

MiDReG: A method of mining developmentally regulated genes using Boolean implications

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We present a method termed mining developmentally regulated genes (MiDReG) to predict genes whose expression is either activated or repressed as precursor cells differentiate. MiDReG does not require gene expression data from intermediate stages of development. MiDReG is based on the gene expression patterns between the initial and terminal stages of the differentiation pathway, coupled with “if-then” rules (Boolean implications) mined from large-scale microarray databases. MiDReG uses two gene expression-based seed conditions that mark the initial and the terminal stages of a given differentiation pathway and combines the statistically inferred Boolean implications from these seed conditions to identify the relevant genes. The method was validated by applying it to B-cell development. The algorithm predicted 62 genes that are expressed after the KIT⁺ progenitor cell stage and remain expressed through CD19⁺ and AICDA⁺ germinal center B cells. qRT-PCR of 14 of these genes on sorted B-cell progenitors confirmed that the expression of 10 genes is indeed stably established during B-cell differentiation. Review of the published literature of knockout mice revealed that of the predicted genes, 63.4% have defects in B-cell differentiation and function and 22% have a role in the B cell according to other experiments, and the remaining 14.6% are not characterized. Therefore, our method identified novel gene candidates for future examination of their role in B-cell development. These data demonstrate the power of MiDReG in predicting functionally important intermediate genes in a given developmental pathway that is defined by a mutually exclusive gene expression pattern.

B-cell differentiation | microarray | gene expression | human | mouse

Precursor cells differentiate to their terminal progeny through a series of developmental intermediates and a network of gene expression changes that gradually establish lineage commitment and the identity of the mature cell type. The identification of genes that are involved in this process has largely been dependent upon the physical isolation and characterization of gene expression patterns within these developmental intermediates. Current methods such as genetic and biochemical experiments to identify developmentally regulated genes are time-consuming, costly, and technically challenging. Array-based approaches examining differential expression between populations are expensive, require highly pure starting populations, and are narrow in scope, as only gene expression levels among the arrayed populations are compared (1–4). Thus, when intermediate steps are unknown for a particular cellular differentiation pathway, the identification of genes that are developmentally regulated in that pathway can be difficult.

In this paper, we present a bioinformatics method called mining developmentally regulated genes (MiDReG), which mines the massive repertoire of publicly available microarray data to identify a specific subset of developmentally regulated genes whose expression patterns change from either low to high or high to low significantly during the course of development. In the case of B-cell development, many important genes including *KIT*,

CD19, and *PAX5* fall in this category. MiDReG does not require that arrays of the intermediate populations exist, only the knowledge of two or more genes within a developmental pathway, of which at least one is expressed in the stem or progenitor and at least one is expressed in the mature lineage. For this study, we chose B-cell development, an already well-studied system, to exemplify and validate MiDReG, but the method is widely applicable to other developmental pathways including those that are poorly characterized.

Results

Previously, we described a method to process and analyze all publicly available microarray gene expression datasets on the Gene Expression Omnibus database, as outlined in Fig. 1A (5). Within these datasets we identified expression relationships between pairs of genes (represented by probesets on the arrays) that follow simple “if-then” rules such as “if gene A is high, then gene B is low,” or more succinctly, “A high \Rightarrow B low” (“A high implies B low”). We call these relationships “Boolean implications.” Fig. 1B outlines the six different types of Boolean implications discovered among the probesets of the human and mouse datasets. More than 60% of the probesets from either mouse or human arrays have more than one thousand Boolean implications (Fig. 1C). We checked for conservation among these Boolean implications by comparing homologous genes between the human and mouse datasets and identified 15,199 human probesets and 10,695 mouse probesets that have corresponding homologs. These human and mouse probesets contain 22 million and 21 million Boolean implications, respectively. Of those, four million Boolean implications (approximately 18%) are preserved between homologous genes of the human and mouse datasets and are thus considered “conserved” (Fig. 1D). Additionally, Boolean implications can also be extended to logical combinations of genes as described in *Methods*. For example, the Boolean implication “ $X \Rightarrow Y$ ” can be discovered where X and Y are either single gene conditions (e.g., A high) or logical combinations of multiple genes (e.g., A high AND B high).

Computational Prediction of Developmental Genes Using Boolean Implications. MiDReG uses Boolean implications to identify developmentally regulated genes. This method is based on the

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expressed in many different cell types unrelated to hematopoiesis and/or B-cell development, including mast cells, bone marrow stromal cells, melanocytes, interstitial cells of Cajal, and thymocyte progenitors in the thymus, or malignant tissues (12), the mutually exclusive relationship between *KIT* and *CD19* is maintained in all the samples. This implication is also conserved between human and mouse datasets.

Having established a clear Boolean implication between *KIT* and *CD19*, we used MiDReG to identify such genes as shown in Fig. 2. To improve the quality of the results, the gene list was filtered by considering only those genes that are identified from these Boolean implications in both humans and mice (i.e., are conserved). The algorithm identified 19 such genes using *KIT* and *CD19* as shown in Fig. 2A. Fig. 2A shows a schematic diagram of the known expression patterns of *KIT* and *CD19* at sequential stages of B-cell differentiation (13). *KIT* is highly expressed in HSCs and multipotent progenitor (MPP^{FL-} and MPP^{FL+}) stages, whereas *CD19* transcripts are not detected in these stages (Fig. 2B). *CD19* is expressed from the fraction B (Fr.B) stage through the germinal center (GC) B-cell stage, whereas *KIT* transcripts are not detected from the Fr.D to the GC stages (Fig. 2B). To determine if the identified genes follow the expected expression patterns, median expression levels from HSCs to MPP^{FL-} and Fr.D to GC stages were computed for 14 genes (see Fig. S1 for purification strategies). The expected expression levels follow a pattern in which the median level from HSC to MPP^{FL-} is less than the median level from the Fr.D to GC stages. Strikingly, 10 out of the 14 identified genes passed this test (false discovery rate = 14.7%) (Fig. 2B). The bottom four genes are not consistent with our prediction because they are expressed in most stages of differentiation including HSC. Thus, our method has a success rate of 71% (10/14) for the prediction of genes that are developmentally regulated during B-cell differentiation.

Validation of B-Cell Precursor Genes Based on *KIT*, *AICDA*, and *CD19*.

Because *CD19* expression begins rapidly after *KIT* expression turns off, relatively few developmentally regulated genes can be identified in the intervening stages. In order to develop a more comprehensive list of B-cell precursor genes, we used the logical combination of both *CD19* and *AICDA* expression as a seed because simultaneous high expression levels of both these genes are specific to GC B cells (14), which are developmentally downstream of mature B cells. In this case Boolean implications are derived from the logical combination of genes “(*CD19* high AND *AICDA* high)” as described in *Methods*. We computed genes *X* such that “*KIT* high \Rightarrow *X* low” and “(*CD19* high AND *AICDA* high) \Rightarrow *X* high.” The list of genes was filtered for being conserved across humans and mice, as before. There are 52 such genes, 8 of which are in common with the 19 genes identified when only *CD19* was used as the (mature expressed gene) seed (Fig. 3A). Whereas it may seem counterintuitive that the addition of *AICDA* to MiDReG would *expand* the list of identified genes, this combination (*CD19* AND *AICDA*) specifies a later stage of development than *CD19* alone, and thus the number of genes that are up-regulated between *KIT*-expressing progenitors and *AICDA*-expressing GC B cells is increased. Several known genes encoding B cell-specific transcription factors were found in this list of 52 genes including *Pax5* and *SpiB* (15, 16). *SYK*, which encodes a tyrosine kinase that is a critical component of B-cell receptor signaling, is also identified (17). Fig. 3B shows the qRT-PCR results of 8 genes including the 3 seed genes as controls (*Kit*, *Cd19*, and *Aicda*) and 5 other selected genes. The qRT-PCR results clearly show that the expression of these genes is established early during B-cell differentiation after the HSC/MPP stages and is maintained stably through the GC B cell. The only exception is that the expression level of *SYK* is low at the GC stage. A detailed list of the predicted B-cell genes with their Affymetrix ID in both human and mouse platform can be found in Table S1.

A MiDReG Prediction Algorithm

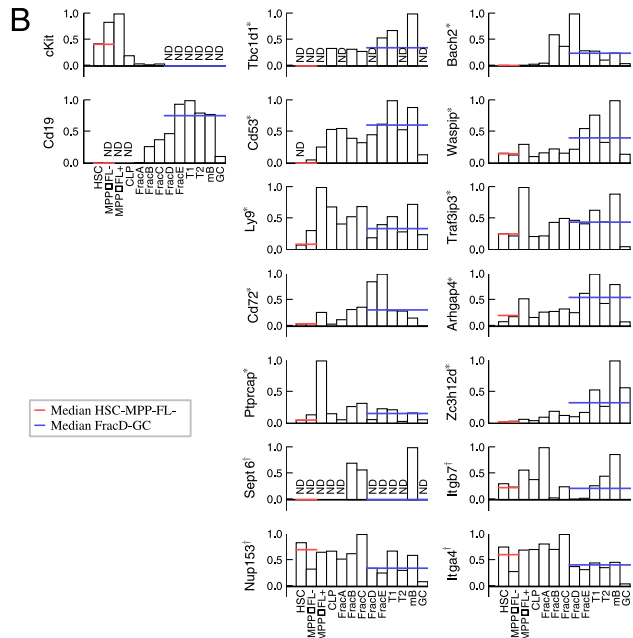
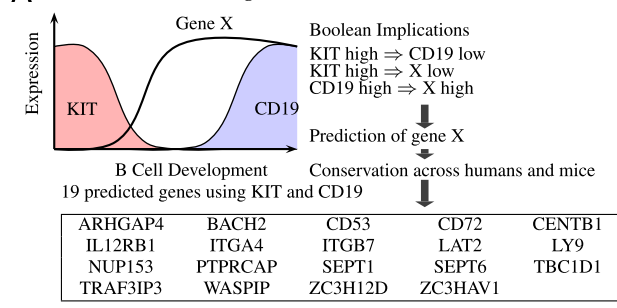


Fig. 2. Validation of B-cell precursor genes based on *KIT* and *CD19*. (A) B-cell precursor genes were predicted by using *KIT* and *CD19* as seed genes. *KIT* is expressed early in the development, and *CD19* is expressed in the mature B cell. The Boolean implication $KIT \text{ high} \Rightarrow CD19 \text{ low}$ indeed reflects this situation. The identified genes turning on between *KIT* and *CD19* are genes *X* such that $KIT \text{ high} \Rightarrow X \text{ low}$ and $CD19 \text{ high} \Rightarrow X \text{ high}$. The list of genes is filtered by intersecting results from both human and mouse datasets. (B) The MiDReG algorithm identified 19 B-cell precursor genes by using *KIT* and *CD19*. Quantitative RT-PCR (qRT-PCR) was performed on 13 purified hematopoietic populations at different stages of B-cell differentiation: HSC, MPP^{FL-} (multipotent progenitors Flk2⁻), MPP^{FL+} (multipotent progenitors Flk2⁺), CLP (common lymphoid progenitors), Frac A (Pre-Pro-B), Frac B (Pro-B), Frac C (large pre-B), Frac D (small pre-B), Frac E (immature B), T1 (Transitional 1), T2 (Transitional 2), mature B, and GC (germinal center B cells). The bar plot shows relative gene expressions from the qPCR result of 16 genes including the seed genes: *KIT* and *CD19*. The gene expressions are displayed as a percentage to the maximum gene expression level. The expression level of *KIT* is high, and none of the *CD19* transcripts are detected from HSC to MPP^{FL+} stages. The expression level of *CD19* is high, and none of the *KIT* transcripts are detected from FracD to GC stages. Therefore, for each of the 14 experimental genes the median expression level from HSC to MPP^{FL-} stages is compared against the median expression level from FracD to GC stages. The results show that 10 out of 14 genes (indicated with *) have higher median expression levels from FracD to GC stages compared to the HSC and MPP^{FL-} stages (FDR = 14.7%). These genes have low expression or turn off at HSC to MPP^{FL-}; then they turn on between MPP^{FL+} to Frac C and are highly expressed in FracD to GC stages. The bottom four genes (indicated with †) did not pass the above test.

Classification of the Predicted B-Cell Genes. To estimate the ability of MiDReG to identify functionally significant genes, we examined the published literature for knockout phenotypes or other evidence of B-cell function among the resulting genes (Fig. 4 and Table S2). The classifications of the identified genes are described

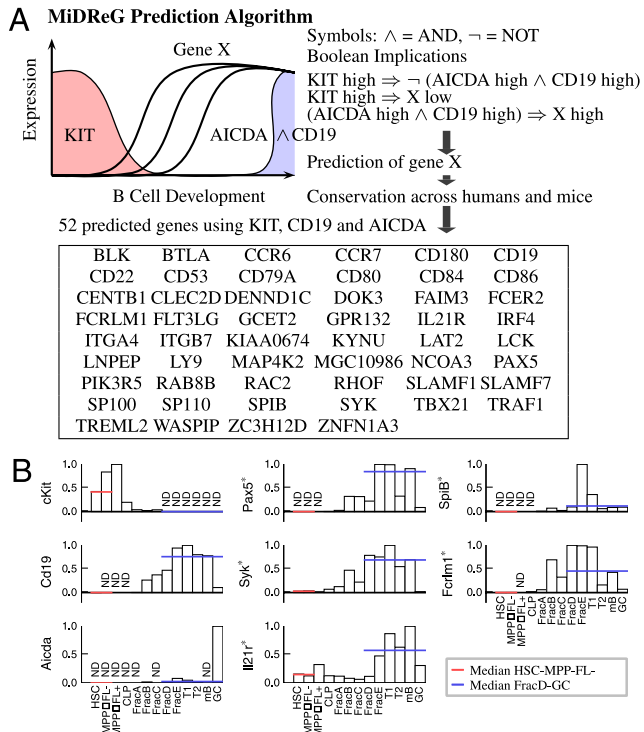


Fig. 3. Validation of B-cell precursor genes based on *KIT*, *AICDA*, and *CD19*. (A) B-cell precursor genes were predicted by using *KIT* as the first seed gene and a combination of *CD19* and *AICDA* as the second seed gene. The list of genes is filtered by using conservation across both human and mouse datasets. The combination of *CD19* and *AICDA* expression levels are specific to a narrow region in the later stages of B-cell development, so the MiDReG algorithm is expected to return more genes than the earlier results using *CD19* only. The MiDReG algorithm predicted 52 B-cell precursor genes by using *KIT*, *CD19*, and *AICDA*. These genes are hypothesized to be expressed after the *c-kit*⁺ progenitor cell stage and remain expressed through *CD19*⁺*AICDA*⁺ GC B cells. (B) qRT-PCR results for *Pax5*, *Syk*, *Il21r*, *Spi-B*, and *Fcrlm1* are shown. The results show that all five genes indicated with * have higher median expression levels from FracD to GC stages compared to the HSC and MPPFL⁻ stages, which suggests that the expression patterns for these genes are indeed stably maintained through GC B cells.

in detail in Table S3. Of the 62 genes identified by MiDReG in Figs. 2 and 3 to be up-regulated in B-cell development, 34 had published B-cell functions (56.5%), and another 5 genes had indirect connections to B-cell function (8.1%), for example. Combined, these data indicate that MiDReG had a 64.6% success rate for predicting genes with known B-cell functions (Fig. 4A). Furthermore, in 11 of the remaining genes (17.7%), we either could confirm expression in B cells (8 genes) or found knockout phenotypes or functions in other tissues and/or pathways similar to B cells [e.g., T cell trafficking, Toll-like receptor (TLR) signaling, cytoskeletal rearrangements]. We would therefore predict that if interrogated, B-cell functions would be identified for many of those genes. Last, only 3 genes were completely unstudied (4.8%), and 8 genes had no role in any pathway related to B-cell function (12.9%). Knockout mice have been published for 41 of the 62 genes, of which 26 genes (63.4%) had an identified B-cell phenotype (Fig. 4B). Also, according to the gene ontology annotations (18), these 62 genes include 26 surface receptors, 15 signal transducers, 10 transcription factors, 9 metabolic genes, 1 cytokine, and 1 unknown (Fig. 4C). Many of the genes identified by MiDReG were related to B-cell receptor signaling, as either cell surface receptors or signal transducers. There were 6 genes related to NF- κ B signaling (*ARHGAP4*, *BTLA*, *CENTB1*, *DOK3*, *TRAF1*, *TRAF3IP3*, and *ZC3H1A1*), of which most were attenuators. There were also four members of the slam family of

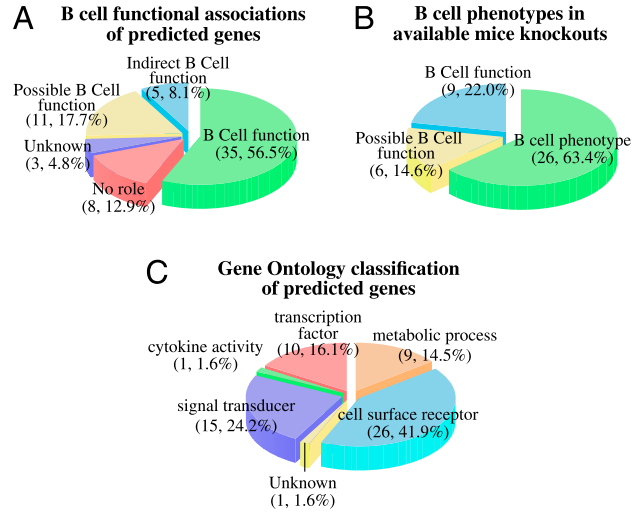


Fig. 4. Classification of the predicted B-cell genes. (A) Predicted B-cell genes are grouped according to reported B-cell functions in the literature. Out of 62 genes, 35 (56.5%) genes are associated with known B-cell function, 5 (8.1%) genes are indirectly related to the B cell through interacting proteins, 3 (4.8%) genes are unknown, 8 (12.9%) genes have other roles, and 11 (17.7%) genes could have a B-cell function based on their expression in the B cell and reported other hematopoietic functions. (B) Predicted B-cell genes with available mice knockouts are grouped according to reported B-cell phenotypes in the literature. Out of 62 genes, 41 genes have been knocked out in mice. Out of these 41 mice knockouts, 26 (63.4%) genes show defects in B-cell function and differentiation, 9 (22.0%) genes are associated with known B-cell function according to other experiments, and 6 (14.6%) genes could have a B-cell function based on their expression in the B cell and reported other hematopoietic functions. (C) Predicted B-cell genes grouped according to gene ontology classification. Out of 62 genes, 26 (41.9%) genes are cell surface receptors, 15 (24.2%) genes are associated with signal transduction, 10 (16.1%) genes are transcription factors, 9 (14.5%) genes are associated with other metabolic process, 1 (1.6%) unknown gene, and 1 (1.6%) cytokine.

surface receptors (*CD84*, *LY9*, *SLAMF1*, and *SLAMF7*). Two genes (*WASPIP* and *GCET2*) are known prognostic markers of B-cell lymphomas (19–21). Of the nine genes with no obvious connection to B-cell function, three belonged to the GLUT4 glucose uptake pathway (*LNPEP*, *RAB8B*, and *TBC1D1*), a pathway by which muscle cells can rapidly uptake glucose in response to insulin signals. In summary, these 62 genes are highly enriched for important B-cell related activities or promising candidates for future functional studies.

Discussion

Comparison with Existing Approaches. The identification of genes that are involved in establishing a cellular lineage can be a technically difficult process. Investigators will often take empirical approaches such as functional genetic screens or biochemical characterizations of their cell type of interest to identify the regulators of fate decisions and lineage commitment. For instance, many of the transcriptional regulators involved in B lineage commitment were first identified as factors that were bound to immunoglobulin enhancer elements in cell lines that were readily available for biochemical studies (15, 22). For myriad technical reasons, however, these types of approaches are often not feasible for the study of a variety of other important developmental processes. In particular, when few of the developmental intermediate steps are known for a particular lineage, the identification of genes involved in lineage commitment and differentiation can be extremely challenging. With the advent of microarray technology, comparisons between two or more populations could reveal developmentally regulated genes (1–4, 23). However, because the purity of the isolated populations is proportional to the quality of

the data, this comparison approach faces the proverbial “chicken-and-egg” problem, because microarrays are needed to identify markers to better purify populations. Whereas this chicken-and-egg problem can be partially solved by repeated rounds of purification and microarray usage, the high cost of microarrays makes this approach somewhat prohibitive. But perhaps the greatest weakness of this approach is the narrow scope, where only closely related populations are compared. In comparisons of more distantly related populations, the number of differentially regulated candidates becomes enormous and unwieldy. However, a complete characterization of differentially regulated genes can be performed once microarrays of the intermediate populations of a developmental pathway are available. Similarly, a thorough characterization of B-cell GC genes has been performed (23). Additionally, there are other approaches to identify important genes by building regulatory networks such as relevance networks (24), the algorithm for the reconstruction of accurate cellular networks (25), Bayesian networks (26), and Inferelator (27). It is not obvious how these methods can be applied to identify genes similar to MiDReG. Our technique takes advantage of Boolean implication relationships mined from the large publicly available repositories of microarray data to identify developmentally regulated genes, even when few of the intermediate stages are known.

Advantages and Limitations of MiDReG. Our method assumes only minimal knowledge of candidate seed genes, by using *c-kit* (*KIT*) as a gene known to be expressed in HSCs and later extinguished and *CD19* as a B-cell-specific gene expressed after the *c-kit*⁺ progenitor stages. MiDReG can identify important genes in B-cell development that are conserved in humans and mice. Therefore, it opens a possibility of translating the complex mouse genetics results to humans. Many of the MiDReG-identified genes have not been analyzed for B-cell function in the literature. Some of these genes have been shown to be expressed in B cells, and they have other hematopoietic function (Table S3). A possible unexpected link between the GLUT4 pathway and B-cell function is described in detail in Table S4. Therefore, these genes are perfect candidates for future B-cell functional experiments. As a test for the power of this method in hematopoietic lineage analysis, in a companion paper MiDReG was used to identify a gene that encodes a cell surface molecule present in cells called common lymphocyte progenitors (CLPs) (28). This gene identified a subpopulation that is committed to the B lineage and is the earliest precursor yet found in that lineage, whereas the other subpopulation is capable of differentiating to T, B, natural killer (NK), and dendritic cells (DCs) (28).

The ability of MiDReG to identify markers of developmental stages in hematopoiesis, which is in many ways a paradigmatic developmental system, opens the possibility of better understanding less well-characterized developmental systems. An important advantage of MiDReG is that it uses all publicly available microarray data and it does not require additional microarrays to be performed on pure populations at the beginning, end, or intermediate stages of the developmental pathways under investigation. Moreover, the genes identified by MiDReG in B-cell development were based on a minimal number of known seed genes; adding additional seed genes can enhance the resolution by broadening or narrowing the scope, as we show elsewhere (28).

One of the important limitations of our method is that it does not identify genes that are expressed only transiently during development, such as *RAG1* and *RAG2*, which are required for antigen receptor recombination but are shut off after productive rearrangement (29, 30). However, genes that are critically important for maintaining B-cell identity, such as *Pax5*, are known not to be transient (31, 32). There are also limitations in identifying conserved Boolean implications using orthologous human and mouse genes, because these are entirely based on the current

annotations. The inaccuracy in annotations will most likely result in important genes missing (false negatives), because our random permutation experiment on BooleanNet shows no conserved Boolean implications. It is important to note that the reliability of the results depends entirely on the choice of the seed genes and the existence and quality of their corresponding probesets on existing microarrays. MiDReG requires at least two probesets that represent developmentally significant seed genes, and those two probesets must have a Boolean relationship. Given the quantity of Boolean implications identified for any given gene (Fig. 1C), we are confident that many developmental pathways will contain multiple seed choices. Indeed, our previous studies demonstrated that logical Boolean implications are made in other developmental systems, such as the mutually exclusive expression relationship between *HoxA13* and *HoxD3* (5). However, we do not know whether such conditions exist for all developmental pathways. Thus, the applicability of MiDReG to any developmental pathway should be approached on a case-by-case basis.

As a tool for gene discovery, MiDReG can complement existing array-based methods by independently identifying candidate genes. As we show for B-cell development, over half of the genes predicted by MiDReG are known to be functionally relevant to B-cell biology. Provided that two seed conditions exist, MiDReG may be able to predict pathway-related genes not only on the differentiation pathways from stem cells to mature cells as we describe here, but also on developmental pathways from pluripotent stem cells to specific tissue or on disease pathways according to malignancy stages or chronic to acute phases. We feel that MiDReG can serve as a useful addition to the toolbox of developmental biologists searching for developmentally regulated genes.

Methods

Data Collection and Preprocessing. Raw data files (“.cel” files) for 4,787 Affymetrix U133 Plus 2.0 human microarrays and 2,167 Affymetrix 430 2.0 mouse arrays were downloaded from Gene Expression Omnibus (33). These array types were chosen because they are widely used and because results from different arrays can be compared more reasonably than results from two-channel arrays. The datasets were normalized and probeset level expressions were generated by using the standard robust multichip average algorithm (Fig. 1A) (34). The human U133 Plus 2.0 platform has 54,677 probesets, and the mouse 430 2.0 platform has 45,101 probesets. Boolean implications between pairs of probesets were extracted from these data (5). A database of all Boolean implications was created for each platform.

MiDReG Algorithm Using Two Seed Conditions. A seed condition is described by using either a single gene logical condition or logical combinations of multiple genes. For single gene seed conditions A high and B high, we first check if there is a Boolean implication $A \text{ high} \Rightarrow B \text{ low}$ between genes A and B. Then, the algorithm identifies all genes C such that $A \text{ high} \Rightarrow C \text{ low}$ and $B \text{ high} \Rightarrow C \text{ high}$ by intersecting the list of genes that have high-low implication with A (e.g., $A \text{ high} \Rightarrow C \text{ low}$) and high-high implication with B (e.g., $B \text{ high} \Rightarrow C \text{ high}$). Optionally, the algorithm filters the candidate C genes by insisting that the implications are conserved across humans and mice. For the conservation analysis, the probesets in U133 Plus 2.0 and mouse 430 2.0 were matched by using the ortholog functional annotation file from the Affymetrix web site.

When logical combinations of multiple genes are used in the seed conditions, the algorithm computes Boolean implications from those seed conditions to all the probesets in the datasets. In order to check if a Boolean implication “seed $A \Rightarrow X \text{ low}$ ” is significant, BooleanNet computes the number of arrays satisfying the following four conditions: (seed A, X low), (seed A, X high), (negation of seed A, X low), and (negation of seed A, X high). Then it checks if (seed A, X high) is sparse by using the statistic described in the BooleanNet paper (5). For the identification of up-regulated genes, MiDReG searches for all genes C such that seed $A \Rightarrow C \text{ low}$ and seed $B \Rightarrow C \text{ high}$. Similarly, for the identification of down-regulated genes, MiDReG computes all genes C such that seed $A \Rightarrow C \text{ high}$ and seed $B \Rightarrow C \text{ low}$.

Validation of MiDReG. The candidate B-cell precursor genes were identified by the MiDReG algorithm by using *KIT* and *CD19* as seed genes. These genes are low when *KIT* is high and high when *CD19* is high. Therefore, these genes are hypothesized to turn on after *KIT* turns off and before *CD19* turns on. To

identify more genes, a similar experiment is repeated by using *KIT* as one of the seed genes and by using high expression levels of both *AICDA* and *CD19* as the other seed. In this case a virtual gene is created whose expression level is high when both *AICDA* and *CD19* expression levels are high. Boolean implications between this virtual gene and other genes are computed in the same way as before (5). The identified genes here are supposed to be expressed after the *c-Kit*⁺ progenitor cell stage and maintained through *CD19*⁺*AICDA*⁺ GC B cells.

Statistical Tests for the Validation of B-Cell Precursor Genes. The qRT-PCR data are arranged as genes in the rows and 13 different stages of B-cell development with three replicates each in the columns. The median expression level from HSC to MPP^{FL} stages is compared against the median expression level from FracD to GC stages. The test is successful if the median expression level from FracD to GC stages is higher than the median expression level from HSC and MPP^{FL} stages. The columns are then permuted randomly 100,000 times while keeping the correlation between genes the same. The percentage of times these random tests exceeds the original number of successes is recorded as the false discovery rate.

Animals. All animal procedures were approved by the International Animal Care and Use Committee and the Stanford Administrative Panel on Laboratory Animal Care. C57Bl/Ka-Thy1.1 mice were derived and maintained at Stanford University. Bone marrow and spleen cells were obtained from mice aged 10–12 weeks.

Antibodies. A complete list of all antibodies used in the study is shown in Table S5.

Fluorescence-Activated Cell Sorting. All cells were sorted and data collected on a BD FACS-Aria (Becton Dickinson). FlowJo software (TreeStar) was used for

flow cytometric data analysis. HSCs, MPPs, CLPs, and Fr.A–E cells were stained and harvested from the marrow as described. T1, T2, and mature B cells were harvested from the spleen, and GC B cells were harvested from the spleens of mice immunized with 100 μg alum-precipitated 4-Hydroxy-3-nitrophenylacetyl hapten conjugated to chicken gamma globulin lysine through amide bonds (Biosearch Technologies) at 14 days postimmunization as previously described (35).

Quantitative PCR for B-Cell Precursor Validation. Cells were sorted into TRIzol (Invitrogen Life Technologies), and RNA was isolated according to manufacturer's instruction. cDNA was synthesized by using the Superscript III kit (Invitrogen Life Technologies) using random hexamers. Amplifications were performed by using SYBR Green (SYBR is a registered trademark of Molecular Probes, Inc.) PCR core reagents (Applied Biosystems), and transcript levels were quantified by using an ABI 7900 Sequence Detection System (Applied Biosystems). The mean ct value of triplicate reactions was normalized against the mean ct value of β-actin. Primers were used at 400 nM. A complete list of primers sequences is shown in Table S6.

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