Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling

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Abstract | The pre-B-cell receptor (pre-BCR) is expressed following the productive recombination of the immunoglobulin heavy chain gene. Signals through the pre-BCR are required for initiating diverse processes in pre-B cells, including proliferation and recombination of the light chain gene, which eventually lead to the differentiation of pre-B cells to immature B cells. However, the molecular mechanisms by which the pre-BCR promotes these processes remain largely unresolved. Recent findings suggest that forkhead box O (FOXO) transcription factors connect pre-BCR signalling to the activation of the recombination machinery. In this Review, we discuss how FOXO transcription factors are regulated by the pre-BCR to allow the progression of the cell cycle and the recombination of the light chain gene.

V(D)J recombination
Somatic rearrangement of variable (V), diversity (D) and joining (J) regions of the genes that encode antigen receptors, leading to repertoire diversity of both B- and T-cell receptors.

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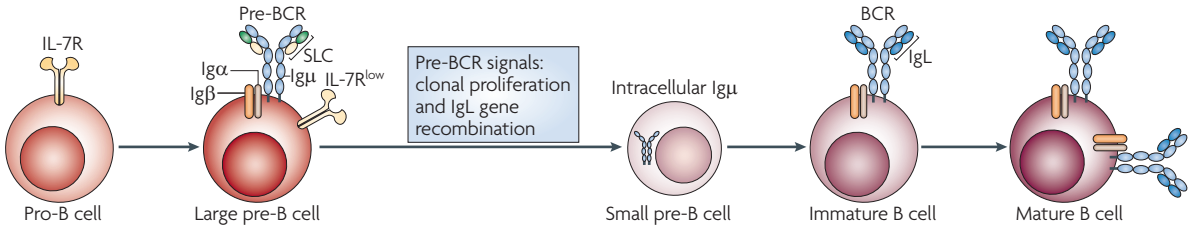
The enormous variability of B-cell receptor (BCR) specificities in the pool of mature B cells is generated during early B-cell development in a process known as V(D)J recombination, in which the variable (V) regions of the immunoglobulin heavy chain (IgH) and immunoglobulin light chain (IgL) are assembled from V, diversity (D), joining (J) and V, J gene segments, respectively[1]. The discovery that IgH and IgL genes are recombined in a sequential manner suggested that developing B cells are tested for successful recombination events at defined checkpoints before progressing to the next step of development. The expression of the pre-BCR emerged as an important checkpoint for IgH recombination following the finding that Igμ, which is the product of a successfully recombined IgH gene, associates with the germline-encoded surrogate light chain (SLC) to form a receptor complex on precursor B cells[2,3]. Therefore, the expression of the pre-BCR indicates that the recombination of the IgH gene was successful and regulates key processes in developing B cells. Once the pre-BCR is expressed on the cell surface, pre-BCR signalling induces a burst of pre-B-cell proliferation, which serves to increase the number of cells that have a successfully recombined IgH gene[4]. In addition, signalling through the pre-BCR is involved in activating the recombination of the IgL gene and is therefore required for the continuation of B-cell differentiation[5]. It is obvious that the pre-BCR-induced cell expansion and the subsequent recombination of the IgL gene require tight regulation to avoid uncontrolled proliferation and genomic instability, which might result in cellular transformation and the development of leukaemia. In this Review, we discuss how pre-BCR signalling switches from inducing cell proliferation to inducing cell differentiation and from inducing downregulation to inducing upregulation of the recombination machinery, resulting in cell differentiation.

The pre-B-cell checkpoint in B-cell development

The development of B cells is characterized by the differential expression of marker proteins and by the stepwise recombination of the immunoglobulin gene loci[6] (FIG. 1). Immunoglobulin gene recombination is initiated by the RAG1 (recombination-activating gene 1)–RAG2 protein complex, which generates double-stranded DNA breaks between gene segments and specific recognition sites that are also known as recombination signal sequences[7,8]. The cleaved DNA ends are then joined by a ubiquitously expressed set of non-homologous end joining proteins, which repair double-stranded DNA breaks irrespectively of sequence homology[9].

B cells in the bone marrow develop from a pool of multipotential stem cells, the immunoglobulin loci of which are in a germline configuration. RAG-mediated immunoglobulin gene rearrangement is first initiated on the IgH locus at the common lymphoid progenitor (CLP) or early pro-B-cell stage of development with D_H to J_H rearrangements followed by V_H to DJ_H rearrangements at later pro-B-cell stages[10]. Productive in-frame



Gene locus	IgH	DJ	VDJ	VDJ	VDJ	VDJ
	IgL	Germline	Germline	VJ	VJ	VJ
Surface immunoglobulin		–	Igμ and SLC (pre-BCR)	–	Igμ (IgH) and Igκ and Igλ (IgL)	Igμ (IgH) and Igκ and Igλ (IgL)

Figure 1 | **The pre-BCR and B-cell development.** Rearrangement of the immunoglobulin heavy chain (IgH) locus is initiated at the common-lymphocyte-progenitor or pro-B-cell stage and, if successful, gives rise to the Igμ chain that is expressed on the cell surface in the form of the pre-B cell receptor (pre-BCR) at the large pre-B-cell stage. Signalling from the pre-BCR provides rapid feedback about the functionality of the recombined Igμ and induces clonal proliferation, downregulation of pre-BCR components and recombination of immunoglobulin light chain (IgL) genes. In-frame IgL gene rearrangements in small pre-B cells result in the expression of a BCR that is composed of two Igμ chains and two Igκ (the κ-chain of IgL) or Igλ (the λ-chain of IgL) chains on immature B cells. These immature B cells are subjected to selection processes and eventually enter the pool of long-living mature B cells. D, diversity; J, joining; IL-7R, interleukin-7 receptor; SLC, surrogate light chain; V, variable.

VDJ recombination gives rise to the Igμ chain, which is expressed by pre-B cells as part of the pre-BCR complex[11]. Similar in structure to the BCR, the pre-BCR comprises two Igμ chains and two SLCs that are associated with the signalling subunits Igα and Igβ. In contrast to a conventional IgL, the SLC is a heterodimer composed of two germline-encoded invariant proteins, VpreB and λ5.

Although it is only transiently expressed, the pre-BCR marks an important checkpoint in B-cell development. Indeed, signals from the pre-BCR provide rapid feedback about the functionality of the recombined Igμ gene, so only pre-B cells that express a signalling-competent receptor can mature further. The importance of the pre-BCR in B-cell development is apparent in mice that have a targeted deletion of distinct pre-BCR components. For example, in *μmt*$^{-/-}$ mice, deletion of the exon that encodes the transmembrane region of Igμ prevents the expression of the membrane-bound Igμ chain during B-cell development[12]. This results in a twofold enrichment of pro-B cells and a complete block of B-cell development beyond this stage. Similarly, B-cell development in mice that lack either Igα or Igβ is arrested at the pro-B-cell stage, although VDJ recombination and intracellular Igμ expression are not affected[13,14]. Deficiency in the SLC component λ5 leads to a block at the pro- to pre-B-cell transition in the bone marrow, resulting in a marked decrease in the number of mature B cells in the periphery[15]. The same phenotype has been reported in mice that lack both VpreB1 and VpreB2, two isoforms of the SLC component VpreB in mice that are encoded by genes which share 98% homology in the coding regions[16]. The fact that B-cell development is not completely blocked in the absence of SLC components (in contrast to RAG1 or RAG2 deficiency) indicates that, under certain conditions, a BCR with a conventional IgL chain can replace the pre-BCR and induce the differentiation of B cells[17]. In addition, several studies have identified receptors that contain Igμ but not the SLC on early B cells, suggesting that the Igμ chain can be expressed on the cell surface in the absence of both the SLC and the conventional IgL chain[18–20]. These SLC-deficient Igμ-containing receptors, in addition to the prematurely expressed IgL chains, might explain the residual B-cell development that can occur in the absence of SLC expression[21]. It is possible that these unusual receptors mimic pre-BCR signalling, thereby allowing further differentiation. However, it should be noted that the partial B-cell developmental rescue that occurs in the absence of SLC is restricted to mice, as B-cell development is severely blocked and accompanied by agammaglobulinaemia in humans that lack expression of the SLC component λ5 (REF. 22). This suggests that both expression and signalling of the conventional pre-BCR are required for initiating human pre-B-cell development.

Pre-BCR signalling leads to the downregulation of λ5 gene expression and, consequently, to the termination of SLC expression[23,24]. Thus, the pre-BCR seems to activate a negative feedback loop that results in the termination of pre-BCR expression and signalling. It might therefore be speculated that the induction of IgL gene rearrangement and B-cell differentiation is induced solely by the termination of pre-BCR signalling and that no additional pre-BCR-specific signals are required for the activation of differentiation. However, accumulating data (discussed below) show that the pre-BCR is actively involved in the initiation of IgL gene rearrangement.

Another important role of pre-BCR signalling is to inhibit V_H to DJ_H recombination at the second IgH allele and to establish allelic exclusion, which is an integral step of B-cell differentiation and a mechanism by which to ensure that BCRs of a single specificity are expressed on the cell surface[1,25] (BOX 1). Available data suggest that a productive V(D)J recombination event at the IgH gene locus and subsequent expression of a pre-BCR result in

Recombination signal sequences
Conserved elements that constitute recognition sites for the V(D)J recombinase proteins, which are encoded by *RAG1* (recombination-activating gene 1) and *RAG2*. They consist of a palindromic heptamer that is immediately adjacent to the coding gene segments — V (variable), D (diversity) or J (joining) — and is separated by a 12- or 23-base-pair spacer from a conserved nonamer sequence.

Non-homologous end joining
The process that joins broken DNA ends without depending on extended homology. Components of this pathway include the proteins Ku70, Ku 80, ARTEMIS, X-ray repair cross-complementing protein 4 (XRCC4), DNA ligase IV and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs).

μmt$^{-/-}$ mice
Mice that carry a stop codon in the first membrane exon of the Igμ constant region. They lack IgM$^+$ B cells, and B-cell development is arrested before the differentiation stage at which IgD can be expressed.

the downregulation of the expression of the RAG1–RAG2 recombination machinery, thereby inhibiting further gene rearrangements and ensuring that only one Igμ is expressed[26]. However, the expression of a functional pre-BCR triggers IgL gene recombination[5] and must therefore also induce the expression of the recombination machinery. This indicates that RAG1 and RAG2 downregulation has to be reversed either by specific pre-BCR signals or, more passively, by the termination of pre-BCR expression. Interestingly, by using a cellular system in which receptor signalling can be induced in a precise temporal manner, pre-BCR expression was found to mediate the expression of the RAG1–RAG2 complex (S.H., unpublished observations) and to promote the accessibility of the IgL gene for recombination[27].

Initiation of pre-BCR signalling

As signalling in B cells has mainly been studied in the context of the BCR, signalling downstream of the pre-BCR is less well characterized. However, available data indicate that similar signalling complexes are formed following the engagement of the BCR and the pre-BCR, which suggests that the two receptors use the same main signal transduction pathways[28].

The main difference between the BCR and the pre-BCR is how signalling is initiated. In contrast to the triggering of BCR signalling by ligand (that is, antigen) binding, it is still unclear whether pre-BCR signalling is ligand dependent or whether the main mechanism for signal initiation is autonomous aggregation of pre-BCR complexes on the cell surface. Possible candidates for pre-BCR ligands have been identified on stromal cells[29,30], but pre-BCR-mediated proliferation and differentiation of pre-B cells have been found not to rely on the bone marrow or fetal liver environment *in vitro*[31]. Further studies have shown that the positively charged non-immunoglobulin portion of λ5 is fundamental for the initiation of pre-BCR aggregation and signalling[32]. Moreover, recent data indicate that the positively charged non-immunoglobulin portion of λ5 is polyreactive and consequently allows the pre-BCR to interact with multiple structures, including DNA, insulin, lipopolysaccharide, galectin and heparan sulphate[29,30,33]. This suggests that, once expressed on the surface, single pre-BCR molecules are immediately aggregated because of the polyreactive potential of the non-immunoglobulin region of λ5, thereby resulting in high-molecular mass pre-BCR complexes, which facilitate signal initiation by recruiting an increased number of important signalling proteins.

SYK proliferation and differentiation

One of the first steps following pre-BCR or BCR engagement is the activation of the SRC-family protein tyrosine kinase LYN and the cytoplasmic tyrosine kinase SYK (spleen tyrosine kinase), both of which phosphorylate target sequences, such as tyrosine residues in immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of Igα and Igβ[34,35]. Binding of SYK to a phosphorylated ITAM promotes the autophosphorylation of SYK, as well as its phosphorylation by other SRC kinases, resulting in an increase in its kinase activity[36–38]. Subsequently, the SYK-mediated phosphorylation of ITAMs of neighbouring pre-BCR or BCR complexes amplifies the signal and initiates a positive feedback loop[39]. Ultimately, SYK has a central role in the activation of pathways that regulate the proliferation and differentiation of pre-B cells, as shown in studies of knockout mice (FIG. 2).

The role of SYK in promoting B-cell proliferation is shown by the phenotype of SYK-deficient B cells, which have a developmental block at the pre-B-cell stage and fail to undergo clonal expansion[40,41]. This indicates

Box 1 | **Allelic exclusion**

During their development, B cells generate diverse antigen receptor specificities by randomly assembling gene segments using V(D)J recombination. However, although each cell has many allelic loci for the different receptor chains (two immunoglobulin heavy chain (IgH) loci and multiple immunoglobulin light chain (IgL) loci), each mature cell ultimately expresses only a single type of receptor. This is accomplished through restriction of antigen receptor gene expression to a single allele in a process referred to as allelic exclusion.

Allelic exclusion is supported by two processes, namely monoallelic activation and feedback inhibition. Monoallelic activation prevents recombination of the gene encoding the receptor from occurring simultaneously on both alleles: that is, one allele undergoes rearrangement before the other one does. The choice of allele that undergoes rearrangement mainly depends on epigenetic marks, which include DNA methylation, histone modification, heterochromatization, locus contraction and positioning of the loci within the nucleus[112]. Feedback inhibition suppresses continuous rearrangements once recombination has been successful.

Two possible mechanisms have been proposed to account for monoallelic activation and, consequently, asynchronous recombination[112]. The stochastic model postulates that the two alleles are equivalent at the time of recombination, leaving the choice between the two to chance. As the successful recombination of variable (V), diversity (D) and joining (J) gene segments is a rare event, it is probable that only one in-frame IgH or IgL gene is produced within a given time frame. Evidence for this model comes from studies using transgenic mice that express green florescent protein (GFP) under the control of an Igκ (the κ-chain of IgL) allele that has not yet been rearranged. Results from these studies indicate low frequency activation and stochastic usage of the two equivalent alleles in pre-B-cells that are undergoing V to J recombination[113]. As the recombination machinery itself can operate at high frequencies[114], it has been proposed that the IgH or IgL alleles compete for a limiting amount of the transcription factors that are required for the rearrangement.

By contrast, the deterministic model postulates that the initial decision for one of the alleles to recombine occurs early during embryogenesis, but then becomes instructive and is maintained throughout development[112]. Consequently, the two alleles are distinguishable at the time of recombination, which predetermines one allele to undergo monoallelic activation.

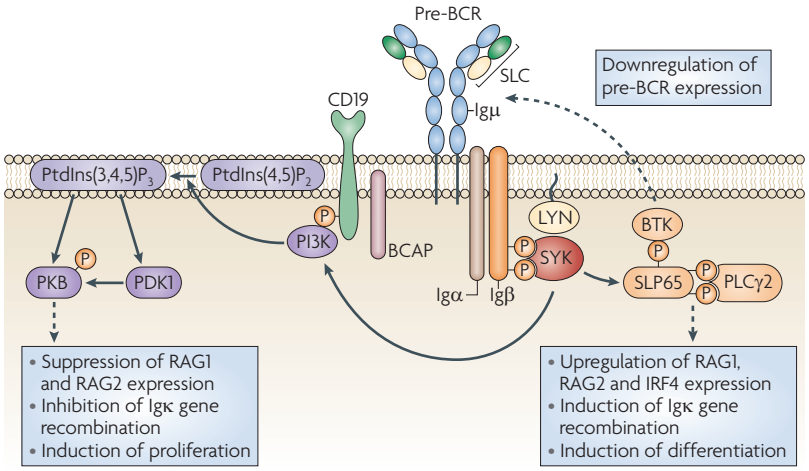


Figure 2 | **The central role of SYK downstream of the pre-BCR.** Pre-B-cell receptor (pre-BCR) engagement results in the activation of SYK (spleen tyrosine kinase), which together with SRC-family protein tyrosine kinases, such as LYN, phosphorylates diverse substrates downstream of the receptor. This triggers signalling pathways that are involved in both proliferation and differentiation of pre-B cells. An important signalling event downstream of SYK that is implicated in cell proliferation and survival is the activation of the phosphoinositide 3 kinase (PI3K) pathway. SYK and SRC-family protein tyrosine kinases phosphorylate the co-receptor CD19 and/or the adaptor protein B-cell PI3K adaptor (BCAP), which recruit and activate PI3K. PI3K activation results in the generation of the second messenger phosphatidylinositol-3,4,5-trisphosphate ($PtdIns(3,4,5)P_3$), which recruits signalling molecules that have a pleckstrin-homology domain to the plasma membrane. These molecules include the serine/threonine kinase protein kinase B (PKB), which is the dominant mediator for controlling cellular proliferation downstream of PI3K, and its activating kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1). Ongoing signalling through the PI3K–PKB axis suppresses recombination-activating gene 1 (RAG1) and RAG2 expression, blocks Igκ (the κ-chain of the immunoglobulin light chain) gene recombination and induces cell proliferation. Activation of another key substrate of SYK, SH2-domain-containing leukocyte protein of 65 kDa (SLP65), then organizes a molecular complex that consists of, among others, Bruton's tyrosine kinase (BTK) and phospholipase Cγ2 (PLCγ2). Signalling by the pre-BCR through this complex results in the expression of RAG proteins and interferon-regulatory factor 4 (IRF4). This results in the opening of the Igκ locus through the binding of IRF4 to the 3′Eκ enhancer and induces Igκ recombination. Ultimately, this drives the differentiation of pre-BCR$^+$ cells to BCR$^+$ cells. $PtdIns(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; SLC, surrogate light chain.

that SYK is required for the transduction of pre-BCR-mediated proliferation signals. The important role of SYK in pre-B-cell proliferation is also supported by the observation that constitutively active or overexpressed SYK functions as an oncogene by promoting growth-factor-independent proliferation of pre-B cells and inhibiting differentiation, including the recombination of the IgL genes[42]. However, the exact mechanism by which constitutively active or overexpressed SYK suppresses differentiation is unclear.

With regard to proliferation, it has been shown that constitutively active SYK induces constitutive phosphorylation and activation of the lipid-modifying kinase phosphoinositide 3 kinase (PI3K), which regulates diverse biological processes, including cell growth, survival, proliferation, migration and metabolism[43–45]. Downstream of the pre-BCR or BCR, SYK and SRC-family protein tyrosine kinases induce phosphorylation of the co-receptor protein CD19 and/or the adaptor protein B-cell PI3K adaptor (BCAP; also known as PIK3AP1), resulting in the recruitment and activation of PI3K[43,46]. Subsequently, PI3K phosphorylates its substrate phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$), thereby generating the second messenger, phosphatidylinositol-3,4,5-trisphosphate ($PtdIns(3,4,5)P_3$)[47]. Several signalling proteins, including the serine/threonine kinase protein kinase B (PKB; also known as AKT) and 3-phosphoinositide-dependent protein kinase 1 (PDK1), contain a pleckstrin-homology domain that binds $PtdIns(3,4,5)P_3$, which allows their recruitment to the plasma membrane[48]. PKB, which becomes activated by PDK1-mediated phosphorylation, is thought to be the main mediator of the PI3K signalling pathway and induces cell proliferation through the inhibition of multiple downstream targets that are involved in the control of the cell cycle[49].

Because SYK activates PI3K downstream of the pre-BCR, tight regulation of pre-BCR-induced signalling is important to avoid abnormal PKB activity, which can result in uncontrolled cellular expansion and malignant transformation. In agreement with this, increased pre-BCR expression is associated with high incidence of pre-B-cell leukaemia in mice that are deficient for the adaptor protein SH2-domain-containing leukocyte protein of 65 kDa (SLP65; also known as BLNK and BASH), which can impair PKB signalling (see later)[4]. Owing to impaired downregulation of λ5 expression, *Slp65*$^{-/-}$ pre-B cells express high amounts of the pre-BCR on their surface and exhibit enhanced proliferation *in vitro*. Moreover, *Slp65*$^{-/-}$ mice develop pre-B-cell leukaemia, and a drastic reduction of SLP65 expression has been observed in B cells from patients with human pre-B acute lymphoblastic leukaemia[50]. These observations suggest that SLP65 has a tumour-suppressor function *in vivo* by reducing B-cell proliferation[51,52]. However, it should be noted that there is a synergism between the pre-BCR and interleukin-7 receptor (IL-7R) in the activation of pre-B-cell proliferation. Therefore, survival and proliferation of early B cells is influenced by IL-7 and its receptor in addition to pre-BCR signalling[53–55] (BOX 2).

SYK is also involved in another important function of the pre-BCR, facilitating IgL chain gene recombination and subsequent differentiation of pre-B cells to immature B cells. An important substrate of SYK that mediates pre-B-cell differentiation is SLP65. SYK phosphorylates SLP65 on multiple tyrosine residues, thereby allowing it to function as a molecular scaffold protein and to provide docking sites for SRC-homology-2-domain-containing signalling mediators, such as Bruton's tyrosine kinase (BTK), phospholipase Cγ2 (PLCγ2) and growth-factor-receptor-bound protein 2 (GRB2)[28,56,57]. By recruiting these signalling mediators, SLP65 nucleates a signalosome that regulates the activation of important cellular responses, including pre-B-cell differentiation.

SLP65 function is required for both the downregulation of λ5, which terminates SLC expression, and the activation of Igκ (the κ-chain of IgL) gene recombination[23]. The SLP65-mediated downregulation of λ5 expression is achieved by the induction of the expression of the transcription factor Aiolos, which leads to efficient silencing of *Igll1*, the gene that encodes

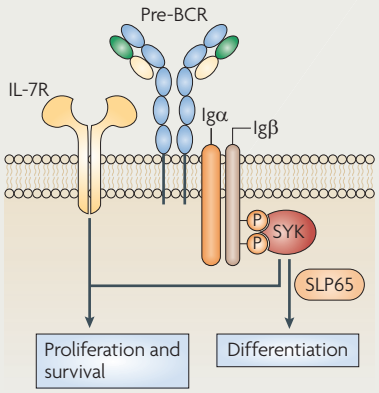
Oncogene
An altered or mutant form of a proto-oncogene, which drive the proliferation of cells. Proto-oncogenes are usually involved in the control of cell growth and division.

Scaffold protein
A protein that assembles various proteins in a signalling pathway into multimolecular complexes for them to interact. Scaffold proteins are usually characterized by a large number of protein-binding domains.

λ5 (REF. 23). The activation of Igκ gene recombination requires the expression of the RAG complex, the attenuation of the cell cycle and the transcriptional activation of Igκ gene[58].

One of the transcription factors that seems to have an important role in VDJ recombination and in regulating the expression of *Rag* genes is forkhead box P1 (FOXP1). Indeed, deletion of *Foxp1* results in a block in the transition from the pro-B-cell to the pre-B-cell stage owing to diminished expression of *Rag1* and *Rag2* (REF. 59). Similarly, E2A proteins have been shown to regulate the expression of several important B-cell transcription factors, including *Rag1* and *Rag2* (REF. 60). Moreover, E2A proteins seem to be required for the regulation of cell cycle progression and the transcriptional activation of the Igκ gene[60]. Transcriptional activation, also known as germline transcription, occurs before VDJ recombination and is important for making the Igκ gene accessible to the recombination machinery. The transcription factors SPI-B and interferon-regulatory factor 4 (IRF4) have been reported to be sufficient for inducing Igκ germline transcripts and therefore for promoting Igκ recombination[61,62]. Interestingly, *Irf4*$^{-/-}$ mice show severe defects in Igκ gene recombination[63,64], and SLP65 is thought to regulate IRF4 expression, as reconstitution of SLP65 function in *Slp65*$^{-/-}$ pre-B cells induces the expression of IRF4 (REF. 23). Together, these data suggest a dual role of SLP65 in the downregulation of SLC expression and the activation of Igκ germline transcription. However, it has been unclear how SLP65 attenuates cell cycle progression and how it activates RAG1 and RAG2 expression. The identification of the FOXO transcription factors as downstream elements of the SLP65-activated signalling cascade may provide the missing link to the regulation of the cell cycle and the activation of RAG1 and RAG2 expression (see below). Available data suggest that SLP65 can switch cell fate from proliferation to differentiation[27,51]. In human pre-B cells, SLP65 has a non-redundant role in inducing differentiation, as patients lacking SLP65 expression have a complete developmental block at the pre-B-cell stage[65]. These findings highlight the importance of SLP65 as a crucial player in orchestrating the differentiation of early B cells.

Box 2 | **The role of interleukin-7**



In addition to the role of pre-B-cell receptor (pre-BCR) signalling, cell fate during early B-cell development is also influenced by interleukin-7 (IL-7) and its receptor (IL-7R), which have a central role in controlling the survival, proliferation and differentiation of B cells (see the figure)[115].

The absence of the IL-7 signal in mice that have a targeted deletion of the gene encoding IL-7 or of the genes encoding components of IL-7R results in an arrest of B-cell development at the pro-B-cell stage and impaired V to DJ recombination[115]. In these mice, only a few B cells mature and populate the periphery, supporting the hypothesis that IL-7 is required for proper cell proliferation during early B-cell development. Several experiments indicate that there is intensive crosstalk between the signalling pathways that are triggered by the pre-BCR and IL-7R. Expression of the pre-BCR by pro-B cells upregulates IL-7R expression on the cell surface, thereby increasing the responsiveness of these cells to IL-7 (REFS 53–55). This results in the selective expansion of pre-BCR$^+$ cells in conditions in which IL-7 concentration is low. At the same time, however, the pre-BCR also induces the differentiation of pre-B cells into a state in which they have reduced IL-7 responsiveness, which probably limits the expansion of pre-B cells during B-cell development *in vivo*[55,116].

Experiments using mice that are transgenic for the surrogate light chain (SLC), in which the pre-BCR is constitutively expressed beyond the pre-B-cell stage, show that prolonged pre-BCR expression does not increase pre-B-cell proliferation. Instead, prolonged pre-BCR expression resulted in a developmental block at the immature B-cell stage[117]. These data suggest that the ability of the SLC to induce cell proliferation might be restricted to large pre-B cells. This is because only these cells express the IL-7R and are therefore responsive to IL-7 (REFS 55,116), whereas cells that are at later stages of B-cell development downregulate the expression of IL-7R and therefore have a decreased proliferative capacity.

The importance of IL-7R for pre-B-cell proliferation is highlighted by data showing that enhanced IL-7R signalling results in the proliferation of pro- and pre-B cells in the bone marrow, as well as the migration of these populations to the spleen, lymph nodes and blood[115]. Of note, most of the mice that have enhanced IL-7R signalling develop pro- and pre-B-cell tumours or bipotent B-cell and myeloid-cell tumours[118,119]. SLP65, SH2-domain-containing leukocyte protein of 65 kDa; SYK, spleen tyrosine kinase.

The PI3K–PKB–FOXO pathway and proliferation

Several lines of evidence point to an important role for the PI3K–PKB signalling pathway in the regulation of IgL gene recombination. For example, inhibition of PI3K promotes the rearrangement of an artificial recombination substrate, which suggests that signalling through PI3K suppresses the recombination machinery[66]. This is in line with data showing that PI3K signalling inhibits both the expression of a *Rag2*–GFP (green florescent protein) reporter gene and the rearrangement of the endogenous IgL gene in bone-marrow-derived B cells[67]. In agreement with this, cultured B cells that lack expression of p85α, the regulatory subunit of PI3K, show increased transcription of *Rag1* and *Rag2* and increased recombination of the IgL gene compared with wild-type control cells[68].

Further support for the importance of the PI3K–PKB signalling pathway in the inhibition of IgL gene recombination comes from recent reports showing that the FOX class O family of transcription factors activates IgL gene recombination[66,69]. The FOXO family, members of which are homologues of the *Caenorhabditis elegans* transcription factor DAF-16 (REF. 70), consists of FOXO1, FOXO3a, FOXO4 and FOXO6 (REF. 71). FOXO proteins regulate cell fate by modulating the expression of genes that are involved in diverse biological processes, including cell cycle arrest[72], apoptosis[73], oxidative stress resistance[74] and longevity[75]. The activity of FOXO proteins is tightly controlled on several levels by posttranslational modifications[76]. Most importantly, PKB-mediated phosphorylation of FOXO proteins inhibits their transcriptional activity by promoting the export of phosphorylated FOXO proteins from the nucleus to the cytoplasm, where these molecules are rapidly degraded[77].

In pre-B cells, FOXO proteins have been shown to induce IgL gene recombination through the activation of RAG complex expression and the induction of a delay in the G_1 phase of the cell cycle[66,69]. Indeed, this role of FOXO proteins is supported by the decreased number

of Igκ-expressing *Foxo3a*$^{-/-}$ B cells *ex vivo*[66]. FOXO1 has a unique role in the development of B cells, as conditional inactivation of *Foxo1* at the early pro-B-cell stage resulted in a complete block in B-cell development[78]. Interestingly, *Foxo1*$^{-/-}$ pro-B cells are highly prone to apoptosis and show normal levels of D_H to J_H gene recombination but impaired V_H to D_HJ_H rearrangement[78]. Based on these data, it was suggested that FOXO1 has an essential role in IgH gene recombination[78]. Inactivation of *Foxo1* at the later pre-B-cell stage of development resulted in normal pre-B-cell numbers, but the percentage of immature and mature B cells was reduced[78]. Interestingly, the spleen and blood of mice in which *Foxo1* was inactivated contained a distinct population of pre-B cells that lacked IgL gene recombination, which is in agreement with an important role of FOXO1 in this process. However, further experiments are required to investigate whether the immature B cells that develop in these mice do so despite *Foxo1* deficiency or whether *Foxo1* inactivation was incomplete at the time point when IgL gene recombination occurred. We think that, in addition to FOXO1, both FOXO3a and FOXO4 are regulated by pre-BCR signals and are involved in the activation of IgL gene recombination, but further investigation is required to fully understand the role of different FOXO proteins in IgL gene recombination.

Experiments with receptor-deficient pre-B cells that were reconstituted with pre-BCR components showed that PI3K, PKB and FOXO proteins (FOXO1, FOXO3a and FOXO4) are regulated by autonomous signalling from the pre-BCR (REF. 66 and S.H. and H.J., unpublished observations). This autonomous signalling, which is triggered by the pre-BCR and activates the PI3K–PKB pathway, seems to depend on SYK but to not involve SLP65. Instead, reconstitution of SLP65 in *Slp65*$^{-/-}$ pre-B cells decreases PKB activity and reduces the inhibitory phosphorylation of FOXO proteins, thereby providing a molecular mechanism by which SLP65 can drive cells from proliferation to differentiation, and thus explaining SLP65 tumour-suppressor function[51]. Of note, many leukaemic cells are characterized by constitutive activation of the PI3K–PKB pathway[44,79], and recent data indicate that inactivation of FOXO proteins (which promotes proliferation and inhibits differentiation) is a crucial event in the pathology of cancer[80–82]. Indeed, the induced deletion of FOXO1, FOXO3a and FOXO4 in mice results in the development of thymic lymphomas and haemangiomas[83].

Interestingly, the PI3K–PKB–FOXO pathway not only has a role in primary Igκ recombination in pre-B cells, but also regulates cell fate decisions in immature B cells that express a BCR with a conventional IgL. Cross-linking of the BCR on these cells results in efficient SLP65-mediated downregulation of PKB activity and the subsequent reduction of FOXO inhibition, thereby inducing secondary IgL gene recombination and receptor editing[66]. In line with these observations, one study[69] showed that the treatment of cultured primary immature B cells with a short hairpin RNA that was specific for FOXO1 interfered with the expression of RAG1 and RAG2 following BCR stimulation.

Receptor editing
A molecular process that involves secondary rearrangements (mostly of the immunoglobulin light chains) that replace existing immunoglobulin molecules and generate a new antigen receptor with altered specificity.

The mechanism by which SLP65 modulates PKB activity is currently unknown. However, it is conceivable that SLP65 impairs PKB signalling proximal to the pre-BCR or BCR by altering the activity of SYK, CD19 and PI3K, and/or even the activity of PKB itself. Alternatively, SLP65 could interfere with the PI3K–PKB signalling pathway through the activation of lipid phosphatases, such as phosphatase and tensin homologue (PTEN) and SH2-domain-containing inositol-5-phosphatase (SHIP), which directly antagonize PI3K through the dephosphorylation of PtdIns(3,4,5)P_3 (REF. 47).

Cell cycle regulation by PI3K, PKB and FOXO

The role of G_1 cell cycle delay for IgL recombination. The observation that FOXO proteins activate the expression of the RAG protein complex and promote the recombination of the IgL gene raises questions about the molecular mechanisms that regulate this process. A prerequisite for V(D)J recombination, which is based on the repetitive cleavage and subsequent reassembly of DNA fragments[1], seems to be a tight linkage to the G_0 and G_1 phases of the cell cycle, as the recurrence of double-stranded breaks during DNA replication or mitosis could compromise genetic integrity. It is therefore tempting to speculate that downregulation of PKB activity and subsequent stabilization of FOXO proteins are required to inhibit the progression from G_1 to S phase of the cell cycle. The tight linkage of V(D)J recombination to the G_1 phase is established mainly at the level of RAG2 protein stability[84]. RAG2 expression oscillates as a function of the cell cycle, accumulating preferentially in the G_1 phase, but markedly decreases following entry in the S phase, and remains low or undetectable until the M phase is completed[85]. Destabilization of RAG2 is initiated by its phosphorylation at the consensus recognition sequence for cyclin-dependent kinases (CDKs), which control entry and progression through phases of the cell cycle. CDK2, which becomes active at the transition from G_1 to S phase, phosphorylates RAG2 at threonine 490 (REF. 84). This phosphorylation induces the translocation of RAG2 from the nucleus to the cytoplasm, where it is degraded by the ubiquitin–proteasome system[85,86]. In line with this observation, a mutation of threonine 490 to alanine prevents the periodic degradation of RAG2 in dividing cells, resulting in the accumulation of double-stranded DNA breaks throughout the cell cycle[87]. In addition to the accumulation and degradation of RAG2, other proteins might participate in the coordination of V(D)J recombination with the cell cycle. For example, the double-stranded DNA breaks that are generated during V(D)J recombination are exclusively repaired by non-homologous end joining, which is the main repair pathway that operates during the G_1 and early S phases of the cell cycle[88].

Cell cycle regulation at the G_1 to S phase transition. Cell cycle progression is controlled by CDKs, which require binding to a cyclin to become catalytically active[89] (FIG. 3). The most prominent CDK in late G_1 is CDK2, which associates with E-type cyclins (cyclin E1 and cyclin E2; encoded by *CCNE1* and *CCNE2*, respectively) and cyclin A (encoded by *CCNA2*). If active, CDK2 promotes

DNA replication and entry into the S phase. In the absence of mitogenic signals, cells use several mechanisms to keep CDK2 in an inactive state to prevent progression from G_1 to S phase. One of these mechanisms is based on limiting the amount of E-type cyclins, the expression of which depends on the activity of E2F transcription factors[90]. In resting cells, E2F is bound by retinoblastoma protein or its family members p107 and p130 (also known as RBL1)[90], and its transactivating function is therefore inhibited. Mitogenic signals, for example, by the RAF–MEK1–ERK (RAF–MAPK/ERK kinase 1–extracellular regulated kinase) signalling pathway or by PI3K, can overcome this block of E2F-mediated transcription through the upregulation of D-type cyclins, which are the activating subunits for the early G_1 phase CDKs CDK4 and CDK6. Active CDK4–D-type cyclin or CDK6–D-type cyclin complexes phosphorylate retinoblastoma, p107 and p130, which dissociate from E2F and thereby enable E2F-dependent transcription. This results in the expression of E-type cyclins and, consequently, to the activation of CDK2, and this is further enhanced by direct phosphorylation of retinoblastoma by CDK2–E-type cyclin complexes in a positive feedback loop[91]. Other targets of E2F, in addition to E-type cyclins, are genes encoding components that are involved in DNA replication.

As well as regulating CDK activation by cyclin synthesis and degradation, CDK–cyclin complexes can also be rendered functionally inactive by associating with CDK inhibitors, which are part of either the inhibitor of cyclin-dependent kinase 4 (INK4) family or the CDK interaction protein (CIP)/kinase-interacting protein (KIP) family (comprising p21, p27 and p57; also referred to as CIP1, KIP1 and KIP2, respectively, and encoded by *CDKN1A*, *CDKN1B* and *CDKN1C*, respectively)[92,93]. The activity of CDK inhibitors is regulated on several levels, including transcription, translation, protein localization and stability[94].

Mechanisms of FOXO-mediated G_1 cell cycle delay. An important target of FOXO as part of its role in the regulation of the cell cycle is the CDK inhibitor p27, which blocks progression from G_1 to S phase by binding to CDK2–E-type cyclin complexes (FIG. 3). p27 has been shown to be activated by FOXO proteins in several cellular systems[72,81,95,96], in which the induction of FOXO transcriptional activity, either through inhibition of the PI3K–PKB signalling pathway or expression of constitutively active FOXO proteins, resulted in increased mRNA encoding p27, increased p27 protein levels and a G_1 cell cycle delay. In addition to the putative regulation by FOXO proteins, the expression of p27 has been shown to be controlled by PKB, as phosphorylation of p27 at threonine 157 by PKB impairs nuclear import of p27 and opposes p27-mediated G_1 cell cycle arrest[97]. So, it is probable that downregulation of PI3K–PKB signalling activates both FOXO proteins and p27, leading to cell cycle arrest.

The available data suggest that FOXO proteins activate the expression of RAG1 and RAG2 and increase the amount of p27 in pre-B cells; p27 is important for stabilizing RAG2 because it inhibits the activity of CDK2–E-type cyclin complexes, which mark RAG2 for ubiquitylation and proteasomal degradation[84,86]. As p27 blocks the cell cycle in the G_1 phase and stabilizes RAG2 expression, it is apparent that induction of p27 is an important step in FOXO-induced pre-B-cell differentiation. The role of p27 in cell cycle progression is further supported by the phenotype of *p27*$^{-/-}$ mice, the lymphocytes of which have enhanced proliferation, resulting in the enlargement of several organs, including the thymus and spleen[98–100]. However, one study showed that increased expression of p27 was not sufficient to activate IgL gene recombination in pre-B cells, although it was sufficient to block the cell cycle at the G_1 phase and to induce accumulation of RAG2 (REF. 66). In addition, B cells from *p27*$^{-/-}$ mice do not show severe abnormalities in early B-cell development (S.H., unpublished observations). This suggests

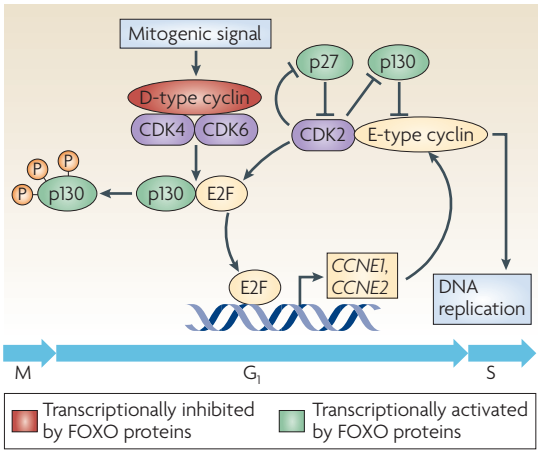


Figure 3 | **Control of cell cycle progression by PKB and FOXO proteins.** Entry and progression through the G_1 phase of the cell cycle is regulated at the level of cyclin-dependent kinase 2 (CDK2), which associates with E-type cyclins (cyclin E1 and cyclin E2) and cyclin A. In non-proliferating cells, CDK2 is controlled by the limited supply of E-type cyclins and by inhibition that is mediated by the cyclin-dependent kinase inhibitor p27. Mitogenic signals cause the upregulation of D-type cyclins, which pair with and activate CDK4 and CDK6. Active CDK4–CDK6–D-type cyclin complexes hyperphosphorylate retinoblastoma proteins (including p130), which detach from E2F transcription factors and enable E2F-mediated transcription. This leads to the transcription of *CCNE1* and *CCNE2* (the genes encoding cyclin E1 and cyclin E2, respectively) and the generation of CDK2–E-type cyclin complexes. In a positive feedback loop, CDK2 inactivates retinoblastoma and p27 to promote further CDK2 activation, which finally culminates in the progression from the G_1 phase of the cell cycle to the S phase. If protein kinase B (PKB) is inactivated, stabilized forkhead box O (FOXO) proteins can influence G_1 cell cycle progression by transcriptional upregulation or repression of several components, including p27, D-type cyclins and the retinoblastoma-family protein p130. Targets that are upregulated by FOXO proteins are shown in green and targets that are downregulated by FOXO proteins are shown in red. Note that p27 is also directly stabilized by PKB inactivation, as it becomes degraded following PKB-mediated phosphorylation.

that other factors can compensate for the loss of p27 and stabilize RAG2, thereby allowing early B-cell maturation. Interestingly, FOXO proteins also activate the expression of the gene encoding the retinoblastoma family protein p130, which, in addition to its function as an inhibitor of E2F, has been identified as a classical CDK2 inhibitor that resembles p27 and might functionally compensate for potential B-cell developmental defects in *p27*$^{-/-}$ mice[101]. Indeed, mice that are deficient for both p27 and p130 have significantly increased proliferation of myeloid, lymphoid and erythroid cells in the spleen, resulting in splenomegaly[102]. As proper cell cycle regulation seems to be mandatory for RAG2 stabilization, IgL gene recombination and differentiation, it would be interesting to analyse early B-cell development and the phenotype of splenic B cells in these mice.

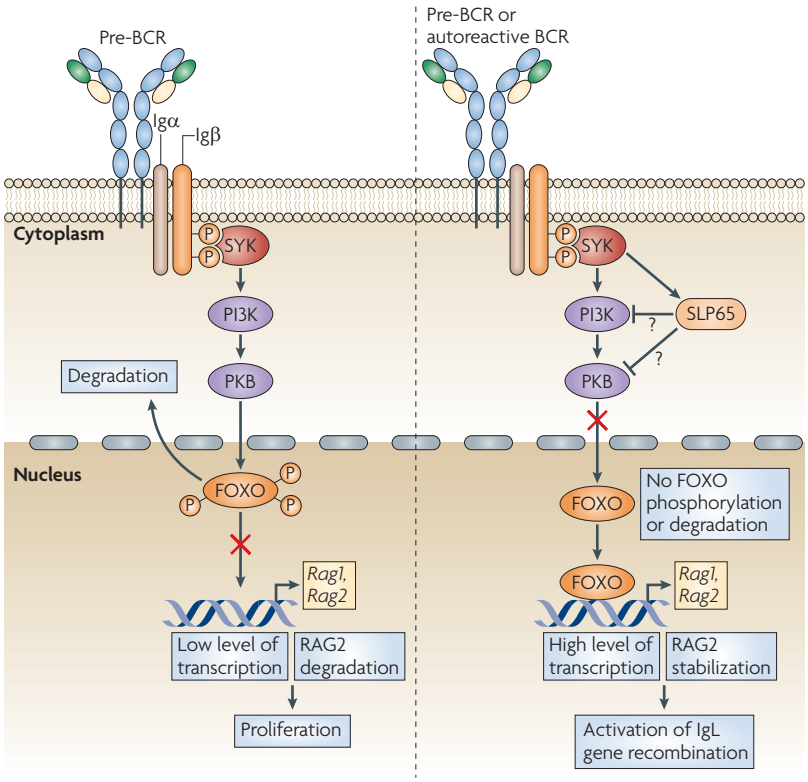


Figure 4 | **Regulation of IgL gene recombination by the PI3K–PKB–FOXO pathway.** Expression of a signalling-competent pre-B-cell receptor (pre-BCR) on the cell surface triggers the phosphoinositide 3 kinase (PI3K)–protein kinase B (PKB) pathway through autonomous signalling that is mediated by SYK (spleen tyrosine kinase). Active PKB phosphorylates forkhead box O (FOXO) proteins, resulting in FOXO protein degradation by the proteasomal pathway. Owing to the constant inactivation of FOXO proteins, cells are primed for proliferation (left panel). Under these conditions, *Rag1* (recombination-activating gene 1) and *Rag2* are only transcribed at low levels, and the RAG1–RAG2 protein complex stays inactive because of the periodic degradation of RAG2. However, signalling through SH2-domain-containing leukocyte protein of 65 kDa (SLP65) counteracts the function of PKB–PI3K signalling, enabling FOXO proteins to activate target genes in the nucleus. This results in the transcription of the *Rag* genes and the stabilization of RAG2 through the attenuation of the cell cycle. Consequently, the RAG1–RAG2 complex initiates immunoglobulin light chain (IgL) gene recombination (right panel). If the resulting BCR is non-autoreactive, tonic signals from the receptor turn off FOXO proteins. Cells with autoreactive BCRs that are cross-linked by self antigens may have a second chance to edit their IgL owing to the fact that FOXO proteins remain active.

D-type cyclins are also targeted by FOXO proteins[103], which interfere with proliferation when constitutively active by repressing protein expression of cyclin D1 and D2 (FIG. 3). D-type cyclins, which are the activating subunit for the early G_1 phase CDKs CDK4 and CDK6, have an important role in promoting the transition from G_1 to S phase through the activation of E2F. Decreased protein expression of D-type cyclins is associated with reduced activity of CDK4, resulting in impaired cellular proliferation. Interestingly, recent data show that the expression of cyclin D3 during B-cell development normally becomes induced in large pre-B cells and is directly downregulated in small pre-B cells, suggesting that cyclin D3 has an important role in the receptor-mediated proliferation of pre-B cells[104]. Indeed, deletion of *CCND3* (which encodes cyclin D3) in mice results in a block of B-cell development at the pro-B-cell to pre-B-cell transition owing to impaired cell cycle progression of the large pre-B-cell population. Further analysis revealed that the expression of the pre-BCR is necessary to stabilize cyclin D3 at the protein level[104]. Based on the fact that SLP65 is an important regulator of pre-BCR signalling, it is tempting to speculate that SLP65 terminates the proliferation of large pre-B cells through the activation of FOXO proteins and, consequently, the transcriptional repression of cyclin D3.

A model for the regulation of IgL recombination

The data described above suggest a model that could explain how primary and secondary IgL gene recombination are regulated in the course of B-cell development (FIG. 4). When initiated at the pro-B-cell stage, successful recombination of the IgH gene results in the expression of Igμ together with SLC and the Igα–Igβ heterodimer on the cell surface. SYK-dependent signals from the pre-BCR then feed into the PI3K pathway through CD19 and/or BCAP, leading to the activation of PKB and, in turn, to the inactivation of FOXO transcription factors. This scenario promotes proliferation and inhibits differentiation. In the absence of SLP65, most cells remain in a proliferative state, which presumably increases the risk of leukaemic transformation. However, in the presence of SLP65 the autonomous receptor signalling that is mediated by SYK and PI3K–PKB is downregulated, which enables FOXO proteins to promote IgL gene recombination. An obvious question in this context is how the SLP65-induced downregulation of PI3K–PKB signalling is delayed to allow a few rounds of pre-B-cell proliferation before IgL gene recombination and B-cell differentiation are activated. It is conceivable that the signalling proteins that are involved in the activation of pre-B-cell proliferation are constitutively expressed in pre-B cells, whereas the expression of important components of the SLP65-organized pro-differentiation complex might need to be initiated by the pre-BCR.

Initial IgL gene rearrangement gives rise to several possible outcomes. First, IgL gene recombination does not always result in the formation of a functional receptor, which is the case if the V to J gene segment assembly is not productive. If so, autonomous

signalling through PI3K–PKB could be predicted to be downregulated or completely shut down owing to the lack of receptor expression. This would maintain FOXO activity and allow further IgL gene recombination. Second, productive IgL gene recombination and successful pairing of IgL with Igμ gives rise to an immature B cell that expresses a BCR on the cell surface. However, as receptor specificity is generated in a random process, this BCR can either be autoreactive or non-autoreactive. If the BCR is autoreactive, cross-linking by self antigens results in constitutive receptor internalization that leads to BCR removal from the cell surface. This situation is reminiscent of the pre-BCR, which seems to be polyreactive and can be cross-linked by different self antigens. Therefore, it is probable that an autoreactive BCR could induce responses that are similar to those triggered by the pre-BCR, including the induction of secondary IgL gene rearrangements in autoreactive B cells. So, signalling from an autoreactive receptor through SLP65 continues to downregulate the PI3K–PKB pathway and, by doing so, it keeps the FOXO proteins in an active state. This enables secondary IgL gene recombination or receptor editing. If the resulting BCR is non-autoreactive, it is then stably expressed on the cell surface, which leads to constant BCR signalling activity. Presumably, these constant signals lead to stable PI3K–PKB activation, thereby resulting in the downregulation of FOXO proteins and the termination of IgL gene recombination. Consequently, this further stabilizes BCR expression and allows the cells to become selected and to migrate out of the bone marrow. How constitutive BCR internalization, compared with stable expression of a non-autoreactive BCR, results in the activation of SLP65 function is an important question that has yet to be answered. However, it is also conceivable that constitutive BCR internalization results in reduced PI3K–PKB activity owing to low BCR expression on the surface and, consequently, in the activation of FOXO proteins in a SLP65-independent manner.

Importantly, rearrangement of the IgL genes, either because of lack of receptor expression or as a consequence of autoreactivity, is not infinite, as in addition to promoting IgL gene recombination, FOXO proteins initiate an apoptotic programme following their activation[105]. In terms of pre-B-cell differentiation and receptor selection, such an apoptotic programme might provide a fixed time frame for IgL gene recombination. This would mean that only those cells that generate a non-autoreactive receptor within a certain time frame are rescued from the FOXO-induced apoptosis, whereas cells that fail to do so are deleted.

Several lines of evidence support this model. Increased PI3K–PKB signalling in the absence of SLP65 would be predicted to compromise primary and secondary IgL gene recombination. Indeed, loss of SLP65 and its downstream signalling elements, such as BTK and PLCγ2, has been shown to impair the transition from the pre-B-cell to the immature B-cell stage, probably as a result of defects in IgL gene recombination and BCR expression[4]. In agreement with this, recent data show that defective IgL gene recombination in SLP65-deficient mice leads to compromised receptor editing and an increased antibody response to immunization with a self antigen[106]. This suggests that SLP65 deficiency leads to the development of autoreactive B cells expressing receptors that would normally be edited in wild-type mice[106]. The same is true for PLCγ2-deficient mice, which display impaired antigen-induced receptor editing both *in vitro* and *in vivo*[107].

A second prediction of this model would be that turning off the PI3K–PKB pathway promotes IgL gene recombination. Indeed, recent studies have shown that the inhibition of PI3K in immature B cells, either by specific inhibitors or by the deletion of cell-surface receptor expression, resulted in the expression of the RAG1–RAG2 recombination machinery and of pro-B-cell-specific genes[67]. Of note, these cells showed evidence of receptor editing, which supports the hypothesis that basal BCR signals inhibit secondary IgL gene recombination through PI3K[67]. Similarly, recent data indicate that PI3K signalling in immature non-autoreactive B cells suppresses RAG1 and RAG2 expression and that the inhibition of PI3K signalling induces RAG1 and RAG2 expression and promotes IgL gene recombination[68]. In agreement with this, immature B cells that are deficient for the regulatory PI3K subunit, p85α, poorly suppress RAG1 and RAG2 expression and undergo excessive receptor editing[68]. Further evidence comes from studies in which BCR signalling has been abrogated at the level of SYK or CD19, both of which are required for optimal activation of the PI3K pathway. Here, loss of SYK promoted receptor editing even in non-autoreactive B cells, which indicates that SYK transmits signals that normally suppress recombination and control IgH and IgL allelic exclusion[108,109]. Similarly, immature *Cd19*$^{-/-}$ B cells do not suppress the expression of RAG1 and RAG2 and undergo intensive receptor editing *in vitro*[110] and *in vivo*[111].

Remaining questions

Recent data have shed some light on the mechanism by which IgL gene recombination is regulated by receptor signalling in developing B cells. The identification of the PI3K–PKB pathway as part of autonomous receptor signalling, which seems to antagonize the differentiation of pre-B cells, and of FOXO proteins, which have a crucial role in activating the RAG recombination machinery, has led to a new model of primary and secondary recombination, as outlined in this Review. However, much remains to be elucidated.

First, the question of how FOXO proteins induce IgL gene recombination needs to be addressed in detail. To activate IgL gene recombination, FOXO proteins have to induce germline transcription. To date, it is an open question whether FOXO proteins directly bind to the IgL chain genes or whether the activation of IgL gene recombination is an indirect effect. An interesting candidate for indirect FOXO function may be IRF4, which is known to bind to transcriptional regulation elements in IgL genes.

Second, the individual role of the different FOXO family members in B-cell development and function has to be investigated. To this end, the target genes of FOXO proteins in developing B cells need to be identified and characterized.

Third, future studies have to clarify the mechanisms by which constitutive BCR internalization activates SLP65 function and subsequent IgL gene recombination in early B cells, and stable BCR expression turns off SLP65-dependent FOXO activation and leads to stable PI3K–PKB signalling. Given that SLP65 functions as a tumour suppressor, understanding the molecular interplay that counteracts proliferation will add to our knowledge of basic signalling and improve our understanding of the mechanisms of cellular transformation and development of cancer.

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DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
BTK | *CCNE1* | *CCNE2* | CDK2 | FOXP1 | FOXO1 | FOXO3a | FOXO4 | FOXO6 | IgH | IgL | IL-7R | p27 | PDK1 | PI3K | PKB | PLCγ2 | RAG1 | RAG2 | SLP65 | SYK

FURTHER INFORMATION
Hassan Jumaa's homepage: http://www.immunbio.mpg.de/home/research/molimmun/jumaa/index.html

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