MK5 activates *Rag* transcription via Foxo1 in developing B cells

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Foxo1 is a critical, direct regulator of *Rag* (*recombination activating gene*) transcription during B cell development and is thus essential for the generation of a diverse repertoire of antigen receptors. Although Foxo1 regulation has been widely studied in many cell types, pathways regulating Foxo1 in B cells have not been fully elucidated. By screening a panel of Foxo1 mutants, we identified serine 215 on Foxo1 as a novel phosphorylation site that is essential for the activation of *Rag* transcription. Mutation of S215 strongly attenuated transactivation of *Rag* but did not affect most other Foxo1 target genes. We show that MK5, a MAPK-activated protein kinase, is a previously unidentified upstream regulator of Foxo1. MK5 was necessary and sufficient to activate *Rag* transcription in transformed and primary pro–B cells. Together, our experiments show that MK5 positively regulates *Rag* transcription via phosphorylation of Foxo1 in developing B cells.

Proper functioning of the adaptive immune system requires that lymphocytes express a diverse repertoire of antigen receptors. The genes encoding these receptors are generated by V(D)J recombination, a process by which various gene segments at the Ig or T cell receptor loci are joined together during development to create novel antigen receptor genes ( Tonegawa, 1983). The first step of the recombination process requires *recombination activating gene* proteins 1 and 2 (RAG1 and RAG2, collectively known as RAG) to generate double-stranded DNA (dsDNA) breaks at the boundaries of a pair of rearranging gene segments. The cleaved gene segments are then ligated together by DNA repair proteins to form coding exons (Schatz and Swanson, 2011). Because this process generates dsDNA breaks, it poses a threat to genomic integrity. Hence, it is essential that RAG activity be regulated in a lineage and stage-specific manner.

RAG activity is tightly linked to B cell development. In *Rag1*- or *Rag2*-null mice, B cell development is completely abrogated (Mombaerts et al., 1992; Shinkai et al., 1992). Starting at the pro–B cell stage, *Rag* is expressed to allow *Ig* heavy chain gene rearrangement. Expression is then down-regulated during a brief proliferative burst, and then up-regulated again at the pre–B stage when the *Ig* light chain loci undergo rearrangement. Once a self-tolerant BCR is successfully generated, *Rag* expression is shut off. Production of an autoimmune BCR results in continued *Rag* expression promoting a process known as receptor editing (Schlissel, 2003; Halverson et al., 2004). This dynamic pattern of *Rag* expression is controlled by a network of transcription factors that includes Foxo1 (Amin and Schlissel, 2008; Dengler et al., 2008).

Foxo1 is a Forkhead family transcription factor that, together with Foxo3a, Foxo4, and Foxo6, constitutes the O subfamily (FoxO). FoxO proteins are conserved from nematodes to mammals and regulate diverse cellular processes including apoptosis, proliferation, differentiation, cell cycle progression, oxidative stress resistance, autophagy, and metabolism. These diverse functions allow FoxO proteins to play central roles in stem cell proliferation, pluripotency maintenance, aging, and tumor suppression (Arden, 2007; Huang and Tindall, 2007; Greer and Brunet, 2008; Salih and Brunet, 2008; Zhang et al., 2011). Foxo1 is required for proper developmental progression as a result of distinct functions at different stages of B cell development. In pro–B cells and B cells undergoing receptor editing, Foxo1 is required for up-regulating *Rag* transcription (Amin and Schlissel, 2008; Dengler et al., 2008).

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Abbreviations used: AMuLV, Abelson murine leukemia virus; ChIP, chromatin immunoprecipitation.
FoxO family members are posttranslationally regulated by various signaling pathways in different cellular contexts. One of the best studied regulators of FoxO is AKT, which phosphorylates FoxO at two conserved serine and one conserved threonine residues, resulting in its nuclear export and sequestration in the cytoplasm (Brunet et al., 1999). Besides AKT phosphorylation, several other posttranslational mechanisms have been shown to regulate FoxO1 activity in various cell types. These regulators modulate FoxO1 activity by subcellular localization, DNA binding affinity, and interaction with binding partners (Calnan and Brunet, 2008). Known regulators of FoxO1 include the deacetylases SIRT1 and SIRT2, class II histone deacetylases, the acetyltransferase CBP/p300, the methyltransferase PRMT1, and various kinases including CDK2, SGK, CK-1, and MST1 (Vogt et al., 2005; Lehtinen et al., 2006; Mattila et al., 2008; Yamagata et al., 2008; Mihaylova et al., 2011). Recently, MK5 (also known as PRAK), a MAP kinase–activated protein kinase, was shown to positively regulate Foxo3a activity in colon cancer cells (Kress et al., 2011). Although these FoxO regulatory pathways have been characterized extensively in various cell types, the regulatory mechanisms of FoxO during B cell development have not been fully elucidated.

We sought to understand how Foxo1 is regulated in B cells. We and others have shown that AKT phosphorylation negatively regulates Foxo1 activity and diminishes Rag transcription in developing B cells (Amin and Schlissel, 2008; Ochiai et al., 2012). However, in the absence of PTEN, an antagonist of the AKT pathway, Rag expression is reduced but not completely abrogated, suggesting that there are AKT-independent pathways regulating Foxo1 activity in B cells (Alkhairi et al., 2012).

To study this question, we took advantage of Abelson murine leukemia virus (AMuLV)–transformed pro–B cells as a model system for early B cell development. Infection of mouse bone marrow with a replication–deficient retrovirus expressing the oncogene v-abl results in transformed B cells that are blocked at the pro– to pre–B transition (Rosenberg et al., 1975). These cells are highly proliferative in a cytokine independent manner but undergo a process that resembles the developmental transition from the pro– to pre–B cell stage upon treatment with the ABL kinase inhibitor STI-571 (Muljo and Schlissel, 2003). This provides a robust model system to study gene regulation during the pro–B to pre–B transition of B cell development. Using the AMuLV-transformed pro–B cells, we discovered a novel phosphorylation site (serine 215) on Foxo1 that regulates Rag transcription. MK5, a likely kinase which phosphorylates Foxo1-S215, is required for full activation of Rag transcription. We confirmed these results in primary B cells, revealing a novel role for MK5 as an activator of Foxo1 and Rag transcription in developing B cells.

**RESULTS**

**Activation of Foxo1 does not depend on AKT activity in AMuLV-transformed cells**

To test the dependence of Rag transcription in AMuLV-transformed cells on Foxo1, we generated Foxo1-deficient AMuLV-transformed cells by transforming bone marrow B cell progenitors from a mouse homozygous for a targeted, floxed allele of Foxo1 (Foxo1<sup>f/f</sup>; Paik et al., 2007). These mice also have an ERT2-Cre fusion gene knocked into the Rosa26 locus (ERCre) such that tamoxifen treatment results in deletion of the floxed allele (Ventura et al., 2007).

Tamoxifen-induced deletion of Foxo1 in these Foxo1<sup>f/f</sup> ERECre AMuLV-transformed cells is extremely efficient, as Foxo1 protein becomes undetectable after 24 h (Fig. 1 a). We used quantitative real-time RT-PCR on RNA purified from Foxo1-deficient cells to ask whether Foxo1 is required for Rag transcription. In the absence of Foxo1, the basal level of Rag transcripts is far lower and Rag induction by STI-571 treatment is severely blunted, indicating that Foxo1 is required for Rag transcription in AMuLV-transformed cells (Fig. 1 b).

Because AKT is known to negatively regulate Foxo1 activity in primary B cells, we asked whether Foxo1 is similarly regulated by AKT in AMuLV-transformed cells. We have previously observed that AKT is expressed but unphosphorylated in AMuLV-transformed cells (Amin and Schlissel, 2008), indicating that it is largely inactive in these cells. This led us to hypothesize that Foxo1 might be regulated by factors other than AKT in AMuLV-transformed cells. We tested this hypothesis by using inhibitors specific for either AKT or PI(3)K, an upstream activating kinase of AKT. We reasoned that if AKT negatively regulates Foxo1 in these cells, inhibiting this pathway will lead to activation of Foxo1, and thus up-regulation of Rag expression. We used Rag<sup>f<sup>GFP/+/</sup>f<sup>GFP/+<sub>LacZ</sub></sup></sub> AMuLV-transformed cells in which a GFP cDNA is knocked into the Rag1 coding region, rendering GFP expression a faithful reporter of Rag transcription (Kuwata et al., 1999). Treatment with inhibitors had no effect on Rag1-GFP expression, whereas treatment with STI-571 robustly induced GFP fluorescence (Fig. 1 c), suggesting that Foxo1 activity is independent of the AKT pathway in AMuLV-transformed cells. We further tested this hypothesis by assessing Foxo1 localization because AKT inhibits Foxo1 activity by sequestering it in the cytoplasm. Using subcellular fractionation, we found that a portion of Foxo1 is in the nucleus in resting AMuLV-transformed cells (Fig. 1 d), further supporting the idea that AKT does not play a role in Foxo1 regulation in AMuLV-transformed cells. The absence of AKT regulation of Foxo1 in AMuLV-transformed cells allowed us to use these cells to search for other modes of Foxo1 regulation.

**Serine 215 is required for Foxo1 transactivation of Rag expression**

We first asked how Foxo1 activity is regulated in AMuLV-transformed cells by measuring Foxo1 protein levels in cells treated with STI-571. Foxo1 protein levels remain unchanged upon STI-571 treatment (Fig. 2 a), suggesting that Foxo1 is activated posttranslationally. Foxo1 is known to undergo posttranslational modification by phosphorylation, acetylation, and methylation at various sites and in different combinations (Calnan and Brunet, 2008). To identify the relevant modifications that regulate Foxo1 activity for Rag transcription, we...
AMuLV-transformed cells. One of these residues, serine 215, was included in the panel because the corresponding serine, conserved among Foxo family members, was shown to be phosphorylated on Foxo3a (Kress et al., 2011). As expected, reconstitution with wild-type Foxo1 induces robust Rag expression (Fig. 2 c). Other Foxo1 mutants tested induced Rag expression to levels similar to reconstitution with wild-type Foxo1 (unpublished data). However, when S215 was mutated to alanine, Rag induction was severely blunted (Fig. 2 c), indicating that S215 is required for Foxo1-induced Rag transcription. Western blot analysis showed that the difference in Rag expression was not a result of differential expression levels of wild-type and mutant Foxo1 in this system (Fig. 2 b).

We next asked whether S215 is required for Foxo1 to transactivate other target genes. Because Foxo1 has different target genes in different cellular contexts, we performed microarray analysis comparing wild-type and Foxo1-deficient cells to identify relevant target genes in AMuLV-transformed cells. One of these residues, serine 215, was included in the panel because the corresponding serine, conserved among Foxo family members, was shown to be phosphorylated on Foxo3a (Kress et al., 2011). As expected, reconstitution with wild-type Foxo1 induces robust Rag expression (Fig. 2 c). Other Foxo1 mutants tested induced Rag expression to levels similar to reconstitution with wild-type Foxo1 (unpublished data). However, when S215 was mutated to alanine, Rag induction was severely blunted (Fig. 2 c), indicating that S215 is required for Foxo1-induced Rag transcription. Western blot analysis showed that the difference in Rag expression was not a result of differential expression levels of wild-type and mutant Foxo1 in this system (Fig. 2 b).
cells. This analysis identified nine genes that are up-regulated and five genes that are down-regulated >2.5-fold by Foxo1 (Fig. 3 a). Expression levels of Rag1 and Rag2 are low in resting AMuLV-transformed cells, and although the decrease in expression upon Foxo1 deletion was confirmed by RT-qPCR (unpublished data), these genes did not make the stringent cutoff in the microarray analysis. We validated the target gene identification by RT-qPCR in Foxo1-deficient and Foxo1-overexpressing AMuLV-transformed cells (unpublished data). When we tested the ability of Foxo1-S215A to regulate these genes, we found that only Rag and Aicda expression are affected by this mutation (Fig. 3 b and not depicted), indicating that S215A does not abrogate overall Foxo1 activity but rather the ability of Foxo1 to regulate a specific subset of gene targets.

### S215 regulates DNA binding of Foxo1
Foxo1 has been shown to bind directly to the Rag locus in developing B cells (Lin et al., 2010; Ochiai et al., 2012), and a DNA binding-defective mutant of Foxo1 fails to induce Rag

| Genes                  | Log2 Fold Change |
|------------------------|-----------------|
| NM_001159557 // Cd36  | 2.421           |
| NM_01033350 // Bank1   | 1.975           |
| NM_133809 // Kmo       | 1.769           |
| LOC100046496 // similar to Igk V-region 24B | 1.655 |
| NM_001042605 // Cd74   | 1.632           |
| NM_023665 // Irf30     | 1.552           |
| NM_178991 // Prkd      | 1.493           |
| NM_013867 // Bcra1     | 1.482           |
| NM_019868 // Sphb      | 1.394           |
| NM_028889 // Ezh1      | -1.370          |
| NM_009645 // Aicda     | -1.386          |
| NM_001042715 // Cdc135 | -1.473          |
| NM_199241 // Semaph6d  | -1.763          |
| NM_009368 // Tgfb3     | -2.250          |
| NM_019739 // Foxo1     | -2.276          |

**Figure 3.** S215 regulates gene-specific transcriptional activity of Foxo1. (a) Foxo1<sup>WT</sup>-ERCre cells were untreated (WT) and treated (KO) with tamoxifen. mRNA was extracted and subjected to microarray analysis. Genes differentially expressed (P < 0.05) by 2.5-fold or greater are shown. Values are displayed as log2 of fold change (%KO – log2 (WT)). (b) Foxo1<sup>WT</sup>-ERCre cells were reconstituted with exogenous Foxo1-WT or Foxo1-S215A. Transcripts of the indicated genes were determined by quantitative RT-PCR. The top panel shows genes regulated by Foxo1-S215. The middle panel shows selected examples of Foxo1-induced genes unaffected by Foxo1-S215A mutation. The bottom panel shows selected examples of Foxo1-repressed genes. P-values were calculated by a two-tailed Student’s t-test. Error bars represent standard deviation of triplicate or quadruplicate PCR assays. *, P < 0.001; **, P < 0.05; ns (not significant) denotes P > 0.05. All data are representative of at least three independent experiments.
with isotype control, we detected robust binding of wild-type Foxo1 at five previously identified binding sites at the Rag locus (Ochiai et al., 2012). The S215 mutation resulted in decreased Foxo1 binding at all five sites (Fig. 4a), suggesting that S215 regulates Foxo1 binding to the Rag locus.

To assess whether S215 regulates Foxo1 binding at other loci, we performed ChIP to examine Foxo1 occupancy at other target genes. We observed decreased binding of S215A expression (Amin and Schlissel, 2008). Together, these data strongly suggest that direct binding to the Rag locus is essential for Foxo1 to transactivate Rag transcription. We thus hypothesized that S215 might regulate Foxo1 activity by modulating Foxo1 binding at the Rag locus.

Figure 4. S215 regulates Foxo1 binding to DNA. (a) Diagram of Rag locus. Black lines indicate approximate locations amplified by primers used in part b. (b) AMuLV-transformed cells overexpressing 3xFlag-tagged wildtype (Foxo1-WT) or S215A mutant Foxo1 (Foxo1-S215A) were analyzed by chromatin immunoprecipitation (ChIP). Recovered DNA was analyzed by quantitative RT-PCR at the Rag locus. IgG is an isotype control performed in parallel with immunoprecipitation by Flag antibody. Diagram shows approximate primer locations in the Rag locus. (c) AMuLV-transformed cells overexpressing 3xFlag-tagged wildtype (Foxo1-WT) or S215A mutant Foxo1 (Foxo1-S215A) were analyzed by ChIP. Recovered DNA was analyzed by quantitative RT-PCR at the indicated loci. IgG control was performed in parallel with immunoprecipitation by Flag antibody. All data are representative of at least two independent experiments.

expression (Amin and Schlissel, 2008). Together, these data strongly suggest that direct binding to the Rag locus is essential for Foxo1 to transactivate Rag transcription. We thus hypothesized that S215 might regulate Foxo1 activity by modulating Foxo1 binding at the Rag locus. We performed chromatin immunoprecipitation (ChIP) to assess Foxo1 occupancy at the Rag locus in AMuLV-transformed cells overexpressing 3xFlag-tagged wild-type or S215A mutant Foxo1. When compared with isotype control, we detected robust binding of wild-type Foxo1 at five previously identified binding sites at the Rag locus (Ochiai et al., 2012). The S215 mutation resulted in decreased Foxo1 binding at all five sites (Fig. 4a), suggesting that S215 regulates Foxo1 binding to the Rag locus.

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MK5 regulates Rag expression in AMuLV-transformed cells

Because MK5 was shown to activate Foxo3a through reversible phosphorylation at S215 (Kress et al., 2011), we asked whether MK5 activates Foxo1 for Rag transcription in AMuLV-transformed cells. We overexpressed an MK5 cDNA in Rag1(+/+) AMuLV-transformed cells (Fig. 5 a) and found that MK5 overexpression increases Rag1-GFP fluorescence when compared with empty vector control (Fig. 5 b). To test whether kinase activity of MK5 is required for Rag transcription, we tested a kinase-dead mutant of MK5 (K51E; Seternes et al., 2002). MK5-KE has no effect on Rag1-GFP fluorescence when overexpressed in Rag1(+/+) AMuLV-transformed cells (Fig. 5 b), strongly supporting the conclusion that MK5 phosphorylates a target protein, most likely Foxo1, to up-regulate Rag expression. We also measured Rag1 transcript level by RT-qPCR in cells overexpressing MK5 to confirm that the Rag1-GFP level accurately reflects Rag1 transcript level (Fig. 5 c). As a control, Western blot analysis was performed to ensure that wild-type and mutant MK5 were overexpressed to similar levels (Fig. 5 a). These data indicate that MK5 is sufficient to activate Rag transcription in AMuLV-transformed cells.

We next asked whether MK5 is required for Rag induction by STI-571. We designed an shRNA targeting MK5 (shMK5). We confirmed the knockdown efficiency by measuring transcript level of MK5 (Mapkapk5) in AMuLV-transformed cells expressing shMK5. shMK5-expressing cells had an ~50% reduction in MK5 transcript level as compared with untransduced cells (parental), or cells expressing a control shRNA against luciferase (shLuc; Fig. 6 a). We further confirmed knockdown efficiency by measuring protein level of 3xFLAG-MK5 when shMK5 was transduced into 3xFLAG-MK5 overexpressing AMuLV-transformed cells (Fig. 6 b). These results demonstrated that shMK5 efficiently knocked down MK5 at both the transcript and protein levels. We then assessed Rag induction by STI-571 in the presence or absence of shMK5 in Rag1(+/+) AMuLV-transformed cells. Rag1-GFP induction by STI-571 was blunted in the presence of shMK5, but not in cells expressing shLuc, or untransduced cells in the same culture (Fig. 6 c). To further assess the requirement of MK5 for Rag expression, we transduced shMK5 into an AMuLV-transformed line that expresses constitutively high levels of Rag (Schulz et al., 2012). Compared with cells expressing shLuc, shMK5-expressing cells had decreased Rag expression, similar to cells expressing an shRNA against Foxo1 (shFoxo1; Amin and Schlissel, 2008; Fig. 6 d). We conclude that MK5 regulates Rag expression in AMuLV-transformed cells.

MK5 activates Foxo1–S215–regulated gene targets

Extensive biochemical and functional analyses performed by Kress et al. (2011) identified MK5 as a bona fide kinase for S215 on Foxo3a. Because of the highly conserved nature of serine 215 and the surrounding sequence among FOXO family members across diverse taxa (Fig. 7 a), based on sequence homology it is likely that MK5 also phosphorylates S215 on Foxo1. To directly show that MK5 activates Foxo1 via S215 in AMuLV-transformed cells, we overexpressed MK5 and used RT-qPCR to measure the expression levels of genes that are sensitive and insensitive to Foxo1-S215A mutation identified in Fig. 3. As expected, MK5 overexpression resulted in up-regulation of Rag1 and Rag2 as compared with empty
vector control (Fig. 7 b). Another Foxo1–S215–sensitive gene, Aicda, was also induced upon MK5 overexpression (Fig. 7 b). However, none of the other Foxo1–regulated but S215–insensitive genes tested were responsive to MK5 overexpression (Fig. 7 b and not depicted), indicating that MK5 regulates expression of the same gene targets as Foxo1–S215. A kinase-dead mutant of MK5 was used as a control in these experiments to ensure that kinase activity of MK5 was responsible for the observed changes in gene expression (Fig. 7 b). These results strongly support the notion that MK5 phosphorylates Foxo1 at serine 215, which results in transcriptional activation of a select subset of Foxo1 target genes.

Figure 6. Knockdown of MK5 blunts STI–571–induced Rag expression. (a) Mapkapk5 transcript levels in untransduced AMuLV-transformed cells (parental), cells expressing shRNA against luciferase (shLuc), and cells expressing shRNA against MK5 (shMK5) were determined by quantitative RT-PCR. Values were normalized to Hprt1 transcript abundance. (b) AMuLV-transformed cells overexpressing 3xFlag-MK5 were infected with retroviruses expressing shRNAs against MK5 (shMK5) or luciferase (shLuc). 3xFlag-MK5 level was determined by immunoblot. GAPDH was used as a loading control. (c) Rag1–GFP cells were infected with retroviruses expressing shMK5 or shLuc, and GFP level was determined by flow cytometry. Cells were labeled with anti-hCD2 (retroviral marker) and gated on hCD2+ cells (expressing shRNA, solid lines) or hCD2− cells (not expressing shRNA; filled histogram). Percentage of GFP+ cells is shown in gray for cells not expressing shRNA (corresponding to shaded histograms), and in black for cells expressing shRNA (corresponding to solid lines). Top and bottom panels show cells untreated and treated with 1 µM STI–571 for 12 h, respectively. (d) Constitutively high Rag expressing AMuLV-transformed cells were infected with retroviruses expressing shRNA against luciferase (shLuc), Foxo1 (shFoxo1), and MK5 (shMK5). Cells were sorted for infection marker, and Rag1 expression levels were determined by quantitative RT-PCR. Values were normalized to Hprt transcript abundance. Error bars represent standard deviation of triplicate or quadruplicate PCR assays. All data are representative of at least three independent experiments.

MK5 requires Foxo1 to activate Rag transcription
To assess the requirement of Foxo1 in MK5–induced Rag expression, we overexpressed MK5 in Foxo1–deficient AMuLV-transformed cells and measured Rag transcript levels by RT-qPCR. Empty vector and Foxo1 constructs were used as controls. As expected, reconstitution of Foxo1–deficient cells with wild-type Foxo1 robustly induces Rag expression as compared with empty vector control, whereas reconstitution with S215A mutant Foxo1 did not (Fig. 8). Overexpression of MK5 failed to induce Rag expression in the absence of Foxo1 (Fig. 8), indicating that Foxo1 is required for MK5 to up–regulate Rag expression.
MK5 is the primary MK family member that regulates Rag expression

MK5 is part of a family of related kinases with two other members, MK2 and MK3. These kinases have been shown to have overlapping targets (Gaestel, 2006; Shiryaev and Moens, 2010; Cargnello and Roux, 2011). We asked whether other MK family members might also be necessary for Rag transcription by generating shRNAs against MK2 and MK3. Knockdown efficiency of two different shRNA constructs against each of the kinases resulted in a 50% decrease in transcript level (Fig. 9 a), similar to the extent of MK5 knockdown by shMK5 (Fig. 6 a). We assayed Rag induction by STI-571 when MK2 or MK3 level was reduced and found that this had no effect on Rag1-GFP induction by STI-571 in Rag1-GFP/± cells (Fig. 9 b). Overexpression of MK2 or MK3 in AMuLV-transformed cells resulted in a moderate but inconsistent up-regulation of Rag expression (unpublished data). Thus, MK5, and not MK2 and MK3, is limiting for Rag transcription and consistently up-regulates Rag expression when overexpressed.

MK5 regulates Rag transcription through Foxo1 in developing B cells

Although AMuLV-transformed cells provide an excellent tool to study signaling pathways and gene regulation in B lineage cells, the transformation process is variable and the behavior of transformed cells may not accurately reflect all aspects of B cell development. To confirm that MK5 is a physiologically relevant regulator of Foxo1-dependent Rag transcription in developing B cells, we infected bone marrow B cells from
Rag1GFP+/ mice with a retrovirus expressing an MK5 cDNA and measured Rag1-GFP levels. We gated on CD19+ and IgM− developing B cells where Rag is actively transcribed. Consistent with our data from AMuLV-transformed cells, MK5 overexpression increased Rag1-GFP levels in developing B cells, whereas no increase in Rag1-GFP expression was observed in cells overexpressing a kinase-dead mutant of MK5 or an empty vector control (Fig. 10a). These data indicate that MK5 kinase activity is sufficient for up-regulation of Rag transcription in primary developing B cells.

We next asked whether MK5 is required for normal Rag transcription by infecting bone marrow B cells from Rag1GFP+/ mice with a retrovirus encoding an shRNA targeting MK5. Knocking down MK5 decreased Rag1-GFP fluorescence in CD19+ IgM− B cells when compared with a luciferase-specific control shRNA (Fig. 10b), suggesting that MK5 activity is required for normal Rag expression in developing B cells.

To further assess the role of MK5 in B cell development, we used the IL-7 withdrawal system to ask whether MK5 is required for Rag induction when B cells differentiate from large cycling pre–B cells to small resting pre–B cells (Johnson et al., 2008). We infected primary pro–B cells from Rag1GFP+/ mice with a retrovirus encoding an shRNA targeting MK5. Knocking down MK5 decreased Rag1-GFP fluorescence in CD19+ IgM− B cells when compared with a luciferase-specific control shRNA (Fig. 10b), suggesting that MK5 activity is required for normal Rag expression in developing B cells.

To ask whether MK5 acts on Foxo1 in primary B cells, we tested whether serine 215 on Foxo1 is required for Rag transcription in developing B cells. We infected bone marrow B cells from Rag1GFP+/ mice with a retrovirus expressing cDNA encoding either wild-type Foxo1 or the Foxo1-S215A mutant and measured Rag1-GFP levels. As expected, wild-type Foxo1 overexpression robustly up-regulated Rag expression when compared with the empty vector control (Fig. 10d). The Foxo1-S215A mutant, however, failed to induce Rag1-GFP fluorescence (Fig. 10d). Collectively these results strongly suggest a positive role of MK5 on Rag transcription in developing B cells, likely through phosphorylation of S215 in Foxo1.
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**Figure 10.** MK5 modulates Rag expression in primary B lymphocytes. (a) CD19-IgM+ bone marrow B cells from Rag1-GFP heterozygous mice were infected with retroviruses expressing MK5 (Thy1.1+, solid lines) or empty vector (Thy1.1+, filled histograms; repeated in each plot for reference) and cultured in 2 ng/ml IL-7 for 3 d. Cells were collected and labeled with antibodies to delineate B cell developmental subsets and mark retrovirus-infected cells. GFP expression was determined by flow cytometry. (b) CD19-IgM+ bone marrow B cells from Rag1-GFP heterozygous mice were infected with retroviruses expressing shMK5 (hCD2+, solid lines) or shLuc (hCD2+, filled histograms) and analyzed for GFP expression by flow cytometry 3 d later. (c) B220+IgM+ bone marrow B cells from Rag1-GFP heterozygous mice were infected with hCD2-marked retroviruses expressing shMK5 (solid line) or shLuc (filled histogram). Cells were cultured for 5 d in the presence of 4 ng/ml IL-7 (IL-7 high), followed by 24 h of IL-7 withdrawal (IL-7 low). GFP expression was analyzed by flow cytometry. Data were gated on hCD2+ cells. (d) B220+IgM+ bone marrow B cells from Rag1-GFP heterozygous mice were infected with hCD2-marked retroviruses expressing empty vector control (filled histogram), wild-type (WT), or mutant (S215A) Foxo1 (solid line) and analyzed for GFP expression by flow cytometry 3 d later. Data were gated on hCD2+ cells. All data are representative of at least two independent experiments.

**DISCUSSION**

Foxo1 regulates Rag expression at multiple stages during B cell development. In common lymphoid progenitors, Foxo1 and EBF form a positive-feedback loop to activate genes important for B lineage specification, including Rag (Mansson et al., 2012). At the pro- to pre-B transition, IL-7 receptor signaling activates the PI(3)K–AKT pathway, resulting in inhibition of Foxo1 activity and, thus, repression of Rag transcription (Ochiai et al., 2012). Attenuation of IL-7 receptor signaling coupled with pre-BCR signaling results in activation of Foxo1 for Rag transcription and subsequent light chain locus rearrangement in the pre-B cell stage (Ochiai et al., 2012). Our study identified a novel pathway that regulates Foxo1 activity for Rag expression at this pro- to pre-B checkpoint of B cell development. By screening the activity of a panel of Foxo1 mutants, we found a novel phosphorylation site (serine 215) on Foxo1 that modulates its activity. This residue is required for optimal DNA binding. However, it does not regulate overall Foxo1 activity but rather transactivation of a specific subset of target genes. We next demonstrated that MK5 is necessary for full activation of Rag transcription and requires the presence of Foxo1. We also showed that MK5 regulates the same set of genes that are sensitive to Foxo1-S215. Finally, we confirmed these results in primary B cells, implicating MK5 as a novel regulator of Foxo1 in Rag regulation during B cell development.

To date, phosphorylation of serine 215 on Foxo1 has not been reported. S215 is a highly conserved residue among FOXO family members across diverse taxa and is phosphorylated on another family member, Foxo3a (Kress et al., 2011). In-depth biochemical and functional analyses performed by Kress et al. (2011) convincingly showed that MK5 is a bona fide kinase of S215 on Foxo3a. We postulate that the highly conserved nature of this residue and the surrounding sequences makes S215 on Foxo1 a likely direct substrate of MK5.

S215 lies in the winged-helix (DNA binding) domain of Foxo. The crystal structure of Foxo1 suggests that S215 may contribute to a hydrogen bond and interact directly with DNA. It is postulated that phosphorylation of this residue interferes with DNA binding by steric hindrance, hence reducing Foxo1 transactivational activity (Brent et al., 2008). However, we and others have shown that phosphorylation of S215 enhances Foxo1-dependent gene expression (Kress et al., 2011). Thus, the mechanism by which S215 phosphorylation regulates Foxo1 transcriptional activity remains unclear. Although our ChIP data suggest a role for S215 to regulate Foxo1 occupancy at target loci, we cannot distinguish whether S215 phosphorylation enhances direct binding to DNA or whether S215-phosphorylated Foxo1 is more efficiently recruited to DNA indirectly by other cofactors. It has been well established that Foxo1 cooperates with diverse binding partners to regulate gene expression (van der Vos and Coffer, 2008). The differential regulation of target genes by S215 may be explained if specific cofactors are required for transactivation of Foxo1 at different loci. Indeed, out of the target genes tested, only Rag and Aida expression levels were affected by this mutation. Given these are both B lineage–specific genes, it is tempting to hypothesize that a B cell lineage factor cooperates with Foxo1, through S215 phosphorylation, to activate transcription at these loci.
whereas other S215-independent Foxo1 gene targets require different cofactors whose association with Foxo1 is not regulated by S215. Consistent with this hypothesis, motif analysis (Bailey et al., 2005) revealed that enriched motifs in Foxo1 binding regions at the Rag and Aida loci found by ChIP-seq analysis (Ochiai et al., 2012) have no overlap with the enriched motifs found at the S215A-insensitive Foxo1 target genes (Fig. S1), suggesting that besides Foxo1 DNA binding, other factors likely play a role in the regulation of the S215 mutation-sensitive and -insensitive genes.

We identified MK5 as an activator of Foxo1 in B cells. MK5 is a 54-kD serine/threonine kinase discovered simultaneously by two groups (New et al., 1998; Ni et al., 1998). To date, few substrates of MK5 have been identified in vitro, with HSP27 and Foxo3α being the only validated targets in vivo (Kress et al., 2011). Other MK family members, MK2 and MK3, have been shown to have overlapping target specificities (Shiryaev and Moens, 2010). HSP27, for example, is phosphorylated by all three MKs but on different serine residues (Kostenko et al., 2009). We observed that knocking down MK2 and MK3 has no effect on Rag expression, suggesting that Foxo1 is an MK5-specific substrate. Indeed, although MK2 and MK3 share 75% sequence homology and have been shown to share similar functions and display redundancy, MK5 is more distantly related (35% homology) and has distinct structure and function. Overexpression of MK2 or MK3 in AMuLV-transformed cells resulted in moderate but inconsistent up-regulation of Rag expression, possibly as a result of pleiotropic effects of these kinases. Thus, although MK2 and MK3 are not limiting for Rag expression, we cannot rule out the possibility that they might have low activity toward Foxo1. Collectively, our results suggest that MK5 is the primary MK family member that activates Foxo1.

The biological functions of MK5 are still under scrutiny. To date, two MK5 knockout mice have been independently generated. However, the knockout mice in different genetic backgrounds display either no obvious phenotype or embryonic lethality, and the reason for this lethality remains unknown (Shi et al., 2003; Schumacher et al., 2004). Recent studies have implicated MK5 in Ras-induced senescence, tumor suppression, rearrangements of the cytoskeleton, cell migration, energy depletion–induced cell growth arrest, angiogenesis, and neuronal differentiation (Gerits et al., 2007b; Sun et al., 2007; Zheng et al., 2011; Yoshizuka et al., 2012a). However, the genuine biological role of MK5 remains elusive, as most of these studies were performed in vitro, and animal studies performed in one knockout mouse have not been reproduced using the reciprocal mouse. So far, no defect in B cell development in either MK5 knockout mice has been reported. Although our study indicates that only MK5 is limiting for Rag activation, we cannot exclude the possibility that the other MK family members may compensate for loss of MK5 in vivo. Further studies involving B cell–specific MK5 deletion and compound deletion of MK5 and other MK family members should be done to address the specific functions of MK5 in B cell development.

Upstream pathways regulating MK5 are also under debate. MK5 was initially discovered as a p38-activated/regulated protein kinase and has been shown to act downstream of p38 during oncogene-induced senescence (Sun et al., 2007; Yoshizuka et al., 2012b). Other upstream regulators of MK5 have been identified in different contexts. During embryonic development, MK5 forms a complex with ERK3 and ERK4 which promotes its activity (Schumacher et al., 2004; Seternes et al., 2004; Aberg et al., 2006; Kant et al., 2006). Activation of ERK3 and ERK4 by p21-activated kinases (PAKs) results in activation of MK5 (De la Mota-Peynado et al., 2011; Délèris et al., 2011). cAMP/PAK has also been shown to activate MK5 for actin remodeling (Gerits et al., 2007a). In AMuLV-transformed cells, treatment with either SB203580 or BIRB 796 (p38 inhibitors) has no effect on STI-induced Rag expression, and treatment with anisomycin or isoproterenol (p38 agonists) do not induce Rag (unpublished data). Furthermore, shRNA knockdown of ERK3, PAK2, and induction of cAMP level by forskolin have no effect on Rag expression (unpublished data). Further studies are required to pinpoint the relevant upstream signaling events regulating MK5–Foxo1 during B cell development.

In conclusion, our results reveal a novel residue on Foxo1 that regulates its transcriptional activity, and we discovered a role for MK5 in B cell development. Besides regulating Rag transcription, Foxo1 also plays distinct roles at other stages of B cell development as well as peripheral B cell function. In fact, Aida, which encodes AID, is essential for class switch recombination upon B cell activation and has been shown to be a Foxo1 target gene in vivo (Dengler et al., 2008). The fact that Foxo1-S215 mutation affected Aicda expression might indicate a more general role for MK5 in the antibody response.

The system used in this study allowed us to examine the function of MK5–Foxo1 in the pro–to pre–B cell transition specifically. Stage-specific deletion of MK5 or Foxo1-S215A knockin mice would be the ideal systems to tease apart MK5–Foxo1 functions at the other stages of B cell development in vivo. Alternatively, systems such as the Id2-overexpressing hematopoietic progenitors, where Id2 withdrawal induces B lineage differentiation from multipotent progenitors (Mercer et al., 2011), could provide insight into the roles of MK5 and Foxo1 in the activation of Rag and other genes during the earliest stages of B lineage commitment. Given the diverse functions of Foxo1 both in and outside the B cell lineage, it will be important to investigate whether MK5 also plays a role in modulating Foxo1 activity in other cellular contexts.

MATERIALS AND METHODS

Chemicals. Wortmannin, AKT inhibitor VIII, 4-hydroxy-Tamoxifen were purchased from EMD Millipore. STI-571 was purchased from Novartis. Recombinant mouse IL-7 was purchased from R&D Systems.

Cell culture. The AMuLV-transformed Foxo1<sup>1631</sup>-ER<sub>Cre</sub> cell line was generated by infection of bone marrow from a mouse homozygous for a floxed Foxo1 allele carrying a tamoxifen inducible Cre allele (femurs were a gift from S. Hedrick, University of California, San Diego, San Diego, CA).
Transformed B cells were cloned and screened for efficient deletion of floxed alleles upon Tamoxifen treatment. A single clone was selected for all experiments. The AMuLV-transformed Rag1-GFP knockin cells were previously described (Amin and Schlissel, 2008). All AMuLV-transformed cell lines were cultured in RPMI 1640 medium supplemented with 5% (vol/vol) FCS, 2 mM t-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 50 mM 2-mercaptoethanol. Primary B cells were cultured in same media for AMuLV-transformed cells, except with 10% FCS and supplemented with 2 mg/ml IL-7. For IL-7 withdrawal experiments, primary cells were cultured in 5 mg/ml IL-7 for 2 d, and then resuspended in media without IL-7 for 1 d before analysis. Phoenix cells used for viral packaging were cultured in DMEM medium supplemented with 5% (vol/vol) FCS, 2 mM t-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, and sodium pyruvate. All cells were grown at 37°C in 5% CO2. All mouse experimentation was approved by the Animal Care and Use Committee of the University of California at Berkeley (Protocol # R253-0313BR).

Retroviral production and infection. Phoenix cells were transfected with retroviral plasmid and VSV-G resuspended in Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Viral supernatant was collected and filtered 24–96 h after transfection. AMuLV-transformed cells were infected by resuspension of the cells in viral supernatant containing 4 mg/ml polybrene (Sigma-Aldrich) and cultured overnight. Cells were then expanded into normal media. Primary cells were infected as in Amin and Schlissel (2008). Cells were analyzed or sorted by flow cytometry 3–4 d after infection.

Gene expression analysis by quantitative real-time PCR. RNA was isolated by lysing cells in TRIzol reagent (Invitrogen). Reverse transcription was performed using MMLV-RT (Invitrogen) or SuperScript III-RT (Invitrogen) with random hexamers according to the manufacturer’s instructions. Quantitative real-time PCR was performed using JumpStart Taq polymerase (Sigma-Aldrich) according to the manufacturer’s protocol and fluorescent labeling with EvaGreen (Biotium). PCR, cycling conditions were 95°C for 4 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. Primer sequences are provided in Table S2.

Expression plasmids. All retroviral plasmids were based on the MSCV retroviral vector (Cherry et al., 2000). Overexpression constructs contain cDNA cloned upstream of an IRES in frame with a surface marker (Thy1.1 or human CD4; Amin and Schlissel, 2008). cDNAs were cloned by PCR amplification using PfX platinum (Invitrogen) according to the manufacturer’s protocol and confirmed by DNA sequence analysis.

Plasmids containing MK5 cDNAs were provided by O.M. Sørensen (University of Tromso, Tromso, Norway). Open reading frames were PCR amplified from those plasmids and cloned into the MSCV retroviral construct upstream of IRES-Thy1.1. A 3xFLAG tag was inserted in frame at the C terminus of MK5.

Wild-type murine Foxo1 was PCR amplified from a cDNA library generated from primary pro– and pre-B cells (Amin and Schlissel, 2008) and cloned into the MSCV retroviral construct upstream of IRES-ICD4. A 3xFLAG tag was inserted in frame at the C terminus. Foxo1 mutants were generated by QuikChange multi-site mutagenesis kit (QIAGEN) according to the manufacturer’s protocol. Primers used for mutagenesis are listed in Table S2. All cDNAs were confirmed by DNA sequence analysis.

shRNAs were expressed using a retrovirus containing human CD2 cDNA followed by a modified human miR-30 microRNA precursor (Steiger et al., 2005). The sequence of shRNA targeting MK5 was obtained from the RNAi Codex database (target sequence: 5’-GGGCTCGACTCTTAAATTGTAA-3’) and cloned into the miR-30 context.

ChIP. ChIP was performed as previously described (Lee et al., 2006). 50 million cells were used for each immunoprecipitation. 5 µg anti-FLAG (M2; Sigma-Aldrich), anti-Foxo1 (Abcam), or IgG control antibody (Santa Cruz Biotechnology, Inc.) was used. Immunoprecipitated was collected using magnetic Protein G Dynabeads (Invitrogen) and washed three times with low salt buffer, once with high salt buffer, and once with LCL buffer as described in (Yu et al., 2000). DNA protein cross-links were reversed, and DNA was extracted using DNA spin columns (QUAGEN) and subjected to quantitative real-time PCR. Primer sequences are provided in Table S2.

Flow cytometry. Single-cell suspensions were prepared from mice or from cultured cells and were labeled with fluorochrome-conjugated antibodies by standard techniques. An FC500 (Beckman Coulter) or LSRII (BD) flow cytometer was used for analysis; a MoFlo or an Influx high-speed cell sorter (Dako) was used for sorting. Data were analyzed with FlowJo software (Tree Star). Dead cells were gated out using forward and side scatter for all analyses. Analysis with primary B cells was done by labeling cells with anti-IgM (IL/41) and anti-CD19 (1D3) antibodies and analyzed by infection marker (anti-hCD2 [RPA-2.10], anti-hCD4 [RPA-T4], or anti-Thy1.1 [OX-7]). Anti-CD19 and anti-Thy1.1 antibodies were obtained from BD, and all other antibodies were obtained from eBioscience.

Subcellular fractionation. Cells were washed in PBS and resuspended in 2–3× volume of NP-40 lys buffer containing 0.5% NP-40, 30% sucrose, 25 mM Tris-HCl, pH 7.5, 2.5 mM KCl, and 7.5 mM MgCl2. Cells were lyse on ice for 10 min. Supernatant (cytoplasmic fraction) was collected after 20 s of centrifugation at top speed. Pellet was washed once with PBS, and lysed in RIPA buffer (same volume as NP-40 lysis buffer used for cytoplasmic extraction) for 10 min on ice. Supernatant (nuclear fraction) was clarified by cen trifugation at top speed for 10 min. Equal volume of cytoplasmic and nuclear fraction was boiled in sample buffer containing SDS for 5 min before immunoblot analysis. Fresh complete protease inhibitor cocktail (Roche) and 1 mM PMSF were added to all lys buffers.

Immunoblot. Cells were lysed in RIPA buffer for 10 min on ice and then centrifuged to clear insoluble material. Protein was quantified with Bradford Protein Assay (Bio-Rad Laboratories). 10–80 µg of protein was boiled for 5 min with sample buffer containing SDS. The lysate was separated by 10% or 15% SDS-PAGE gel and then transferred to PVDF-FI (Millipore) membranes. Membranes were blocked in 5% milk and labeled with primary and secondary antibodies according to the manufacturer’s instructions. Blots were analyzed with the Odyssey Infrared Imaging System (LI-COR Biosciences). Anti-Foxo1 (L27) and anti-GAPDH (D16H11) antibodies were obtained from Cell Signaling Technologies, anti-FLAG (M2) antibody was obtained from Sigma-Aldrich, anti-Lamin B1 (ab16048) and anti-Histone H3 (di-/tri-methylated lysine 4; ab6000) antibodies were obtained from Abcam, and anti-Axin (C-11) was obtained from Santa Cruz Biotechnology, Inc. Infrared dye–conjugated secondary antibodies were purchased from Molecular Probes.

Microarray analysis. Three independent replicates of Foxo1 ΔERCre cells treated and untreated with 1 µM Tamoxifen for 2 d were collected. RNA was isolated with TRIzol reagent (Invitrogen), and further purified by the RNeasy Mini kit (QIAGEN). Samples were submitted for analysis to the UCSF genomics core facility. Affymetrix GeneChip Mouse Gene 1.0 ST Arrays were used. Differential gene expression analysis was performed using the GenePattern platform (Broad Institute). Microarray dataset was deposited to NCBI GEO repository (GSE46031).

Online supplemental material. Fig. S1 shows Foxo1 binding peaks at S215A-sensitive and -insensitive genes enrich for different motifs. Table S1 shows Foxo1 mutants and primers used for mutagenesis. Table S2 shows quantitative RT-PCR primers. Online supplemental material is available at http://www.jem.org/content/full/jem.20130498/DC1.

We thank Dr. O.M. Seternes (University of Tromso, Norway) for MK5 plasmids, Dr. Y. Kendels and S. Hedrick (University of California, San Diego) for femurs from Foxo1 ΔERCre mice, and H. Nolla and A. Valeros (University of California, Berkeley) for help with cell sorting. We thank W. Huang (U.C. Berkeley) for technical assistance.
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