg poly di-dc and final concentrations of 15 mM HEPES pH 7.9, 80 mM NaCl, 15 mM KCl, 0.02 mM EDTA, 1 mM DTT, and 3% glycerol. Five

picomoles of probes were end labeled with T4 polynucleotide kinase followed by purification with G-50 columns (Roche #100609). Complexes were allowed to form for 10 minutes with competitors prior to addition of radiolabeled probes. After probe addition, samples were incubated for an additional 30 minutes. All incubations were carried out on ice. Complexes shown in Fig, 5B were resolved by 4% PAGE, run at constant 350 volts for 4hours. Complexes shown in Fig. 5C and 6C were resolved by 6% PAGE run at constant 350 volts for 2.5 to 3.5 hours. All gels were run at 4^{o}C with 0.5x TBE gels and 0.25x TBE running buffer. 4% gels were prerun for 30 minutes. Quantification was performed by Phosphorimager and ImageQuant 5.2 analysis. Runx1 N-terminal antibody was from Calbiochem (Catalog #PC284). Runx1 antibody against amino acids 231-245 was from Active Motif (Catalog #39000). Pan-Runx antibody was a kind gift from Masanobu Satake (Tohoku University, Sendai, Japan). Ikaros (sc-13039) and Myb (sc-516) antibodies used in gel shift assays were from Santa Cruz.

Western Blots:

Nuclear extracts were mixed with 2x Laemmli sample buffer, boiled, and then run on 8% SDS-PAGE. Gels were transferred to Immobilin (Millipore) by semi-dry transfer. Blots were blocked with 5% milk in TBS-T (Tris-buffered saline, 0.5% Tween-20) then incubated overnight with primary antibody at 1:3000 dilution. After washing, blots were incubated for 90 minutes with secondary antibody at 1:3000 dilution, washed, then incubated with substrate (SuperSignal, Pierce #1859675 and #1859674). Substrate was drained from blots then blots were exposed to film. Primary Runx1 antibody PC284 was from Calbiochem. Sp1 antibody was from Santa Cruz (sc-59).

ChIP assays:

ChIP assays were performed as recommended by Upstate Biotechnology (Millipore). Briefly, 2-3 x 10^7 cells were fixed with 0.33-1% formaldehyde for 10-30 minutes, and then lysed in 0.8 ml with protease inhibitors. Lysate was sonicated to produce an average fragment size of ~ 250 bp. Lysate (130 μl) was diluted and used for each ChIP sample with 9 μg of antibody. Crosslinking was reversed by overnight incubation at 68°C. Proteinase K digests were for 30 minutes at 55°C. DNA was purified by ethanol precipitation and resuspended in 100 μl water. Analysis of recovered DNA was performed by SYBR green based QPCR with an AB 7900HT. One μl of purified DNA was used per 10 μl PCR reaction, in triplicate. Whole thymi from $Rag2^{-/-}$ mice were excised and thymocytes were recovered by cutting and scraping thymic lobes through steel mesh. Thymocytes were then immediately fixed and processed for ChIP assays. Approximately $4x10^6$ thymocytes were used per ChiP. Runx ChIPs performed with an equal mix of antibodies from Calbiochem and Active Motif (above). Rabbit Ig (sc-2027) and GABPα (sc-22810) antibodies were from Santa Cruz. Primer pairs used for analysis of ChIP enriched DNA by QPCR are: CE1-F AGCTCAGCTGGATGTTACAGG and CE1-R AGATGGTCACACATCCCAAAG; -2kb-F TTCTCACATCCCAGACCATTC and -2kb-R CGCCAGCAGTTGTAGTTCTTC; -2.8kb-F GCAGCTCACTGCTCCAAGTT and -2.8kb-R GAGACGGGGAGTGGGTATGT; CE3-F TGGAGCTCTGAGGGGCCTAA and CE3-R GGCTGGGAAAGCTGACCATAA; -8.4kb-F AGAGGAGCTGACATTGGCATAC and -8.4kb-R TGAGCCTCTGAAGTGGCTTTAT; CE4B-F AGCAAAGCCTGTGGGAGATT and CE4B-R ATACCTTGGAGGCCTGTGCT; CE4A-F GGAAGCAGCTCTTGTCCTTGG and CE4A-R TCACCTCCTGGCCACATCACT; CE5-F

GCTCTGAAAAGCACCGTTTCC and CE5B-R CTGTGTTGGACCTGCAAGGAG T; -11.8kb-F CTCTGCCCGCTCTTAACCTT and -11.8kb-R GATCTGACACGGGGATGAAA; CE76-F CACACGGAGTCAGAGCGGGCAG and CE76-R AGGAAAGAGGAAGCCATGGGGAGA; CE8-F AGGCAGAGCACACATGCTTC and CE8-R CTTCTGGGCAGGGTCAGAGT; CE9-F CAGGAGAGGCAGGAGGAAGGA and CE9-R AGAGAGCAGAGCACTTCATGGCT; -17.8kb-F CTGGACAAGTGGAAGGTGACA and -17.8kb-R TCAGAGGGCTTCAAAGTGGA; CD4-F TGACGGAAGGGAGGATGTAG and CD4-R AGTGGGTGGGAGCTCTGTAA; MEF2C-F AGCACACTCAGCCTGCTCTAC and MEF2C-R GGTGTAAAGGTGCTTCCTTCC; IL-7Rα-F GTCTGAGCAAAAGGATTGCTG and IL-7Rα-R GGAGCTTCAGGGAATACCAAG.

Results

The PU.1 URE is a stage specific T-cell enhancer

A 2.2-kb PU.1 promoter fragment could not drive reporter expression in myeloid cells in a chromatin context, but was able to do so when joined to the 3.5 kb URE fragment. This URE failed to enhance promoter driven expression in mature T-cells (18). In order to elucidate the dynamic mechanism of PU.1 silencing during early T-cell development, we tested the regulatory function of the URE in a more immature T-cell line representing the DN3 stage, i.e. the developmental state in which endogenous PU.1 expression is actively being silenced. We made reporters with the 2.2 kb PU.1 promoter, with or without the conserved regulatory regions of the URE (Fig. 1A). These reporters were named L98 and L1, respectively, and tested for activity by transient transfection into a mature myeloid cell line, Raw264.7, and an immature DN3 like T-cell line, Adh.2C2. As expected, we observed L98 reporter activity enhanced by ~14 fold relative to L1, in a myeloid cell line (Fig. 1B). We also transiently transfected a more mature T-cell line, EL4, and showed that here L98 failed to show enhanced activity relative to L1, as expected from previous reports (Fig. 1D). Unexpectedly, however, the L98 construct containing the URE also reproducibly showed ~3 fold enhanced reporter expression in the immature T-cells (Fig. 1C). These results suggest that the activity of the URE is not exclusively repressive in T-cells, but rather developmental stage dependent.

Cell type-specific patterns of DNase hypersensitivity associated with conserved regions outside the URE

To look for other cis regulatory elements that may contribute to PU.1 silencing in immature T-cells, we used multigenome sequence alignments to identify more conserved noncoding elements across the ~50 kb PU.1 mouse locus (Fig. 1A). Besides the conserved elements of the promoter (CE1) and the two previously identified within the URE, which we have termed CE9 and CE8, our alignments revealed other conserved regions, mapping from about -12.5 kb to -7.5 kb upstream of the *Sfpi1* transcriptional start site. These were named as CE7, CE6, CE5, CE4 (A+B), and CE3.

To assess whether any of these regions might show cell type-specific differences in accessibility or transcription factor engagement, a range of hematopoietic cell lines including myeloid (32D), multipotent progenitor (FDCP-mix and EML-c1), and pro-T cell lines (Adh.2C2) were tested to map DNase hypersensitive (HS) sites across the 5' flanking region and first two introns of *Sfpi1* (Fig. 1E, F, and Fig. S1). One DNase HS site related to the promoter was only formed in PU.1 expressing cells, as earlier reported (18). A HS site at -14kb was previously identified (18) to mark the URE and was detected in all the hematopoietic cell lines tested (Fig. 1E, band at 9.6 kb from SphI site). Also confirmed were two reported sites in the second intron, which were seen in both PU.1-expressing and –nonexpressing cell types (1)(Fig. 1F; see Fig. S1). In addition, five new sites were found. Two novel DNase HS sites formed to varying extent in all the cell lines tested (Fig. 1A & E, black arrows). The first of these was found at -12.3 kb, near CE7 and CE6. This site is just downstream of the boundary of the 3URE fragment. The second cell type-nonspecific HS site was seen at -8.8 kb, between CE4A and CE4B. Of more interest, two other upstream DNase HS sites were detected only in PU.1 expressing

cell lines (Fig. 1A and F, red arrows). These two HS sites were found at -10.8 kb and -7.4 kb (5 kb and 1.6 kb from the SphI site in Fig. 1E), flanking CE5 and CE3, respectively. Notably, we also detected a doublet of DNase HS sites specific to immature T-cells, at -8.5 kb (Fig. 1A and 1F, blue arrow; Fig. 1E right, bands around 2kb from the SphI site), and not seen in any of the PU.1-expressing cell types. We hypothesized that a regulatory feature associated with *Sfpi1* silencing could be near this region.

Identification of a novel cell type-specific PU.1 regulatory element with T lineage repressive activity

Reporters were made as shown in Fig. 1A to test if any conserved elements from CE7-CE3 might have regulatory activity independent of the URE in immature T-cells. The L7-6 construct was made to combine the *Sfpi1* promoter with a 2 kb region including CE7 and CE6, where an overlapping non cell type-specific HS was found in myeloid and T-cells (Fig. 1A). L7-6 showed enhanced and nonspecific activity in both myeloid and immature T-cell lines, with ~2× increased activity in transient transfection assays (Fig. 1G, black bars) as compared to L1(Fig. 1G, gray bar). In contrast, a construct containing the promoter plus the new conserved elements from CE5 through CE3, L5-3, showed activity that was clearly cell type-specific. L5-3 (Fig. 1G, blue bars) was strongly repressed in the Adh.2C2 cells as compared to L1 (Fig. 1G, gray bar). Strikingly, the same L5-3 construct was found to have enhanced activity in Raw264.7cells, with CE5-3 increasing promoter-driven reporter expression by ~4x. In summary, the conserved sequences CE9-CE6 span aregion of ~4 kb and contain multiple regulatory elements that increase promoter activity in both myeloid and immature T-cells. In contrast, the ~3 kb

region containing CE5-CE3 was found to mediate cell type-specific activating or repressive regulatory function.

The regulatory elements in CE5-3 not only showed lineage-specific effects on promoter activity, but also strongly modulated the combined activity of the promoter and the URE. These effects were strongest when the reporters were stably integrated and expressed from a chromatin context in myeloid and immature T cell lines. We stably transfected linearized reporters containing elements CE9-CE6 (L9-6) or a longer sequence extending further to include CE9-CE3 (L9-3). The L9-6 reporter efficiently expressed luciferase when stably integrated into chromatin, as expected (Fig. 2A), both in myeloid cells and in our DN3 like immature T-cell line to similar levels (Fig. 2B). The CE9-6 enhancer activity was hematopoietic specific, as the L9-6 reporter generated >100 fold increase in luciferase expression in both myeloid and immature T-cells compared to nonhematopoietic NIH 3T3 fibroblasts (Fig. 2C).

The addition of the CE5-CE3 region to L9-6 to make the L9-3 construct yielded sharply different results. This construct gave an ~8 fold increase in reporter expression in myeloid cells as compared to L9-6 (Fig. 2A). In the T-cells, however, addition of CE5-CE3 repressed reporter expression to a level comparable to the background level in NIH 3T3 fibroblasts (Fig. 2B & C). These data show that the CE5-CE3 cis-regulatory region can contribute to a >500 fold difference in reporter expression between myeloid and immature T-cells.

Mapping of a bipartite region necessary for silencing in a chromatin context and a core silencer sufficient for silencing in transient assays

DNAse HS mapping revealed a pan-hematopoietic HS site between CE4A and CE4B, as well as a T lineage-specific HS site just downstream of CE4B (Fig. 1A, E, F). This suggested that the CE4A-B region might be involved in the T-cell silencing effect. As shown in Fig. 2B, deletion of CE4A and CE4B did abolish the repressive function within the L9-3 construct. To map regions within CE5-CE3 that contribute to the cell type-specific regulatory function, we made more reporters combining individual conserved regions together with the 2.2 kb PU.1 promoter and then tested their function in transient transfection assays. While the dynamic range of these assays is less than in the stable transfections, these rapid surveys yielded results that could be verified in the stable transfectants.

This functional mapping showed that the myeloid enhancing activity and the pro-T cell silencing activity are mediated by different cis-regulatory sequences. The CE5 region confers myeloid-specific enhancer activity. As shown in Fig. 3B, CE5 (in construct L5) was able to enhance promoter activity by ~7-fold in myeloid cells, but by only 1.5 fold in the immature T cells (Fig. 3C). In contrast, all the repressive activity in immature T cells mapped to the CE4 region. Results with the L4A construct showed that the CE4A region alone could repress *Sfpi1* promoter activity in the immature T cells (Fig. 3C), but with little or no effect in the myeloid cells (Fig 3B). The CE4B region did not confer independent regulatory function in transient assays (Fig 3B and 3C), though it contains one reported Stat3 site implicated in *Sfpi1* induction by cytokines (12). However, the presence of both CE4A and CE4B was necessary for full silencing when

integrated stably in a chromatin context (Fig. 3D), indicating that the CE4A-B region is a bipartite T-specific silencer.

The CE4A region spans ~450 nt in which the central ~120 nt are most conserved. To map the sequences within the CE4A region that are vital for silencing, we truncated this 450 nt region and made reporters with these truncations flanked by the URE elements and promoter(Fig. 4A). These experiments showed that a minimal conserved core is necessary and sufficient for repression in the transient assays (Fig. 4B). Construct L98+4A-5, containing only the minimal core silencer region of ~120 nt from CE4A, was sufficient to repress the enhancer activity of the CE9-CE8 region in immature T-cells (Fig. 4B).

Scanning mutagenesis analysis of the core silencer

The CE4A core silencermapped to a peak of conservation identified by the multigenome alignment (Fig 1A). The precise nucleotide alignment is shown in Fig. 4C, with asterisks marking nucleotides that are 100% conserved amongst eleven organisms, and with predicted transcription factor binding sites shown (see Materials and Methods). To unmask the most influential repression sites in an unbiased way we carried out scanning mutagenesis and tested for loss of repression in transfection assays, mutating 6-nt blocks (M1to M15, Fig. 4C) across the core CE4A silencer region within the reporter construct L98+4A-5. The first four mutants examined,M1-M4, did not affect the repressive function of the core silencer (Fig. 4D). However, mutantsM5-M9 caused a loss of core silencer function (Fig. 4D). These mutants span a region of 30 nt across the largest conserved block within the core silencer (Fig. 4C). Another mutant, M13, also blocked core silencer activity (Fig. 4D). Close examination of the sequences where

mutations abolished core silencer function showed that 5/6 mutations, M6-M9, and M13, overlap sequences with ~90% similarity to the canonical Runx binding motif, (Py)G(Py)GGT (Fig. 4C, red boxes). Mutant M5 does not overlap a predicted Runx site, but crosses a conserved site, “site X”, predicted to contain an Ets family target site (Fig. 4C, blue box).

Identification of T-cell specific protein complexes

To identify transcription factors vital for the T-cell-specific repressive activity of the CE4A core silencer, probes spanning the CE4A core region were used in gel shift assays with nuclear extracts from Adh.2C2 and Raw264.7 cells to determine the nature of cell type-specific protein-DNA complexes. Complexes were identified by mobility, and by cross-probe competition and antibody treatments. These assays showed that at least three regions of the CE4A+B elements could nucleate cell type-specific protein-DNA complexes that differed qualitatively when formed with T or myeloid extracts (Fig. 5C). The cell type specificity of the complexes formed with these probes contrasted with those detected by the CE8 region of the URE, where similar patterns of complexes were formed with extracts from T and myeloid cells alike (Fig. S2)

Fig. 5B shows that a large probe spanning the whole critical repression region (CE4A-P6) could nucleate a single complex from Adh.2C2 extracts. This large, slow-migrating complex (T1) depended on binding to two distinct regions, as defined by competition with mutant and wildtype competitors. One critical site was element X (compare CE4A-P6 and CE4A-P6m5) and the other was the region of predicted Runx site CBF3 (compare CE4A-P3 and CE4A-P3m7a). To define the distinct component complexes that might contribute to the large T1 complex and to identify those that might

be cell type specific, gel shift assays were then carried out with a tiling array of smaller CE4A probes. Two distinct complexes formed on these smaller probes which were T cell specific: one named A3, and a larger, slow-migrating complex named A1 (Fig. 5C, lanes 1, 3, 5). Using probe CE4A-P3, which formed both complexes, Fig. S3A shows that the sequence requirements for these two complexes can be dissociated. Complex A3 mapped to element X, which is mutated in CE4A-P3m5a (lanes 1-4), while complex A1 depended on the region of the CBF3 site that is mutated in CE4A-P3m7a (lanes 1-4).

Because the full silencing activity in a chromatin context depends on region CE4B as well as CE4A, we carried out a screen for potential T-cell-specific binding complexes to CE4B probes too. Of seven CE4B probes tested, only two formed complexes and only probe CE4B-P3 formedcell type-specific complexes(Fig. S3B). This probe was also found to form a very slow moving complex, designated B1, which was T-cell specific (Fig. 5C, lane 11, red arrow; Fig. S3B). Strikingly, the CE4B B1 complex was similar to the large A1 complex formed with CE4A, based on cross competition and mutational analysis (Fig. S3A, lanes 1, 2, 5; Fig. S3C, lanes 1, 2, 4). Both complexes depended on the integrity of predicted Runx sites (Fig. S3A, lanes 1-3 & 5-7; Fig. S3B, lanes 1 vs 6 and 7; Fig. S3C). As these are likely to be higher-order complexes with multiple protein components, we also tested them for the presence of additional factors predicted to bind nearby (Fig. 4C, Fig. S3E). Both A1 and B1 complexes were super-shifted by antibodies against Ikaros, though not by anti-Myb (Fig. S3A, lanes 9-10; S3D, lanes 2-3).

Together, the analyses suggest that the T-cell specific bands A1 and B1 represent redundant T-cell specific complexes dependent on predicted Runx sites while A3 is a T-cell specific complex that depends on site X. The higher-order T1 complex could thus be

a composite of A1 and A3. The possibility that the A1 and A3 complexes on CE4A interact at the protein level was supported by the ability of a pan-Runx antibody to interfere not only with T1 and A1 but also with A3 (Fig. S4, lane 5; Fig. 5B, lane 10). The composition and precise role of the complexes depending on the X site are still unresolved (data not shown). However, further experiments strongly support direct involvement of Runx1 protein in the T-cell specific repression activity of the bipartite silencer, as described below.

CE4A silencer contains multiple nonconsensus Runx binding sites that contribute to T-cell specific complexes

Although similar to Runx sites, the core silencer defined by mutations does not contain precise matches to the Runx consensus sequence (Py)G(Py)GGT, and more evidence was needed to determine whether Runx proteins could be part of complexes A1, B1, and T1. Pan-Runx antibodies shifted or inhibited these complexes and Runx1-specific antibodies seemed to crosslink them, but the slow initial mobility of these complexes made the results difficult to interpret (data not shown). Therefore, the binding of Runx proteins to CE4A was confirmed by competition in gel shifts against a validated Runx-dependent silencer sequence, the *Cd4* silencer (31)(Fig. 6, Fig. S5).

The *Cd4* silencer probe contains two canonical Runx motifs (Fig. 6B) and formed a strong band with nuclear extract prepared from Adh.2C2 cells (Fig. S5, black arrows). The complex is competed by cold *Cd4* probe, and this competition depends on the two Runx motifs (Fig. S5A, lane 3). The band can also be super-shifted with Runx1-specific antibodies, but not control Ig, confirming that the *Cd4* probe gel shift complex contains Runx1 (Fig. S5, red arrows). Competition for this complex thus affords an assay for

Runx1 binding to other sequences. Probe CE4A-P1, though outside the core region required for silencing, does contain a canonical Runx motif, CBF1, and competed with the *Cd4* probe for Runx binding (Fig. S5A). CE4A-P2 lacks the canonical Runx motif and did not compete for Runx binding. Competitor probe CE4A-P3 competed for Runx binding (Fig. S5A) but when the potential Runx site CBF3 was mutated (Fig. 5A, CE4A-P3m7a), it could no longer compete for the *Cd4* silencer Runx complex (Fig. S5A, lane 6 vs. 7). However, this is not the only site in the CE4A region where Runx could bind. CE4A-P4 contains predicted Runx sites CBF4 and 5 that also competed with the *Cd4* probe for Runx binding (Fig. S5A). Note that the Runx sites in all these probes, except for CE4A-P1, map to the locations of mutations that damaged repression in Fig. 4, i.e. mutants M6- M9. The predicted CBF6 sequence in probe CE4A-P5, which is disrupted by mutant M13, also competed with the *Cd4* probe for Runx binding (Fig. S5A). When a longer probe that spanned the M5-M13 regions of CE4A was used as a competitor, mutations in at least four distinct sites were needed to abolish all Runx competition activity (Fig. S5C, "M-all"). The CE4B-P3 probe also competed against *Cd4*, again indicating that this region has a Runx binding site (Fig. S5A, lane 10). These results suggest not one but multiple potential Runx binding sites in the functionally vital regions of the bipartite silencer.

The one mutation that affected repression in transient assays without sequence similarity to a Runx site was the M5 mutant, a 6-bp mutation which spans the X site (Fig. 4C). Consistent with the specificity of the assay, smaller mutations in the region of the X site did not prevent the CE4A-P3 oligo from competing for Runx binding to the *Cd4* silencer probe (Fig. S5B, lanes 5-7). Thus, the X site per se, and specifically its Ets site-like GGAA core, is not required for the binding of Runx to the CBF3 site. However, the

full six-nucleotide M5 mutation also destroys the ability to compete away the *Cd4* Runx complex (Fig. S5B, lane 4), implying that this sequence is important to stabilize Runx binding to the flanking CBF3 site. Thus, all of the mutations that abolished core silencer function in the scanning mutagenesis assay are associated with the sites of Runx complexes in vitro.

Quantitative impact of Runx levels on binding to the CE4A silencer

Runx1 was implicated in the regulation of PU.1 expression already and was shown to bind to three canonical Runx sites in the CE8 element. These CE8 sites are organized as a doublet of sites with ~50 nt separating them from another single Runx site. We therefore asked whether the ability of Runx1 to bind CE8 is distinct from its ability to bind CE4.

Western Blot analysis of Adh.2C2 and Raw264 nuclear extracts showed that these T-cells have ~4x more Runx1 protein than the myeloid cells (Fig. 6A). Note also that the Runx sites in the functionally important regions defined by mutants M6-M9, and M13 all deviate from the consensus, (Py)G(Py)GGT, whereas CE8 has consensus sites. Thus, we hypothesized that occupancy of the repression-linked Runx sites in CE4A-4B might require higher Runx expression levels than the activating or bivalent sites in CE8.

We examined the binding affinities of the CE8 and CE4A-P3 Runx sites by titrated and quantified competitions against the *Cd4* Runx probe. As shown in Figure 6C, cold *Cd4* probe competed against itself in a dose-dependent way. The CE8-P1 and CE8-P3 probes both competed fully for Runx binding. When the Runx sites were mutated in CE8-P1m1 and CE8-P3m1, competition was lost. The *Cd4*, CE8-P1, and CE8-P3 probes all reduced *Cd4* probe Runx binding by ~80% at 20 fold excess under these conditions,

and eliminated binding to below the threshold of detection when used at 100 fold excess (Fig. 6D). In contrast, the CE4A-P3 probe was a weaker competitor. This is notable as it includes not only the highest-scoring Runx site across the functionally important silencer region, but also the adjacent X site which seemed to enhance Runx binding. The CE4A-P3 competitor reduced the *Cd4* complex by only 65% at 20 fold excess and by only 79% at 100 fold excess. Taken together, these data suggest that the most dominant Runx site in CE4A indeed has a weaker binding affinity than the Runx sites in the URE.

Runx protein perturbations and Runx binding site mutations abolish silencer function

We used two approaches to determine whether Runx1 itself was functionally important for the silencing mediated by CE4A in the T-cell context. First, we used co-transfections of a Runx1 dominant negative expression construct (d190) (32) together with the reporters to compete against endogenous Runx1 (Fig. 7A). This Runx1 DN construct includes the full Runx1 DNA binding domain but lacks the C-terminal effector domains associated with silencing and transactivation. Second, we used transfection of morpholino antisense oligonucleotides to knock down Runx1 protein levels (Fig. 7B). In both assays, the effects on silencing mediated by the CE4 region were assessed by comparison with effects on activity of L98 in the absence or presence of the silencer (the full CE5-CE3 region or CE4A alone). We compared the effects of these Runx1 antagonists with effects of full-length, wildtype Runx1 and of morpholinos against the Runx complex partner, CBFβ. In addition, these results were compared with effects of a "dominant negative" mutant form of another candidate silencing factor, Ikaros (25), and with effects of a morpholino against PU.1 itself.

Fig. 7A shows that competition with Runx1 DN blocked repression mediated by the CE4 silencer. There was clear enhancer activity of the CE9-CE8 URE in the T-cells in this assay (Fig. 7A, lanes 3, 4 vs. lanes 1, 2) which was eliminated by addition of CE5-CE3(Fig. 7A, lanes 11, 12 vs lanes 1, 2). Cotransfection of full length Runx1 had no effect on this silencer activity, nor did the dominant-negative “Plastic” mutant of Ikaros (lanes 15-18). However, cotransfection of the Runx1 DN relieved silencer activity in a dose dependent way (Fig. 7A, red bars, lanes 13, 14). The repression-alleviating effect of the Runx1DN was also detectable when assayed with the L98 reporter, consistent with evidence that endogenous Runx1 can be repressive at CE8 as well (15); however this effect was much weaker than when the CE5-CE3 region is present.

Endogenous Runx1 protein in the Adh.2C2 cells could be knocked down by transfection with an antisense morpholino (Fig. 7C). This treatment also blocked silencer activity of CE4A, as measured by the ratio of expression driven by L98 plus the CE4 element to expression driven by L98 alone (Fig. 7B). As Runx1 binds DNA in a heterodimer with CBFβ, a morpholino was made to block CBFβ expression and this also relieved some repression (Fig. 7B). All these effects were cell type specific. In contrast, a morpholino against PU.1 had no effect, nor did a control morpholino against the inverse of the Runx1 sequence.

Finally, we confirmed the role of the Runx sites in stable transfections by testing the L9-3mut-Runx reporter in which all predicted Runx sites across CE4A-B were mutated. We also evaluated the impact of the M5 mutation in L9-3 and in combination with mut-Runx (L9-3 M5 mut-Runx), as the X site stabilized Runx1 binding in vitro. As shown in stably transfected cells, the L9-3mut-Runx reporter, with or without the M5 mutation, could no longer be silenced in Adh.2C2 cells (Fig. 7D). In contrast, the L9-3

M5 construct which retains all Runx sites but has the M5 mutation, was still moderately silenced.

Runx1 binds to the CE4A core silencer in vivo specifically in immature T-cells

These results raised the question why Runx1 could exert repression via CE4 in T-cells but not in myeloid cells. The competitive titration assays predicted that recruitment of Runx1 to the silencer element might depend on the higher level of expression found in T-cells more than recruitment to the URE, but this remained to be verified in vivo. Therefore, we carried out ChIP assays against Runx1 on chromatin from Raw264.7 and Adh.2C2. The results confirmed that Runx1 binding on CE4A is cell type specific in vivo (Fig. 8A, B). No strong Runx1 binding was detected across any part of the PU.1 upstream region in the myeloid cells (Fig. 8A, right), consistent with evidence that it may act there in a hit and run style (14). In contrast, there was a strong peak of Runx1 binding at CE4A in the immature T-cell line, stronger than its binding to the URE (Fig. 8B, right). The disparity between strong Runx1 binding at CE4A and weaker signals at CE8 in the T-cells was unexpected in view of the established activity of Runx at CE8 and the open chromatin at CE8 in T and B cells alike (13), but this was highly reproducible. Both T and myeloid cells showed similar binding of the Ets family factor GABPα to CE8 (left panels, Fig. 8A, B), confirming that protein-DNA complexes could be detected at CE8 at least as efficiently in the T-cells as in myeloid cells, if they were there. Thus, in immature T-cells but not in myeloid cells Runx1 is selectively recruited to CE4A, even more than to the URE, consistent with a role in T-cell specific silencing activity of this element in vivo.

Finally, to verify whether Runx1 binding to CE4 occurs in normal T-cell precursors at the stage when they first turn off PU.1 expression; we carried out ChIP assays on chromatin of primary thymocytes from $Rag2^{-/-}$ mice. These are populations in which >90% of cells are blocked in vivo at the pro-T-cell DN3 stage and have newly silenced *Sfpi1*. Control Ig did not generate strong relative enrichment of any region examined. However, a mix of Runx1 specific antibodies strongly enriched for CE4Ain $Rag2^{-/-}$ thymocytes, at least as strongly as CE8 (Fig. 8C). The magnitude of enrichment was comparable to the enrichment of the *Cd4* silencer in these cells, an internal positive control because Runx1 is known to be repressing *Cd4* at this stage of development (31) (Fig. 8C). Thus, Runx1 binds to CE4A preferentially and lineage-specifically under physiological conditions within primary T-cell precursors.

Discussion

We have identified multiple novel conserved elements of *Sfpi1* that can mediate developmentally lineage-specific transcriptional regulation. The nature of these elements contrasts with the roles proposed for the promoter and the URE, which are essentially bifunctional. Thus, dedicated regulatory modules also exist that can contribute to *Sfpi1* regulation in a lineage-restricted way. The new elements seem specific not only for context-dependent activity but also for valency of function, activation versus repression. They correspond to conserved sequences and are thus likely to have been evolutionarily selected for these functions. Although the factors that bind these new modules include ones like Runx1, which also binds to three conserved sites in the URE, our results show that the new element CE4A can provide these factors with an alternative site organization and an alternative selection of interaction partners. Thus, at CE4A and CE4B, Runx

factors participate in cell type-specific complexes that they do not generate at CE8. These alternative complexes, organized by binding to the distinct cis-regulatory DNA "scaffolds", are likely to explain why the impact of Runx1 binding at sites like CE4A can be focused on mediating a repressive transcriptional response.

Identification of novel regulatory elements was needed to explain the early repression of *Sfpi1* during T-cell development, because the *Sfpi1* URE elements CE9 and CE8 retain their enhancer function in an immature DN3 like T-cell line even though they lose enhancer function in more mature T cells. The residual expression of PU.1 observed in UREΔ hematopoietic cells (27) already implied the existence of additional positive regulatory sites outside the URE. Our results in fact identify at least two novel, conserved cis-elements: one that can act as an enhancer in myeloid cells at about -10 kb, CE5, as well as the bipartite CE4A+4B element that mediates profound silencing in immature T-cells, ~9 kb upstream of the *Sfpi1* transcriptional start site. At least CE4A appears to be fully T-cell specific in its function and a selective mediator of negative regulation.

Discovery of CE4 and the ability of this element to exert dominant silencer activity over continued URE enhancer function in immature T-cells together offer an elegant explanation for how the dual functionality of the URE is temporally switched in T-cell development. Our results show that Runx proteins, specifically Runx1 in these immature T cells, are dose-limiting for the effect of this silencer, that Runx1 binds to the CE4A and CE4B regions, and that its sites in the CE4A conserved element are crucial for repressive function of the silencer. In vivo, Runx1 rises to its highest level in immature T cells at the DN3 stage, just as PU.1 expression is first shut off (19, 30). T cells then continue to express one or more of the Runx family members throughout their continued development and mature function, potentially preserving the silence of PU.1 by active

repression. Even if this were not the case, the Runx-dependent silencing of the *Cd4* gene provides a precedent for a hit-and-run silencing mechanism that Runx proteins can use for lasting effects (31).

Nevertheless, Runx1 is clearly not T-cell specific, and so its effectiveness at the CE4 silencer must be subject to other conditions. Its selective recruitment to the CE4 repression module in T-cells likely depends both on increase of Runx1 beyond a dose-dependent threshold, from our evidence that the silencer Runx1 sites are relatively weak, and also on interaction with a T-cell specific repression cofactor. The evidence for the latter is indirect, based on two findings. First, Runx1 is recruited to the CE4 element in T-cells more strongly than to the CE8 element, in spite of the fact that individual Runx binding sites in CE8 are "stronger" by criteria of match to the consensus and in vitro gel shift competition. This could be explained if Runx1 interacts with a partner that binds at CE4 but not at CE8. The other finding, from our mutagenesis screen and gel shift complex analysis, suggests that a T-cell specific partner might bind at the "site X" region of CE4A. This site does show an effect on Runx binding affinity as well as an effect on repression in the context of the whole silencer. Clearly, the factor or complex that binds here is of great interest. One motif within site X suggests that a T-cell specific Ets-family factor could be involved, but to date this has been impossible to confirm even with a large range of antibodies that have been tested in both gel shift and ChIP assays (M. Zarnegar, unpublished data). However, note that the factor that binds to this site must still collaborate with a critical mass of Runx1 binding in order to mediate repression, and that in the most rigorous stable transfection assays, the multiplex Runx sites can mediate silencing in the absence of the X element.

Formally, the T-cell specific recruitment of Runx factors to CE4 could depend on the removal of an antisilencing factor. Against this possibility is the evidence that the CE4 region shows more “open” histone marks in T-cells than in myeloid or B-cells (M. Zarnegar, unpublished results). The CE4 region is marked by H3K4m1 and H3K4me2 marks even in mature $CD4^+$ human T cells, as defined by the ChIP-seq studies (4) (Fig. S6). Nevertheless, it is interesting that the CE4B region includes a Stat3 binding site shown to mediate *Sfpi1* activation (11). The Stat3 site does not overlap but is adjacent to the sites needed for complex B1 formation, and possibly Stat3 mobilization by growth factor receptors such as Flt3 or gp130 could prevent assembly of the silencer complex until a key stage of T cell development, and until inflammatory cytokines are absent.

In a larger sense, the characterization of the Runx-dependent CE4 silencer provides a prototype for other functionally dedicated, developmentally specific cis-regulatory elements that may collaborate with the URE and the promoter to shape the complex expression pattern of PU.1. For example, we have identified a new myeloid enhancer at -10.3 kb, CE5. There is an additional conserved cluster, CE6 and CE7, located from -12.5 to -12 kb that can mediate enhancer activity in a non cell type-specific way in transient assays, but may have more restricted functions in a chromatin context (M. Zarnegar, unpublished results). Additional lineage-specific elements may refine PU.1 expression in other cell types. Recent evidence from others shows that in erythroid cells GATA factors may modulate PU.1 gene expression through binding to another conserved site at -17.8 kb, upstream of the URE (7). Although T-cell GATA-3 should also recognize these sites, in vivo GATA-3 does not appear to bind to the -17.8 site or promoter-associated GATA sites in immature T cells as they silence PU.1 expression (M. Zarnegar & Jingli Zhang, unpublished results), so these sites may be specific for

regulation in erythroid and megakaryocytic lineages. Thus, the complex lineage-specific regulation of *Sfpi1* is not played out simply through transcription factor interactions at the URE and the promoter, but also through lineage-specific intermodular interactions between the URE and a variable set of other conserved regulatory elements.

Acknowledgments

We are grateful to Masanobu Satake (Tohoku University, Sendai, Japan) for donating pan-Runx antibody and to Janice Telfer (University of Massachusetts, Amherst) for the Runx1 DN construct. We also wish to thank Jingli Zhang and Georgi Marinov, for sharing data before publication; and Rochelle Diamond, for help isolating primary mouse tissues and excellent lab management. This work was supported by grants from the USPHS, R21 DK073658 and R01 CA90233, and by funds from the Louis A. Garfinkle Memorial Laboratory Fund, the Al Sherman Foundation, and the Albert Billings Ruddock Professorship to E.V.R.

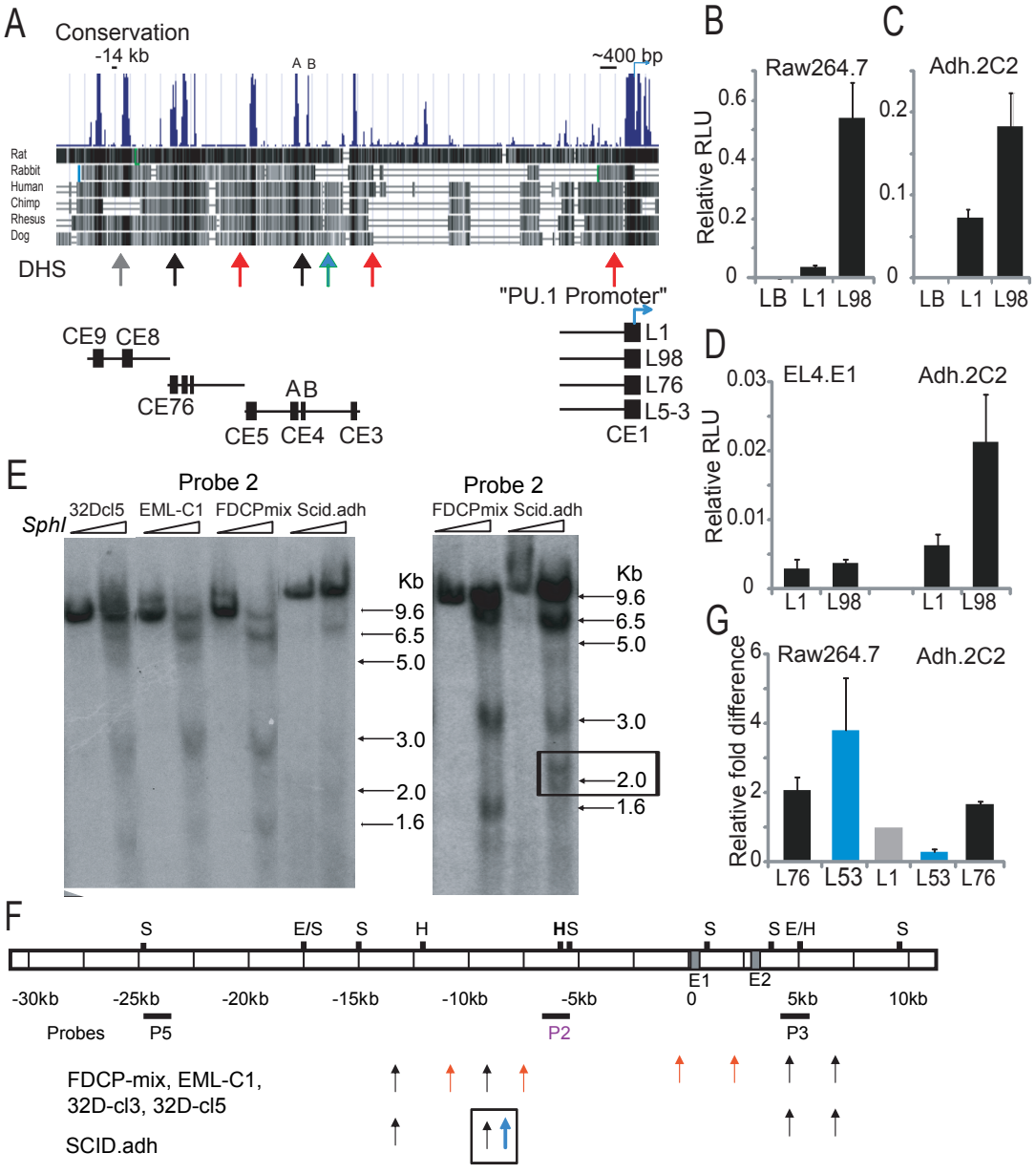
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Figure 1.

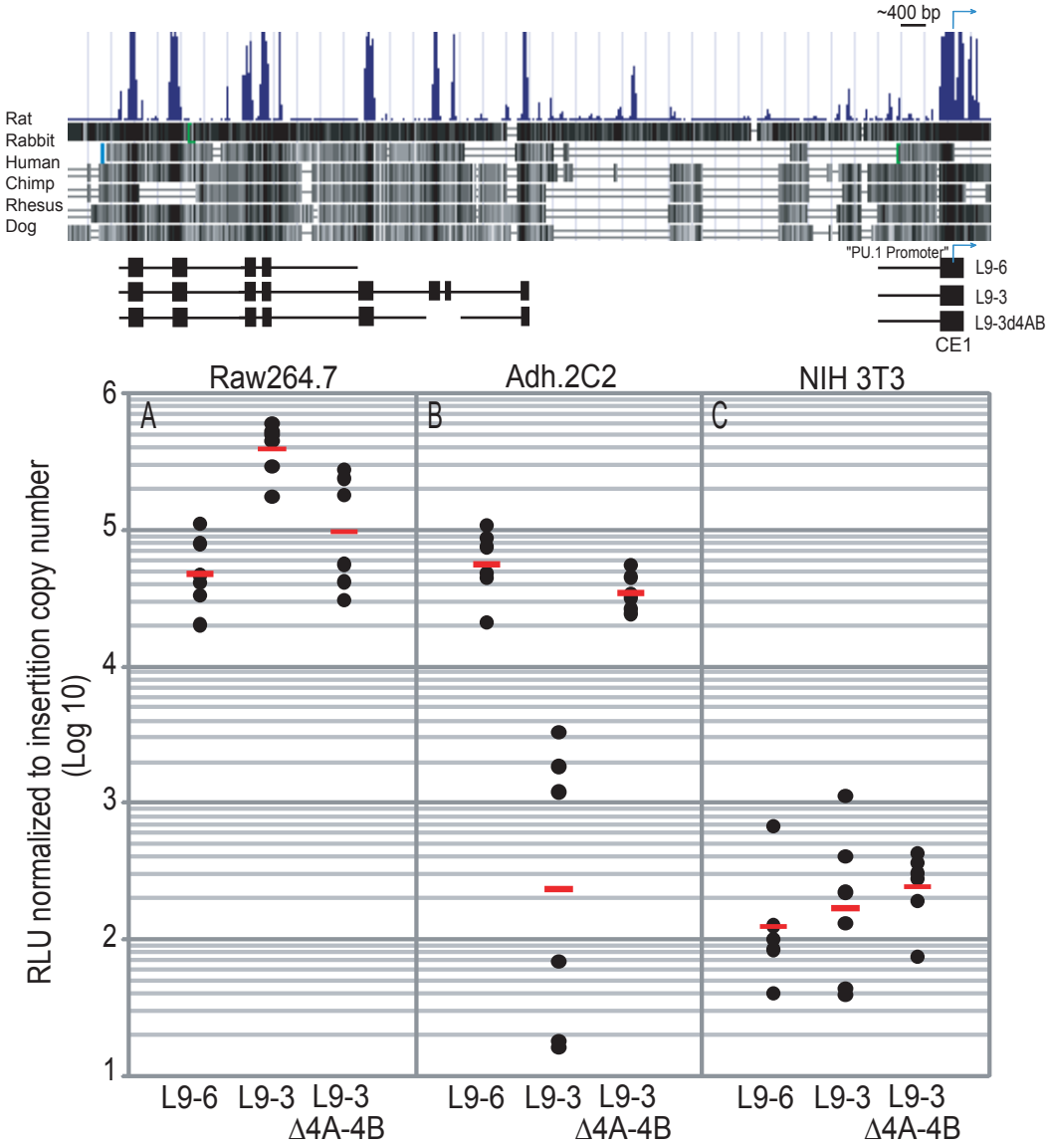
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Figure 2.

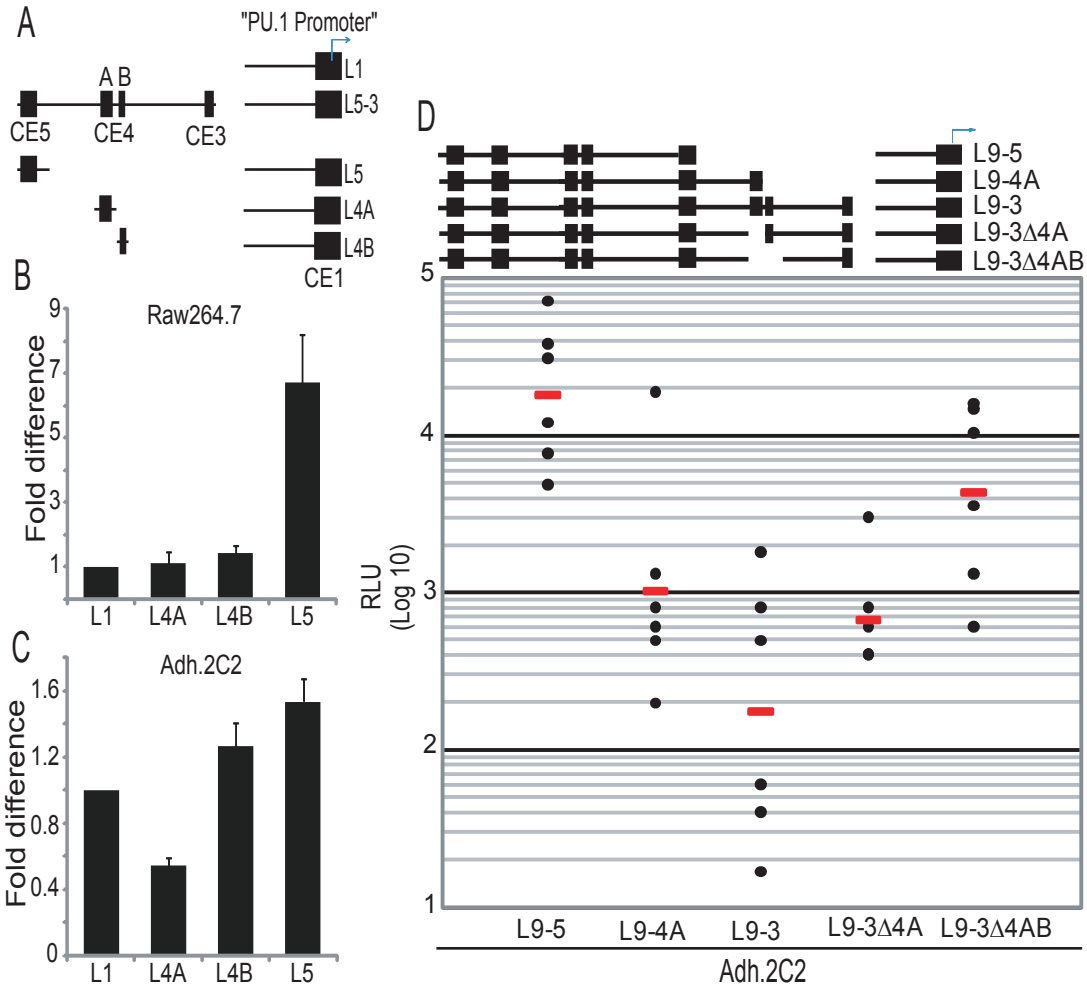
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Figure 3.

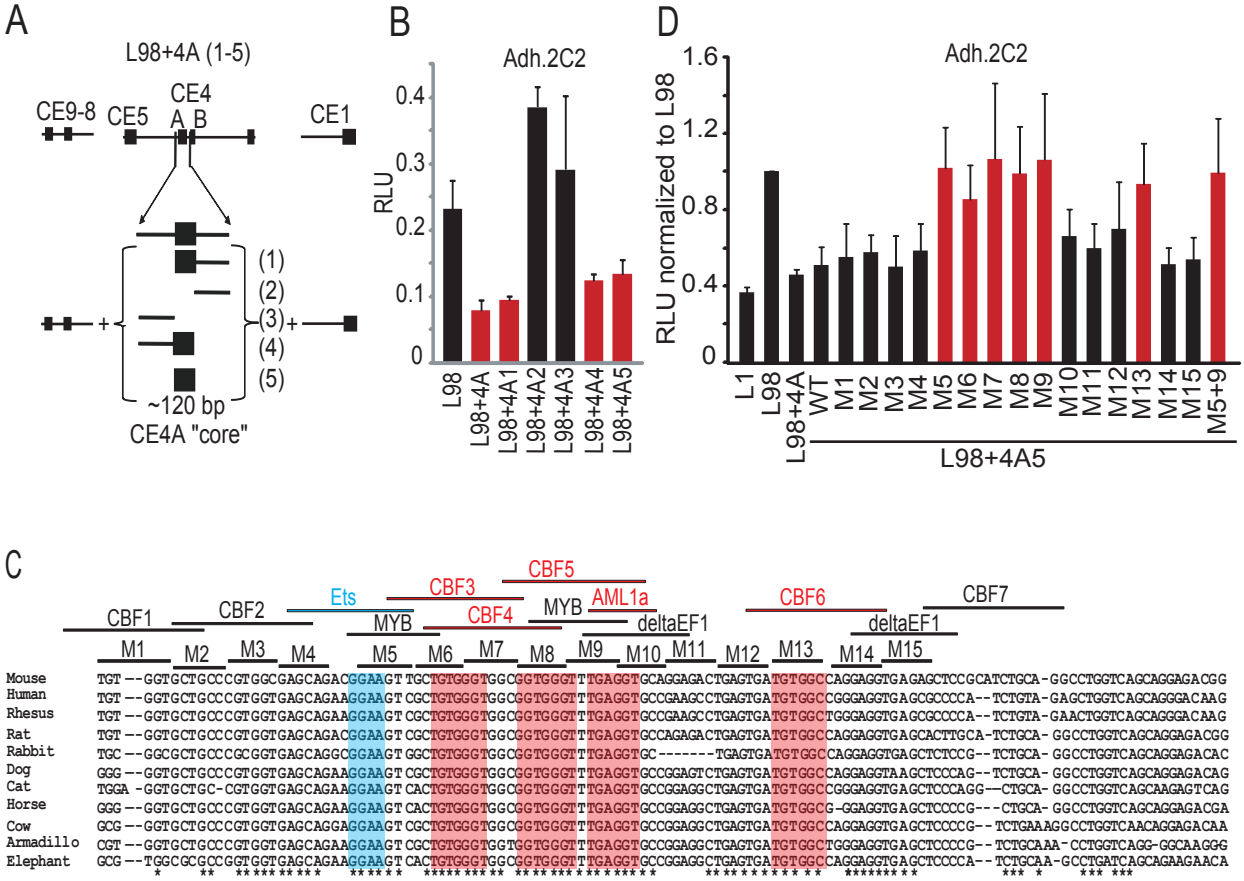
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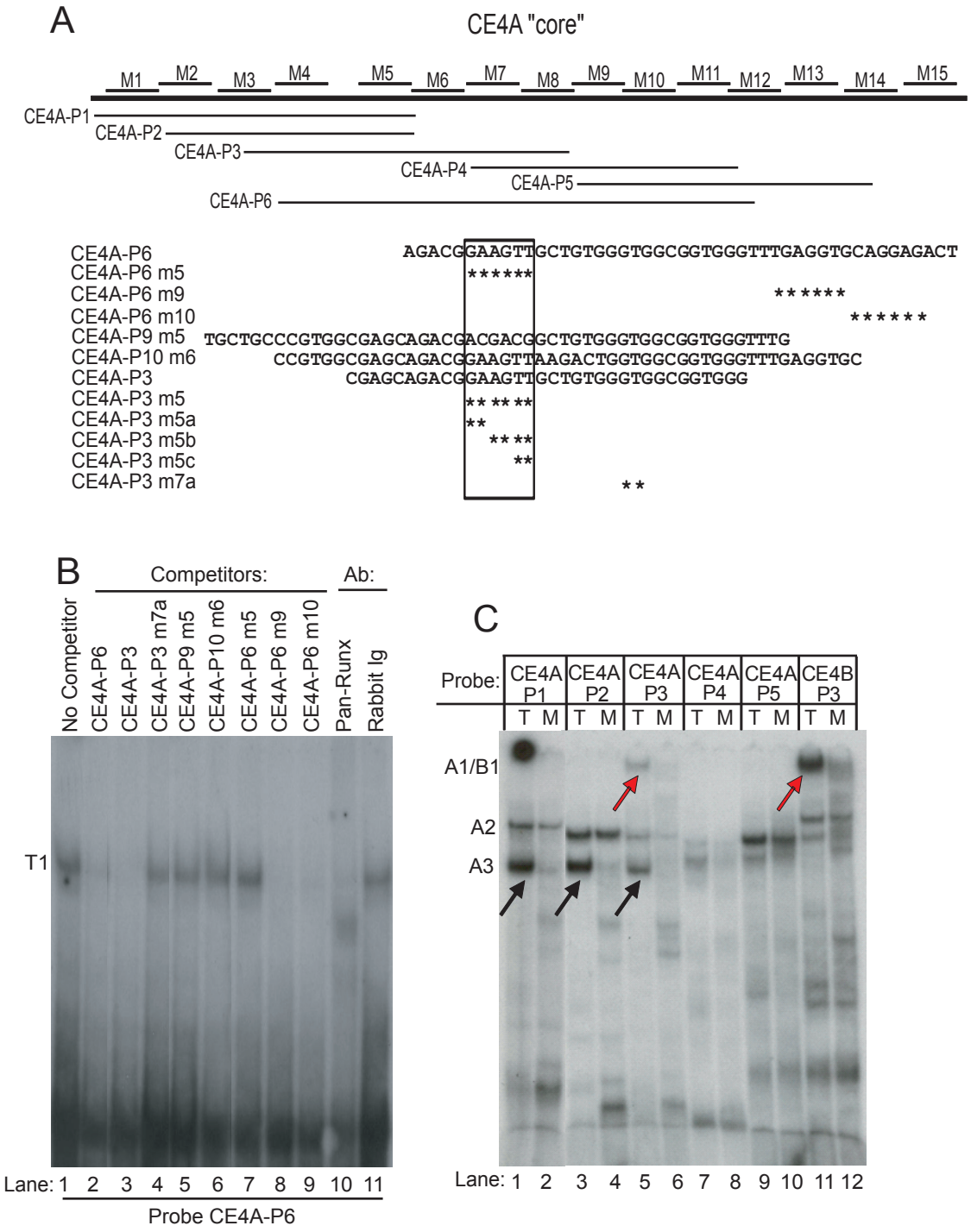
Figure 4.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Lane: 1 2 3 4 5 6 7 8 9 10 11 12

Figure 5.

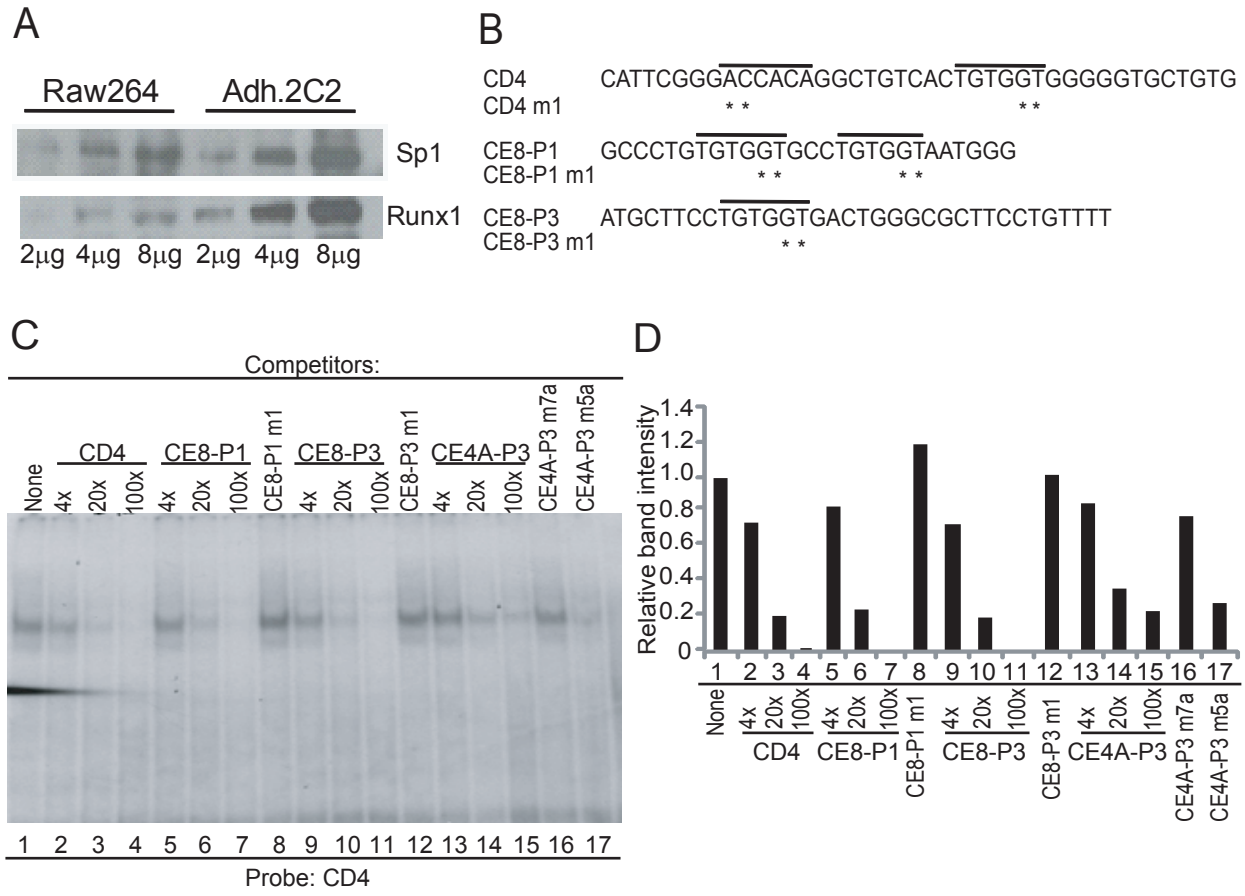

A
Raw264
Adh.2C2
Sp1
Runx1
2μg 4μg 8μg 2μg 4μg 8μg
B
CD4 CATTCGGGACCACAGGCTGTCACTGTGGTGGGGGTGCTGTG
CD4 m1
CE8-P1 GCCCTGTGTGGTGCCTGTGGTAATGGG
CE8-P1 m1
CE8-P3 ATGCTTCCTGTGGTGACTGGGCGCTTCCTGTTTT
CE8-P3 m1
C
Competitors:
None
CD4
CE8-P1
CE8-P3
CE4A-P3
4x
20x
100x
CE8-P1 m1
CE8-P3 m1
CE4A-P3 m7a
CE4A-P3 m5a
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Probe: CD4
D
Relative band intensity
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
None
CD4
CE8-P1
CE8-P1 m1
CE8-P3
CE8-P3 m1
CE4A-P3
CE4A-P3 m7a
CE4A-P3 m5a

Figure 6.

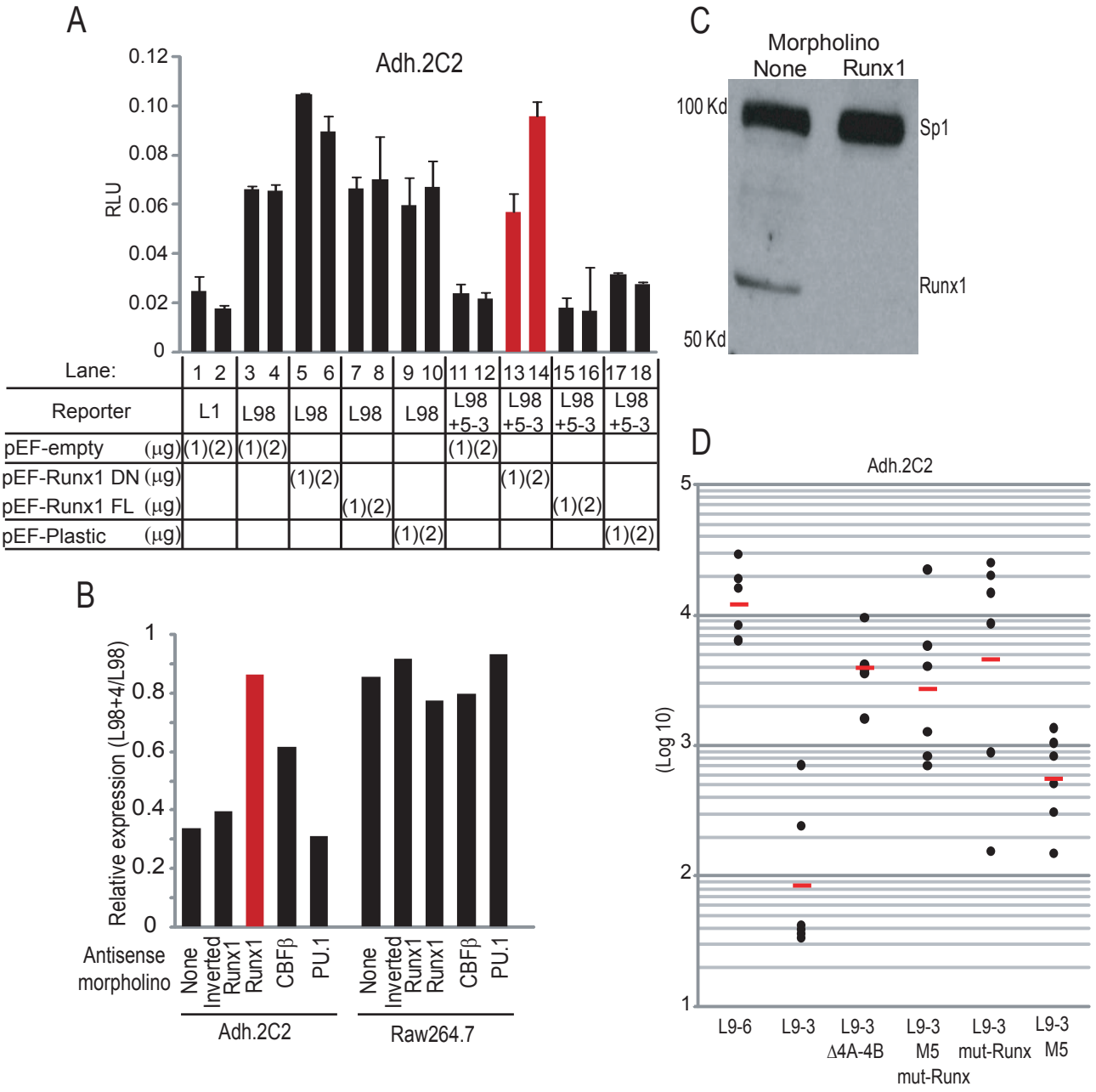

A
Adh.2C2
RLU
0.12
0.10
0.08
0.06
0.04
0.02
0
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Reporter L1 L98 L98 L98 L98 L98 +5-3 L98 +5-3 L98 +5-3 L98 +5-3
pEF-empty (μg) (1)(2) (1)(2) (1)(2) (1)(2)
pEF-Runx1 DN (μg) (1)(2) (1)(2)
pEF-Runx1 FL (μg) (1)(2) (1)(2)
pEF-Plastic (μg) (1)(2) (1)(2)
B
Relative expression (L98+4/L98)
1
0.8
0.6
0.4
0.2
0
Antisense morpholino
None
Inverted Runx1
Runx1
CBFβ
PU.1
Adh.2C2
Raw264.7
C
Morpholino
None
Runx1
100 Kd
50 Kd
Sp1
Runx1
D
Adh.2C2
(Log 10)
5
4
3
2
1
L9-6
L9-3
L9-3 Δ4A-4B
L9-3 M5 mut-Runx
L9-3 mut-Runx
L9-3 M5

Figure 7

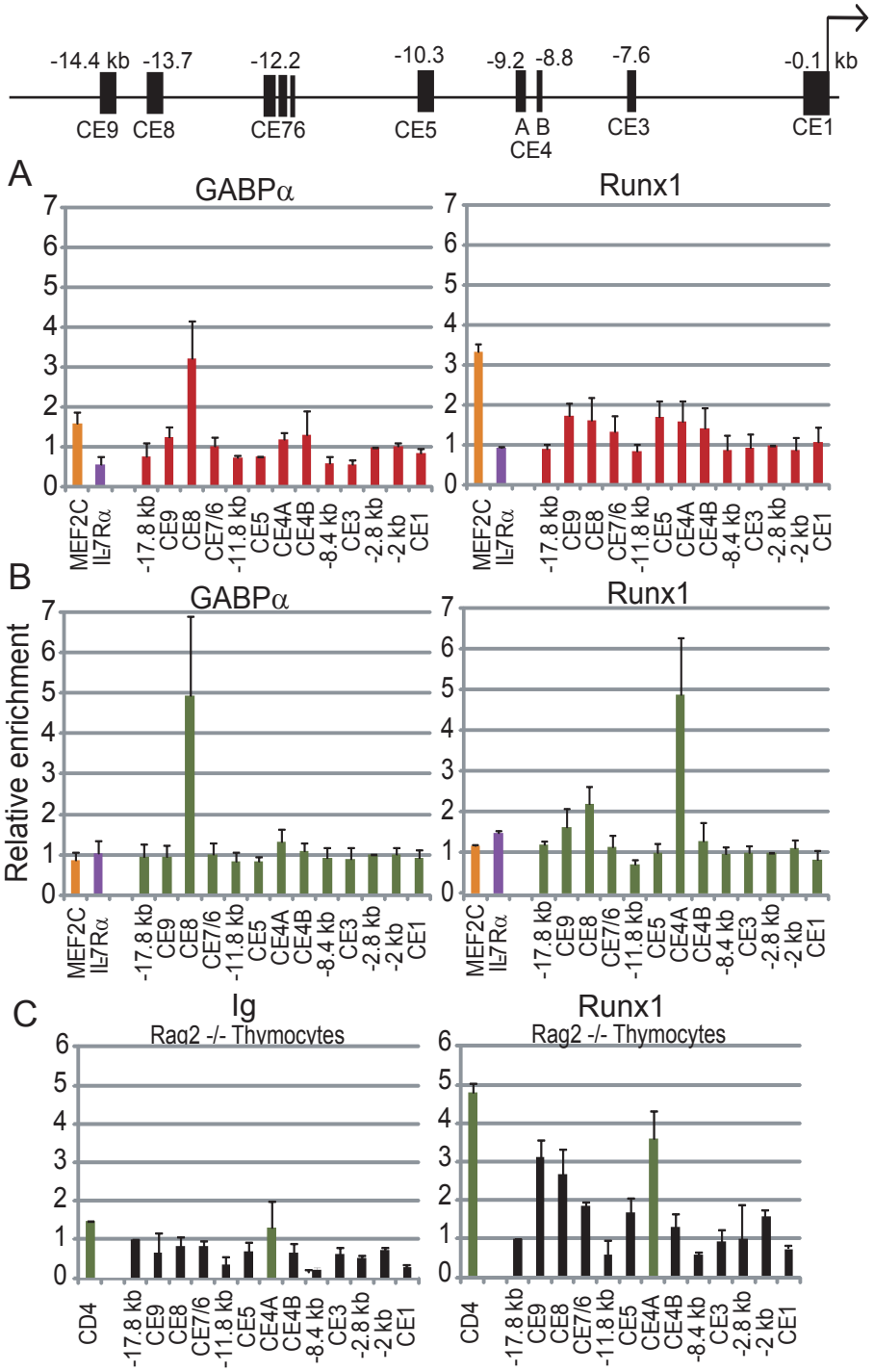

-14.4 kb
-13.7
-12.2
-10.3
-9.2
-8.8
-7.6
-0.1 kb
CE9
CE8
CE76
CE5
A B
CE4
CE3
CE1
A
GABPα
Runx1
B
Relative enrichment
C
Ig
Rag2 -/- Thymocytes
Runx1
Rag2 -/- Thymocytes
MEF2C
IL7Rα
-17.8 kb
CE9
CE8
CE7/6
-11.8 kb
CE5
CE4A
CE4B
-8.4 kb
CE3
-2.8 kb
-2 kb
CE1
CD4

Figure 8.

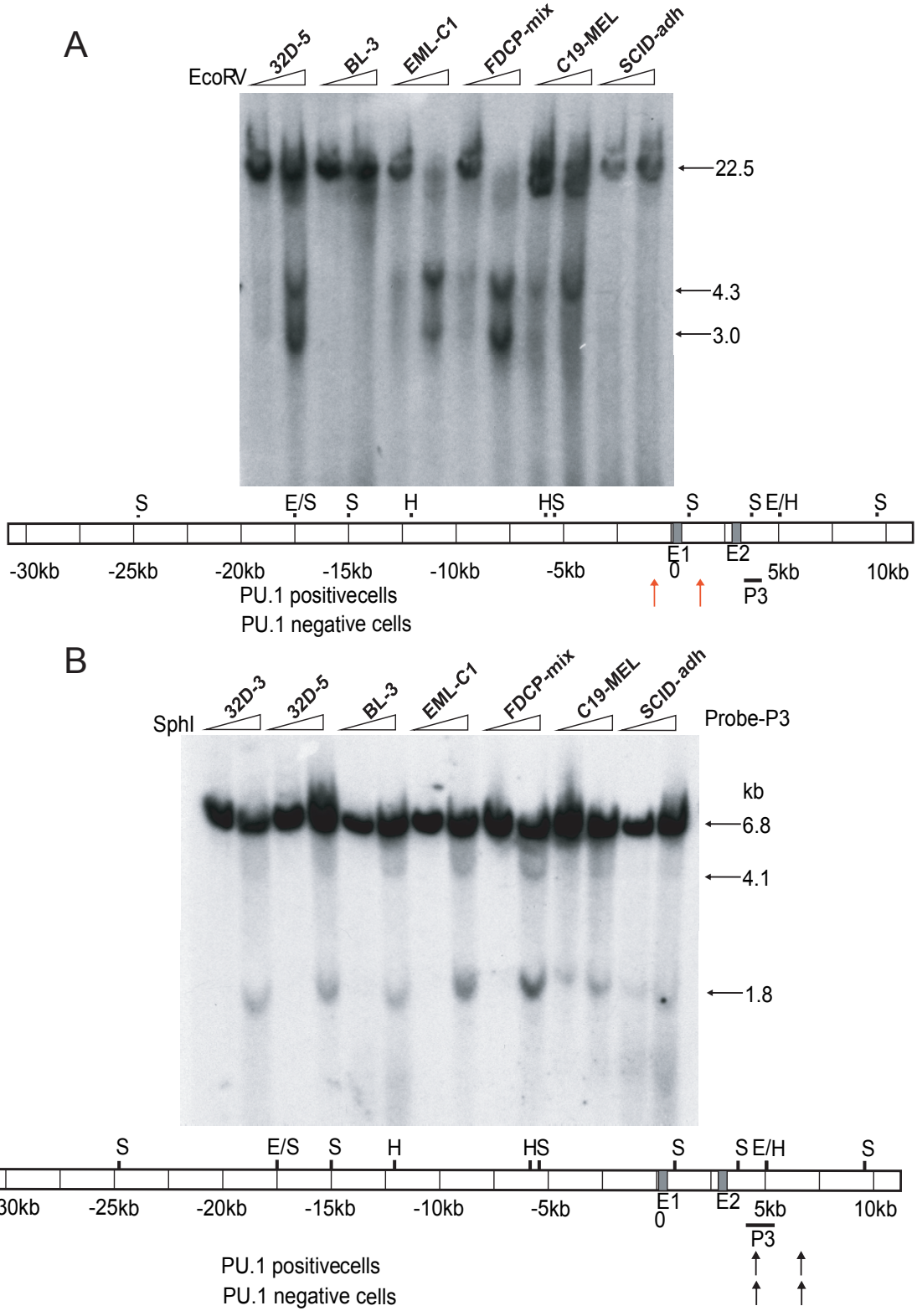
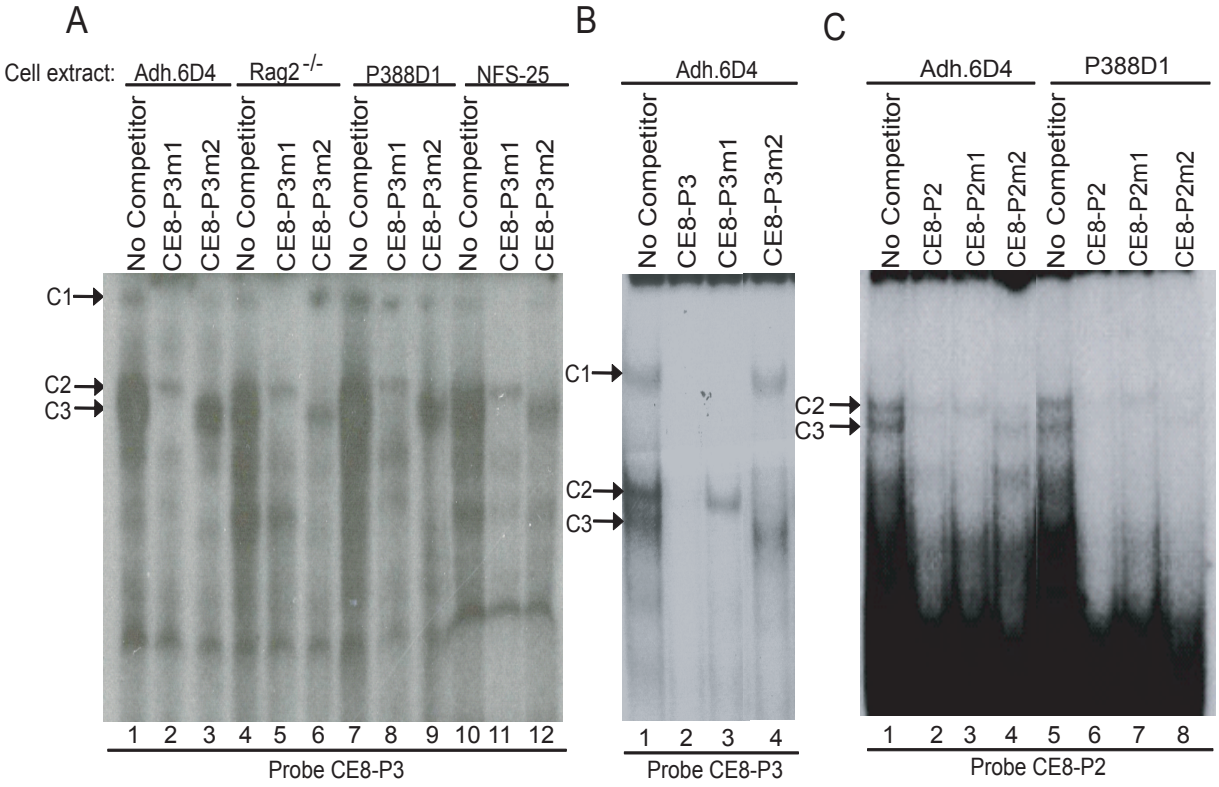

A
EcoRV
32D-5
BL-3
EML-C1
FDCP-mix
C19-MEL
SCID-adh
22.5
4.3
3.0
S
E/S
S
H
HS
S
S E/H
S
E1
E2
-30kb
-25kb
-20kb
-15kb
-10kb
-5kb
0
5kb
10kb
P3
PU.1 positivecells
PU.1 negative cells
B
SphI
32D-3
32D-5
BL-3
EML-C1
FDCP-mix
C19-MEL
SCID-adh
Probe-P3
kb
6.8
4.1
1.8
S
E/S
S
H
HS
S
S E/H
S
E1
E2
-30kb
-25kb
-20kb
-15kb
-10kb
-5kb
0
5kb
10kb
P3
PU.1 positivecells
PU.1 negative cells

Figure S1

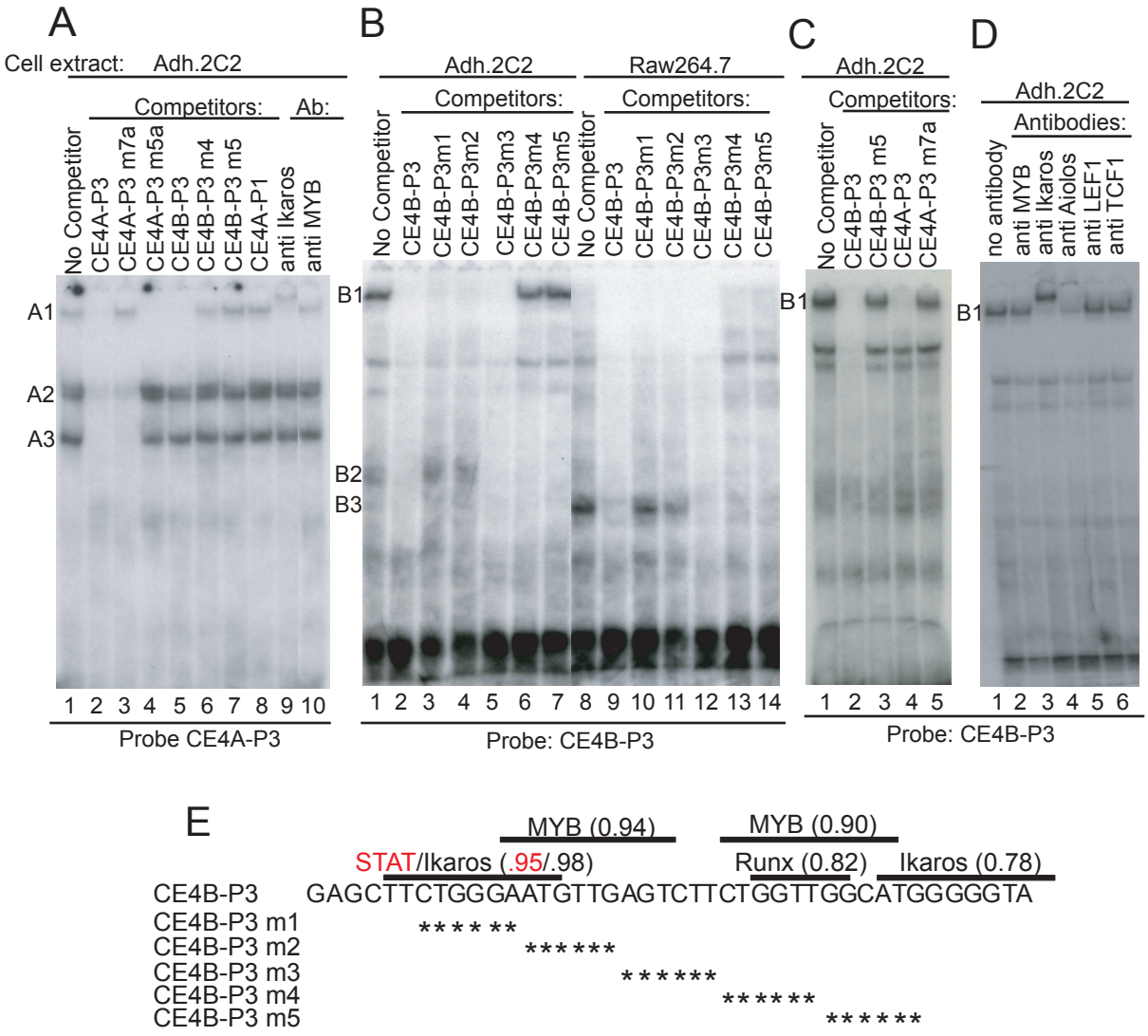


D

```
CE8-P3      ATGCTTCCTGTGGTGACTGGGCGCTTCCTGTTTT
CE8-P3 m1                 * *
CE8-P3 m2          * *                    * *

CE8-P2      ATGCTTCCTGTGGTGACTGGGCGCTTCC
CE8-P2 m1                 * *
CE8-P2 m2        *   *
```

Figure S2.


A
Cell extract: Adh.2C2
Competitors:
Ab:
No Competitor
CE4A-P3
CE4A-P3 m7a
CE4A-P3 m5a
CE4B-P3
CE4B-P3 m4
CE4B-P3 m5
CE4A-P1
anti Ikaros
anti MYB
A1
A2
A3
1 2 3 4 5 6 7 8 9 10
Probe CE4A-P3
B
Adh.2C2
Raw264.7
Competitors:
No Competitor
CE4B-P3
CE4B-P3m1
CE4B-P3m2
CE4B-P3m3
CE4B-P3m4
CE4B-P3m5
B1
B2
B3
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Probe: CE4B-P3
C
Adh.2C2
Competitors:
No Competitor
CE4B-P3
CE4B-P3 m5
CE4A-P3
CE4A-P3 m7a
B1
1 2 3 4 5
Probe: CE4B-P3
D
Adh.2C2
Antibodies:
no antibody
anti MYB
anti Ikaros
anti Aiolos
anti LEF1
anti TCF1
B1
1 2 3 4 5 6

E
MYB (0.94)
MYB (0.90)
STAT/Ikaros (.95/.98)
Runx (0.82)
Ikaros (0.78)
CE4B-P3 GAGCTTCTGGGAATGTTGAGTCTTCTGGTTGGCATGGGGGTA
CE4B-P3 m1 ******
CE4B-P3 m2 ******
CE4B-P3 m3 ******
CE4B-P3 m4 ******
CE4B-P3 m5 ******

Figure S3.

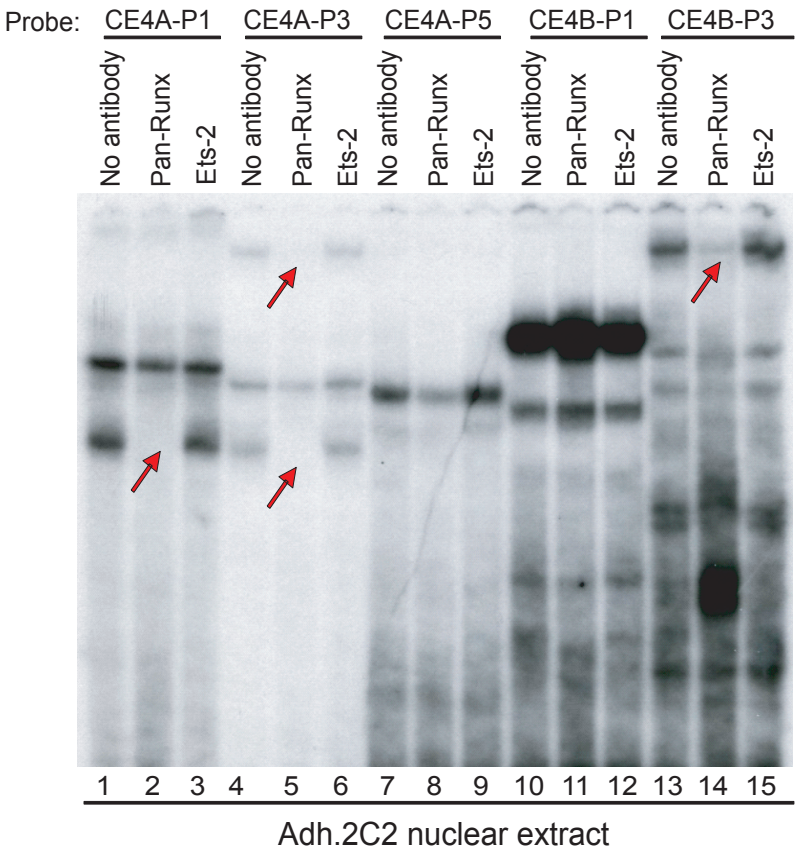
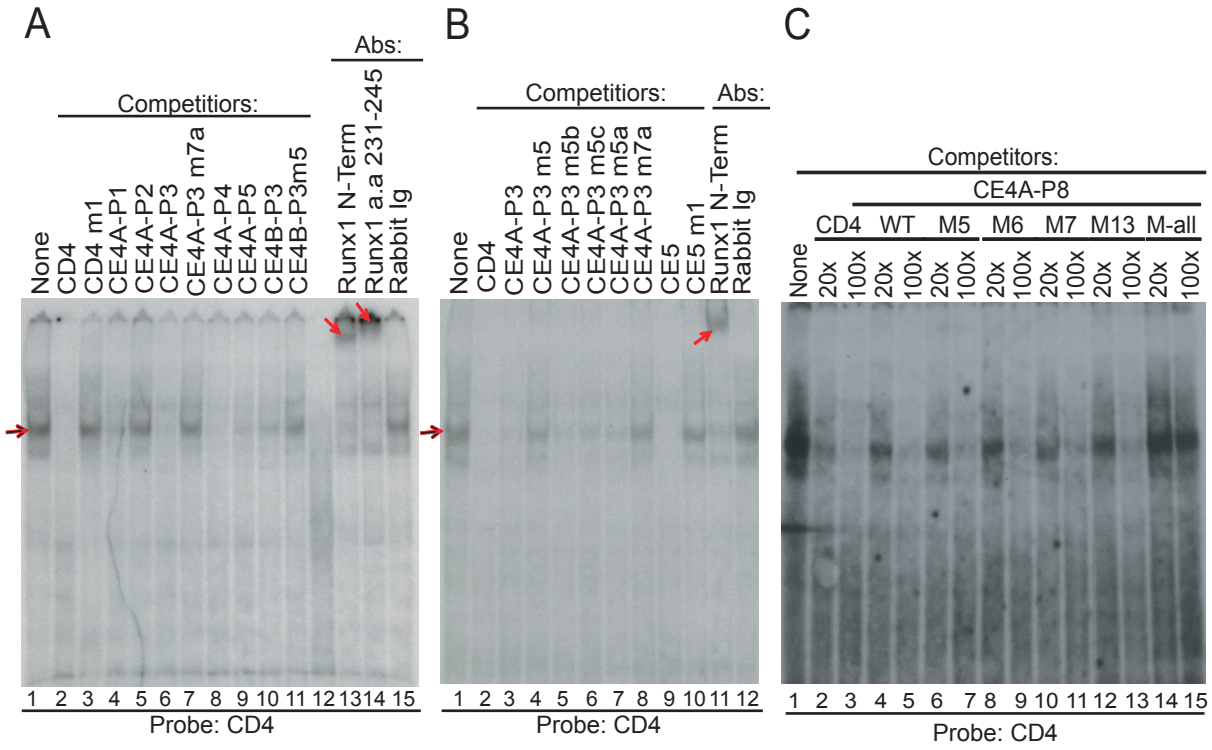

Probe:
CE4A-P1
CE4A-P3
CE4A-P5
CE4B-P1
CE4B-P3
No antibody
Pan-Runx
Ets-2
No antibody
Pan-Runx
Ets-2
No antibody
Pan-Runx
Ets-2
No antibody
Pan-Runx
Ets-2
No antibody
Pan-Runx
Ets-2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Adh.2C2 nuclear extract

Figure S4.


A
Abs:
Competitiors:
None
CD4
CD4 m1
CE4A-P1
CE4A-P2
CE4A-P3
CE4A-P3 m7a
CE4A-P4
CE4A-P5
CE4B-P3
CE4B-P3m5
Runx1 N-Term
Runx1 a.a 231-245
Rabbit Ig
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Probe: CD4
B
Competitiors:
Abs:
None
CD4
CE4A-P3
CE4A-P3 m5
CE4A-P3 m5b
CE4A-P3 m5c
CE4A-P3 m5a
CE4A-P3 m7a
CE4A-P3
CE5
CE5 m1
Runx1 N-Term
Rabbit Ig
1 2 3 4 5 6 7 8 9 10 11 12
Probe: CD4
C
Competitors:
CE4A-P8
CD4
WT
M5
M6
M7
M13
M-all
None
20x
100x
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Probe: CD4

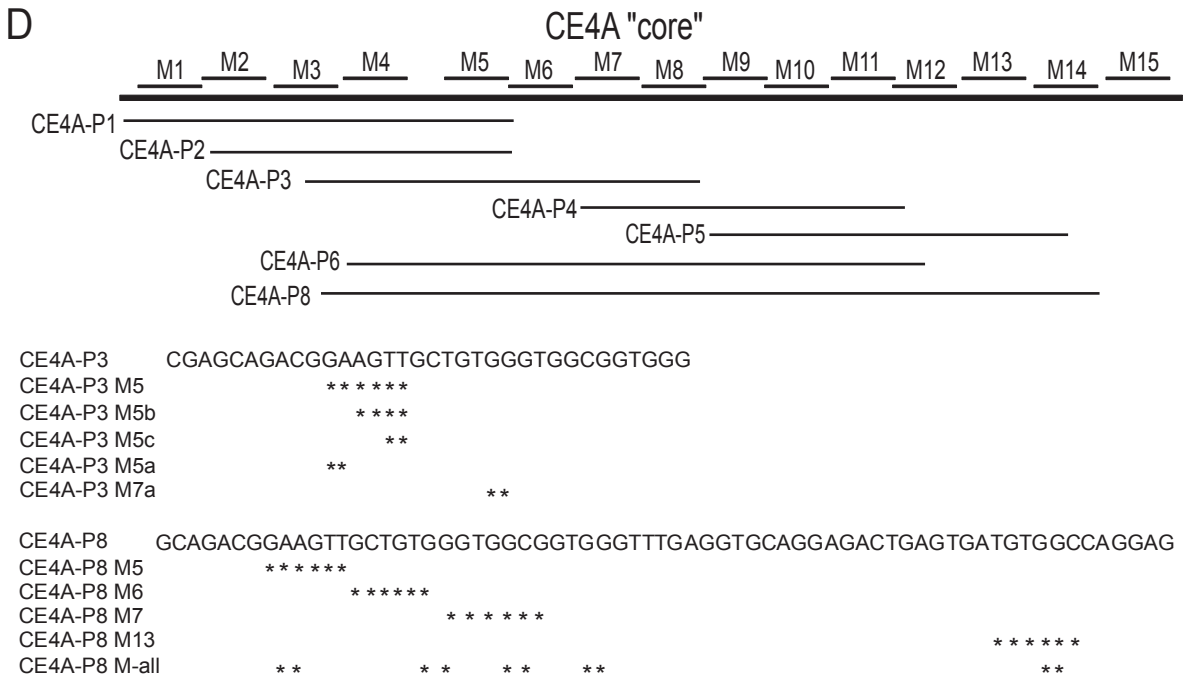

D
CE4A "core"
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
M11
M12
M13
M14
M15
CE4A-P1
CE4A-P2
CE4A-P3
CE4A-P4
CE4A-P5
CE4A-P6
CE4A-P8
CE4A-P3 CGAGCAGACGGAAGTTGCTGTGGGTGGCGGTGGG
CE4A-P3 M5 ******
CE4A-P3 M5b ****
CE4A-P3 M5c **
CE4A-P3 M5a **
CE4A-P3 M7a **
CE4A-P8 GCAGACGGAAGTTGCTGTGGGTGGCGGTGGGTTTGAGGTGCAGGAGACTGAGTGATGTGGCCAGGAG
CE4A-P8 M5 ******
CE4A-P8 M6 ******
CE4A-P8 M7 ******
CE4A-P8 M13 ******
CE4A-P8 M-all ** ** ** ** **

Figure S5.

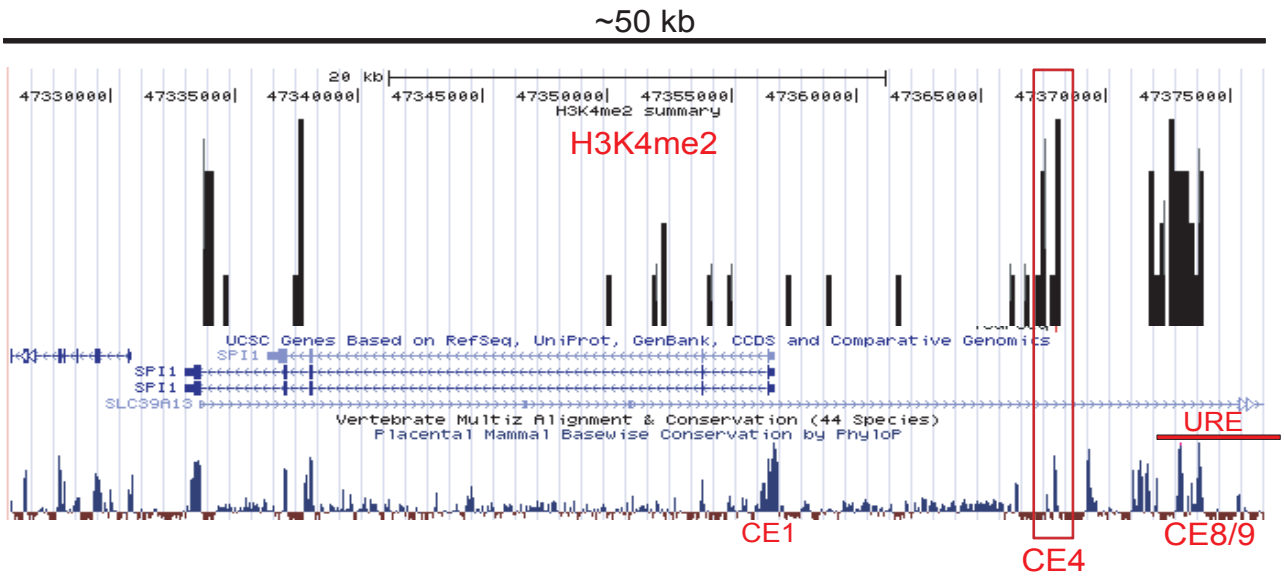

~50 kb
20 kb
47330000
47335000
47340000
47345000
47350000
47355000
47360000
47365000
47370000
47375000
H3K4me2 summary
H3K4me2
UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics
SPI1
SPI1
SPI1
SLC39A1S
Vertebrate Multiz Alignment & Conservation (44 Species)
Placental Mammal Basewise Conservation by PhyloP
URE
CE1
CE4
CE8/9

Figure S6

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Figure legends:

Figure 1. *Sfpi1* upstream region contains novel cis regulatory elements. (A) *Sfpi1* multigenome alignment from exon 1 to ~15 kb upstream. Schematic of regions used in reporters are shown. (B and C) The CE9-CE8 (URE) region is an enhancer in the myeloid cell line Raw264.7 and in the immature DN3-like Adh.2C2 pro-T cell line. The Renilla luciferase expressing control vector pRL-CMV was used as an internal standard. The empty pGL3-basic was used as a control, LB. The average RLUs of triplicates from a representative experiment is shown with standard deviations. (D) The CE9-CE8 region does not possess enhancer activity in a mature T cell line, EL4. Data shown are the averages of three independent experiments performed in duplicate with standard deviations. (E, F) Novel DNase I HS sites are identified. Probe 2 southern blot of SphI digested DNA from nuclei of indicated cell lines, -/+ DNase I, is shown. Bands are defined by sites of DNase I sensitivity. Right panel shows T-cell specific doublet of HSs at ~2 kb from Sph I site (box). Schematic summarizes HS mapping with various probes. S=Sph I, H=Hind III, E=EcoRV. (G) The L5-3 reporter shows lineage-specific activity.

RLUs normalized to L1. The average RLUs of triplicates from a representative FuGENE transfection experiment is shown with standard deviations.

Figure 2. The CE5-3 region has cell type specific regulatory functions in stably transfected cells. A schematic of the reporters used is shown. Data reported as RLU/insert copy number. (A) Extension of L9-6 to include the CE5-3 region increases activity in RAW264.7 myeloid cells. (B) Inclusion of the CE5-3 region silences activity in immature Adh.2C2 T-cells. (C) Background activity of reporters in the nonhematopoietic NIH/3T3 fibroblast cell line. Dots represent independent wells containing multiple founders and bars show the geometric mean for the wells.

Figure 3. T-cell and myeloid cell type-specific regulatory activities map to distinct regions within the CE5-3 fragment. (A) A diagram of the CE5-3 truncations used in transient transfection experiments is shown. (B and C) Myeloid enhancer activity mapped to the CE5 region. T-cell silencer activity mapped to the CE4A region. Representative experiments with RLUs normalized against L1 and standard deviations are shown. (D) Both CE4A and CE4B contribute to silencing in a chromatin context. Diagram of reporters used for stable cell lines is shown. Data points are as described in Fig. 2. Data are shown as RLU.

Figure 4. T-cell silencer activity mapped to a conserved core region (A) A diagram of reporters with CE4A truncations flanked by the CE9-CE8 enhancer and the promoter is shown. (B) The L98+4A5 reporter contains the core silencer sufficient for silencing (red bar). Data shown is from a representative transient experiment performed in duplicate

with Adh.2C2 T-cells. (C) A multigenome alignment of the CE4A core silencer is shown. Nucleotides conserved in all eleven organisms are marked by an asterisk. Six nucleotide blocks mutated in scanning mutagenesis analysis are labeled (M1-M15). Sequences with >0.925 similarity to TRANSFAC predicted binding sites are shown. (D) Scanning mutagenesis reveals multiple sites contributing to core silencer activity. Data shown are averages from four or more independent Adh.2C2 transfections with standard deviations. RLUs normalized against L98 activity. Red bars mark reporters with mutations blocking silencer activity

Figure 5. The CE4 region nucleates T-cell specific protein complexes in vitro. (A) Schematic shows alignment of probes relative to the core silencer. Wild type probe sequences shown with asterisks marking bases mutated in competitors. (B) Mapping of sites contributing to “T1” complex formation on probe CE4A-P6 by Adh.2C2 nuclear extracts. (C) Multiple CE4A probes form T-cell specific complexes in vitro. Probes were incubated with nuclear extracts from Adh.2C2 (T) or Raw264.7 cells (M). T-cell specific complexes A1/B1 and A3 are indicated by arrows.

Figure 6. The nonconsensus CE4A-P3 Runx site has weaker binding affinity than the consensus sites of CE8. (A) Western blot of Runx1 and Sp1 protein. The same blot was probed for Runx1 then Sp1. (B) Sequences of *Cd4* and CE8 oligos are shown. Asterisks mark bases mutated in competitors. (C, D) CE4A-P3 binds Runx more weakly than CE8 probes. The CD4 silencer probe was used to assay Runx1 binding potential. Competitors were used at 100 fold excess except where indicated. Band intensity was quantified with

a phosphorimager and background was subtracted with data plotted as relative intensities normalized against lane 1.

Figure 7. Runx perturbations abolish silencing activity in the immature T-cells, Adh.2C2. (A) Cells were nucleofected with PU.1 reporters, plus 1-2 μg of indicated plasmids. Only cotransfection with the Runx dominant negative expression vector relieved silencing (red bars). (B) Antisense morpholino knockdown of endogenous Runx1 blocks silencing (red bar). Data from two independent experiments, in duplicate, were averaged and shown as a ratio of L98+4/L98 reporter activity. (C) Western blot of whole cell lysates from Adh.2C2 cells transfected with or without Runx1 morpholino, showing Runx1 loss. (D) Runx sites are essential for silencing in a chromatin context in stably transfected Adh.2C2 cells. L93 mut-Runx: all predicted Runx sites in CE4A-B region mutated. L93 M5, L93 with M5 mutation. L93 mut-Runx M5, mutations combined. Data plotted as in Fig. 3D.

Figure 8. Runx1 binds the CE4A silencer region in vivo in T-cells. A schematic of the PU.1 upstream region with conserved elements and their approximate location is shown. (A, B) ChIP assays in Raw264.7 cells (A, red) and Adh.2C2 (B, green). Left panel shows ChIP analysis with antibody against the Ets factor GABPα. Right panel shows Runx1 ChIP. Results shown are from three or more independent experiments. (C) ChIP assays with Ig failed to enrich any region in $Rag2^{-/-}$ thymocytes (left panel). ChIP assays demonstrate Runx1 binding to the CE4A core silencer in primary thymocytes (right panel). Runx binding to the CD4 silencer is shown for comparison (green bars). Results

shown are from two independent experiments. Standard deviations are shown (error bars.)

Figure S1. DNase HS mapping detects HS sites in the first two *Sfpi1* introns. (A) Pro-T-cell line scid.adh lacks HS sites flanking exon 1 (E1). Probe 3 (P3) was used in a southern blot of EcoRV digested DNA from nuclei of indicated cell lines, -/+ DNase I. Bands correspond to sites of DNase I sensitivity. Size markers are shown. Schematic depicts HS mapping results with Probe 3. Red arrows indicate HS sites detected in PU.1 expressing cells and correspond to the bands in the southern blot. S=Sph I, H=Hind III, E=EcoRV. (B) Nonspecific DNase HS sites detected across intron 2 in PU.1 expressing cells and nonexpressing pro-T-cells (scid.adh). Probe 3 was used in a southern blot of Sph I digested DNA, -/+ DNase I, as described above.

Figure S2. The CE8 region does not form cell type specific bands in vitro. (A) Pro-T-cell line Adh.6D4, $Rag2^{-/-}$ mouse thymocytes, P388D1 myeloid cells, and NFS-25 pre-B-cells all nucleate the CE8 probe in vitro with complexes equivalently abolished by competitions. (B) The sequence requirements for complex formation can be separated. When the Runx site is mutated in CE8-P3m1 (B, lane 3), complexes C1 and C3 are lost, indicating the Runx site is not needed to form bands C1 and C3, but is required for complex C2 (also A, lanes 2, 5, 8 and 11). Mutating the Ets sites in CE8-P3m2 abolishes C2 (B, lane 4; A, lanes 3, 6, 9, and 12), indicating complexes C1 and C3 require Ets sites. (C) A shorter CE8 probe lacking the three prime Ets site is similarly nucleated by pro-T-cell and myeloid extracts. Probe CE8-P2 does not form complex C1 (lanes 1 and 5)

indicating it requires both Ets sites. (D) The sequences of probes and competitors are provided. Asterisks indicate nucleotides mutated in competitors.

Figure S3. The CE4B region forms a pro-T-cell specific complex in vitro and can cross compete with the T lineage specific A1 complex formed by the CE4A region probe. (A) Sequence requirements for T lineage complex formation on probe CE4A-P3 can be separated. Competition cannot abolish complex A1 when the Runx site is mutated (lane 3), indicating the Runx site is needed for A1 formation. Complexes A2 and A3 cannot be abolished by competition with the probe when the Ets site is mutated (lane 4). Competition with a probe from the CE4B region eliminates complex A1 and this competition depends on the CE4B probe's Runx like site (lanes 6-8). Ikaros antibody (sc-13039) can supershift complex A1 (lane 9). (B) The CE4B-P3 probe nucleates a pro-T-cell specific complex, B1 (B, lane 1). Complex B1 does not depend on the STAT site previously shown to contribute to PU.1 expression (lanes 3 and 4). In contrast, the myeloid complex B3, and the weaker T lineage complex B2, do require the STAT site for formation of complexes (lanes 3 and 4, and 10 and 11). Complex B1 depends on the sequences spanning nonconsensus Runx and Ikaros sites (lanes 6 and 7). Complex B1 can be supershifted by Ikaros antibody (lane 17). Probe CE4A-P3 can be used in competition to eliminate complex B1 formation and ability to compete requires the Runx site (lanes 24 and 25). (C) The sequence of probe CE4B-P3 is shown with asterisks indicating mutated nucleotides. TRANSFAC predicted transcription factor binding sites are shown with their matrix similarity score in parenthesis.

Figure S4. Pan-Runx antibody inhibits complex formation on multiple probes nucleated by Adh.2C2 pro-T-cell extract (red arrows, lanes 2, 5, and 15). Ets2 antibody (sc-351) had no effect on any complex.

Figure S5. Multiple Runx sites are present across the CE4A core silencer region. A *Cd4* silencer reference probe was used to assay for Runx binding potential. (A) The strong complex nucleated by the Cd4 probe is dependent on Runx sites (lane 3) and can be supershifted by anti Runx1 antibodies (lanes 13 and 14). The CE4A probes (Fig. 5C) were used to compete with the *Cd4* probe for Runx protein binding. CE4A-P3 cannot abolish complex formation when its Runx site (CBF3 site) is mutated (lane 7). (B) The CE4A-P3 probe's site "X" is needed to compete with the *Cd4* probe for Runx binding, but the nts needed for the overlapping Ets site and CBF3 site(see Fig. 4C) are dispensable (lane 4 vs 5 and 7). (C) All Runx sites across the CE4A-P8 competitor must be mutated to abolish competitive inhibition of the *Cd4* probe Runx complex formation. Compare CE4A-P8 M-all (lane 15) with lanes 7, 9, 11, and 13. (D) The CE4A-P8 probe is shown in relation to other CE4A probes. The CE4A-P3 and CE4A-P8 probe sequences are provided and asterisks indicate mutated nucleotides in competitors.

Figure S6. $CD4^+$ T-cell ChIP-seq experiments suggest open chromatin across CE4. Histone 3 lysine 4 dimethylation (H3K4me2) ChIP-seq data is borrowed from Barski et al. (2007) (http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcell.aspx) CE4 is marked by H3K4me2 to a similar extent as the URE elements CE8 and CE9.

Chapter 3

PU.1 autoregulation mediated through cell-type-specific PU.1 recruitment to multiple novel *Sfpi1* cis regulatory modules

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This chapter represents a manuscript describing the PU.1 autoregulation through lineage restricted recruitment and binding of transcription factors to novel cis regulatory modules.

ABSTRACT

PU.1, a critical transcription factor in multiple hematopoietic lineages is upregulated during specification of granulocyte-macrophage progenitors, and is dynamically regulated thereafter. PU.1 is most elevated in monocyte derived lineages, particularly macrophages. If PU.1 is under expressed, myeloid hyperproliferation occurs and terminal differentiation of macrophages is severely blocked. This suggests maintenance of higher PU.1 expression after myeloid specification is critical both to facilitate normal development and to prevent neoplasia. Maintenance of PU.1 expression is thought to depend in part on PU.1 autoregulation through its promoter and URE, a previously described compound conserved region with regulatory functions in multiple blood lineages. However, the URE cannot account for all PU.1 regulation in myeloid cells, nor is PU.1 the only Ets factor able to act through the URE. We now present evidence for another complex of conserved noncoding elements that appear to mediate cell type-specific PU.1 dependent enhancement of *Sfpi1* transcription in myeloid cells. We show that the novel regulatory regions are additional myeloid specific RNApol II nucleation centers and that they do not bind a broad number of non-PU.1 Ets factors, in contrast to the URE. We also show that Ikaros binds these new regulatory elements, and the URE, and collaborates with PU.1 to regulate *Sfpi1* transcription in macrophages.

Introduction

PU.1, encoded by the *Sfpi1* gene, is a differentially regulated pleiotropic Ets family transcription factor utilized in the lineage decisions of many hematopoietic cell types (22, 29). Initially expressed at relatively uniform levels in hematopoietic stem cells and early progenitors, PU.1 levels rise in granulocyte macrophage progenitors (GMPs) but fall in pro-pre-B-cells (23).

GMPs enter various lineage specification programs through context dependent combinatorial use of PU.1, sometimes accompanied by modulation of PU.1 expression level. PU.1 expression continues to rise in GMP derived monocytes, with elevated PU.1 expression levels needed to overcome MafB to specify a dendritic cell fate (4). In osteoclasts, also derived from monocytic progenitors, PU.1 but not other Ets family factors can collaborate to activate the beta three integrin promoter in cooperation with NFATc1 (5, 9). In contrast to monocytes, granulocyte lineages such as mast cells and basophils have lowered PU.1 expression relative to GMPs (3, 10). Together, these studies suggest that dynamic PU.1 expression together with unique PU.1 specific functions not shared by other Ets factors are important to monocyte lineages. How PU.1 expression is modulated, elevated, or maintained in monocytes is not fully understood.

PU.1 expression in the hematopoietic compartment is dependent on Runx1 (16, 24), yet Runx1 does not appear to occupy its target sites within known PU.1 regulatory regions in more mature myeloid cells. This implies that PU.1 expression may rely on other factors to drive expression upon lineage specification after transcriptional competence is first established in hematopoietic stem cells (HSCs) (16). Many non Runx factors have been shown to bind the *Sfpi1* promoter and its previously characterized

URE, a compound enhancer element located at about -14 kb upstream of the *Sfpi1* transcriptional start site. Some of the factors thought to positively contribute to PU.1 expression through the promoter and URE include Sp1, C/EBPα, Octamer factors, AP1, Elf-1, and Fli-1 (6, 8, 16, 25, 34). While all of these factors seem to play important roles in regulating *Sfpi1* transcription, one of the most interesting contributors to *Sfpi1* transcriptional control may be PU.1 itself.

PU.1 has long been thought to regulate its own expression in myeloid cells as it has been shown to occupy target sites within the *Sfpi1* promoter and URE (8, 25, 30). Additionally, mutation of PU.1 target sites reduces URE enhancer activity (15, 25). However, the potential PU.1 autoregulation through these URE sites has not been formally verified. These target sites may be bound by other Ets family transcription factors, such as Elf-1 or Fli-1, and in PU.1's absence these factors may be responsible for *Sfpi1* transcriptional output through the URE (15, 16, 25). Nonetheless, PU.1 is able to occupy at least one distal URE target site in myeloid cells that remains unoccupied by any Ets family factor in B-cells (16). This suggests the existence of lineage specific mechanisms able to control PU.1's ability to occupy specific target sites and potentially autoregulate. Furthermore, such finely controlled myeloid versus lymphoid lineage specific recruitment of PU.1 to its own cis regulatory elements likely contributes to observed differences in myeloid versus B-cell PU.1 expression levels. Therefore, a deeper understanding of how PU.1 is transcriptionally regulated is needed.

The hypothesis that lineage specific PU.1 autoregulation might involve myeloid versus lymphoid lineage context dependent recruitment to the URE is complicated by studies showing that the URE region is dispensable for PU.1 expression (28). Mice with

the URE deleted (UREΔ) might still benefit from autoregulation mediated in part through the *Sfpi1* promoter, but the promoter cannot drive expression in a chromatin context on its own (21). Additionally, while deletion of the URE perturbed normal PU.1 transcriptional output, proper developmental expression of PU.1 remained largely intact (17, 27, 28). Ultimately, the dispensability of the URE arouses some critical regulatory questions. Specifically, it remains unclear whether PU.1 is necessary or sufficient for its own expression in more mature myeloid cells and it is unknown what other regulatory elements may contribute to PU.1 expression and what other factors are relevant to these elements. Moreover, it is difficult to explain the biochemical mechanisms limiting PU.1 autoregulation if dynamic PU.1 expression is controlled from a cis-regulatory region like the URE through which other Ets factors may also act. If PU.1 is necessary for its own expression after its transcription is first initiated in HSCs, then the reduced but continued expression of PU.1 in UREΔ mice predicts unidentified and potentially more restricted PU.1 target sites may be within additional *Sfpi1* regulatory modules.

We recently identified novel *Spfi1* noncoding conserved elements located from about -12.5 to -7.5 kb upstream of the *Sfpi1* transcriptional start site, just downstream of the previously characterized -14 kb URE (Zarnegar et al., submitted). One of the conserved elements in this region, CE5, located at about -10.3 kb, was relatively inactive in an immature T-lineage cell line in which the URE maintained enhancer function. In contrast, the CE5 module demonstrated robust myeloid enhancer activity in transient transfections or when stably integrated into chromatin (Zarnegar et al., submitted). An additional compound element, CE7-CE6, located at about -12.3 kb, may also contribute to *Sfpi1* transcriptional output. Together, these novel elements may be the sources of regulatory control mechanisms affecting dynamic PU.1 expression by acting as additional

transcription factor nucleation centers able to confer lineage specific functions that may also explain how developmentally regulated PU.1 expression is sufficiently maintained in UREΔ animals.

Here, we present evidence that the myeloid enhancer CE5 and the CE7-CE6 region (CE7/6 hereafter) are myeloid restricted PU.1 and RNApol II target elements. We provide evidence that these regions are restricted in their capacity to bind other Ets family members, unlike the URE, which appears to be a nonspecific enhancer able to associate with RNApol II in non cell-type-specific ways. We also demonstrate that Ikaros binds to the CE5 and CE7/6 regions, and even binds to the URE, in vivo. RNApol II and Ikaros occupancy of these PU.1 regulatory regions may be facilitated at least in part by PU.1 itself, in a context dependent fashion with myeloid and lymphoid cells showing differential abilities to recruit PU.1 and Ikaros to specific cis-regulatory modules. Reporter assays validated the ability of Ikaros to contribute to transcriptional activation through these modules in a myeloid cell line. Most importantly, we provide evidence that PU.1 itself is strictly required for the enhancer activity of a DNA fragment harboring CE7, CE6, and CE5. Collectively, our data indicates that PU.1 collaborates with Ikaros in a myeloid specific multimodular positive autoregulatory loop that has the potential to contribute to the establishment and/or maintenance of high PU.1 expression in the myeloid compartment.

Materials and methods

Cell culture

Adh.2C2 and NFS-25 cells were grown in RPMI media supplemented with 10% fetal bovine serum, penicillin/streptomycin/glutamine, non essential amino acids, sodium pyruvate, and 2-ME. Raw264.7 cells were grown in DMEM media with 10% fetal bovine serum and penicillin/streptomycin/glutamine.

Cloning of reporters and expression constructs:

PU.1 DNA was obtained by PCR using the BAC RP23-20F9, obtained through BACPAC resources of CHORI (bacpac.chori.org). Reporter constructs were made by cloning PU.1 regulatory sequences into Promega's pGL3-basic vector. Detailed maps of reporters and their construction are available upon request. The Ikaros dominant negative, Plastic, was synthesized by GenScript based on published sequence (26) and cloned into pEF1.

Transfections and luciferase assays

In some experiments, cells were transiently transfected with FuGENE 6 reagent at a FuGENE:DNA ratio of 3:1. Cells were harvested ~30-48 hours post FuGENE transfection. Cells were cotransfected with pRL-CMV and lysates were analyzed using Promega's Dual Luciferase system. 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 Blasticidin for their duration in culture, beginning one day post transfection. Cotransfection experiments were performed by Nucleofaction (Lonza/Amaxa) using Solution-V kits and program D-32 for RAW264.7 cells or program D-19 for NFS-25 cells. The following morpholino antisense oligos were ordered from Gene Tools, Inc., anti-PU.1 (E2) GAGGACCAGGTACTCACCGCTATG; anti-PU.1 (E1) GTAGTGAAGCCCCAGTACTCACAGG; Standard Control oligo CCTCTTACCTCAGTTACAATTTATA.

Transcription factor binding site predictions

TRANSFAC analysis was used to predict potential transcription factor binding sites. Biobase's (https://portal.biobase-international.com/cgi-bin/portal/login.cgi) TRANSFAC suite's MATCH tool was used for the analysis. Matrix similarities >0.9 were shown in black and selected predictions between 0.9 and 0.8 were shown in gray.

Gel Shift Assays

Nuclear extracts were prepared by hypotonic swelling in buffer A, followed by NP40 lysis, nuclei pelleting, and extraction with buffer C containing protease inhibitors (Roche #11873580001). Buffer A: 10 mM HEPES pH 7.9, 60 mM KCl, 1 mM DTT, 0.1 mM

EDTA, 0.1 mM EGTA, followed by addition of NP40 to 0.625%. Buffer C: 20 mM HEPES pH 7.9, 0.4 M NaCl, 1mM EDTA, 1mM EGTA, 1 mM DTT. Protein was quantified by the Bradford method. Gel shifts were performed with ~6 μg extract in 30 μl volume containing 1.5 μg poly di-dc and final concentrations of 15 mM HEPES pH 7.9, 80 mM NaCl, 15 mM KCl, 0.02 mM EDTA, 1 mM DTT, and 3% glycerol. Five picomoles of probes were end labeled with T4 polynucleotide kinase followed by purification with G-50 columns (Roche #100609). Complexes were allowed to form on ice for 10 minutes with competitors prior to addition of radiolabeled probes. After probe addition samples were incubated for an additional 30 minutes on ice. Complexes were resolved by 6% PAGE, run at constant 300 volts for ~3 hours. All gels were run at 4^{o}C with 0.5x TBE gels and 0.25x TBE running buffer. Anti-PU.1 antibody was from Santa Cruz (sc-352). Anti-Ikaros antibodies, sc-13039, sc-9861, and sc-9859 are from Santa Cruz. Anti-Ikaros antibody #39291 is from Active Motif.

Retroviral Infections:

Viral sups were prepared by transfection of pMX-PU.1-IRES-hCD8 plasmid into Phoenix packaging cells with FUGENE 6 reagent. Virus containing media was collected at 48-72 hours post transfection. Adh.2C2 cells were subsequently infected with PU.1 virions using the TAKARA RetroNectin method.

Western blots and RNA processing:

Nuclear Extracts were mixed with 2x Laemmli sample buffer, boiled, and then run on 8% SDS-PAGE. Gels were transferred to Immobilon (Millipore) by semi-dry transfer. Blots were blocked with 5% milk in TBS-T then incubated overnight with primary antibody. After washing, blots were incubated with secondary antibody, washed, then incubated with substrate (SuperSignal, Pierce #1859675 and #1859674) and exposed to film. Ikaros and Sp1 antibodies were obtained from Santa Cruz (sc-13039 and sc-59). Western showing PU.1 knockdown performed using anti-PU.1 antibody (sc-5948) and anti-Ets-1 (sc-350) as a control. 32Dcl5 cells were grown in RMPI media supplemented with IL-3. Total RNA from cell lines were isolated using Trizol Reagent and manufacturer's protocol. For PU.1 RNA analysis, one microgram total RNA was used to synthesize cDNA using the Superscript III system (#18080-400). cDNA was diluted and analyzed by QPCR, in triplicate. Genes of interest were normalized to GAPDH.

ChIP assays:

ChIP assays were performed according to Upstate's protocol. 2-3 x 10^7 cells were fixed with 1% formaldehyde for 15-30 minutes. Glycine was used to stop fixation, followed by washing. Cells were then lysed in 0.8 mls SDS lysis buffer with protease inhibitors. Lysate was sonicated to produce an average fragment size ~ 250 bp. 130 μl lysate was diluted and used for each ChIP with 9 μg anitbody. Crosslinking was reversed by overnight incubation at 68^0C. Samples were difested with Proteinase K for 30 minutes at 55^0C. DNA was purified by ethanol precipitation and resuspended in 80-100 μl water.

Analysis of recovered DNA was performed by SYBR green based QPCR with an AB 7900HT. 1 μl of purified DNA was used per 10 μl PCR reaction, in triplicate, in 384 well plates. Primer pairs used for analysis of ChIP enriched DNA by QPCR are: CE1-F AGCTCAGCTGGATGTTACAGG and CE1-R AGATGGTCACACATCCCAAAG; -2kb-F TTCTCACATCCCAGACCATTC and -2kb-R CGCCAGCAGTTGTAGTTCTTC; -2.8kb-F GCAGCTCACTGCTCCAAGTT and -2.8kb-R GAGACGGGGAGTGGGTATGT; CE3-F TGGAGCTCTGAGGGGCCTAA and CE3-R GGCTGGGAAAGCTGACCATAA; -8.4kb-F AGAGGAGCTGACATTGGCATAC and -8.4kb-R TGAGCCTCTGAAGTGGCTTTAT; CE4B-F AGCAAAGCCTGTGGGAGATT and CE4B-R ATACCTTGGAGGCCTGTGCT; CE4A-F GGAAGCAGCTCTTGTCCTTGG and CE4A-R TCACCTCCTGGCCACATCACT; CE5-F GCTCTGAAAAGCACCGTTTCC and CE5B-R CTGTGTTGGACCTGCAAGGAG T; -11.8kb-F CTCTGCCCGCTCTTAACCTT and -11.8kb-R GATCTGACACGGGGATGAAA; CE76-F CACACGGAGTCAGAGCGGGCAG and CE76-R AGGAAAGAGGAAGCCATGGGGAGA; CE8-F AGGCAGAGCACACATGCTTC and CE8-R CTTCTGGGCAGGGTCAGAGT; CE9-F CAGGAGAGGCAGGAGGAAGGA and CE9-R AGAGAGCAGAGCACTTCATGGCT; -17.8kb-F CTGGACAAGTGGAAGGTGACA and -17.8kb-R TCAGAGGGCTTCAAAGTGGA; CD4-F TGACGGAAGGGAGGATGTAG and CD4-R AGTGGGTGGGAGCTCTGTAA; MEF2C-F AGCACACTCAGCCTGCTCTAC and MEF2C-R GGTGTAAAGGTGCTTCCTTCC; IL-7Rα-F GTCTGAGCAAAAGGATTGCTG and IL-7Rα-R GGAGCTTCAGGGAATACCAAG. Antibodies from Santa Cruz that were

used for ChIPs include: GABPα (sc-22810), Ets-1 (sc-22802 and sc-111), Elf-1 (sc-631). Anti-Ikaros ChIPs were performed with an equal mix of sc-13039 and Active Motif #39291. RNApol II ChIPs were performed using ABCAM (ab5408).

Statistical Analysis of ChIP Data:

One way ANOVA analysis was used to analyze ChIP enrichment data. ANOVA was performed with all regions to determine if a significant difference in enrichment was present between regions being analyzed. 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 until a group of regions generated a $p > 0.1$. The regions remaining in the test group that lacked a statistically significant enrichment difference were then used a control group against which to compare the removed "regions of interest" individually. The resulting p-values were adjusted using the Bonferroni correction method and regions with $p < 0.0001$ were marked by asterisks in ChIP data figures.

Results

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

Once PU.1 expression is initiated in the hematopoietic compartment, its expression diverges as lymphoid-myeloid multipotent progenitors differentiate to become myeloid cells or lymphoid cells (1, 23). Changes in PU.1 expression levels continue as myeloid and B lineage cells mature, with relative PU.1 expression in pro-pre-B cells several fold reduced versus myeloid cells. In contrast, PU.1 expression in developing T-cells shuts off at the commitment stage (23). We recently described characterization of a ~3 kb DNA fragment, CE5-CE3, which was found to possess cell-type-specific regulatory functions. The CE5-CE3 fragment was a potent silencer in immature T-cells with silencing activity precisely mapping to the CE4 region (Fig. 1A)(Zarnegar et al., submitted). We also demonstrated that CE5, on its own or as part of the CE5-CE3 fragment, was a myeloid enhancer able to augment reporter expression in conjunction with the PU.1 promoter (Zarnegar et al., submitted). Since the CE5-CE3 fragment could account for myeloid versus T-lineage divergence of PU.1 expression, we sought to expand our understanding of the CE5-CE3 fragment's enhancer specificity by testing its function in a PU.1 expressing lymphoid lineage, the pre-B cell line NFS-25.

Several luciferase reporters were constructed as depicted in Fig. 1A, with fragments of the *Sfpi1* upstream region joined to a ~2.2 kb fragment containing the *Sfpi1* basal promoter element, CE1. These reporters were transiently transfected into NFS-25 pre-B-cells and RAW264.7 monocyte/macrophage cells. As shown in Fig. 1B, the L5-3 reporter generated similar luciferase expression compared to L1, the promoter only

reporter, but L5-3 produced strong enhanced reporter expression in RAW264.7 cells. This result indicated that the CE5-CE3 fragment contains myeloid restricted enhancer activity. In contrast to the CE5-3 fragment's cell type-specific regulatory function, the conserved URE elements CE9 and CE8 within the L98 reporter could act to enhance promoter driven luciferase expression in both the pre-B and myeloid cell lines, as expected from previous reports (Fig. 1B)(27).

To further confirm the myeloid specific enhancer activity of CE5-CE3, we tested its function when integrated into chromatin using stable cell lines. Linearized reporters containing the URE and the novel region CE7/6 without the CE5-CE3 fragment (L9-6) or with it (L9-3), were stably transfected into NFS-25 and RAW264.7 cells. A linearized control luciferase plasmid harboring a blasticidin resistance cassette was cotransfected with the *Sfpi1* reporters. L9-6 generated equivalently strong luciferase expression in both cell lines, but CE5-CE3 only enhanced reporter activity in the RAW264.7 cells (Fig. 1C). These results confirmed that the CE5-CE3 fragment only contains enhancer function in myeloid cells.

The myeloid specific CE5 enhancer is an additional RNA polymerase II nucleation site

Recent work has shown that the URE may function as a nucleation center directing *Sfpi1* transcription through the assembly of the RNA polymerase II containing pre initiation complex, which could produce local enhancer initiated sense and anti-sense noncoding RNAs (15). Those RNA transcripts may contribute to regulation of PU.1 expression levels (13). Since deletion of the URE does not block all PU.1 expression, and

the CE5-CE3 fragment could enhance reporter expression independently of the URE, we considered the possibility that the myeloid enhancer activity of CE5 might involve cell-type-specific recruitment of RNA pol II.

We performed RNA pol II ChIP assays and found cell-type-specific occupancy patterns across the *Sfpi1* upstream region. RNA pol II was found associated with the URE element CE8 in all three cell types examined, including immature T-cells that have silenced PU.1 expression (Fig. 2A-C). While the URE was associated with RNApol in PU.1 expressing and nonexpressing cell lines, RNApol II was detected at the proximal promoter element CE1 only in the PU.1 expressing myeloid (Fig. 2A) and pre-B cells (Fig. 2B). RNA pol II was also found associated with the URE element CE9 in pre-B and myeloid cells (Fig. 2A, 2B). Most notably, and in stark contrast to the proximal URE enhancer, RNApol II binding was detected at CE5, and also at CE7/6, but only in the myeloid cells (Fig. 2A). These results support a role for CE5 in myeloid specific PU.1 regulation and also suggest CE7/6 may contribute to myeloid specific *Sfpi1* transcriptional control.

PU.1 cis-elements CE5 and CE7/6 contain numerous conserved target sites predicted to bind Ets and Ikaros family factors

After verifying the myeloid enhancer specificity of the CE5-CE3 fragment and detecting RNA polymerase II binding to CE5, we sought to identify additional factors that could bind to CE5 and contribute to its regulatory function. Since we also detected myeloid restricted RNA polymerase II association with the CE7/6 region, we included that region in our analysis. We conducted multigenome alignments of the CE5 and CE7/6

regions and then subjected sequences spanning the conserved peaks to TRANSFAC analysis to identify potential transcription factor binding sites (Fig. 3). Predicted sites with average matrix similarities >0.9 and present in all six species used in the alignments are shown. Some conserved sites with matrix similarities less than 0.9 are also shown (in gray) and only hematopoietic expressed transcription factor target sites are presented.

The CE5 and CE7/6 regions have densely packed and overlapping predicted transcription factor target sites. CE7/6 and CE5 contain several conserved core binding factor sites which are target sequences for the Runx family of transcription factors that has been associated with PU.1 regulation previously (16, 19, 24). The CE5 enhancer also has sites predicted to bind C/EBP family members, AP1, and Sp1, factors previously shown to regulate *Sfpi1* transcription through other elements (6, 25, 34). Intriguingly, the most frequently predicted sites were target sequences belonging to members of the Ets and Ikaros family of transcription factors. Most of these sites share the core nucleotides GGA(A) and are overlapping Ikaros/Ets sites. However, some sites were predicted to be either PU.1/Ets or Ikaros sites, but not both (Fig. 3, boxes).

The *Sfpi1* regions CE5 and CE7/6 show myeloid restricted PU.1 recruitment

Since PU.1 is thought to autoregulate and PU.1also has been shown to contribute to production of URE derived RNA transcripts (15), we hypothesized that PU.1 may also bind to PU.1 target sites in CE5 and CE7/6 to contribute to RNA polymerase II recruitment and PU.1 autoregulation. Therefore, we sought to detect PU.1 occupancy of these modules in various hematopoietic lineages by ChIP analysis using a high PU.1

expressing myeloid cell line, RAW264.7 macrophages; a low PU.1 expressing pre-B-cell line, NFS-25; and a DN3-like pro-T-cell line that lacks PU.1 expression, Adh.2C2.

PU.1 ChIP assays revealed cell-type-specific PU.1 occupancy patterns across the *Sfpi1* upstream region. Previous reports suggested PU.1 could bind to its own promoter and autoregulate (7). Our ChIP analysis did detect PU.1 at the promoter region (Fig. 4A, CE1), but that binding was relatively weak. In contrast, very strong PU.1 binding was detected at the URE elements CE9 and CE8 in the myeloid cells (Fig. 4A). We also detected PU.1 association with two known PU.1 target genes, *Mef2c* and *IL7Rα* (Fig. 4A, orange and purple bars) (11, 31). Significantly, very strong PU.1 binding was also detected at CE5 and CE7/6 in the myeloid cells (Fig.4A). While PU.1 associated strongly with CE8, *Mef2c*, and *IL7Rα*, no other *Sfpi1* element was strongly enriched in the pre-B-cells (Fig. 4B). As expected, no region was enriched by anti-PU.1 ChIP with the PU.1 negative immature T-cell line, Adh.2C2 (Fig. 4C). Collectively, these results indicate that the myeloid enhancer CE5 is a cell-type-restricted PU.1 target element.

Forced expression of PU.1 facilitates RNApol II recruitment to *Sfpi1* regulatory elements in immature T-cells, but PU.1 is insufficient to initiate a positive autoregulatory loop

The immature T-cell line Adh.2C2 does not express PU.1, but when retrovirally infected with PU.1, some Adh.2C2 cells gain myeloid characteristics (12). We therefore asked where ectopic PU.1 could bind in Adh.2C2 cells and whether PU.1 might alter RNApol II recruitment to the *Sfpi1* locus in these cells. To investigate this question, we

infected Adh.2C2 cells and performed ChIP assays to examine the capacity of PU.1 and RNApol II to bind regulatory modules in these immature T-cells.

Cells were infected with PU.1 virions that also generate coexpression of human CD8 to mark infected cells. A representative CD8 staining of infected cells at the time of cell harvesting is shown (Fig. 5A). After infection, cells were expanded for 48-72 hours to provide enough cells for ChIP experiments. We first examined PU.1 mRNA levels in the infected Adh.2C2 cells and compared them to uninfected Adh.2C2, RAW264.7, and NFS-25 cells. Figure 5B shows that infected cells expressed very high levels of PU.1, even more than RAW264.7 macrophages. We next examined where ectopic PU.1 could bind in infected Adh.2C2 cells. Despite having more PU.1 than myeloid cells, PU.1 binding was restricted in Adh.2C2 cells. As indicated in Figure 5C, anti-PU.1 ChIP assays with infected cells allowed for strong enrichment of CE9, CE8, *Mef2c,* and *IL7Rα.* Significantly, PU.1 could not bind CE5 in these cells. Note that the cells being used in these ChIP assays are not all $PU.1^{+}$ ($hCD8^{+}$ in Fig. 5A) so the enrichments reported in Fig. 5C and 5D are under representations of actual binding relative to the total non infected cells. We next looked to see if PU.1's occupancy of CE9 allowed for detection of RNApol II as well. As shown in Figure 5D, RNApol II associated with CE9 only in the presence of PU.1 (green bars for PU.1 infected cells versus black bars for normal immature T-cells). The relative enrichment of other regions by RNApol II ChIP also correlated with regions with detectable PU.1 binding. Together, these results show that PU.1 binding and its facilitation of RNA pol II recruitment is cell-type-restricted to a subset of target regions.

ChiP assays with PU.1 infected immature T-cells implied concomitant binding of PU.1, and RNA pol II at CE9. PU.1 and RNApol II could also bind CE8, and to a lesser extent, could also be detected at CE4. As these cells now had three regulatory modules occupied by factors with potential positive transcriptional regulatory effects, and PU.1 is thought to be autoregulatory, we asked if ectopic PU.1 expression could affect endogenous *Sfpi1* transcription. We analyzed endogenous PU.1 expression (Fig. 5E), but found infected Adh.2C2 cells were still lacking endogenous PU.1 mRNA. This result suggests that either PU.1 alone is incapable of overcoming a dominant silencing mechanism to establish a multimodule autoregulatory loop in these immature T-cells, or that PU.1 autoregulation through CE7-CE5 is also needed.

Elf-1, Ets-1, and GABPα can bind a limited subset of PU.1target sites but are excluded from occupying the CE5 and CE7/6 modules

At least two possible explanations for the absence of PU.1 binding to non CE8 *Sfpi1* target sites in B-cells exist. First, some target sites may be bound by other factors in B cells, out competing PU.1. Alternatively, targeting of PU.1 to non CE8 modules may be combinatorial and require additional binding to sites flanking PU.1 interacting regions. Both possibilities were explored through additional ChIP analyses.

As the immature T-cells lack PU.1 (Fig. 5B), we performed ChIP assays with a broad range of hematopoietic expressed Ets family members in an attempt to detect non PU.1 occupancy of either CE5 or CE7/6 in lymphoid cells. ChIP assays with numerous Ets family members (Ets-2, NERF, SAP1A, NET, TEL, Erg, and Elk-1) failed to enrich *Sfpi1* upstream regions in any cell line (data not shown). However, three Ets family

members known to be expressed in all hematopoietic lineages examined, Elf-1, Ets-1, and GABPα (2), demonstrated restricted binding to PU.1 target sites.

Elf-1 was previously shown to be able to bind to CE8 in addition to PU.1 (25). Elf-1 ChIP assays were performed and confirmed that Elf-1 could bind the proximal URE enhancer element, CE8, and at least one of the control PU.1 targets (*Mef2c* or *IL7Rα*) in each cell line examined (Fig. 6A-C). Importantly, Elf-1 could not significantly bind the myeloid enhancer CE5, nor could Elf-1 be detected at CE7/6 in any of these cell lines. We next examined Ets-1 binding across the *Sfpi1* upstream region. Like Elf-1, Ets-1 was found to have lineage restricted access to PU.1 target elements. Ets-1 did not strongly bind any *Sfpi1* upstream region in myeloid cells (Fig. 6D). In contrast, Ets-1 could occupy multiple sites in pre-B-cells, including the CE10 region, a region PU.1 fails to bind (Fig. 6E). Additionally, CE1 could be bound by Ets-1 in the pre-B-cells, although Ets-1 failed to bind CE1 in myeloid cells. Notably, Ets-1 could not bind any region in the immature T-cells, nor could Ets-1 associate with CE5 or CE7/6 in the pre-B-cells (Fig. 6E, 6F). Finally, we investigated GABPα binding to *Sfpi1* elements as GABPα is an Ets factor argued to bind the same sites through which PU.1 regulates some target genes (11). We performed GABPα ChIP assays and found that GABPα could associate with CE8 in myeloid and lymphoid cells, and could also bind to *Mef2c* in the PU.1 expressing cell lines (Fig. 6G-I). Like Ets-1 and Elf-1, no GABPα binding to PU.1 target sites in CE5 and CE7/6 could be detected.

Together, the Elf-1, Ets1, and GABPα ChIP results indicate that the majority of PU.1 target sites within *Sfpi1* regulatory modules, especially CE5 and CE7/6, are not promiscuous Ets family sites and preferential PU.1 occupancy of these sites may be

dictated by yet unknown biochemical mechanisms in myeloid cells. Since we performed a nearly exhaustive search for Ets factor binding to PU.1 target regions in myeloid and lymphoid cells, but were unable to detect any occupancy of CE7/6 or CE5, except by PU.1, we next turned to a non Ets factor expressed throughout the hematopoietic compartment.

Ikaros binds CE7/6 and CE5 in myeloid but not lymphoid cells and Ikaros recruitment is not dose dependent

TRANSFAC analysis predicted numerous Ikaros sites within the CE5 and CE7/6 regions. Ikaros is a bifunctional transcription factor able to activate or repress transcription and Ikaros is known to compete with the Ets family factor Elf-1 for the same sites in some contexts (32). While most of the predicted Ikaros sites overlap potential Ets sites that are not specifically predicted to bind PU.1, some flank Ets family sites explicitly predicted to bind PU.1 (Fig. 3, boxes). We considered the possibility that Ikaros may be binding these regions in myeloid cells in collaboration with PU.1. Alternatively, Ikaros could be bound to these modules in lymphoid cells, perhaps suppressing enhancer function by preventing Ets factor binding. To address these possibilities, we investigated the ability of Ikaros to associate with the *Sfpi1* upstream regions.

ChIP analysis revealed lineage specific patterns of Ikaros binding to *Sfpi1* cis-elements. Consistent with the PU.1 and RNApol II ChIP results, we found CE5 and CE7/6 were also myeloid restricted in their ability to bind Ikaros (Fig. 7A). In contrast to myeloid specific Ikaros occupancy at CE5 and CE7/6, the URE elements CE9 and CE8

were both comparably associated with Ikaros in myeloid and pre-B-cells (Fig. 7A, 7B). Additionally, Ikaros binding to CE10 was detected and like RNApol II, association with CE10 was restricted to the pre-B-cells (Fig. 7B and Fig. 2B). In the immature T-cells, Ikaros could also associate with the proximal URE element CE8 (Fig. 7A) but was not detected at the distal URE enhancer CE9. Given that Ikaros was relatively weakly associated with some regions in immature T-cells compared to the PU.1 expressing myeloid and B-cells, we considered the possibility that Adh.2C2 cells might be lacking sufficient Ikaros protein expression. We therefore examined Ikaros protein levels in nuclear extracts from myeloid cells and immature T-cells. Adh.2C2 cells had higher levels of Ikaros protein, implying that the absence of Ikaros binding to *Sfpi1* regulatory elements is not dosage restricted in these cells (Fig. 7D). Since ectopic PU.1 expression was able to alter RNApol II recruitment to *Sfpi1* regions, we considered the possibility that some Ikaros binding to *Sfpi1* regions might also depend on the presence of PU.1. We therefore examined Ikaros binding in PU.1 infected Adh.2C2 cells. Indeed, Ikaros binding to CE9 and also to *IL7Rα*, like RNApol II, was strongly increased in the presence of PU.1 protein (Fig. 7E). Collectively, these results demonstrate that CE5 and CE7/6 are myeloid restricted Ikaros target elements and suggest that PU.1 binding may be facilitating Ikaros occupancy, in addition to RNApol II, in highly context dependent ways.

The CE7/6 and CE5 regions can nucleate formation of PU.1 and Ikaros protein-DNA complexes in vitro

PU.1 and Ikaros ChIP analysis showed all-or-none occupancy of the CE5 and CE7/6 regions in myeloid cells. To formally demonstrate these factors can concomitantly occupy these modules, we designed several DNA probes for use in gel shift assays (Fig. 3, red bars). Probes CE5-P2 and CE76-P4 were designed to span DNA sequences with specifically predicted PU.1 target sites adjacent to additional Ikaros sites (Fig. 3, boxes). These probes were then used in gel shift assays. Since the ChIP analysis indicated cell-type-specific occupancy of CE5 and CE7/6 in vivo, we examined the ability of Adh.2C2 (T), RAW264.7 (M), and NFS-25 (B) cells to nucleate protein complexes on probes CE5-P2 and CE76-P4. RAW264.7 extract nucleated complexes on both probes, labeled M1 and M2 (Fig. 8A). In contrast, extracts from the T- and B-cells produced distinct banding patterns on either probe. Notably, complex M1 was not formed by the T or B extracts.

The nature of the myeloid M1 and M2 protein complexes was examined more closely with probe CE76-P4. As shown in Fig. 8B, competition with cold probe could eliminate bands M1 and M2 formed by probe CE76-P4 (Fig. 8B, lane 1 vs 2), demonstrating probe/complex specificity. Significantly, probe CE5-P2 could cross compete with the CE76-P4 probe (Fig. 8B, lane 1 vs 7) to eliminate band M1 and M2 formation, confirming that the CE5 and CE7/6 M1 and M2 bands represent redundant myeloid specific protein complexes formed by two distinct *Sfpi1* regulatory modules. We also subjected the M1 and M2 complexes to antibody treatments to test for the presence of PU.1 and Ikaros protein. Significantly, anti-PU.1 antibody super shifted the myeloid specific M1 complex (Fig. 8B, lane 13). Complex M2 was also supershifted by

anti-PU.1 antibody (Fig. 8B, lane 13). One of the anti-Ikaros antibodies appeared to crosslink Ikaros with probe CE76-P4 to form a novel band (Fig. 8B, lane 14, red arrow). More significantly, an anti-Ikaros antibody inhibited the formation of complex M2, but appeared not to affect M1 (Fig.8B, lane 17). This Ikaros antibody also inhibited complex M1 formed by the CE5-P2 probe (data not shown). Also noteworthy, none of the other CE5 or CE7/6 probes could competitively inhibit complex M1 or M2 at a high 250 fold excess relative to labeled probe even though most have predicted Ikaros/Ets sites (see Fig. 3). In contrast to other CE5 and CE7/6 probes, a competitor probe from CE4A, a region ChIP assays showed could weakly associate with PU.1 and Ikaros in PU.1 infected Adh.2C2 cells, could competitively eliminate bands M1 and M2 (Fig. 8B, lane 11), but not when the Ets site in CE4Am1 is mutated (Fig. 8B, lane 12). Collectively, these gel shift analyses critically show that the M2 complex is formed at least in part by PU.1 and Ikaros, thus demonstrating that Ikaros and PU.1 are not simply competing for the same sites but can concomitantly occupy regions within both CE5 and CE7/6 regulatory modules.

PU.1 is required for CE5 enhancer activity

The fundamental question of whether the myeloid specific PU.1 recruitment to CE5 and CE7/6 can actually contribute to myeloid enhancer activity remained to be addressed. As the in vivo and in vitro PU.1binding ability of CE5 and CE7/6 appeared to be redundant, we constructed an additional reporter, L75, with a ~2.5 kb DNA fragment from CE7-CE5 joined to the PU.1 promoter to test these modules' combined dependence on PU.1 regulatory function in myeloid cells. To analyze PU.1's regulatory role, we

employed an antisense morpholino knock down strategy to perturb PU.1 regulatory function in RAW264.7 cells. We first tested two morpholinos designed to target different PU.1 exon/intron boundaries to see if they could knockdown PU.1 protein levels. As shown in Figure 9A, either morpholino targeting PU.1 transcripts could eliminate detectable PU.1 protein expression as anylyzed by Western blot. We then cotransfected *Sfpi1* reporters into RAW246.7 cells with anti-PU.1 E2 or control morpholionos. Transfection of L98 with PU.1 E2 morpholino did affect reporter activity, lowering URE driven luciferase expression ~40% (Fig. 8B). However, as this URE enhancer activity remained moderately strong in these cells, other factors appear to independently drive expression through the URE elements CE9 and CE8. In stark contrast, knockdown of PU.1 completely destroyed the enhancer function of the CE7-CE5 fragment (Fig. 8B). These results demonstrate that the URE is PU.1 responsive but independent, while the CE7-CE5 fragment is strictly dependent on PU.1 for enhancer activity.

Ikaros contributes to the enhancer function of both the URE and CE7-CE5 regions in myeloid cells

The potential regulatory contribution of Ikaros was examined last. We cotransfected L1, L98, and L75 reporters into myeloid cells with empty pEF plasmid, pEF-Runx1, or pEF-Plastic. Plastic is a version of Ikaros harboring a point mutation that inhibits DNA binding while maintaining the ability to heterodimerize with wild type Ikaros family members (26). As shown in Figure 9B, cotransfection with the dominant negative Ikaros version Plastic strongly reduced the activity of the L98 and L75 reporters, demonstrating Ikaros is a functionally relevant positive regulator of *Sfpi1* in

macrophages. In contrast to the myeloid cells, cotransfection of L98 with Plastic in the pre-B-cells did not inhibit reporter activity. Thus, Ikaros' positive regulatory contribution through the URE, and not just binding to the myeloid enhancer CE5 is cell-type-specific.

DISCUSSION

We have characterized a new myeloid restricted *Sfpi1* enhancer element that provides a preferential PU.1 nucleation center able to recruit RNApol II and mediate cell-type-specific PU.1 autoregulation. Discovery of another *Sfpi1* enhancer element was required to explain how dynamic PU.1 expression could be produced because the well-established *Sfpi1* URE elements CE9 and CE8 confer similar levels of reporter expression in high PU.1 expressing macrophages and low PU.1 expressing B-cells. Until now, dynamic control of PU.1 dosage has been argued to be mediated through the URE. Although we have identified some of the factors that bind to CE5 and CE7/6, including RNApol II, PU.1, and Ikaros, these factors also bind the URE, but their occupancy of the URE is not all-or-none dependent on PU.1. In contrast, the CE7-CE5 region appears to have a more restricted combinatorial all-or-none occupancy that depends on preferential use of PU.1, and not other Ets family members. Thus, the *Sfpi1* CE5 enhancer module appears to have evolved to depend specifically on cell-type-specific context dependent PU.1 recruitment to provide autoregulatory control of PU.1 dosage even where the PU.1 binding and autoregulatory contributions at the URE may be subject to competition with other Ets family members.

Formally, it is possible that the observed myeloid restricted PU.1 occupancy of CE5 is due to limited chromatin accessibility or is itself a consequence of PU.1 dosage. Evidence against these interpretations comes from three sources. First, it has been shown by others that bone marrow derived lympoblasts are marked by H3K4me1 and H3K4me2 in ChIP-seq studies from Dr. BE Bernstein's lab and available through ENCODE (Fig. S1). Second, when we examined the PU.1 expression levels in our cell lines by RNA analysis we found that the NFS-25 pre-B-cells had ~8 fold less PU.1 mRNA compared to the RAW264.7 macrophages, as expected. Despite lower PU.1 expression in pre-B-cells, two known PU.1 target genes, *IL7Rα* and *Mef2c* (11, 31) were strongly enriched to a similar extent as in the myeloid cells. Lastly, forced high level expression of PU.1 in immature T-cells did not generate detectable PU.1 binding to CE5. These results imply that the relative absence of PU.1 binding to CE7-CE5 in pre-B-cells is not the result of inadequate PU.1 dosage or chromatin accessibility and instead indicates that additional factors influence PU.1 and Ikaros recruitment.

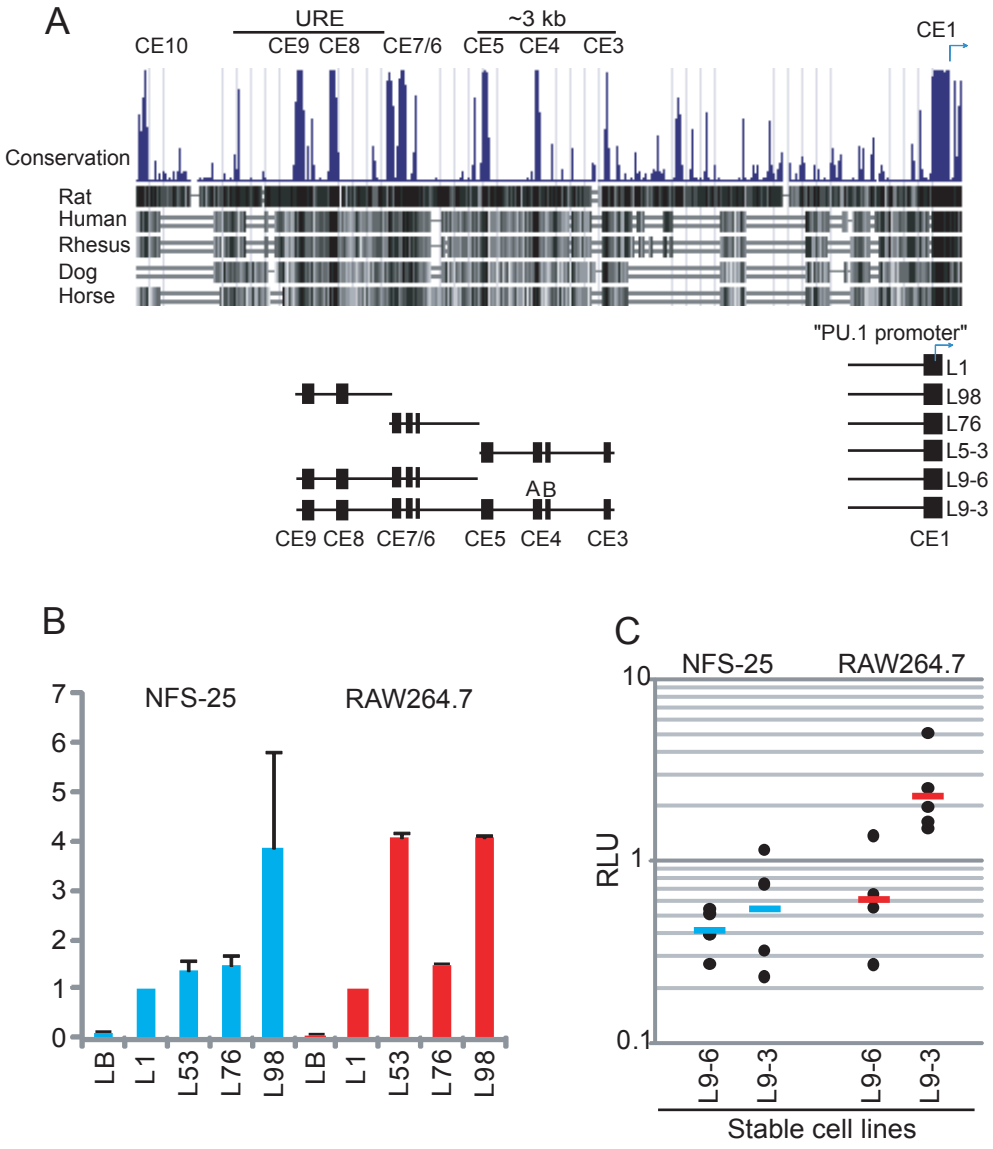
Our results showing all-or-none cell-type-specific PU.1 and Ikaros binding to CE5 also distinguishes this regulatory module from other PU.1 and Ikaros target elements present in genes such as *Mef2c* and *IL7Rα.* These PU.1 regulated modules were equally bound by PU.1 in pre-B-cells and myeloid cells, but only pre-B-cells had Ikaros associated with the *IL7Rα* promoter. This is an intriguing result for two reasons. First, *IL7Rα* is a known PU.1 target gene but is not expressed in myeloid cells. We analyzed *IL7Rα* mRNA levels in our cell lines and verified that the pre-B-cells do express *IL7Rα* while RAW264.7 macrophages do not (data not shown). Thus, PU.1 is not sufficient for regulating *IL7Rα* transcription. Secondly, the absence of Ikaros binding to *IL7Rα* in

myeloid cells suggests that an additional factor regulates its recruitment to the *IL7Rα* promoter independently of PU.1 occupancy. As ectopic PU.1 expression was sufficient to recruit Ikaros to the distal URE enhancer, CE9, in immature T-cells, these results illustrate that cis-regulatory elements have evolved multiple combinatory mechanisms to discriminate context dependent use of PU.1 for lineage restricted functions. In contrast to *IL7Rα*, *Mef2c* is reportedly expressed by both myeloid and B-cells (31) and was occupied by Ikaros in both RAW264.7 and NFS-25 cells. Taken together with the *IL7Rα* results, this suggests that PU.1 is not the limiting transcriptional input controlling *IL7Rα* expression. In fact, Ikaros expression has been correlated with *IL7Rα* regulation previously (35). Our results are therefore consistent with other reports and suggest that Ikaros may be required for *IL7Rα* expression, with PU.1 and Ikaros acting in parallel with other factors to positively control *IL7Rα* transcription in a B-cell specific fashion.

Our functional results in transient transfect experiments showing that Ikaros can collaborate with PU.1 in cell-type-specific contexts may have important implications. We showed that Ikaros contributes to URE enhancer activity in myeloid cells, but while Ikaros also was found to bind the URE in pre-B-cells, it did not appear to contribute to URE regulatory function. This result indicates that Ikaros' regulatory contribution and not just binding is dictated by mechanisms yet to be identified. Interestingly, Ikaros has been suggested as a suppressor of PU.1 expression in multipotent progenitors that have not yet been specified to become lymphoid or myeloid lineages (30). Additionally, it has been recently shown that the Ikaros family member Eos can directly interact with PU.1 and MITF, which leads to repression instead of activation of some PU.1 target genes in myeloid progenitors (18). Our results here suggest the possibility that Ikaros too, may

have the capacity to modulate *Sfpi1* transcriptional control differently in distinct lineages or at varied developmental stages depending on context dependent collaborations. And like Eos, it may be possible that Ikaros can block or change the directionality of PU.1 autoregulation in some contexts.

Like PU.1, Ikaros expression has also been shown to be dynamically regulated as HSCs differentiate. While the mechanisms controlling Ikaros expression are not understood, an Ikaros GFP reporter mouse has correlated Ikaros and PU.1 expression and shown that like PU.1, Ikaros reaches its highest levels in GMPs (2). Ikaros is thought to be dispensable for most myeloid cell development while being essential for lymphocyte development (14). However, both Ikaros and PU.1 are required for generating high PU.1 expressing dendritic cells (29, 33). Additionally, it is worth noting that Ikaros mutant mice suffer from myeloid hyperplasia which is consistent with dysregulated PU.1 expression and a role in positively regulating *Sfpi1* transcriptional output after myeloid specification (14, 28).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L76
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AB
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CE1
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Stable cell lines

Figure 1.

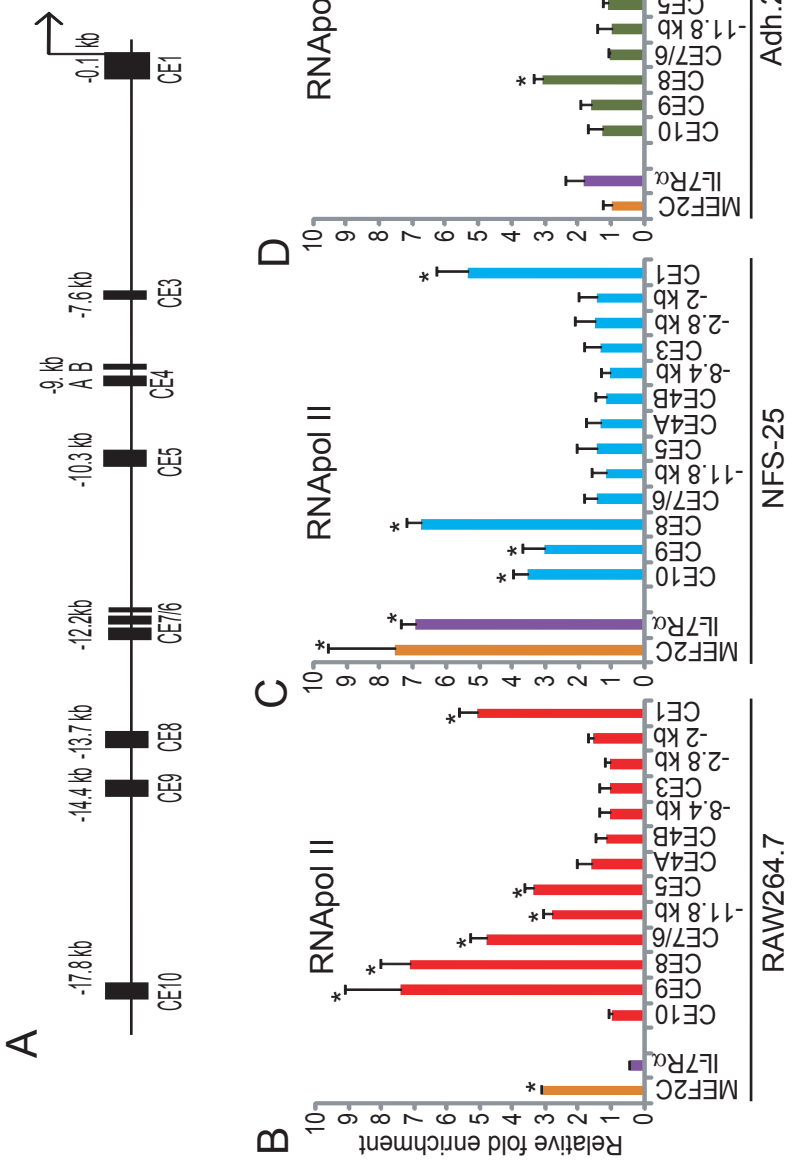
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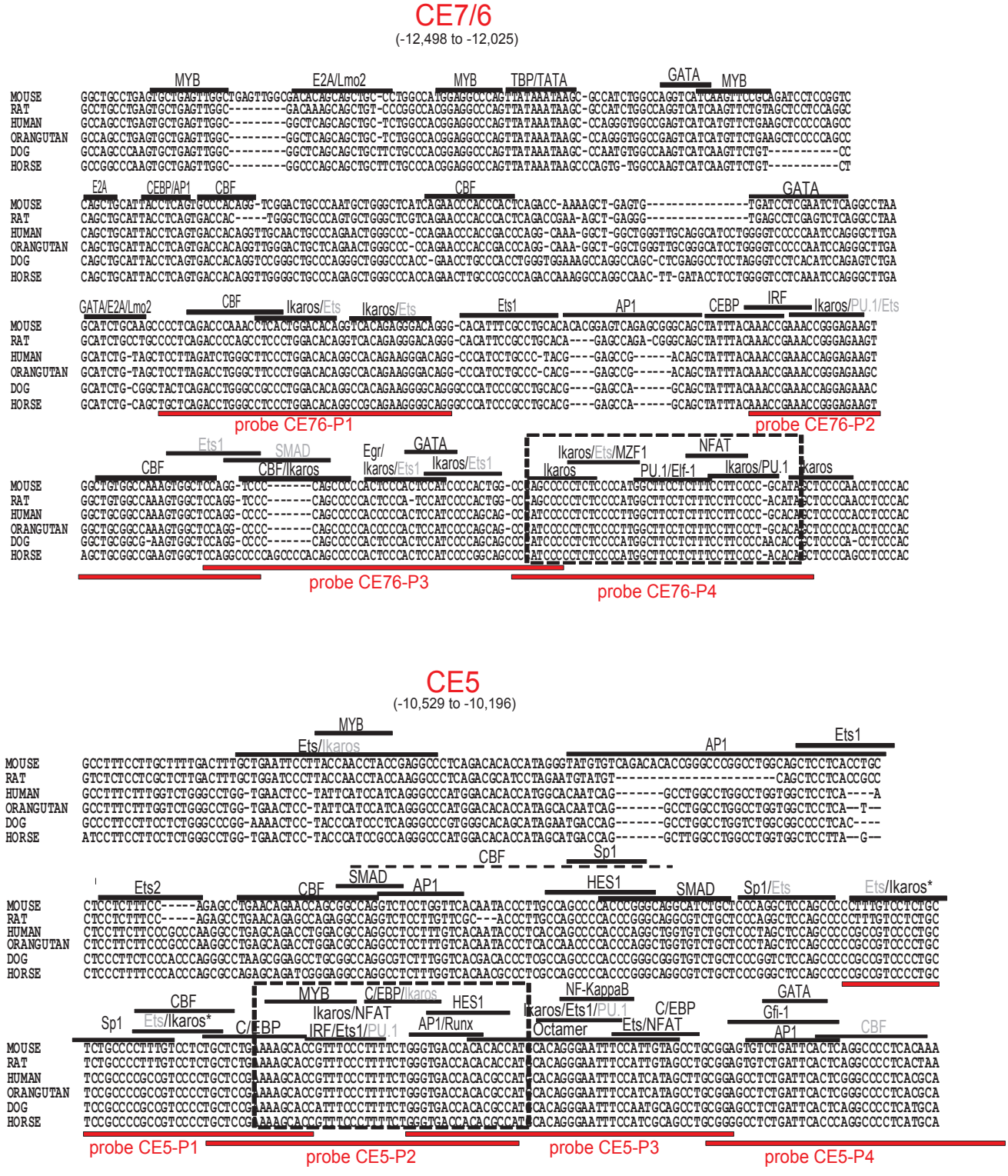
Figure 2.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DOG
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E2A
CEBP/AP1
CBF
CBF
GATA
GATA/E2A/Lmo2
CBF
Ikaros/Ets
Ikaros/Ets
Ets1
AP1
CEBP
IRF
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SMAD
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ORANGUTAN
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CBF
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AP1
HES1
SMAD
Sp1/Ets
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Sp1
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probe CE5-P1
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probe CE5-P3
probe CE5-P4

Figure 3.

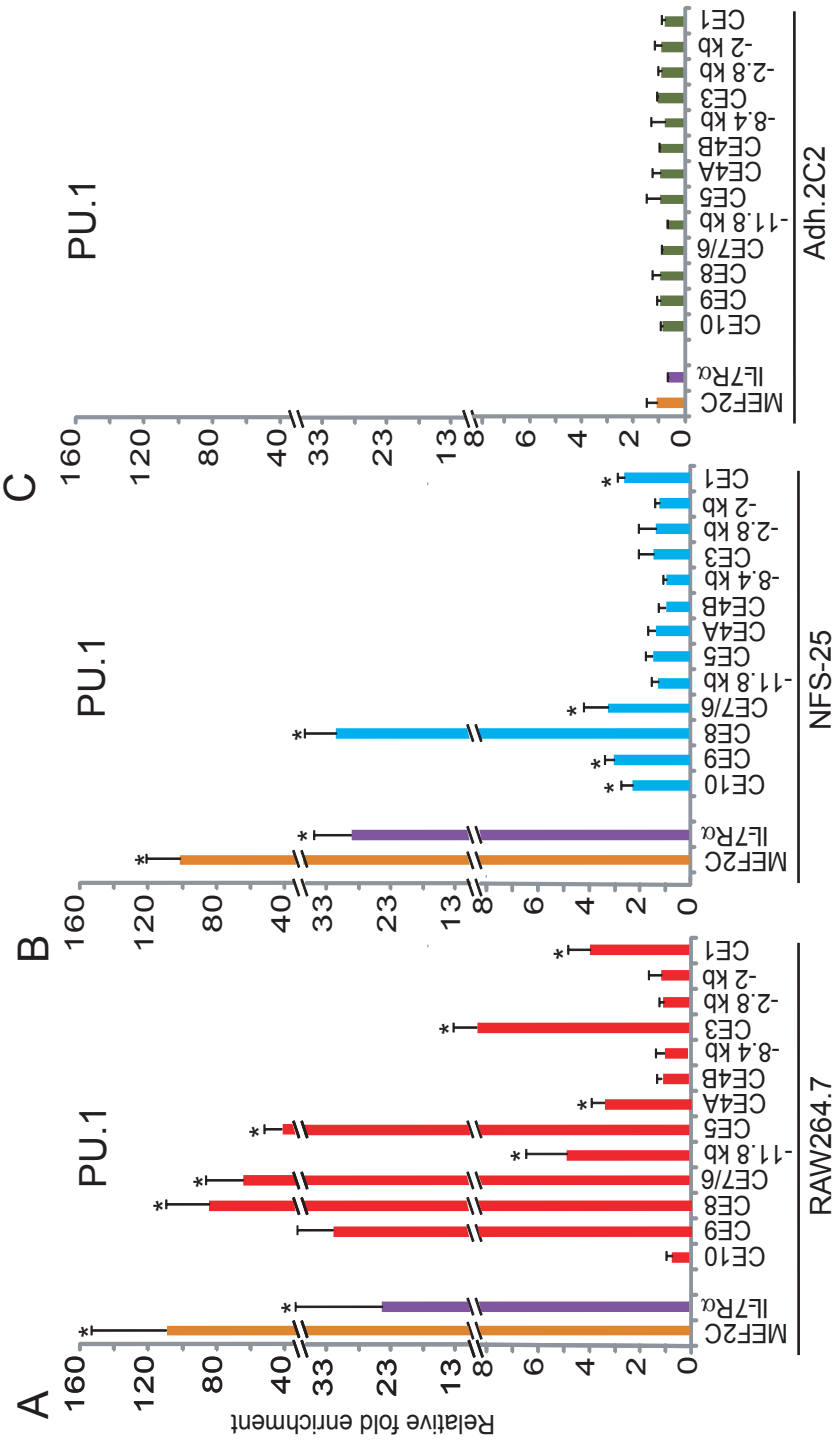
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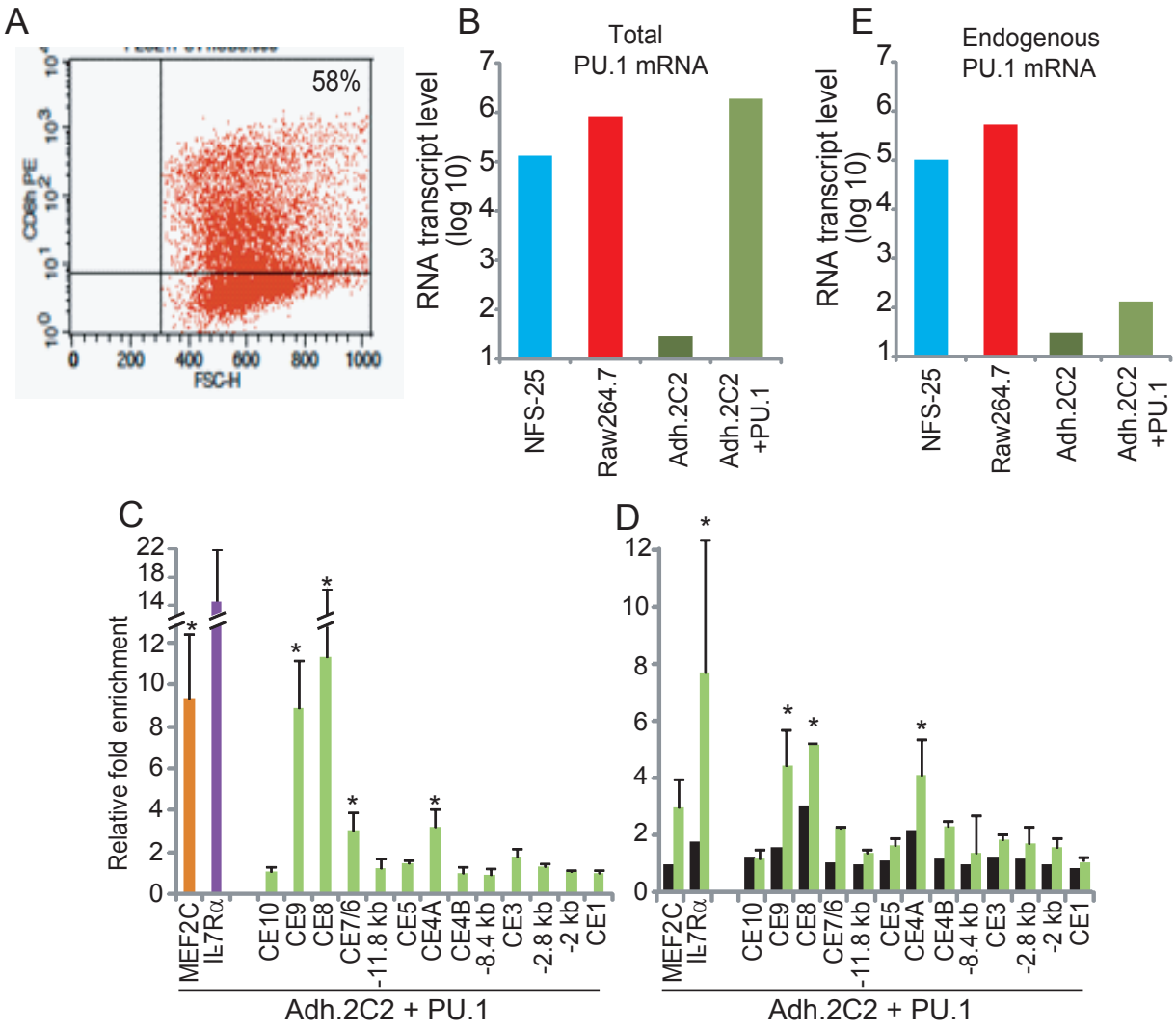
Figure 4.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Raw264.7
Adh.2C2
Adh.2C2
+PU.1
E
Endogenous
PU.1 mRNA
RNA transcript level
(log 10)
NFS-25
Raw264.7
Adh.2C2
Adh.2C2
+PU.1
C
Relative fold enrichment
MEF2C
IL7Rα
CE10
CE9
CE8
CE7/6
-11.8 kb
CE5
CE4A
CE4B
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CE3
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CE7/6
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CE4B
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CE3
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CE1
Adh.2C2 + PU.1

Figure 5.

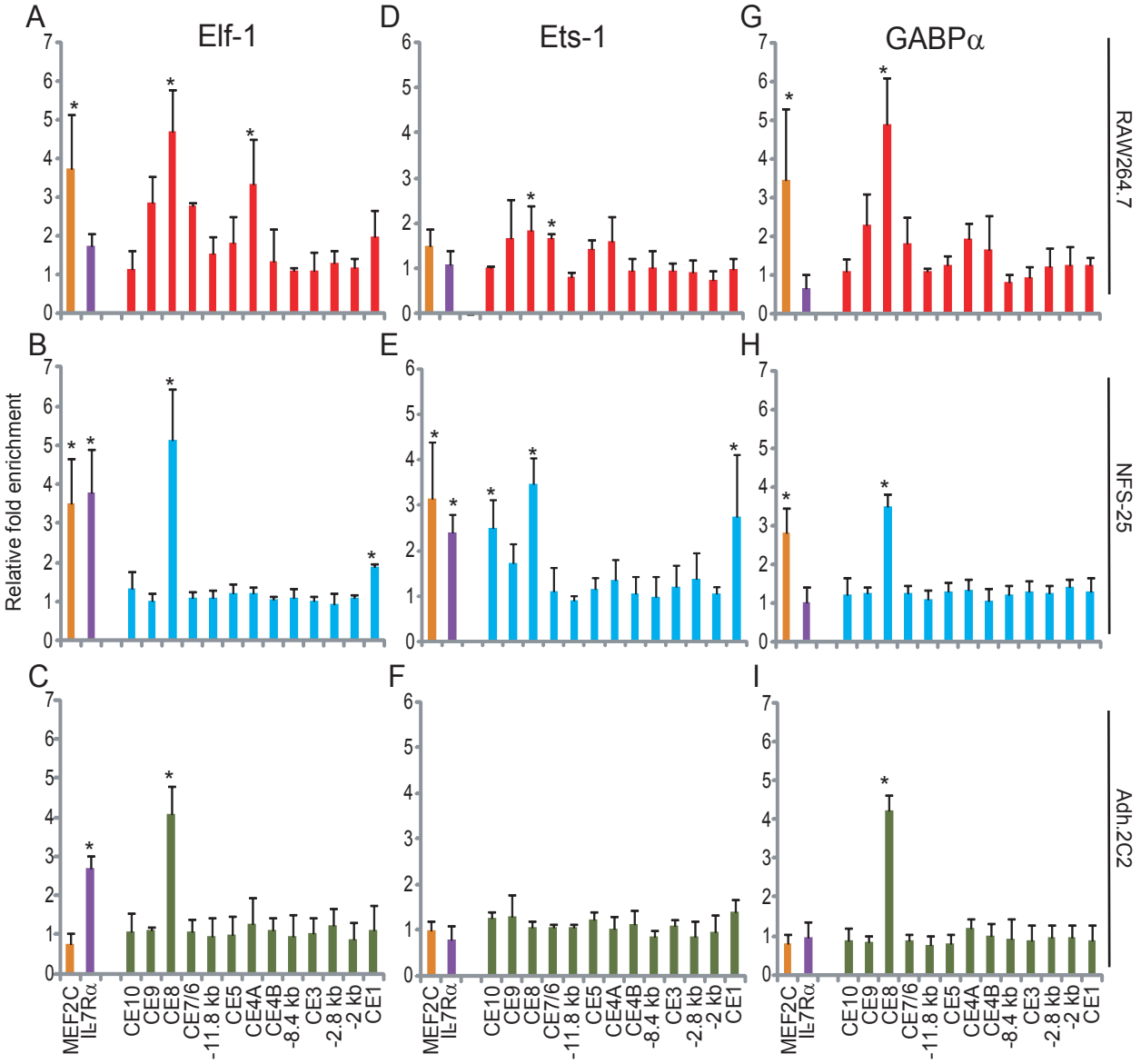
A
Elf-1
D
Ets-1
G
GABPα
RAW264.7
B
E
H
NFS-25
Relative fold enrichment
C
F
I
Adh.2C2
MEF2C
IL7Rα
CE10
CE9
CE8
CE7/6
-11.8 kb
CE5
CE4A
CE4B
-8.4 kb
CE3
-2.8 kb
-2 kb
CE1

Figure 6.

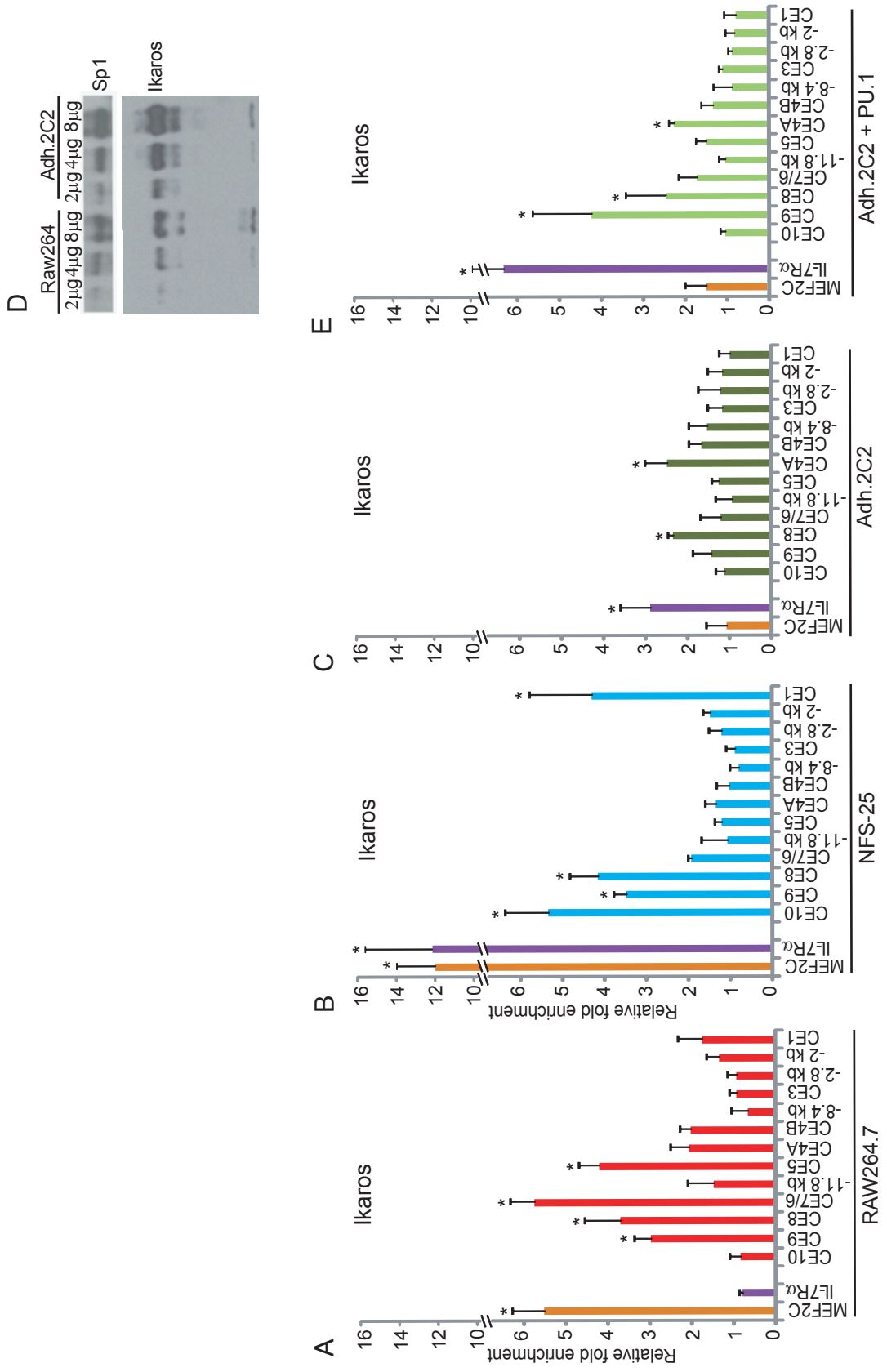
A
Ikaros
Relative fold enrichment
RAW264.7
B
NFS-25
C
Adh.2C2
D
Raw264
Adh.2C2
2µg 4µg 8µg 2µg 4µg 8µg
Sp1
Ikaros
E
Adh.2C2 + PU.1
MEF2C
IL7Rα
CE10
CE9
CE8
CE7/6
-11.8 kb
CE5
CE4A
CE4B
-8.4 kb
CE3
-2.8 kb
-2 kb
CE1

Figure 7.

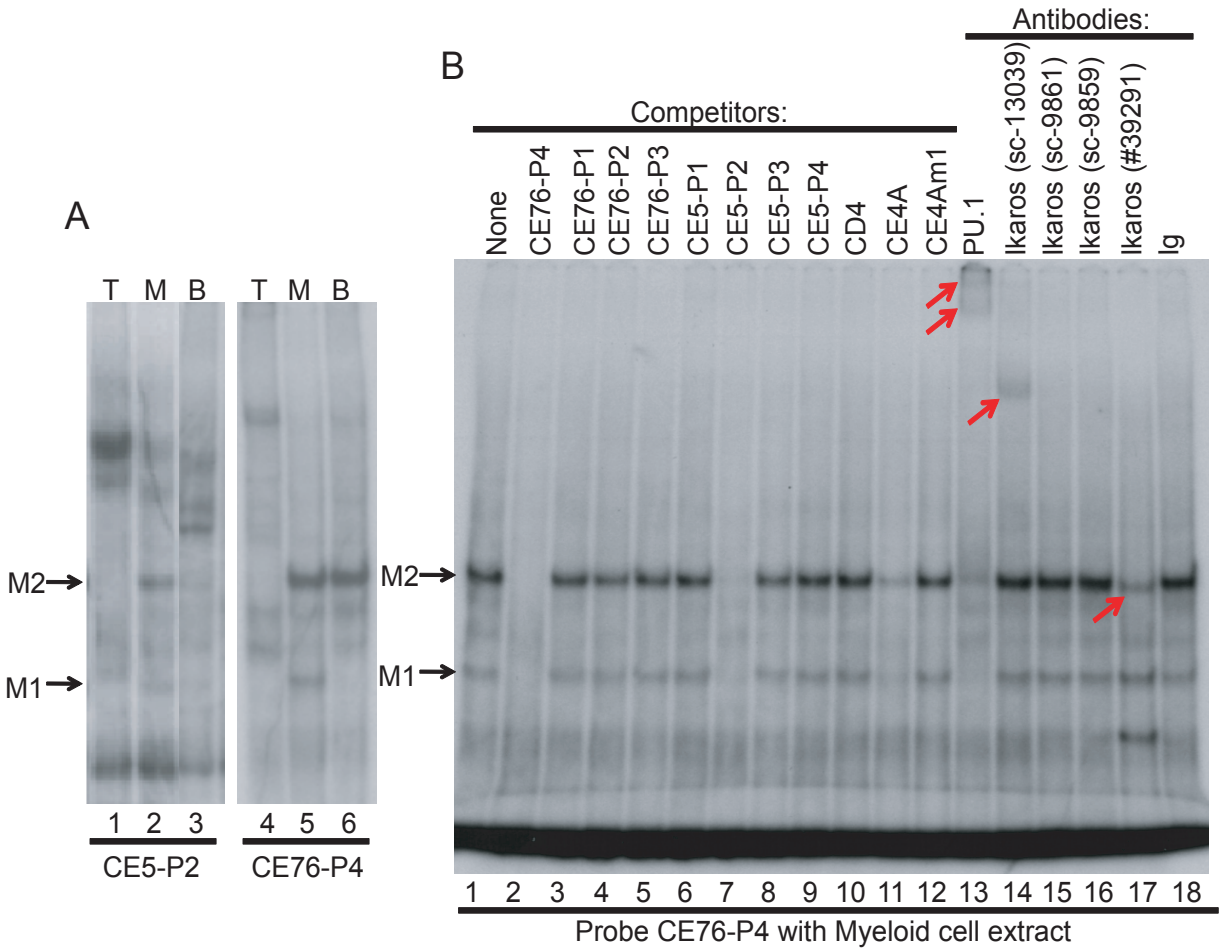
A
T M B T M B
M2
M1
1 2 3
CE5-P2
4 5 6
CE76-P4
B
Competitors:
None
CE76-P4
CE76-P1
CE76-P2
CE76-P3
CE5-P1
CE5-P2
CE5-P3
CE5-P4
CD4
CE4A
CE4Am1
Antibodies:
PU.1
Ikaros (sc-13039)
Ikaros (sc-9861)
Ikaros (sc-9859)
Ikaros (#39291)
Ig
M2
M1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Probe CE76-P4 with Myeloid cell extract

Figure 8.

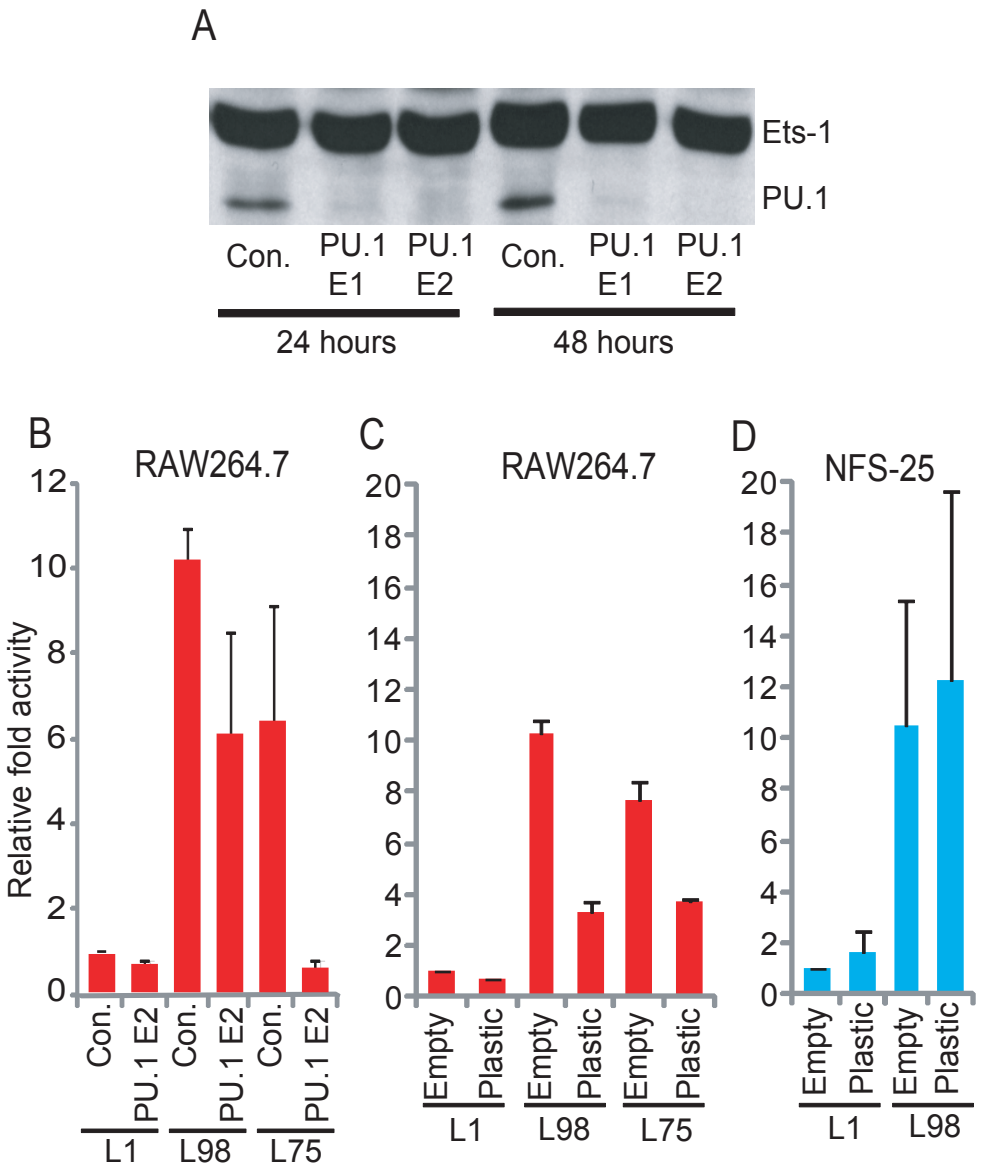
A
Ets-1
PU.1
Con.
PU.1 E1
PU.1 E2
Con.
PU.1 E1
PU.1 E2
24 hours
48 hours
B
RAW264.7
Relative fold activity
Con.
PU.1 E2
L1
L98
L75
C
RAW264.7
Empty
Plastic
L1
L98
L75
D
NFS-25
Empty
Plastic
L1
L98

Figure 9.

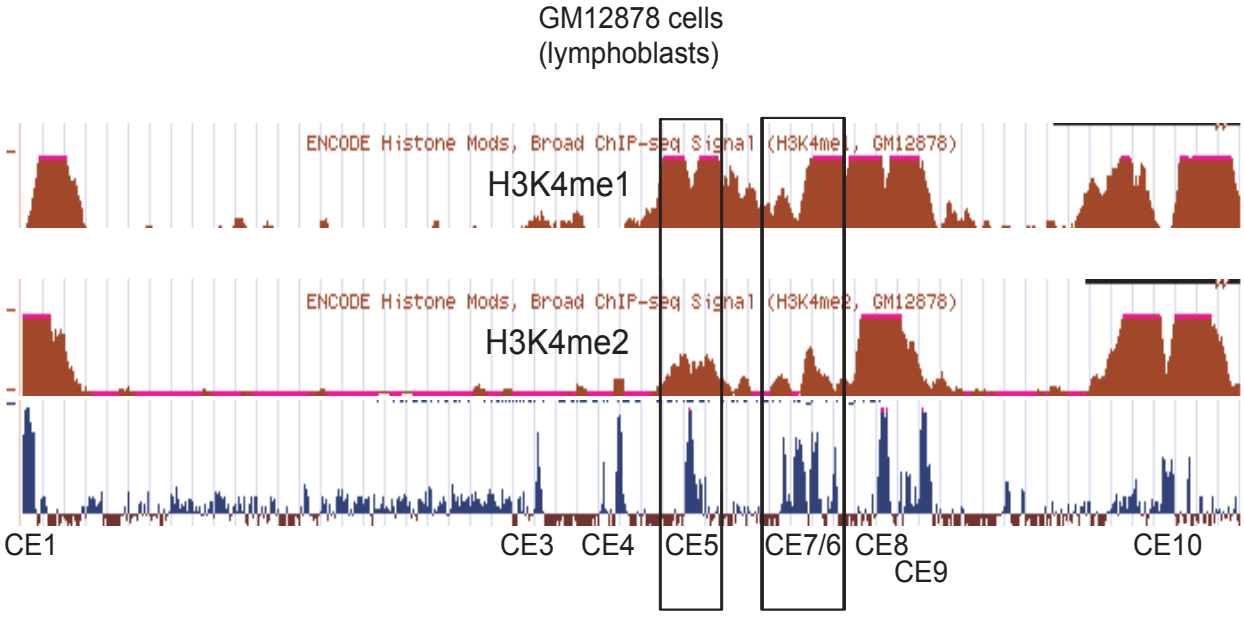
GM12878 cells
(lymphoblasts)
ENCODE Histone Mods, Broad ChIP-seq Signal (H3K4me1, GM12878)
H3K4me1
ENCODE Histone Mods, Broad ChIP-seq Signal (H3K4me2, GM12878)
H3K4me2
CE1
CE3
CE4
CE5
CE7/6
CE8
CE9
CE10

Figure S1.

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Figure Legends:

Figure 1. The CE5-CE3 fragment is a myeloid restricted enhancer and does not have B-cell regulatory activity. (A) A schematic showing a multigenome alignment of the ~18 kb upstream region of the *Sfpi1* gene with peaks of conservation is shown (UCSC genome browser.) Regions contained in luciferase reporters are depicted. (B) Transient transfection assays showing *Sfpi1* reporter activity in NFS-25 pre-B-cells and RAW264.7 macrophages. Data represents 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. (C) The CE5-CE3 fragment is a myeloid specific enhancer in a chromatin context. NFS-25 pre-B-cells and Raw264.7 macrophages were stably transfected with the L9-6 and L9-3 constructs and a control luciferse reporter conferring Blasticidin resistance. Bars represent the geometric mean of five independent mixed pools (dots) of stably transfected cells 30 days post transfection under continuous selection. Data are reported as relative light units (RLU).

Figure 2. The myeloid enhancer CE5 is a cell-type-specific RNApol II nucleation center. (A) A schematic of regions examined in the ChIP assays is shown. The PU.1 transcriptional start site is depicted by the arrow. Conserved Elements are labeled below and approximate locations of forward primers used for QPCR analysis are labeled above. (B) RNApol II binds CE5 and CE7/6 in RAW264.7 cells. Data shown are from three independent experiments. (C) RNApol II binds to the URE in NFS-25 cells, but not to CE5 or CE7/6. Data shown are from three independent experiments. (C) RNApol II also

binds to the proximal URE enhancer CE8, in immature T-cells. Data shown are from four independent experiments. Orange and purple bars in each graph show data for positive control regions of reported PU.1 target genes *Mef2c* and *IL7Rα*. 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 represent standard deviations. Asterisks mark peaks with statistically significant enrichment ($p < 0.0001$). See Materials and Methods for details of statistical analysis. Primer pairs used for each region labeled on the x-axis.

Figure 3. Multigenome alignments and transcription factor target site prediction analysis of the CE7/6 and CE5 regions are shown. The mouse sequence used is shown in parenthesis. TRANSFAC analysis was performed through the Biobase TRANSFAC suite's MATCH tool. 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 Factor Binding 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. Boxes surround sequences with specifically predicted PU.1 target sits having adjacent Ikaros target sites. Red bars underneath sequences represent DNA probes designed for gel shift assays in Figure 8.

Figure 4. ChiP analysis reveals cell-type-restricted PU.1 binding to *Sfpi1* upstream regions. PU.1 ChIP assays were performed and reported as described earlier. ChIP data shown are the average fold enrichment from two independent experiments for immature T-cells (A), five independent experiments for NFS-25 pre-B-cells (B) and three independent experiments for RAW264.7 myeloid cells (C).

Figure 5. Forced expression of PU.1 in immature T-cells permits enhancer restricted PU.1 recruitment and facilitates RNApol II binding. (A) Flow cytometric analysis of PU.1 infected immature T-cells at the time of harvesting for ChIP analysis. Cells were stained with anti hCD8-PE antibody to mark infected cells coexpressing hCD8 from the pMX-PU.1-IRES-hCD8 vector. (B) PU.1 RNA expression analysis demonstrates high PU.1 levels in infected cells. RNA levels were analyzed by QPCR analysis. PU.1 levels were normalized against GAPDH. Data is plotted on a log (10) scale. (C) PU.1 ChIP experiments are shown as described previously. Data shown are from five independent infections/ChIP experiments. (D). RNApol II ChIP assays with PU.1 infected immature T-cells (Green bars) were performed and reported as described earlier. Black bars represent noninfected ChIP data from Figure 2C. RNApol II data are from two independent experiments. (E) Forced PU.1 expression is not sufficient to reestablish endogenous PU.1 mRNA transcription in immature T-cells (Adh.2C2). A primer pair that cannot recognize virally introduced transcripts was used as described earlier.

Figure 6. Non PU.1 Ets family factors are restricted in their ability to bind PU.1 target sites. (A-C) Elf-1 ChIP assays were performed as described in Fig. 2. Data shown are

from three independent experiments for each cell line. (D-F) Ets-1 ChIP assays, as described previously. Data shown are from three independent experiments for each cell line. (C) GABPα ChIP assays, as described previously. Data shown are from three or more independent experiments. Graphs with green bars represent data from immature T-cells (also labeled Adh.2C2 on right axis). Graphs with blue bars are from pre-B-cells (NFS-25). Graphs with red bars are from myeloid cells (RAW264.7).

Figure 7. Ikaros binds the URE region in all cell lines but only binds the CE5 enhancer in myeloid cells. (A-C) Ikaros ChIP assays shown as previously described. Adh.2C2 (A) Data are from four independent experiments. NFS-25 (B) and Raw264.7 (C) Data are from three independent experiments. (D) Western blot of Ikaros and Sp1 protein. 2-8 micrograms of nuclear extract from Adh.2C2 and Raw264.7 cells were separated on an 8% SDS-PAGE gel. This Blot was had been previously probed for Sp1 (Zarnegar et al., submitted). That blot was stripped and reprobed here with anti-Ikaros antibody (sc-13039). (E) PU.1 facilitates Ikaros binding to the distal URE element CE9. PU.1 infected Adh.2C2 cells were used for ChIP assays as described earlier. Data shown are from two independent experiments.

Figure 8. The CE5 and CE7/6 regions nucleate cell-type-specific myeloid complexes in vitro and contain PU.1 and Ikaros. (A) Probes CE5-P2 and CE76-P4 (see Fig. 3) were radiolabeled and incubated with nuclear extracts from Adh.2C2 cells (T), RAW264.7 cells (M), or NFS-25 cells (B). Complexes were resolved, dried, and then exposed to film. Myeloid specific complexes marked by black arrows and labeled M1 and M2. (B)

PU.1 and Ikaros are shown to be part of the myeloid specific complexes. Probe CE76-P4 was used as described above. Competitors (see Fig. 2) were used at 250 fold molar excess. Note that all probes and competitors used were mouse sequences. 4 mg of antibodies were used as labeled.

Figure 9. PU.1 and Ikaros perturbations demonstrate that both factors contribute to *Sfpi1* regulatory activity in myeloid cells. (A) Anti-PU.1 morpholinos (E1 and E2) knockdown PU.1 protein expression. Morpholinos (2 picomoles) were transfected into 32Dcl5 myeloid cells by nucleofection. Cell samples were harvested at 24 and 48 hours then analyzed by Western. (B) PU.1 knockdown abolishes L75 reporter activity. RAW264.7 cells were transfected with *Sfpi1* reporters and 2 picomoles of standard control morpholino (Con.) or antisense morpholino targeting PU.1 as indicated. Data shown are from three independent experiments performed in duplicate and reported as fold difference relative to L1 + Con. (C) Ikaros contributes to myeloid enhancer activity. Raw264.7 cells were cotransfected with *Sfpi1* reporters and empty pEF or pEF-Plastic (dominant negative Ikaros). (D) Ikaros does not contribute positively to URE enhancer activity in NFS-25 pre-B-cells. Data shown are from three independent experiments performed in duplicate. Error bars show standard deviations. Data are shown as fold difference relative to L1+pEF.

Figure S1. ChIP-seq data (Broad/MGH ENCODE track) showing H3K4me1 and H3K4me2 demonstrates chromatic accessibility of CE7-CE5 in immature B-cell like bone marrow derived lymphoblast cells (GM12878).

Chapter 4:

Conclusions

Introduction

The previous chapters described the discovery of novel regulatory regions that can form cell-type-specific transcription factor assemblages able to provide mechanistic explanations for how *Sfpi1* can be dynamically regulated in different hematopoietic lineages. In chapter 2, a dedicated Runx1 dependent silencer was found that could provide cell-type-specific PU.1 silencing in early T-lineage cells. In chapter 3, a novel myeloid enhancer was characterized and shown to provide PU.1 dependent and context specific autoregulation, which extends our understanding of how PU.1 positive feedback may be critical and lineage restricted. We also revealed Ikaros to be a new direct *Sfpi1* regulatory input that appears to have lineage specific functions.

The next sections will discuss some implications of this work with respect to gene regulatory networks and will attempt to integrate findings into newer network models. Runx1 and PU.1 will be discussed first in the context of the HSC GRN. A discussion of Ikaros and what role it may play in *Sfpi1* regulation and PU.1 related regulatory circuitry will follow. Lastly, a discussion of how the newly discovered regulatory elements imply the existence of yet other context determining functional contributors will be provided.

Identification of Runx1 dependent *Sfpi1* silencing and PU.1 dependent autoregulation enrich our understanding of the recursive HSC network circuitry

Runx1 is a pivotal regulator of blood development. As discussed in chapter 1, Runx1 deficiency results in a lack of definitive hematopoiesis. That deficiency is due at least in part to lack of PU.1 expression. While Runx1 is required for *Sfpi1* transcriptional initiation, chapter 2 reported how Runx1 is also crucial for silencing PU.1 expression in the T-lineage. The dual role Runx1 plays is not novel, nor is it the only member of the HSC gene regulatory network that is linked to activation and repression of key HSC nodes. Runx1's pleiotropic target PU.1 can function to activate or repress another core HSC GRN node, SCL. PU.1 can regulate SCL through binding to its promoter and/or silencer (Bockamp et al. 1998; Le Clech et al. 2006). Not to be outdone, Runx1 has the potential to feedback into SCL too, as the AML1-ETO translocation product can turn off SCL (Yeh et al. 2008). Thus, the relative expression of Runx1 and PU.1 may also control SCL dosage and contribute to overall network stability.

PU.1's pleiotropic functions may also be relevant to its own balanced expression in HSCs. For example, although we showed Ikaros contributes to activation of PU.1in a mature myeloid lineage, Ikaros appeared nonfunctional in B-lineage cells even though bound to one of the same enhancer elements as in myeloid cells. This indicated that Ikaros' function varies with cell-type differences and not just DNA context. A similar situation with PU.1was discussed in chapter 1 with respect to the regulation of CD68. In myeloid cells, IRF-4 could alter the directionality of PU.1 regulatory effects through binding to CD68. Furthermore, the Ikaros family member Eos can repress some myeloid

target genes of PU.1 through direct interaction with PU.1. However, it may do so only when PU.1 is also collaborating with MITF for transcriptional control (Hu et al. 2007). These studies are critical in the context of the HSC network because they suggest that PU.1 autoregulation may not necessarily be positive. Indeed, PU.1 may potentially be required for negative autoregulatory modulation of *Sfpi1* transcriptional output, possibly in conjunction with Ikaros.

Other components of the hemangioblast network subcircuit discussed earlier, Fli-1 and GATA2, are also potential contributors to *Sfpi1* transcription in multiple contexts, possibly in HSCs too (Hoogenkamp et al. 2007; Chou et al. 2009). Intriguingly, PU.1 has been shown able to occupy the Runx1 +23 enhancer in a myeloid progenitor cell line, and PU.1can also regulate Fli-1 (Nottingham et al. 2007; Stark et al. 1999). Moreover, PU.1 can cooperate or antagonize GATA2 transcriptional activity and affect its expression, (Walsh et al. 2002). Additionally, Ikaros can bind to the GATA2 promoter, possibly in cooperation with GATA1 (Bottardi et al. 2009). These studies raise the possibility that Ikaros may also indirectly affect PU.1 expression through context dependent modulation of GATA2. Intriguingly, in chapter 3 it was shown that Ikaros can bind to the -18 kb CE10 element in pre-B-cells. This region also binds GATA1 in megakaryocyte erythroid progenitors (MEPs) and may be involved in erythroid silencing of PU.1 expression. It is conceivable that Ikaros may be bound to CE10 with GATA1 and from that element both factors may contribute to context dependent *Sfpi1* regulation in MEPs after HSCs begin to differentiate due to GATA1 activation.

The HSC network gets even more complicated when one considers the noncoding micro RNA, miR-27a. Mir-27a blocks translation of Runx1, but since Runx1 itself

regulates miR-27a, at least in MEPs, a quasistable Runx1 protein level can be maintained with other factors contributing to a rheostat function (Oren-Ben Ami., 2008). As the miR-27a regulatory region contains predicted Ets and GATA sites, there appears to be layer after layer of regulatory options in HSCs and immediately downstream progenitors. Taken together, these potential interactions necessitate a reimagining of the network circuitry underlying the emergence and maintenance of HSCs, with Fli-1 at the top of the hierarchy and PU.1 at the bottom (figure 1). Moreover, the dual functionality of all these factors, with the ability to activate or repress targets in highly context dependent ways, provides a complicated framework for an extraordinarily recursive network that provides ample avenues for feedback to maintain, remap, or outright break the network to direct or restrict lineage specification and commitment.

An HSC subcircuit containing PU.1 and Ikaros may control lineage fate choices

In the preceding section, Ikaros was discussed in relation to PU.1 in a recursive gene regulatory network that may act to maintain HSCs in an undifferentiated state. However, Ikaros was not included in the network diagram (figure 1). Instead, Ikaros will be presented in a separate subcircuit that may be important for facilitating escape from the HSC stable state to initiate lineage fate choice decisions. This undertaking is being performed as Ikaros and PU.1 have recently been proposed to be part of a recurring regulatory network controlling myeloid versus B lineage choice (Spooner et al. 2009). In Spooner's proposed gene network, two factors are thought to suppress PU.1 expression, Ikaros and Gfi-1. In light of discovering Ikaros bound to *Sfpi1* regulatory modules in

both myeloid and B-cells, discussed in chapter 3, an updated network consisting of PU.1 and Ikaros will be proposed. Before doing so, a short discussion of the evidence for Ikaros and Gfi-1 regulation of *Sfpi1* transcriptional output will be provided.

Does Ikaros regulate *Sfpi1* transcriptional output in HSCs and early progenitors?

Whereas chapter 3 discussed Ikaros as a novel positively acting direct regulator of *Sfpi1* in the context of myeloid specific PU.1 autoregulation, other recent work has also offered evidence proposing Ikaros is a modulator of PU.1 expression (Spooner et al. 2009). However, that work claims Ikaros is a suppressor of *Sfpi1* transcription. The work claims that Ikaros may suppress PU.1 expression at the time when MPP specification is being decided between myeloid and lymphoid lineage choice, specifically the myeloid versus B lineage decision. This hypothesis was based on a comparison between wild type MPPs and Ikaros$^{-/-}$ MPPs. In the comparison provided, Ikaros$^{-/-}$ MPPs were shown to have elevated FcγRII/III and *Csf1R* surface markers. This comparison is problematic as the wild type MPP data shown does not include high FcγRII/III or *Csf1R* expressing cells even though such cells are a known subset of total MPPs. Why those cells are not present in the normal MPP population being analyzed was not accounted for. Additionally, it has been reported that the population of high expressing FcγRII/III cells in total MPPs are reduced in Ikaros deficient animals (Yoshida et al. 2006). Accordingly, the comparison presented in Spooner et al. (2009) is not only inappropriate and invalid, but also inconsistent with other reports. Consequently, the aforementioned link between

Ikaros and *Sfpi1* regulation was improperly postulated. Nonetheless, the ChIP assays discussed in chapter 3 do affirm that Ikaros can bind to numerous *Sfpi1* regulatory modules and the role Ikaros might play when associated with those distinct cis-elements at different developmental stages remains to be explored.

While Ikaros may negatively affect PU.1 expression in some contexts, like PU.1, Ikaros expression is dynamic during lineage specification of HSCs to LMPPs and GMPs. The mechanisms controlling Ikaros expression are not understood, but an Ikaros GFP reporter mouse has provided great insight into Ikaros expression patterns within early progenitor populations. Ikaros expression correlated with PU.1 expression, with GFP^+ progenitors showing higher levels of PU.1 transcripts (Yoshida et al, 2006). Additionally, the level of GFP also more finely correlated with the level of PU.1, with intermediate levels in cells thought to correspond to CMPs and and higher GFP in GMPs (Yoshida et al, 2006). PU.1 is required for the formation of both CMPs and GMPs, and PU.1 expression is upregulated in GMPs as previously described (Nutt et al, 2005). Taken together, these studies support a hypothesis that Ikaros might positively contribute to PU.1 expression in myeloid cells, a hypothesis validated in results reported in chapter 3.

Does Gfi-1 constrain PU.1 autoregulation and expression in an early acting HSC subcircuit?

Another reported suppressor of *Sfpi1* transcriptional control that may be part of a recurring Ikaros and PU.1 network is Gfi-1. One of the mechanisms proposed for Gfi-1

antagonism of *Sfpi1* transcription is inhibition of PU.1 autoregulation through the *Sfpi1* URE (Spooner et al. 2009). With the discovery of the CE7-CE5 enhancer modules able to provide restricted PU.1 autoregulation in addition to the URE, the role of Gfi-1 in relation to PU.1 function and expression merits reexamination.

Two mechanisms for negative regulation of PU.1 by Gfi-1 are offered in the literature. Already mentioned, the first mechanism proposed is that Gfi-1 inhibits PU.1 autoregulation by competing with PU.1 for binding to the *Sfpi1* URE. However, the expression patterns of Gfi-1 and PU.1 make such a mechanism problematic. Both Gfi-1 and PU.1 are expressed together in HSCs and both are at their highest levels in GMPs (Zeng et al., 2004; Nutt et al., 2005). Futhermore, Gfi-1 must also outcompete othet Ets factors that can also bind to the URE. As discussed in chapter 1, C/EBPα is critical for specification of GMPs and C/EBPα is thought to regulate both PU.1 and Gfi-1 (Yeamanns et al. 2007; Dahl et al. 2003). Additionally, it should be noted here that Gfi-1 indirectly regulates STAT3 activity, sensitizing cells to STAT3 activation (Yeamanns et al. 2007; Dahl et al. 2003). As also discussed earlier, not only can C/EBPα and STAT3 activate PU.1 expression, they also can potentially cross regulate. Gfi-1 may actually facilitate that cross regulation which would be predicted to increase PU.1 expression. Even if Gfi-1 does repress PU.1 expression in early progenitors, potential suppression in myeloid cells must be blocked or compensated for in order to allow *Sfpi1* transcriptional output to rise as GMPs are specified.

The original argument for Gfi-1 opposition to PU.1 in a neutrophil versus monocyte/macrophage subcircuit reportedly originated with the observation of increased PU.1 expression in RNA from bone marrow cells of Gfi-$1^{-/-}$ mice that also have an

increased number of GMPs (Hock et al., 2003; Laslo et al., 2006). However, C/EBPα expression is also increased in those mice (Hock et al. 2003). Consequently, it is unclear if elevated PU.1 expression is due to loss of negative regulatory constraint or a gain in GMPs due to increased C/EBPα that may also provide too much activation of *Sfpi1*, even where STAT3 activation might be impaired due to loss of Gfi-1.

Another mechanism postulated for Gfi-1 inhibition of PU.1 may be similar to the way GATA1 can block PU.1 protein function. A recent study showed that Gfi-1 can physically interact with PU.1 and suggested that this physical interaction might suppress PU.1 transcriptional activity (Dahl et al., 2007). Part of the evidence for this claim is based on loss of CD64/FcγRI expression as GMP like progenitors become neutrophils. However, CD64/FcγRI is a dual PU.1 and STAT1 dependent gene (Aittomaki et al., 2002). As neutrophils continue to express PU.1, loss of CD64 expression could be due to more restricted cytokine/STAT1 signaling as cells differentiate. Consistent with this, CD64 expression is lost when cells differentiate into neutrophils and is only rexpressed with interferon gamma activation of STAT1 (Bovolenta et al., 1998). That report clearly indicates that PU.1 remains competent to regulate CD64 in normal Gfi-1 and PU.1 coexpressing neutrophils. Consequently loss of CD64 expression cannot be due to a block of PU.1 function by Gfi-1 as originally interpreted.

While it is unlikely that Gfi-1 directly constrains PU.1 expression and transcriptional activity in mature myeloid cells, the potential for binding to the URE in competition with PU.1 in early progenitors does remain an interesting possibility. In fact, once PU.1 expression has risen, PU.1 secondary targets repress Gfi-1 expression which would prevent any competitive inhibition of PU.1 autoregulation through the URE in

more mature cells (Laslo et al. 2006). It remains an intriguing possibility that Gfi-1 dependent suppression may provide a developmental stage dependent limit on URE enhancer function in HSCs with the ability to constrain *Sfpi1* transcription also dependent on relatively lower PU.1 expression in HSCs versus GMPs. If so, then how could *Sfpi1* expression ever be augmented as HSCs undergo differentiation into GMPs? This question is more vexing when one considers that Ikaros is also argued to suppress PU.1 expression. Moreover, Ikaros, Gfi-1, and PU.1 are all coexpressed in HSCs before *Sfpi1* transcriptional output is dramatically altered in committed lymphoid and myeloid cells.

Discovery of the myeloid specific CE5 enhancer offers a solution to the above dilemma. In fact, CE5 also has two conserved C/EBPα binding sites flanking a site able to bind PU.1 in vitro. It is possible that CE5 is also C/EBPα responsive are plays a role in C/EBPα mediated PU.1 expression and GMP specification as discussed in chapter 1. Importantly, and in contrast to the URE, the PU.1 target site in CE5 does not appear to be sensitive to potential competition by Gfi-1. Thus, even if the URE's regulation of *Sfpi1* transcriptional output is suppressed, C/EBPα and/or context dependent PU.1 input into CE5, and possible CE7/6, may more than compensate for Gfi-1 constraint to drive higher PU.1 expression during GMP specification.

Mef2c is another recursively wired node in an early HSC subcircuit controlling lineage choice decisions

In chapter 3, PU.1 and Ikaros ChIP assays showed these factors could bind to *Mef2c* in both myeloid and B-cells. Mef2c is expressed in HSCs as well (Schuler et al.

2008). Moreover, loss of Mef2c has been linked to defective lymphoid specification (Stehling-Sun et al. 2009). A possible contributing mechanism to defective lymphoid development may be lowered Ikaros expression reported in *Mef2c* deficient cells (Stehling-Sun et al. 2009). In light of the finding that Ikaros directly binds *Mef2c*, it appears that Mef2c may be a middle man providing indirect PU.1 regulation of Ikaros, with Ikaros feedback wired back into *Mef2c* and *Sfpi1*. In addition, Ikaros expression was also linked to Gfi-1 expression (Spooner et al. 2009). Taken together, we have a basis for postulating a four node regulatory subcircuit (figure 2A).

Building an HSC subcircuit that may control both PU.1 dosage and lineage specification

In the previous sections, regulatory associations were discussed that provide a potential framework for the construction of a new gene regulatory network subcircuit. At the core of this subcircuit are four nodes, *Sfpi1*/PU.1, Mef2c, Ikaros, and Gfi-1 (figure 2A). Note that while Ikaros may activate *Sfpi1* in myeloid cells, Ikaros may be neutral or even a suppressor when bound to the *Sfpi1* URE. Additionally, while Gfi-1 may inhibit PU.1 expression, it can potentially augment STAT3 transcription of C/EBPα and/or *Sfpi1*. As the C/EBPα node can also regulate Gfi-1, *Sfpi1*, and STAT3, we can add it to the growing subcircuit along with STAT3. We now have a six node circuit of recursively wired factors all expressed in HSCs. Note that these nodes can be considered a part of the larger HSC network presented earlier (figure 1). Together, and in the absence of strong differentiating signals, this network subcircuit construction may provide

potentially competing and multidirectional control of moderate PU.1 expression levels (figure 2B).

In chapter 1 of this thesis, several cytokine receptors were discussed that can activate STAT3. Some of those cytokine signaling pathways, like Flt3, also activate C/EBPα, perhaps dependent on STAT3 activation. If sufficient STAT3 and C/EBPα activation is achieved, GMP specification might occur and PU.1 expression should rise. This specification may involve activation of myeloid determinants such as *IL6Rα* mediated Id expression which will block the E2A family of B-cell promoting factors (discussed in chapter 1). Other predicted consequences of network dynamics include sufficiently high PU.1 expression to block residual Gfi-1 constraint. Additionally, C/EBPα in combination with high PU.1 expression may be needed to activate additional myeloid determinants that allow the combinatorial all-or-none lineage-restricted CE7-CE5 PU.1 autoregulation described in chapter 3. Once established, that PU.1 autoregulation could provide a regulatory loop that no longer requires C/EBPα or other STAT3 activation signals, thus locking in a myeloid fate and promotion of continued myeloid differentiation (figure 2C).

In the absence of myeloid promoting conditions, or within a B-cell promoting environment, signaling pathways may allow for activation of E2A in HSCs/MPPs to prime a lymphoid fate (figure 2D)(Dias et al. 2008). Together with low PU.1 expression, PU.1 and E2A can activate EBF (Medina et al. 2004). Once sufficiently expressed, EBF inhibits C/EBPα expression and blocks activation of higher PU.1 expression, thus promoting a B-cell program while blocking myeloid developmental programming (Pongubala et al. 2008). While Gfi-1 expression may initially require C/EBPα

expression, secondary B-cell determinants may alter Ikaros regulation of several targets, including Gfi-1. For example, in chapter 3 we found that Ikaros was bound to *IL7Rα* in pre-B-cells, but not myeloid cells. Thus, a B-cell determinant, possibly EBF, E2A, or their target(s), could then facilitate that binding. Similarly, a B-cell determinant might allow continued Gfi-1 expression to continue to constrain PU.1 expression until B-cell commitment is achieved. Alternatively, once C/EBPα expression and potential PU.1 activation is neutralized, Gfi-1 constraint and expression may no longer be required. It is also possible that continued restraint of high PU.1 dosage is instead mediated by B-cell context dependent Ikaros input into *Sfpi1* regulatory modules, which may eventually fix PU.1 expression to the familiar lower B-cell level.

Identification of what myeloid and B-cell determinants control where PU.1 and Ikaros can bind is obviously of great interest. It is unknown how many additional collaborators might dictate when and where these factors bind nor is it known to what extent cell-type-specific post translation modification of PU.1 protein might alter choice of collaborators when myeloid and B-cell factors have overlapping expression in these lineages. Examining PU.1 and Ikaros complexes more closely should help clarify how these factors are regulated and will allow further refinement of this network while providing far greater understanding of how context dependent use and regulation of PU.1 is fully achieved.

Unresolved questionns regarding Runx1 silencing of *Sfpi1* in T-cells

Many lingering questions remain to be addressed and more experiments are needed to resolve how T-cells terminate PU.1 expression. One question remaining to be asked is to what extent does Runx or CE4 maintain active *Sfpi1* silencing? The *Sfpi1* locus does not appear to have chromatin marked by silencing related histone modifications such as H3K27me3. This suggests that continued *Sfpi1* silencing may be ongoing throughout the later T-cell developmental stages. Interestingly, PU.1 expression has been reported in Th2 cells and may regulate GATA3 function therein (Chang et al. 2005). Also intriguing, Th2 cell expansion is enhanced by Gfi-1 (Zhu et al. 2006). This raises the possibility that Gfi-1 is functioning to sensitize cells to STAT3 signaling which may allow low level PU.1 expression in some cells where Runx expression is not adequate to maintain active repression of *Sfpi1* transcription. While STAT3 deficiency does not affect early T-cell development, STAT3 does affect mature T-cell signaling through IL-6, IL-21, and IL-2. As Runx1 is repressed in Th2 cells, CE4B or other STAT binding modules may be in a state of chromatin accessibility and available for STAT3 activation of PU.1 expression.

A more immediate question is why is Runx1 repressive in T-cells when bound to CE4? Runx1 is expressed throughout the hematopoietic compartment and it is unlikely to be sufficient for silencing. Idetification of what factor may confer a silencing function to Runx binding is still needed. Interestingly, Ikaros was also found associated with CE4A in immature T-cells. Moreover, pre-B-cells and immature T-cells formed Runx and Ikaros related complexes on CE5 in vitro. Although ChIP assays failed to detect in vivo binding of CE5 by these factors in lymphoid cells, it remains possible that they do

bind in vivo and block occupancy by other factors that might inappropriately provide enhancer function. While the Ikaros dominant negative Plastic did not affect silencing in our assays, it is possible Ikaros is present as a cofactor and part of a repressive complex independent of DNA binding, similar to how the Ikaros family member Eos could turn a PU.1 containing activation complex into a repressive complex.

Recruitment of Runx1 to regulatory elements may depend on combinatory interactions with Ets factors in very specific contexts. While we could not detect Ets factor interactions with CE4 in vivo in immature T-cells, it may be possible that many of the antibodies are simply not viable for use in ChIP assays and are thus false negative results. It also appeared that the Ets site in CE4 might not be required per se, although an overlapping site does seem to stabilize Runx binding. As Runx and Ets interactions may cooperatively alter DNA interactions, it may be that an Ets factor really is involved but its contact points could be altered across the CE4 module.

In megakaryocytes, the Ets factor Fli-1, which can also bind to the *Sfpi1* URE in myeloid cells interacts with and cooperates with Runx1 to activate target genes (Huang et al., 2009). Significantly, Fli-1 could only interact with Runx1 when dephosphorylated upon activation of megakaryocyte differentiation. This indicates posttranslational control can be critical to development, independent of obvious shifts in transcription factor expression profiles. In this way, PU.1/Ets interactions with Runx1 might occupy the URE or CE4 in highly controlled contexts. It is also possible that Runx binding to CE4 is facilitated by loss of a competing Ets factor in conjunction with an increase in Runx protein level through transcriptional or posttranslational mechanisms. Other mechanisms may affect Runx DNA binding as well. For example, myeloid differentiation is blocked

by the PU.1 target CDK6 that can interfere with Runx1 DNA binding, perhaps able to inhibit only non cooperative concentration dependent Runx1 binding (Fujimoto et al., 2007). Whereas active recruitment of Runx1 through combinatory mechanisms may be possible, T-cells could use interactions across multiple lower affinity Runx sites within CE4 to give sensitivity to changes in Runx protein levels that subsequently generate stable repressive complexes, but only when Runx is at a sufficient level of availability and not inhibited by Runx1 interfering factors like CDK6 or out competed by an Ets factor across the Runx stabilizing region of CE4, the M5 region discussed in chapter 2..

Cis-regulatory elements define context and function

Throughout this thesis, three pervasive hematopoietic players have been discussed Runx1, PU.1, and Ikaros. Remarkably, all three factors are pleitropic transcription factors that can activate and repress transcription in various contexts. Moreover, these factors are known to perform both activating and repressive functions within the same cells at the same time. This remarkable achievement is not ultimately dictated by the factors intrinsic properties. Instead, their diverse and evolved context specific functions are the result of dedicated context dependent cis-elements like CE4 and CE5, which can act together with bifunctional and nonspecific cis elements like the *Sfpi1* URE. The contributions described herein provide enhanced insight not only for our understanding of *Sfpi1* transcriptional control, but also presents a complicated and archetypical example of how multiple cis-regulatory elements work together to dictate transcriptional output and the specific roles of bifunctional transcription factors that are used repeatedly and recursively to drive development gene regulatory networks.

PU.1 and Runx1 integrated into highly recursive core HSC GRN

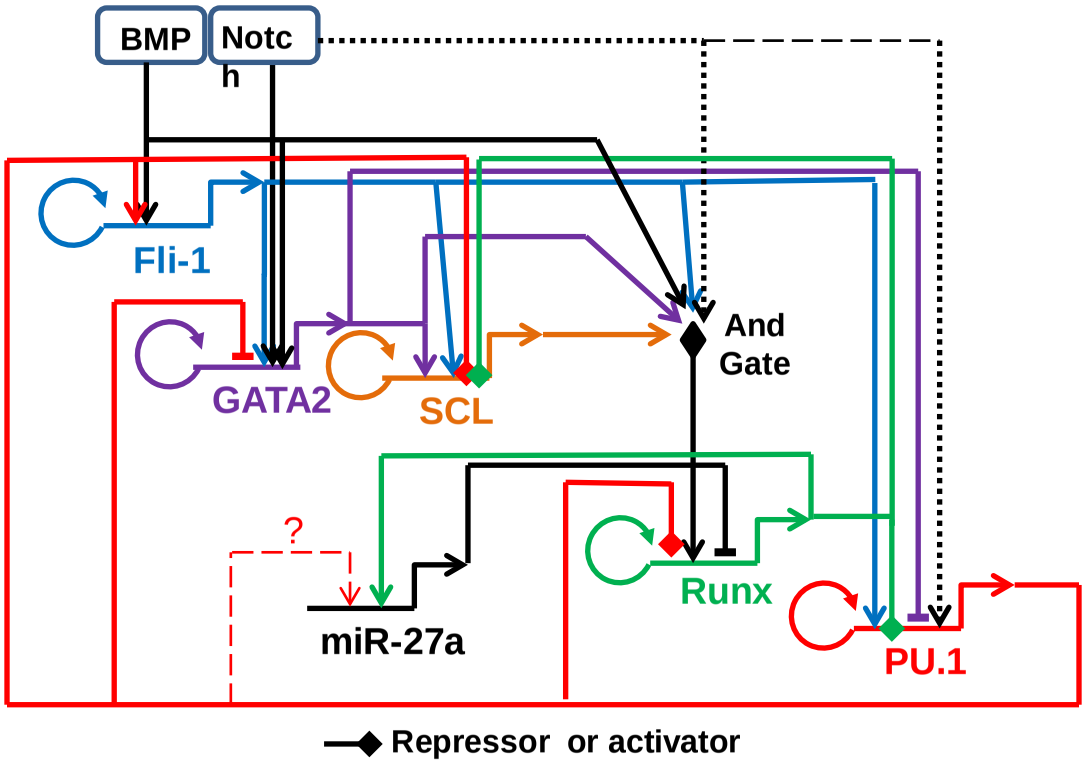

Figure 1. HSC core network model. Data from: Pimanda et al., 2007; Nottingham et al., 2007; Burns et al., 2005; Robert-Moreno et al., 2005; de Pooter et al., 2006; Gering et al., 1998; Kobayashi-Osaki et al., 2005; Oren-Ben Ami et al., 2008; Walsh et al., 2002; Stark et al., 1999; Bockamp et al., 1998; Le Clech et al., 2006; Yeh et al., 2008

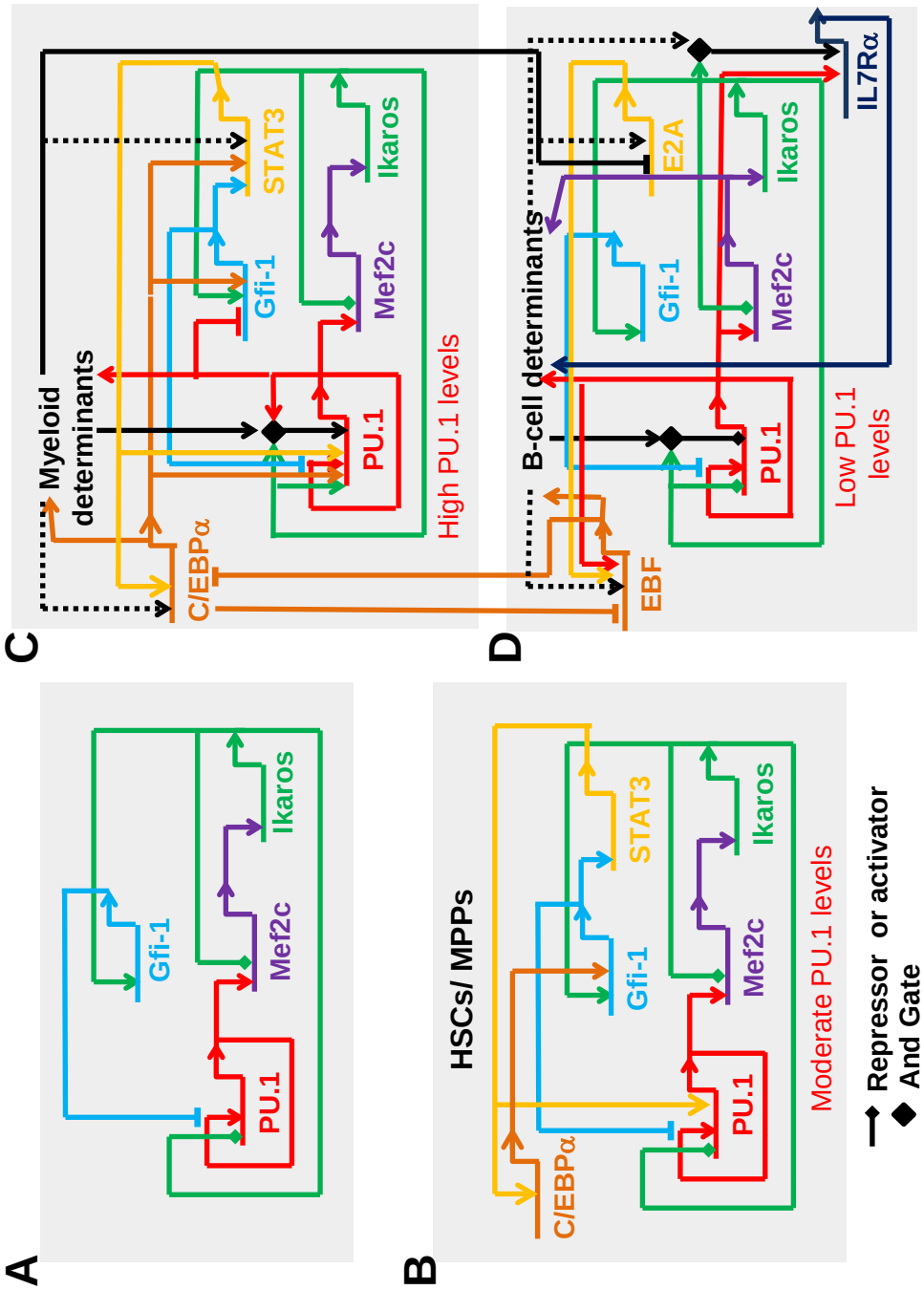

Figure 2. PU.1 HSC subcircuit underlying PU.1 dosage and myeloid versus B lineage choice .

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