REVIEW

The making of a lymphocyte: the choice among disparate cell fates and the IKAROS enigma

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Lymphocyte differentiation is set to produce myriad immune effector cells with the ability to respond to multitudinous foreign substances. The uniqueness of this developmental system lies in not only the great diversity of cellular functions that it can generate but also the ability of its differentiation intermediates and mature effector cells to expand upon demand, thereby providing lifelong immunity. Surprisingly, the goals of this developmental system are met by a relatively small group of DNA-binding transcription factors that work in concert to control the timing and magnitude of gene expression and fulfill the demands for cellular specialization, expansion, and maintenance. The cellular and molecular mechanisms through which these lineage-promoting transcription factors operate have been a focus of basic research in immunology. The mechanisms of development discerned in this effort are guiding clinical research on disorders with an immune cell base. Here, I focus on IKAROS, one of the earliest regulators of lymphoid lineage identity and a guardian of lymphocyte homeostasis.

The ability of differentiating cells to turn genes on and off in a precise manner is a fundamental mechanism in metazoan development. Both the timing and magnitude of gene activation and repression contribute to the precision by which cellular specialization is achieved. Paradigms of this process are provided by the hemolymphoid system, the caretaker of organismal homeostasis. Both multipotency and self-renewal are built-in features of not only the earliest hematopoietic progenitors but also lymphocyte differentiation intermediates and mature effector cells. These allow for the initial generation and sustained maintenance of a diverse repertoire of specialized immune cells over one's life span. Key to this developmental process is a select group of cell type-specific transcription factors responsible for changing gene

expression according to differentiation requirements. This regulatory network is recycled at discrete steps of differentiation acting within distinct epigenetic landscapes, a combination that is likely responsible for disparate cellular outcomes. Mutations in individual network components are frequently encountered in immune cell malignancies and immune cell-based disorders, thereby not only generating insights into their role in lymphocyte homeostasis but also providing strategies for clinical intervention in human disease. Better understanding of the transcriptional and epigenetic mechanisms that control immune cell development and function can also improve the design of immunotherapies, including those targeting cancers.

Lymphocyte differentiation is uniquely dependent on a handful of sequence-specific DNA-binding factors that influence lymphoid lineage transcriptional outcome from the hematopoietic stem cell (HSC) to the terminally differentiated T and B effector cells. Evolutionarily conserved members of the basic helix-loop-helix (i.e., E2A and HEB), the early B-cell factor (i.e., EBF1), the pair box (i.e., PAX5), the Forkhead box (i.e., FOXO1), the effectors of WNT signaling (i.e., TCF1 and LEF1), the Runt domain (RUNX1), and the Krüppel and class IV zinc finger (i.e., IKAROS, BC11b, and GATA4) groups constitute two core transcription factor networks that serve as signatures of B-cell and T-cell differentiation. Loss of function of individual regulators arrests differentiation at distinct steps, arguing for nonredundant stage-specific requirements.

Notable among these factors is the IKAROS gene family responsible for both the onset of lymphocyte differentiation and its functional outcome. Acquisition of lymphoid lineage differentiation potential, transition from a highly proliferative to a quiescent T-cell or B-cell precursor, and regulation of antigen receptor-mediated proliferative responses in T cells and B cells and effector cell function are dependent on IKAROS proteins. In this

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review, I attempt to integrate IKAROS's role in lymphocyte differentiation with recent studies on IKAROS's functional participation in lymphoid-specific as well as "extralinear" transcription and epigenetic networks. I discuss how insight into both the mechanisms by which IKAROS regulates and is in turn regulated can provide new approaches to alter the outcome of immune cell disorders originating from distinct steps in this intricate developmental pathway.

Structure and function of the IKAROS gene family

IKAROS (*Ikarf1*) is the founding member of a family of four genes that includes AIOLOS (*Ikarf3*), HELIOS (*Ikarf2*), and EOS/DEDALOS (*Ikarf4*) (Fig. 1A; Georgopoulos et al. 1992; Hahm et al. 1994, 1998; Morgan et al. 1997; Kelley et al. 1998; Honma et al. 1999). The IKAROS family members encode proteins that are structurally and functionally similar. These share a highly conserved N-terminal DNA-binding domain comprised of four Krüppel-type zinc finger motifs (Fig. 1A). The middle two of the N-terminal zinc finger motifs (F2 and F3) make sequence-specific contacts to DNA through the *a/gGGAA* core motif that is also recognized by other types of transcription factors, such as ETS, NFκB, STAT, RBPj/κ, and TEAD, suggesting a functional interplay between these factors during differentiation (Molnar and Georgopoulos 1994; Hu et al. 2016). The two outer zinc fingers (F1 and F4), although not directly engaged in DNA binding, also contribute to IKAROS activity. This is supported by the phenotypes caused by their deletion, which, although milder than those caused by loss of the DNA-binding zinc fingers (F2 and F3), still negatively impact T-cell and B-cell differentiation (Georgopoulos et al. 1994; Winandy et al. 1995; Schjerven et al. 2013; Arenzana et al. 2015).

Proteins of the IKAROS family share a second highly conserved Krüppel-type zinc finger domain that is located at the C terminus and is engaged in protein interactions between family members (Sun et al. 1996; McCarty et al. 2003). IKAROS proteins use their zinc finger motifs to directly interact with the transcriptional corepressor SIN3B and the ATP-dependent nucleosome remodeler Mi-2β (CHD4), a key regulator of hematopoiesis (Fig. 1A; Koipally et al. 2002; Yoshida et al. 2008). IKAROS and AIOLOS proteins, but not other family members, use the second zinc finger motif to interact with the E3 ligase adaptor CEREBLON when bound by immune modulatory drugs (IMiDs) (Matyskiela et al. 2016; Petzold et al. 2016). Additionally, IKAROS proteins have a PEDLS motif that binds the corepressor CtBP (Fig. 1A; Koipally and Georgopoulos 2000; Perdomo and Crossley 2002).

The organization of IKAROS zinc finger motifs into two separable and functionally distinct domains is similar to HUNCHBACK, encoded by a GAP gene that regulates early patterning in the *Drosophila* embryo by initiating *Hox* gene repression, a process that is subsequently maintained by the Polycomb complex (Qian et al. 1991; Muller and Bienz 1992; Shimell et al. 1994). HUNCHBACK

Figure 1. IKAROS family: protein structure and function. (A) Diagrammatic representation of the coding exons (2–8) of the IKAROS gene family highlighting conserved domains involved in DNA-binding and protein interactions. Sites of post-translational modifications that are of functional consequence are shown. The Krüppel-type zinc finger motifs of the IKAROS family are color-coded for their role in DNA binding (F2 and F3 [red]: direct DNA contact; F1 and F4 [orange]: contribution to DNA binding) or protein interactions (F5 and F6 [purple]). The IKAROS DNA-binding core motif selected by zinc fingers F2 and F3 is underlined on the IKAROS DNA-binding motifs selected at high frequency in IKAROS chromatin immunoprecipitations in T-cell and B-cell precursors. A serine- and threonine-rich conserved region that is phosphorylated during the S phase by casein kinase II is shown on exon 8 (pink circles). Conserved sites of phosphorylation at the linkers of the DNA-binding zinc finger motifs during the M phase are shown (blue circles). Sites of IKAROS interaction with the transcriptional corepressors CtBP and SIN3, the chromatin remodeler Mi-2β, the immune modulatory drug (IMiD)-bound CRBN adaptor (*) of the CRL4 E3 ligase complex, and protein phosphatase 1 (PP1) are depicted. Lysines involved in sumoylation and influencing IKAROS–protein interactions are indicated (black circles). (B) Chromatin distribution of the nucleosome remodeling and histone deacetylase (NuRD) complex in lymphocytes in the presence (*left*) or absence (*right*) of IKAROS DNA binding induced by either IKAROS deletion (Δ IK) or phosphorylation (IK-PP). In the presence of IKAROS, the NuRD complex is targeted at IKAROS-binding sites in the vicinity of lymphoid genes. In the absence of IKAROS, NuRD redistributes to genes involved in metabolism and cell cycle that are aberrantly induced in IKAROS-deficient lymphocytes.

maintains competence of neural progenitor cells and contributes to specification of early born neuronal cell fates (Isshiki et al. 2001; Novotny et al. 2002), much as IKAROS acts at multiple stages of immune cell development. IKAROS also regulates progenitor competence and early born cell fates in the mammalian nervous system (Elliott et al. 2008; Tran et al. 2010; Alsio et al. 2013). Like IKAROS, HUNCHBACK uses the N-terminal zinc fingers to bind DNA and its C-terminal zinc fingers to dimerize (McCarty et al. 2003). HUNCHBACK is also engaged in direct functional interactions with the *Drosophila* homolog of Mi-2β (*dmi2*) (Kehle et al. 1998). Due to these

structural and functional parallels, HUNCHBACK and the IKAROS family have been considered orthologs.

Post-translational modifications

IKAROS family members also share a highly conserved serine- and threonine-rich region located at the protein's C-terminal half (Fig. 1A). Phosphorylation of this region by casein kinase II (CKII) occurs during the G1-S transition and is responsible for reducing the DNA-binding activity of IKAROS proteins (Gomez-del Arco et al. 2004). This phosphorylation event may also promote protein degradation through an associated PEST motif and can be negatively regulated by protein phosphatase 1 (PP1) (Popescu et al. 2009). Additional IKAROS phosphorylation events that involve the serine and threonine residues at the N-terminal zinc finger linker regions occur at the M phase of the cell cycle and also interfere with DNA binding (Fig. 1A; Dovat et al. 2002). Thus, as lymphocytes move through the cell cycle, there appears to be a progressive reduction in IKAROS DNA-binding activity that is conferred by distinct phosphorylation events. In support of a functional consequence for this IKAROS regulation process, overexpression of normally expressed IKAROS DNA-binding isoforms arrests both lymphoid and non-lymphoid cells at the G1 phase, suggesting that unregulated IKAROS binding to DNA inhibits cell division and can be detrimental to both lymphocyte differentiation and function (A Molnar and P Gomez-del Arco, unpubl.).

In multiple myeloma (MM), a neoplasm of high-affinity bone marrow-residing plasma cells, when cells are treated with IMiDs such as lenalidomide, IKAROS and AIOLOS become *de novo* targets of the CRL4-CEREBLON (CRL4^{CRBN}) E3 ubiquitin ligase complex. This results in IKAROS and AIOLOS protein degradation and interferes with MM cell growth (Gandhi et al. 2014; Kronke et al. 2014a,b). The second DNA-binding zinc finger (F2) in IKAROS and AIOLOS binds to the hydrophobic pocket of CEREBLON (the E3 ligase adaptor) when it is occupied by lenalidomide (Fig. 1A; Matyskiela et al. 2016; Petzold et al. 2016). Since the IKAROS-CEREBLON-IMiD interaction is inhibited when IKAROS is bound to DNA, modifications such as phosphorylation may precede IKAROS degradation by the CRL4^{CRBN} complex (Petzold et al. 2016). IKAROS proteins are also modified by sumoylation at two lysine residues that flank the N-terminal zinc finger domain (Fig. 1A). IKAROS sumoylation is not responsible for protein degradation but prevents interactions with Mi-2 β and SIN3B (Gomez-del Arco et al. 2005).

The ability to control IKAROS protein activity through post-translational modifications is likely to be critical for the controlled proliferative expansion of lymphocyte precursors and functional output of mature T cells and B cells. There are instances where IKAROS family members are differentially targeted by post-translational modifications. For example, unlike IKAROS and AIOLOS, HELIOS, which is expressed in the T-cell but not the B-cell lineage, is not targeted for degradation by the CEREBLON-IMiD complex, setting up for a potential differen-

tial regulation of the IKAROS gene family in T cells versus B cells.

Using or hijacking the nucleosome remodeling and histone deacetylase (NuRD) complex?

In lymphocytes, IKAROS proteins are stable components of the NuRD complex (Fig. 1B; Kim et al. 1999; Sridharan and Smale 2007). The NuRD complex is unique, especially in lymphocytes, as it can modulate access to nucleosomes through Mi-2 β , restrict chromatin through histone deacetylases (HDAC1-2), and, with the addition of IKAROS proteins, target chromatin in a sequence- and lineage-specific manner (Kim et al. 1999; Zhang et al. 2011). Indeed, in T-cell and B-cell precursors, Mi-2 β is highly enriched at IKAROS-binding sites in the vicinity of transcriptionally active lymphoid genes (Zhang et al. 2011; Schwickert et al. 2014). The presence of the NuRD complex at these active regulatory sites may serve as a harbinger for their shutdown at a later differentiation stage (Whyte et al. 2012; Yamada et al. 2014). Intriguingly, in T-cell precursors, loss of IKAROS causes a redistribution of Mi-2 β from lymphoid genes to genes engaged in cell cycle and metabolism, correlating with their induction (Zhang et al. 2011). These findings can explain why loss of IKAROS causes an increase in T-cell proliferation, whereas loss of Mi-2 β results in the opposite phenotype (Winandy et al. 1995; Avitahl et al. 1999; Williams et al. 2004; Naito et al. 2007). Nonetheless, the ability of IKAROS to bind nucleosomes *in vitro* and chromatin *in vivo* is dependent on Mi-2 β (Kim et al. 1999; I Hazan, unpubl.). Thus, although there is synergy in some aspects of IKAROS and Mi-2 β activity, there is also antagonism that may originate from differences in downstream targets as defined by the presence or absence of IKAROS (Fig. 1B). Although Mi-2 β nucleosome remodeling and IKAROS sequence-specific DNA binding may support access to lymphoid genes and lymphoid differentiation in multipotent progenitors, sequestering the NuRD complex at the IKAROS sites may also prevent unwanted induction of a growth-supporting gene expression program (Fig. 1B). Regulation of IKAROS DNA binding such as discussed in the previous section may allow for a controlled genome-wide redistribution of the NuRD complex in support of proliferative expansion of both lymphocyte precursors and mature lymphocytes.

Expression and immune cell phenotypes

Although similar in function, IKAROS family members differ in expression (Kelley et al. 1998), a property that ultimately defines the distinct impact that their loss of function brings to differentiation. IKAROS (*Ikzf1*) is strongly expressed from the HSCs to mature T and B effector cells and is the only family member that is necessary at both the onset and later stages of lymphocyte differentiation. There is an extensive redundancy of regulatory elements at the *Ikzf1* locus, with two promoters and six enhancers providing locus control region activity during hemolymphopoiesis (Yoshida et al. 2013; Perotti et al.

2015). *Irf1* enhancers feature enrichment sites for several key transcriptional regulators of the hematopoietic and lymphoid system. Occupancy of *Irf1* enhancers by the E-box proteins (E2A and HEB), RUNX1, and TCF-1 indicates that these may contribute to IKAROS induction in the HSCs, with changes in their expression likely contributing to changes in *Irf1* transcription during differentiation (Yoshida et al. 2013). Still, it remains unclear whether and how these factors impact *Irf1* expression. Studies in leukemic pre-B cells have shown that retinoids up-regulate *Irf1* expression and imply a role for the retinoic acid receptor family in IKAROS gene regulation and leukemia treatment (Churchman et al. 2015).

AIOLOS (*Irf3*) is the only lymphoid-restricted member of the IKAROS family that is induced in lymphoid lineage commitment. Notably, both AIOLOS and IKAROS expression is further increased during the post-proliferative preantigen receptor-regulated steps of T-cell and B-cell differentiation and after antigen receptor-mediated lymphocyte activation, indicating an escalated need for the IKAROS family at these developmental and immune cell activation checkpoints as discussed in later sections. Phenotypes detected in *Irf3*-deficient mice are in line with the higher AIOLOS expression in mature and recirculating B cells (Wang et al. 1998; Cortes and Georgopoulos 2004). Furthermore, the combined AIOLOS and IKAROS deficiencies exacerbate immune cell defects reported by loss in either factor (Cortes et al. 1999; Joshi et al. 2014).

HELIOS (*Irf2*) expression is low in the HSCs, extinguished in B-cell precursors, and specifically increased in T-cell precursors and regulatory T cells (Kelley et al. 1998; Thornton et al. 2010; Akimova et al. 2011). The only time when HELIOS is detected in B-cell precursors is after loss of IKAROS as part of a feedback mechanism that also augments AIOLOS expression (Joshi et al. 2014; Schwickert et al. 2014). Loss of HELIOS does not result in any major immune cell phenotypes, most likely due to redundancy with IKAROS and AIOLOS (Cai et al. 2009).

The IKAROS family is also expressed in epithelial cell progenitors. IKAROS is expressed in neuro-epithelial progenitors of the developing striatum, cortical and retinal progenitors, and the developing pituitary (Georgopoulos et al. 1992; Ezzat et al. 2006; Agoston et al. 2007; Martin-Ibanez et al. 2010; Alsio et al. 2013). In addition to neuro-epithelial cells, HELIOS is also expressed in epidermal keratinocytes (Kelley et al. 1998). Finally, EOS/DEDALOS (*Irf4*) is expressed throughout the immune and neuro-epithelial system, albeit at low levels. In contrast to the dramatic defects observed in the immune system upon IKAROS and AIOLOS deficiency, the reported defects in other tissues associated with loss in IKAROS gene family members are subtle, suggesting that these may play a less central role outside the hematopoietic system.

Priming lymphoid cell identity and repressing a stem cell phenotype

The HSC is the root of all hematopoietic and lymphoid lineages. In line with its capacity for multilineage differ-

entiation, the HSC compartment is transcriptionally primed for genes engaged in erythroid, myeloid, and lymphoid differentiation (Fig. 2; Mansson et al. 2007; Ng et al. 2009; Guo et al. 2013; van Galen et al. 2014). Transcriptional priming is defined by low levels of gene expression that are not yet functional and is frequently associated with restricted but not silent chromatin and with activity of factors that function as pioneers in lineage differentiation (Bernstein et al. 2006; Mikkelsen et al. 2007; Zaret and Carroll 2011).

IKAROS is a key contributor to lymphoid lineage transcriptional priming in the HSC and its immediate progeny, the lympho-myeloid primed multipotent progenitor (LMPP) (Ng et al. 2009). IKAROS-dependent transcriptional priming in the HSC and LMPP includes genes encoding membrane receptors and signaling factors such as the *Il7r*, *Flt3*, *Cd79b*, *Notch1*, *Btla*, *Clnk*, *Ltb*, and *Ccr9*, which are key regulators of early T-cell and B-cell differentiation (Fig. 2). Transcriptional priming of chromatin organizers and transcription regulators, such as *Satb1*, *Mef2c*, *Runx2*, *Sox4*, *Foxp1*, *Hdac9*, and *Ets1*, as well as components of the antigen receptor and recombination machinery, such as *Dntt*, *Rag1*, *Cd79b*, *Igj*, and *Ig-6* and $\text{Ig}\mu$ sterile transcripts, is also dependent on IKAROS. Loss in transcriptional priming of these key regulators of lymphocyte differentiation deprives IKAROS-deficient multipotent progenitors of the capacity for lymphocyte differentiation, resulting in the generation of myeloid-only progenitors (Yoshida et al. 2006). Similar to IKAROS, E2A supports lymphoid lineage priming in the HSCs and the LMPPs (Fig. 2; Dias et al. 2008). The overlap in deregulated genes between E2A and IKAROS mutant multipotent progenitors and the proximity of IKAROS- and E-box-binding sites on chromatin suggest cooperation between these two families of transcription regulators in setting up the epigenetic blueprint for lymphocyte differentiation (Dias et al. 2008; Zhang et al. 2011; Hu et al. 2016).

Priming of lineage-specific genes is not mutually exclusive with expression of genes that support stem cell properties. However, expression of HSC-specific genes is gradually attenuated, as expression of lymphoid-specific genes is increased in lineage-restricted progenitors (Fig. 2). This prevents extensive lymphoid progenitor self-renewal and association with cellular environments that support a cancer stem cell phenotype. In addition to its role in lymphoid lineage transcriptional priming, IKAROS is also engaged in the negative regulation of HSC-enriched genes (Ng et al. 2009). Genes that belong to pathways that direct HSC interactions with a specialized niche, quiescence, and the ability to undergo self-renewing divisions are elevated in IKAROS-deficient HSCs and in downstream progenitors such as the LMPP and the granulocyte monocyte progenitor (GMP), where expression of these genes is normally extinguished (Ng et al. 2009).

Thus, in the most primitive multipotent progenitors and prior to any lineage restriction, IKAROS serves two distinct roles. It primes expression of lymphoid-specific genes, possibly by setting a permissive chromatin environment, thereby working as a pioneer factor for lymphocyte differentiation. It also attenuates expression of HSC-

Figure 2. Developmental transitions in the lymphoid lineage. Schematic representations of major events that control early B-cell and T-cell differentiation are shown in parallel. Lineage transcriptional priming is depicted for the HSC, lympho–myeloid primed multipotent progenitor (LMPP), common lymphoid progenitor (CLP), and early thymic progenitor (ETP). The role of IKAROS, E2A, EBF1, and TCF-1 in transcriptional priming is indicated with arrows. Examples of lymphocyte differentiation-promoting genes that are transcriptionally primed by IKAROS in the HSC compartment are shown. The effect of deficiency in IKAROS, AIOLOS, E2A EBF1, PAX5, IRF4, GATA3, TCF-1, and BCL11B is depicted with red bars during developmental progression. IL-7R, Notch1, pre-B-cell receptor (pre-BCR), and pre-T-cell receptor (pre-TCR) signaling is depicted with squiggly arrows. (BLP) B-cell lymphoid progenitor; (DN1–4) CD4⁺CD8⁺ double-negative 1–4 T-cell precursors; (DP) CD4⁺CD8⁺ double-positive thymocytes; (CD4⁺SP and CD8⁺SP) single-positive thymic T cells.

specific genes, preventing an aberrant propagation of stem cell properties into lineage-restricted progenitors.

Lymphoid lineage commitment: an IKAROS time out

Loss of IKAROS in the HSCs interferes with the emergence of early lymphoid lineage-primed and -restricted progenitors such as the LMPPs and the common lymphoid progenitors (CLPs) (Fig. 2; Allman et al. 2003; Yoshida et al. 2006). However, after lymphoid lineage potential is established in these progenitors, IKAROS is not required for further commitment into the T-cell or B-cell lineages (Fig. 2).

Upon entry into the thymus, LMPP-derived early thymic progenitors (ETPs) respond to NOTCH1 signaling by rapidly eliminating the potential for B-cell differentiation (for review, see Rothenberg 2014; De Obaldia and Bhandoola 2015). Induction of TCF-1 and GATA-3 promotes survival and proliferation of early double-negative T-cell precursors (DN1 and DN2) (Germar et al. 2011; Weber et al. 2011). NOTCH1 signaling, TCF-1, GATA-3, and RUNX1, working together at the DN2 stage, up-regulate BCL11B, which further restricts differentiation into the $\alpha\beta$ T-cell lineage (Ikawa et al. 2010; Kueh et al. 2016).

In a parallel process in the bone marrow, a subset of CLPs (also known as B lineage-restricted progenitors [BLPs]) under the influence of IL-7R signaling, E2A, and HEB induce expression of FOXO1 and EBF1 (Inlay et al. 2009; Lin et al.

2010; Welinder et al. 2011). These factors set up a self-promoting feed-forward loop that establishes B-cell identity while eliminating differentiation into other affiliated cell lineages (Nutt et al. 1999; Rolink et al. 1999; Pongubala et al. 2008; Lin et al. 2010; Treiber et al. 2010; Nechanitzky et al. 2013; Boller et al. 2016). FOXO1 and EBF1 support each other's expression, while EBF1 also induces PAX5 that further augments EBF1 (Roessler et al. 2007; Decker et al. 2009; Inlay et al. 2009; Mansson et al. 2012).

Establishment of these core transcription factor networks in T-cell and B-cell precursors is critical for raising the activities of respective signaling pathways required for early lineage transitions (Nutt et al. 1999; Treiber et al. 2010; Nechanitzky et al. 2013; Boller et al. 2016). Key components of the pre-T-cell receptor (pre-TCR) and pre-B-cell receptor (pre-BCR) signaling pathways (recombination factors) as well as transcription and recombination at the antigen receptor loci (*Igh* and *Tcrb*) are examples of genes whose transcription is supported by these early T-cell and B-cell transcription factor networks (Schebesta et al. 2007; Treiber et al. 2010; Welinder et al. 2011; Mansson et al. 2012).

Thus, although key signaling and recombination factors, hallmarks of lymphocyte differentiation, are primed in the HSCs by IKAROS and the E-box proteins, they reach operational level only after a select combination of transcription factors is induced in early T-cell and B-cell precursors.

The preantigen receptor checkpoint: IKAROS is back in the game

After restriction into the T-cell and B-cell lineages is in place and concomitant with assembly of the respective preantigen receptor signaling complexes comes a massive proliferative expansion with production of T-cell and B-cell precursor pools from which the mature T-cell and B-cell repertoires are selected (Fig. 2). Pre-TCR in combination with the Notch1 receptor is responsible for the proliferative expansion of DN thymocytes and differentiation into the double-positive (DP) stage, whereas pre-BCR in combination with IL-7R is responsible for the expansion and differentiation of pre-B-cell precursors (Fig. 2).

Although loss of IKAROS does not prevent the transition from the proliferative DN3 to the quiescent DP, it causes an aberrant reactivation of NOTCH1 signaling in DP thymocytes during the TCR selection process (Dumortier et al. 2006; Kleinmann et al. 2008; Gómez-del Arco et al. 2010). NOTCH1 expression in DP is directly repressed by IKAROS working at both canonical and alternative NOTCH1 promoter sites (Gómez-del Arco et al. 2010). Aberrant induction of NOTCH1 at this stage of differentiation causes rapid leukemic transformation.

In contrast to T-cell differentiation, loss of IKAROS arrests B-cell differentiation at the proliferative large pre-B cell and prevents transition to the quiescent small pre-B

cell (Fig. 2; Heizmann et al. 2013; Joshi et al. 2014; Schwickert et al. 2014). This is not just a block in differentiation but also a dramatic gain in cell adhesion that is manifested not only in vivo but also upon IKAROS deletion in established wild-type pre-B-cell cultures, indicating a continued requirement for IKAROS repression of the mechanisms that support this cellular property (Joshi et al. 2014; Hu et al. 2016).

Normal large pre-B cells display low-frequency interactions with bone marrow-derived stroma with limited self-renewal (Fig. 3). IKAROS-deficient large pre-B cells show remarkable stable stromal interactions and the ability to self-renew (Joshi et al. 2014). Mutant pre-B cells attached to stroma display a squamous-like phase-dark morphology with numerous filipodia and do not migrate. Unlike normal pre-B cells that differentiate upon stromal detachment, IKAROS deficient pre-B cells undergo an anoikis-type of cell death. Taken together, these cellular properties indicate that loss of IKAROS in pre-B cells awakens properties that are normally displayed by cells dependent on cell–matrix interactions for growth and survival.

Notably, IKAROS-deficient adherent pre-B cells express increased levels of pre-BCR. However, signaling through the pre-BCR is compromised due to loss in the key downstream tyrosine kinases SYK, LYN, FYN, and BLK (Joshi et al. 2014; Schwickert et al. 2014). Instead, integrin combined with IL-7R signaling supports the adhesion-

Figure 3. IKAROS regulation of enhancers in pre-B cells. IKAROS is uniquely required for the highly permissive chromatin of superenhancers (SE) of genes required for pre-B-cell differentiation. At the same time, IKAROS directly represses poised or inactive enhancers of genes that support an epithelial cell-like phenotype. The PRC2 complex is also present at the promoters of the IKAROS-repressed genes. Among the repressed IKAROS targets are extralinear “master transcription regulators” that, upon IKAROS loss, cooperate with B-cell transcription factors to provide a de novo superenhancer landscape that supports a dramatic increase in self-renewal (circular arrows), integrin-mediated signaling, cell adhesion, and glucocorticoid resistance seen in IKAROS-deficient pre-B cells. These are also properties of high-risk *IKZF1* mutant pre-B-cell precursor acute lymphoblastic leukemia (B-ALL) that also feature constitutive activation of tyrosine kinases such as BCR–ABL or the JAK2–PDGFR signaling complex.

mediated survival and proliferation of IKAROS-deficient pre-B cells, a process that is sensitive to inhibition of the focal adhesion tyrosine kinase (FAK), an intracellular mediator of integrin signaling (Joshi et al. 2014).

The role of IKAROS in promoting pre-B-cell differentiation and preventing excessive self-renewal is reminiscent of its role in the HSC compartment. In stem cells, multipotent progenitors, and committed large pre-B cells there is a significant overlap in genes that are negatively regulated by IKAROS and belong to pathways that support cell–substrate adhesion and interactions with the microenvironment (Ng et al. 2009; Joshi et al. 2014). In contrast, the majority of IKAROS gene targets that are derepressed upon IKAROS loss in the HSCs and large pre-B cells remains unchanged in IKAROS-deficient DN and DP thymocytes (Zhang et al. 2011). These mutant thymocytes show no stromal-dependent growth or self-renewal properties. Instead, survival and proliferation of IKAROS-deficient thymocytes are dependent on aberrant induction of Notch1 signaling (Dumortier et al. 2006; Kleinmann et al. 2008; Gómez-del Arco et al. 2010). Thus, although preantigen receptor checkpoints during both T-cell and B-cell differentiation use IKAROS, they do so through distinct molecular mechanisms that underscore the unique properties and environment of T-cell and B-cell precursors.

IKAROS-positive and IKAROS-negative regulation of enhancers in B-cell precursors

Given the conservation in IKAROS-based transcriptional mechanisms prior to and after B-cell lineage commitment, a study on the IKAROS *modus operandi* in pre-B cells has provided key insight into the regulation of this developmental process and the origins of high-risk pre-B-cell precursor leukemias (Hu et al. 2016). IKAROS chromatin distribution studies in large pre-B cells have revealed that this factor is engaged mostly at enhancer sites classified as active or poised/inactive by their immediate chromatin environment (Fig. 3; Hu et al. 2016).

Active enhancers bound by IKAROS are also occupied by B-cell transcription factors, such as EBF1, PAX5, E2A, and IRF4, implicating their functional interaction at this developmental stage. Clustering of IKAROS-bound enhancer sites, strong enrichment by B-cell transcription factors and the Mediator complex, and a highly permissive chromatin environment suggested a superenhancer status for these regulatory domains (Hu et al. 2016). Both the role of associated genes as key regulators of pre-B-cell differentiation and their strong level of expression validated this hypothesis. Notably, these pre-B-cell superenhancers were functionally defined by IKAROS, as both their highly permissive chromatin and strong effect on transcription were attenuated upon IKAROS loss despite the continued presence of other B-cell transcription factors and the Mediator complex at these sites (Fig. 3). Thus, IKAROS functions as part of a B-cell transcription factor network that associates with pre-B-cell superenhancers, and is essential for their activity.

The majority of IKAROS-associated enhancers in the large pre-B cell can be classified as inactive or poised (Hu et al. 2016). This is based on the relative paucity of permissive histone modifications; low occupancy by B-cell transcription factors, the Mediator, and RNA polymerase II complexes; and the limited expression of associated genes (Fig. 3). Loss of IKAROS at many of these poised enhancers leads to their rapid induction. Several of these now active enhancers contribute to a *de novo* superenhancer network that is affiliated with genes and pathways that are prevalent in nonlymphoid cells.

A banned network of extralinearage and B-cell transcription factors

Among the genes that are repressed by IKAROS in pre-B cells is a group of transcription factors that are key regulators of cell identity in nonlymphoid cells (Fig. 3; Hu et al. 2016). LMO2 and HOXB5–7 are expressed at an earlier stage of hematopoiesis, while TBX19, LHX2, TEAD1/2, and YAP1 are expressed in a variety of stem cells and progenitor cells of nonhematopoietic origin. As several of these factors support their own expression and that of the others, loss of IKAROS sets in motion a powerful feed-forward cross-regulatory loop that underscores the rapid induction of both “extralinearage” transcription factors and their downstream targets (Fig. 3).

The network of inactive enhancers occupied by IKAROS in pre-B cells is also heavily occupied by these “extralinearage” transcription factors when IKAROS is depleted (Fig. 3). A wild card in this cryptic regulatory network is the B-cell transcription factors that are normally excluded from the repressed enhancer landscape of large pre-B cells. In the absence of IKAROS, B-cell transcription factors gain access to these sites and, together with the “extralinearage” transcription factors, induce an “epithelial-like” gene expression program. Thus, IKAROS not only represses expression of “extralinearage” transcription factors but also restricts the function of B-cell transcriptional regulators to lineage-appropriate gene targets. Whether this is a direct or indirect effect on IKAROS on B-cell transcription factor redistribution remains to be seen. Nonetheless, unleashing these two activities rapidly induces a hybrid B–epithelial-like cell identity (Hu et al. 2016).

IKAROS and Polycomb repression of a B-cell–epithelial transition

Genes that are normally repressed by IKAROS in pre-B cells are also occupied by the Polycomb complex, and loss of IKAROS correlates with Polycomb eviction (Hu et al. 2016). However, IKAROS is located mostly at enhancers, whereas Polycomb proteins are present mostly at the promoters of these genes. A physical interaction between IKAROS and Polycomb is not seen either on or off chromatin, suggesting that the functional interplay between these two factors is indirect. Instead, the level of *de novo* enhancer activation appears to drive Polycomb

eviction. In support of this hypothesis, genes associated with de novo superenhancers display the most Polycomb eviction (Hu et al. 2016). Thus, IKAROS and Polycomb may contribute independently to the repression of a stem-epithelial cell program in large pre-B cells. This is reminiscent of the HUNCHBACK–Polycomb repression of *Hox* genes in the *Drosophila* embryo, with HUNCHBACK initiating and Polycomb maintaining repression during development.

The IKAROS fall and rise of high-risk pre-B-cell precursor leukemias

Loss of IKAROS reprograms the epigenetic and transcriptional landscape in support of an “altered” pre-B-cell identity with properties such as stromal adhesion, self-renewal, and drug resistance that are similar to those described in high-risk human pre-B-cell precursor acute lymphoblastic leukemias (B-ALLs) with *IKZF1* mutations (Martinelli et al. 2009; Mullighan et al. 2009). However, IKAROS-deficient pre-B cells are not leukemic. In fact, they are more sensitive to apoptosis caused by stromal detachment compared with their wild-type counterparts (Joshi et al. 2014). For leukemic transformation, a second event that supports the survival of IKAROS-deficient pre-B cells both on and off stroma has to occur.

In human high-risk B-ALL, activating mutations in tyrosine kinases affiliated with growth factor receptor signaling are frequently encountered (Roberts et al. 2014). IKAROS mutations that produce protein variants that are unable to bind DNA but can dimerize and interfere with the activity of functionally intact IKAROS family members are also enriched in high-risk B-ALL (Sun et al. 1996; Iacobucci et al. 2008a,b; Dupuis et al. 2013). The constitutively active form of the ABL kinase produced by the Ph⁺ chromosome translocation almost always accompanies IKAROS mutations encountered in high-risk B-ALL (Mullighan et al. 2008; Martinelli et al. 2009). In line with these genome-wide association studies, in a va-

riety of mouse models, *Ikzf1* mutations have been shown to accelerate the development of pre-B-cell leukemias in the context of *BCR-ABL1* (Virely et al. 2010; Schjerven et al. 2013).

Analysis of the signaling and transcription profiles of high-risk B-ALL samples has provided evidence for a conservation of IKAROS-mediated repression of cell adhesion signaling and a compelling rationale for the use of small molecule inhibitors for IKAROS gene targets in the treatment of human disease. FAK is a key effector of integrin signaling that is repressed by IKAROS in large pre-B cells (Joshi et al. 2014). A combination therapy with inhibitors for the ABL and FAK shows promise in reducing adhesion properties and leukemia-initiating frequencies in human B-ALL (Churchman et al. 2016). Thus, previously established protocols combined with recent insights into the IKAROS-based signaling and transcriptional networks in pre-B cells can provide more effective approaches to target high-risk B-ALL.

Ikeros regulation of mature lymphocyte antigenic responses

The role of the IKAROS gene family in the mature B-cell compartment is highlighted by AIOLOS studies (Fig. 4). Unlike the lack of phenotype at the early stages of B-cell differentiation, loss of AIOLOS in mature B cells causes an increase in BCR-mediated proliferative responses (Wang et al. 1998). AIOLOS restricts BCR signaling while supporting FcγR inhibitory signaling, thereby raising the threshold for antigen-mediated B-cell activation. Similar to AIOLOS in mature B cells, IKAROS in mature T cells raises the threshold for activation in response to TCR stimulatory and costimulatory signals (for review, see Avitahl et al. 1999; Cortes et al. 1999; Winandy et al. 1999). Additionally, mature B cells and T cells with combined *Ikzf1* and *Ikzf3* mutations show a further increase in antigen-mediated proliferative responses, indicating that the two factors work together to control the magnitude

Figure 4. Long-lived mature B cells are dependent on the IKAROS family. AIOLOS and IKAROS raise the threshold for antigenic stimulation in mature B cells. Both B-cell proliferative responses and T-cell-mediated B-cell differentiation into a germinal center (GC) reaction are augmented by AIOLOS deficiency. Although affinity maturation in the germinal center or production of short-lived high-affinity plasma cells in the spleen are not affected, the long-lived high-affinity plasma cells that reside in the bone marrow are absent. Notably, MM, the malignant counterpart of this plasma cell population, is also dependent on AIOLOS and IKAROS. Treatment with IMiDs such as lenalidomide induces CRL4^{CRBN}-mediated degradation of AIOLOS and IKAROS and compromises the ability of MM cells to grow. Other long-lived mature B cells such as peritoneal B-1a cells and marginal zone B (MZ B) cells are also affected by AIOLOS deficiency. Thus, the IKAROS family is critical for maintenance of terminally differentiated B cells, possibly by supporting their ability to self-renew (circular arrows).

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of an immune response (Cortes et al. 1999; M Cortes, unpubl.).

Phenotypes that range from expansion of peripheral B cells to an aberrant increase in germinal centers, serum IgG, IgE, and autoantibodies to B-cell lymphomas are manifested in the aging AIOLOS-deficient mice (Wang et al. 1998). However, the long-lived high-affinity antibody-secreting B cells that reside in the bone marrow (McHeyzer-Williams and Ahmed 1999), responsible for rapid recall to antigenic stimulation, are absent (Cortes and Georgopoulos 2004). Since AIOLOS is not required for the generation of the short-lived high-affinity plasma cells in the spleen, the defect could be due to an inability of high-affinity plasma cells or of their immediate precursors to survive in the bone marrow microenvironment.

Long-lived high-affinity plasma cells that are dependent on AIOLOS activity are the source of MM, a devastating B-cell malignancy (Anderson and Carrasco 2011). Notably, IMiD-induced degradation of AIOLOS and IKAROS adversely affects the cellular fitness of MM cells (Kronke et al. 2014a; Lu et al. 2014). One can speculate that the IKAROS- and AIOLOS-based regulatory pathways that modulate pre-B-cell–bone marrow stromal interactions to provide a balance between self-renewal, survival, and differentiation may also be involved in the longevity of bone marrow plasma cells and their malignant counterparts. Further exploration of these IKAROS targeted pathways in bone marrow plasma cells is warranted.

In addition to long-lived plasma cells, peritoneal B-1a cells, marginal zone B cells and recirculating B cells are also absent in AIOLOS-deficient mice (Fig. 4; Wang et al. 1998). An aberrant increase in BCR signaling that adversely affects development of these B cells or their long-term maintenance may be responsible for the preferential loss of these long-lived nonconventional B cells.

Future perspectives

Understanding and manipulating the IKAROS-based regulatory mechanisms remains a challenge for both the lymphocyte biologist and clinicians dealing with lymphoid neoplasms branded with either IKAROS-inactivating mutations (i.e., B-ALL) or an increase in IKAROS activity (i.e., MM).

IKAROS-based transcription activation and repression networks are well established; however, it is still unclear how IKAROS chooses between different functions and chromatin locations. Establishing the order of events that ensue upon IKAROS loss, such as B-cell transcription factor redistribution at normally IKAROS-repressed enhancer sites simultaneously or after prior accumulation of “extralinear” transcription factors at these sites, and how this impacts Polycomb eviction from these genes may provide a new paradigm for lineage regulation. This line of study may also yield new therapeutic targets for high-risk leukemias.

Analysis of IKAROS gene targets in pre-B cells has provided new therapeutic strategies that are currently in clinical trials for B-ALL. Regulatory mechanisms that control

IKAROS at the protein level are already used for the treatment of MM. Nonetheless, resistance frequently develops during these treatments, and insights into the IKAROS mechanism of action in these cells may also provide alternative methods to treat these devastating diseases. Finally, one should not forget that a major role of the IKAROS family in mature lymphocytes is to control their ability to respond to antigenic stimulation by raising the bar for both B-cell and T-cell activation. If one could alter IKAROS activities on demand in T-cell effectors, one could potentially improve on immunotherapy-based approaches and be better prepared for the race against cancer.

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