How Hematopoiesis Research became a Fertile Ground for Regulatory Network Biology as Pioneered by Eric Davidson

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Key points:

- Gene regulatory networks explain programs of developmental change
- Nodes in gene regulatory networks respond to combinatorial inputs from transcription factors via cis-regulatory elements
- Causal gene regulatory network models must be based on systematic perturbation tests and molecular evidence
- Lessons about developmental system behavior based on embryology lead to powerful insights about hematopoiesis

Abstract

Purpose of review: this historical perspective reviews how work of Eric H. Davidson was a catalyst and exemplar for explaining hematopoietic cell fate determination through gene regulation.

Recent findings: Researchers studying blood and immune cells pioneered many of the early mechanistic investigations of mammalian gene regulatory processes. These efforts included the characterisation of complex gene regulatory sequences exemplified by the globin and T/B cell receptor gene loci, as well as the identification of many key regulatory transcription factors through the fine mapping of chromosome translocation breakpoints in leukaemia patients. As the repertoire of known regulators expanded, assembly into gene regulatory network models became increasingly important, not only to account for the truism that regulatory genes do not function in isolation, but also to devise new ways of extracting biologically meaningful insights from ever more complex information. Here we explore how Eric H. Davidson's pioneering studies of gene regulatory network control in non-vertebrate model organisms have had an important and lasting impact on research into blood and immune cell development.

Summary: The intellectual framework developed by Davidson continues to contribute to hematopoietic research, and his insistence on demonstrating logic and causality still challenges the frontier of research today.

Keywords: Gene regulatory network; Causal modeling; Transcription factor; Cis-regulatory element

Introduction

Cellular decision making underpins early development and adult haematopoiesis

The haematopoietic system has long served as a paradigm of how a hierarchically organised differentiation system might mediate the long-term maintenance of adult tissues that are characterized by a high turn-over. Haematopoietic stem cells (HSCs) at the top of this hierarchy serve as an emergency reservoir as well as making a small but steady contribution to the more rapidly proliferating downstream progenitor populations. At the molecular level, this hierarchy is underpinned by cellular decision making processes, which are balanced to ensure that multipotent progenitors give rise to the appropriate numbers of downstream mature cells (for review see [1]).

Cellular decision making also lies at the heart of early development, where, following fertilization of the egg, cellular diversity is rapidly generated through a process of rapid cell division accompanied by the establishment of distinct gene expression programs. Such alterations in gene expression critically depend on the deployment of lineage-specific transcription factors (TFs), or more typically small combinations of such TFs. Of note, TFs of, for example, the Gata, Ets and bHLH families play important roles in early development as well as adult haematopoiesis [2,3]. It is important however to recognize that there are important differences between early embryonic development and adult tissue maintenance. For example, multipotent cells in the embryo such as epiblast or neuromesodermal progenitors only exist for a specified time window, after which they will have turned over

into downstream progeny. In contrast, adult stem cells self-renew as well as generate differentiated derivatives.

The building blocks for regulatory network models

A network model may be defined simply as a model of a set of data, whereby the network model provides a flexible way of representing the individual objects as well as their relationships. The objects in such models are commonly referred to as nodes, and their relationships as edges. Network models are commonly used to interpret and simulate highly complex systems. The question therefore arises what specific entities could form the nodes for network models that would prove useful to advance our understanding of biology. Together with Roy Britten, Eric H. Davidson published a visionary paper in 1969, presenting a theoretical framework for how distinct sequences in the genome may communicate with each other through sequence-specific trans-acting regulators, then envisioned as mobile RNA molecules [4]. The interactions (or edges in network terminology) between the DNA sequences and mobile regulators would thus define the inner workings of a network capable of decoding the regulatory blueprint present within the genome, and thus ensure cell type and condition-specific gene expression programs. As emphasized by Davidson over the following decades, this kind of gene regulatory network model did not simply aim to represent correlations, but rather was intended to show causal mechanisms that either drive system state change or maintain system stability [5,6].

Following on from the 1969 theory paper, research across many laboratories identified DNA-binding proteins as key mediators of converting DNA-encoded regulatory information into cell type-specific gene expression programs. Many of these so-called transcription factor (TF) proteins were first identified by haematopoiesis researchers, either by mapping the breakpoints of recurrent chromosome translocations in leukemia patients, or through biochemical fractionation of proteins binding to regulatory sequences of the haemoglobin or immunoglobulin genes (e.g. [7-9]).

While the identification of each of these individual TFs undoubtedly represented a major advance, another step-change was needed to integrate DNA regulatory sequences and TF proteins into regulatory network models. At a practical level, this required the vision to embrace long-term experiments to systematically analyse all the individual components of the machinery that drive expression of individual genes. Here again, Eric Davidson and co-workers led the way, using sea urchin development as an experimentally tractable model system, with the overall goal of decoding the hardwiring of metazoan development through comprehensive characterisation of the organization and function of genomic regulatory systems [10-12]. Below we outline some of the broadly relevant messages that came from these pioneering studies.

Insights from regulatory network models of sea urchin development: the sea urchin system

Davidson developed gene regulatory network models aiming for full explanation of the highly ordered process, reproducible in space and time, through which a complex embryo develops from a single fertilized egg [11,13,14]. The sea urchin embryo was a particularly clear system in which to attempt this, because future tissue "territories" of the free-living larva become distinct within the first 6 cell divisions, prior to any cell migration. The embryo partitions into ≥10 different territories before gastrulation [15,16], each distinguished by different combinations of newly-expressed TFs. Thus, in this system, the entire diversification of embryonic regions can be transformed into the question of how regulatory genes get activated in the correct combinations, in the correct places, times, and orders.

The gene regulatory network models developed by Davidson and colleagues explained how genomically encoded regulatory systems could: create multiple cell types from one cell; produce complex tissues in an invariant geometry from a single cell; and convert transient signals into more lasting stable states with predictable timing, entirely through the ability of TFs to regulate each other's expression. The foundation for this analysis was the nature of cis-regulatory elements that control gene expression. Network explanations were made possible by the fact that key genes in most systems are regulated simultaneously by multiple "upstream" factors, often with distinct positive or negative roles (early examples: [17,18]). Rule sets for expression of a given gene in some domain are physically embodied in genomic cis-regulatory sequences that comprise enhancers, i.e., clustered, specific binding sites for the factors that need to work coordinately, while the same gene may be expressed under different conditions by using a different cis-regulatory element. The sea urchin system is an exceptionally tractable one for molecular embryology, and this enabled the Davidson team

to isolate cis-regulatory elements linked to specific network genes, demonstrating that they drive reporter gene expression in specific spatial and temporal patterns in the whole embryo, and proving that their activities depend on direct DNA binding from multiple TFs [17,19,20].

Network logic embodied in molecular biology

Combinatoriality: Because cis-regulation responds to multiple inputs, any given gene in the network can respond to logical combinations (AND, OR, ANDNOT) of existing TFs [21,22]. This logic is crucial for explaining how complexity increases as cells divide in an embryo. For example, it means that a gene may require both an inherited factor, which defines its "lineage" criterion for expression, and an independent signal-dependent factor that is activated only if the cell is adjacent to a particular neighbour. As embryonic cells divide in a stereotyped pattern, this causes predictable divergence of the gene expression and fate of one daughter cell from the other.

Distinct rules mediated by distinct cis-regulatory elements: The same gene can be regulated by different cis-regulatory elements, not only to enable expression in different cell types under different control, but also enabling the gene to be activated initially by one set of conditions and then maintained in the same cell lineage by different conditions. Specific early examples came from the sea urchin system [23,24], as well as from *Drosophila* blastoderm and wing discs [25,26]. For example, a cis-element switch enabled a gene first activated by a transient signal to be maintained afterwards, independent of the initial signal,

by direct or indirect positive autoregulatory feedback. Therefore, not only mapping of factor binding sites in one “minimal” regulatory element, but rather defining the whole system of regulatory elements for a gene, is needed to account for that gene’s developmental domain(s) of expression.

Robustness, not parsimony: Certain sets of regulatory genes are co-expressed in a sustained way in the sea urchin embryo to stabilize a developmental state [27,28]. Factors reinforce each other’s expression by direct positive cross-regulation, using redundant “additive OR” logic. This cross-regulation was shown to have a deep evolutionary history [27,28]. Although certain individual connections were direct in one species but indirect in another, the gene set as a whole remained positively cross-regulating across long evolutionary times [29]. These gene network circuits decisively refute the idea that evolution selects mainly for parsimony. Instead, these recursive, redundant system architectures for locking down developmental states are selected for highly reproducible performance and avoidance of failure [30].

Repression: A dominant feature of the embryonic gene network model in the sea urchin was the prominence of transcriptional repression, which was recognized early as the central mechanism for setting boundaries between programs active in different territories [24]. Davidson and colleagues identified multiple examples of sequence-specific, regulated repression, sometimes under switch-like modulation by signalling pathways [11,15,16]. To date, evidence from the sea urchin and *Drosophila* embryo systems still provides clearer

insights into the molecular basis of gene-specific repression than most available mammalian data.

Network architecture, not master regulators: Importantly, gene regulatory network architecture can cause a factor's regulatory impact to appear to be the reverse of its biochemical activity. The sea urchin system illustrated that repression relationships could be nested to create a "double negative gate". This circuit enables a ubiquitous activator to turn on a complex program only in one embryonic cell lineage [31,32]. Whereas this program is silenced in most of the embryo by a first repressor, within the selected region a second repressor blocks expression of the first repressor, allowing the ubiquitous activator to trigger the program (Fig. 1A). Thus, through the double-negative gate, network architecture can produce a pattern of gene expression that does not match the expression patterns of any of its direct activators. This is a caveat for purely correlation-based network inference.

Information processing by the regulatory genome in hematopoiesis

It became clear from the 1990's that hematopoiesis could only be explained by the actions of hematopoietic TFs if one could account for the orderly developmental control of expression of the factors themselves. Davidson (Fig. 1B) strongly encouraged the enterprise of mapping the regulatory sequences that controlled the genes encoding these factors. Successful research in several groups, prior to whole-genome mapping, identified stem and progenitor-cell associated regulatory elements for SCL (*Tal1*) [33-36], *Gfi1* [37], *Gata2* [38-42], Runx1 [43] and *Lmo2* [44], regulatory elements with erythroid-associated activity for

Gata1 [45], and elements important for regulation of PU.1 (*Spi1*) in distinct myeloid and lymphoid contexts [46-55]. Each of these genes was shown to respond to inputs delivered via distinct cis-regulatory elements in different developmental contexts, confirming the generality of rules previously established in the invertebrate embryos. While these mapping studies based on classic, functionally-monitored methods may not have been comprehensive, they yielded strong insights about the regulatory switches that determine expression patterns for pivotal genes.

Examples of regulatory models of haematopoiesis inspired by Eric Davidson

Because of the rapid progress characterising key hematopoietic TFs, it became clear early that hematopoietic programmes must be determined by regulators combinatorially [56]. Very few hematopoietic TFs are strictly lineage-specific in expression; almost all play roles in multiple hematopoietic lineages. Furthermore, while different cell lineages may express different members of a given TF family, family members generally share DNA-binding specificity. Thus, from the "viewpoint" of the DNA sequence, an isolated GATA site might equally mediate control by GATA1 in erythroid or mast cells, by GATA2 in stem cells, or by GATA3 in T cells. In reality, lineage specificity must emerge from the unique combinations of TFs expressed in different cell types. The mobilization and impacts of these different combinations require explanation by a gene regulatory network.

The Davidson group itself focused primarily on logic models of gene regulatory networks, with implicit [11], or explicit timing [13]. The most directly comparable hematopoietic

regulatory network models may well be a series of models developed by Roger Patient's group, with the overall aim of encapsulating key processes that regulate early developmental haematopoiesis in *Xenopus* (summarized in [57]). Starting from a gene regulatory network logic model relating the known regulators of erythroid development [58], the Patient group collected and integrated spatiotemporal expression and signalling data coupled with comprehensive perturbation screens in *Xenopus* embryos. The resulting models do not reach the same degree of temporal or whole organism coverage achieved with the sea urchin work, and direct cis-regulatory information did not constitute a major component of the *Xenopus* work. Nevertheless, interesting parallels emerged including early repression and stepwise cascades of combinatorial TF interplay during the process of establishing and then stabilising cell fates [57].

Differentiation of hematopoietic progenitors from an embryonic hemangioblast or hemogenic endothelium must begin with a robust regulatory "launching pad". Pimanda et al. recognized that in early multipotent hematopoietic progenitors, the cis-regulatory elements of several of the key regulatory genes share target sequences for the TFs encoded by the other genes in the set [38]. In a mutually supportive triad, GATA2, SCL, and Fli1 maintained each other's expression through direct positive cross-regulation. Further work revealed that this triad was at the core of a larger network, also including Lmo2, Lyl1, Runx1, and Erg, in a core heptad, and three other factors. These frequently bound together to the same regulatory elements, including those controlling their own expression [59,60], in a mechanism strongly evocative of the densely interconnected cross-regulatory circuits seen in certain conserved embryonic gene regulatory networks [28]. A comprehensive model of

this network was established, anchored in molecular biology, by testing each factor-bound cis-regulatory element for functional hematopoietic activity, determining the roles of binding sites within those elements by mutation, and then using the results to construct a dynamic Bayesian network model to account for the cells' regulatory state [60](Fig. 1C). The strong performance of this model in predicting experimental observations showed the feasibility of predictive gene network modelling in this system, and also revealed substantial robustness to withstand single factor perturbations reminiscent of the robustness of sea urchin regulatory networks.

The Graf and Thieffry groups extended a related approach to pathways for hematopoietic cell identity change, using TF binding maps in B and myeloid cells and regulatory perturbation effects on B-cell and myeloid transcriptomes to explore these divergent developmental pathways. They constructed an asynchronous Boolean regulatory network model, which performed well in accounting for cytokine-driven lymphomyeloid differentiation, requirements for different TFs in setting distinct fates, and the ability of C/EBP family TFs to transdifferentiate B lineage cells to myeloid fates [61].

Component subcircuits in hematopoietic differentiation

Many gene regulatory network-centered analyses of hematopoiesis have focused on identifying roles of particular network subcircuits, rather than seeking comprehensive predictiveness. For example, driving the transitions in several lineages are feed-forward circuits, which are used in many biological contexts [62] besides developmental gene

regulatory networks. Examples are the E2A-EBF1-Pax5 circuitry promoting B-cell specification (rev. by [63]); the GATA1-FOG1 (Zfpm1) and GATA1-KLF1-FLI1 circuitry that operate during megakaryocyte-erythroid differentiation [64-66]; and the Notch signalling-TCF1-GATA-3-Bcl11b cascade driving early T-cell lineage commitment (rev. by [67,68])(Fig. 1D). Notably, in these developmental contexts, feed-forward circuits have also generally been found to drive an output repressing a lineage alternative as well as an output activating the favoured programme.

Hematopoietic lineage choice by bipotent precursors is often described as an example of bistable switch architecture based on balanced mutual repression, and driven to irreversibility by the positive autoregulation of each of the "contestants". This simple choice-making circuit architecture promotes dichotomous irreversible outcomes, with stochastic timing, based on equilibrium solutions to simple ordinary differential equations with very few variables. Thus, such a model has been popular to explain the instability of bipotent developmental intermediates and to explain how the fate decisions of single cells become irreversible. Mutual repression-based switches have been invoked for the PU.1-GATA1 opposition in myeloid vs. erythroid lineage choice [69-71](Fig. 1E), and for a Gfi1-Egr/Nab opposition circuit that converts modestly different PU.1 and C/EBPα ratios into dichotomous granulocyte vs. macrophage outcomes [72]. More recently, using single-cell transcriptome analysis, fluorescent TF expression reporters, and regulatory gene perturbation, mutual Gfi1-Irf8 opposition has been shown to be perhaps even more central for the granulocyte-macrophage lineage choice [73].

The prevalence of mutual repression subcircuit models for hematopoietic lineage choice contrasts with the circuits described by Davidson in sea urchin embryos, where such subcircuits were rare. If this difference is real, it could reflect a difference between the deterministic timeclock of a developing embryo and the stochasticity of choices governed by bistable mutual repression. Interestingly, on closer inspection, the mutual repression-based switch models are simpler than the hematopoietic reality. For example, if the PU.1-GATA1 antagonism were absolute, mast cells, basophils, and eosinophils would not exist. Indeed, several lines of evidence increasingly indicate more conditionality, and thus more regulatory participants, in the PU.1—GATA1 relationship than a simple stochastic winner-take-all opposition [74-77], reflecting the fact that even single cells in hematopoiesis face more than two potential choices.

Competing feed-forward mechanisms are often embedded within a more complex architecture together with bistable switch circuits, as proposed in the Gfi1/Egr-Nab based granulocyte-macrophage choice model [72]. For example, in later T lymphocyte development, cells use embedded feed-forward and cross-inhibition circuit motifs for the $CD4^+$ helper vs. $CD8^+$ killer decision within the thymus and the T_H1 vs. T_H2 effector subtype decision in the periphery [78-81]. These are irreversible but non-deterministically programmed developmental choices regulated by environmental signals, which are clearly advantageous for hematopoietic functions in adult organisms.

Challenges and caveats: the importance of underlying biological differences

Despite the powerful influence of Davidson's approaches, there are differences between the network models needed to account for non-vertebrate embryogenesis and for hematopoietic systems in postnatal mammals. These are noted briefly here; for a more detailed review, see [82]. Key differences concern dose dependence and timing.

As Davidson's group built increasingly complete models of the sea urchin embryo developmental gene regulatory network, their theoretical work increasingly stressed the Boolean (digital) quality of the network behaviour. A temporally synchronized Boolean model by Peter et al., modeling regulatory genes simply as being "off" or "on", already seemingly approached a complete, temporally accurate account of the generation and patterning of both mesoderm and endoderm in sea urchin embryos [13]. However, the underlying set of assumptions was problematic for parallels with hematopoiesis, since hematopoietic TFs have repeatedly been found to work on their targets in level-sensitive ways.

The developmental genetics field has long exploited the near-equivalence between heterozygous mutant embryos and wildtype control embryos, but in mammalian hematopoiesis many regulatory loci are haploinsufficient, e.g. [83-86]. Such twofold gene dosage differences affect the distribution of fate decisions among nominally equivalent starting cells or the kinetics of progression from progenitor to differentiated state. PU.1, although required in myeloid and lymphoid programmes alike, has strongly differential effects on these programmes depending on its expression level [87-89]. Furthermore, elevated expression of transcription factors in hematopoiesis does not simply flip a

physiological bistable switch. Some TF gain-of-function perturbations drive cells into an unanticipated alternative fate: e.g., overly high levels of the T-cell TF, GATA-3, drive T-cell precursors to generate mast cells instead [90]. These relationships are understandable based on incoherent (self-antagonizing) feedforward circuits, common in systems biology [62], but are not readily represented in classic topological gene network models (for a workaround applicable to some cases, see [91]).

Step timing in embryonic networks, which change state as soon as new TFs or signal-dependent modifications of TFs appear [13,92], seems very different from that in networks governing hematopoietic differentiation, which drive programmes that change slowly over many days. In postnatal hematopoietic precursors, even after experimental introduction or knockout of a TF, it often takes more than a day for changes in specific target gene expression to become measurable. This makes direct, indirect, and feed-forward dependent effects hard to disentangle. The slow response may be due in part to epigenetic state inertia, as discussed elsewhere [82,93]. However, it is also possible that hematopoietic gene regulatory networks themselves are more highly buffered against state changes. For example, in mutual repression-based hematopoietic choices, cells take days to select their fate [72], a marked contrast from the embryonic boundaries that are established by repression within minutes or hours.

Conclusions

In haematopoiesis research as well as many other fields, the use of the term “regulatory network” far exceeds the actual number of studies that include genuine experiments and/or analyses that would be required to gain new insights at the regulatory network scale. At least two relevant take-home messages seem to follow from this observation. Firstly, there is broad recognition that regulatory network analysis is a powerful approach to advance from a descriptive to a mechanistic understanding of biological processes. Secondly, actual regulatory network studies are not a small add-on to an existing body of work, but instead represent a substantial undertaking that is not for the faint-hearted.

Notably, many of the principles that underlie cellular decision making processes apply broadly across biological systems. Hematopoiesis researchers therefore have been able to benefit from pioneering studies such as the work by Davidson and co-workers on regulatory network control of sea urchin development. Against the backdrop of a scientific environment characterized by increasing specialization, there is an important lesson here on the enormous benefits that come from cross-fertilization and crossing artificial boundaries between diverse fields, with important implications for researchers as well as research funders, across all fields of hematopoiesis research and beyond.

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Conflicts of Interest

EVR is a member of the Scientific Advisory Board for Century Therapeutics and has been a consultant for A2 Biotherapeutics. BG has been a consultant for Autolus, and the BG lab has received funding from Astra Zeneca, Novo Nordisk and Autolus.

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Multiple genes can be expressed coordinately in complex organisms even though they are not arranged in operons as in bacteria. To explain this, Britten and Davidson proposed that certain special genes in higher organisms could encode trans-acting regulators that would act on multiple loci in parallel (gene battery) via shared cis-regulatory sequences. While the identities of the regulators were not correctly predicted, the logic was highly influential.

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This paper provided the first comprehensive gene regulatory network model for development of multiple embryonic tissues from a fertilized egg, based on "bottom up" empirical data about cross-regulation of >40 regulatory genes. Not a correlation-based cluster model, every link in this network represented an experimentally demonstrated causal relationship. This model was revised, refined and extended until Davidson's death.

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This paper formally tested how close the gene network for sea urchin development came to completeness, by constructing a Boolean model to simulate the known network operations to test whether it could account for observed regulatory dynamics embryo-wide. Vector equations were based on perturbation data and cis regulatory mapping. Explicitly realistic step time clocks and intra-embryonic cell proximities were included in the successful model.

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This paper provided the first evidence that the hematopoietic stem/progenitor state is stabilized by a "kernel"-like circuit of regulatory genes whose products directly stimulate each other's expression. The circuit model was based on identification of GATA2, FLI1 and SCL as three transcription factors that directly bind together at key cis-regulatory DNA elements previously shown to be functionally important for each of the genes.

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This paper showed the importance of tracking multiple transcription factors by parallel ChIP-seq analyses. The results substantiated the direct cross-regulation of a core circuit of hematopoietic regulatory genes, as their products bound together at known cis-regulatory elements. However, the frequency of highly clustered binding also suggested that transcription factors affect each other's site choices and may not "read" DNA autonomously.

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This paper modeled the multifactor gene regulatory network underlying the pathway of B lineage cell conversion to myeloid cells by forced expression of C/EBP transcription factors. Using ChIP-seq and experimental perturbation data to define network edges, a discrete-valued logical model was used to simulate outcomes of sustained or pulsed C/EBP exposures. In silico perturbations identified factors within the system affecting outcomes.

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Single cell RNA-seq and supervised clustering with a well-curated set of genes were used to create a framework for analysis of granulocyte vs. macrophage fate. Fluorescent reporter alleles of key regulators *Gfi1* and *Irf8* were used to define the cellular pathways of lineage decision. Perturbations in the single-cell gene expression trajectory when either *Gfi1* or *Irf8* was mutated revealed the gene regulatory network that actually controls the lineage choice.

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FIGURE LEGEND

Figure 1. Gene regulatory network circuits in context. (A) Double negative gate, used for sea urchin embryonic skeleton specification [31]. Skel: in skeletogenic precursors. Non-Skel: in

rest of embryo. Horizontal lines with bent arrows: genes. Arrows: positive regulation. Bar-end lines: negative regulation. Grayed out: inactive/non-expressed. (B) Eric Davidson, 2010, in Berlin. (C) Core regulatory network for hematopoietic stem and progenitor cells [60], with inputs to genes transduced via discrete cis-regulatory elements (rectangles). Note dense positive cross-regulation. (D) Feed-forward circuit, example from pro-T cell lineage commitment [68]. (E) Mutual repression-based bistable circuit, GATA1 vs. PU.1 [69].