Published in final edited form as:
Genes Immun. 2013 January ; 14(1): 7–12. doi:10.1038/gene.2012.47.

Reciprocal regulation of *Rag* expression in thymocytes by the zinc-finger proteins, Zfp608 and Zfp609

NP Reed[1], **MA Henderson**[1], **EM Oltz**[2], and **TM Aune**[1,3]

[1]Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN, USA

[2]Department of Pathology, Washington University School of Medicine, St Louis, MO, USA

[3]Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA

Abstract

Recombination-activating gene 1 (Rag1) and Rag2 enzymes are required for T cell receptor assembly and thymocyte development. The mechanisms underlying the transcriptional activation and repression of *Rag1* and *Rag2* are incompletely understood. The zinc-finger protein, Zfp608, represses *Rag1* and *Rag2* expression when expressed in thymocytes blocking T-cell maturation. Here we show that the related zinc-finger protein, Zfp609, is necessary for *Rag1* and *Rag2* expression in developing thymocytes. Zfp608 represses *Rag1* and *Rag2* expression indirectly by repressing the expression of *Zfp609*. Thus, the balance of Zfp608 and Zfp609 plays a critical role in regulating *Rag1* and *Rag2* expression, which may manifest itself not only during development of immature thymocytes into mature T cells but also in generation of the T-cell arm of the adaptive immune system, which does not fully develop until after birth.

Keywords

Zfp608; Zfp609; Rag; immune system; thymus

INTRODUCTION

The assembly of antigen receptor genes by T and B lymphocytes represents one of the fundamental features of the adaptive immune response in vertebrates. This process is required to generate enormous diversity in the immunoglobulin and T cell receptor repertoire, which is necessary to recognize virtually any encountered pathogen.[1–3] The processes involved in the ordered assembly of antigen receptor genes by V(D)J recombination are catalyzed by the recombination-activating gene (RAG)-1/RAG-2 enzymes and are unique to precursor T and B lymphocytes.[4–7] However, the mechanisms

Correspondence: Professor TM Aune, Department of Medicine, Vanderbilt University School of Medicine, MCN T3219, 1161 21st Avenue South, Nashville, TN 37232, USA. tom.aune@vanderbilt.edu.

CONFLICT OF INTEREST The authors declare no conflict of interest.

Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)

that control the activation and repression of *Rag* genes during precursor lymphocyte and fetal development remain unclear.[8,9] These precisely orchestrated pathways are critical to the development of a functioning adaptive immune system.

In contrast to most organs, the adaptive immune system does not fully develop until after birth (3–4 weeks postpartum in mice and 2–4 years in humans).[10–13] The reasons for this delayed development remain unclear. One possibility is that development of a fully functional adaptive immune system *in utero* or early in postnatal life would trigger deleterious responses to maternal antigens. Alternatively, the repertoire of self-antigens in fetal or neonatal development may be vastly different than those expressed in adults. Thus, efficient immune development in neonates may lead to the production of potential autoreactive clones in adults, which may also produce a highly skewed immunoglobulin and T cell receptor repertoire. Therefore, active mechanisms may be in place to delay development of the adaptive immune system until later in postnatal life.

We developed a congenic strain (ZORI (*Zfp608* overexpressing Rag insufficient)) with defects in *Rag1* and *Rag2* expression, thymocyte maturation and peripheral T-cell homeostasis.[14,15] This mutation maps to a locus on chromosome 18 that contains a single known gene, *Zfp608*. In wild-type mice, *Zfp608* is highly expressed in neonatal thymus but is extinguished after birth. In contrast, ZORI mice sustain thymocyte expression of *Zfp608* throughout life. The ZORI mutation produces a thymocyte-intrinsic developmental defect. Overexpression of a *Zfp608* complementary DNA in thymocytes by transient transfection severely impairs *Rag* expression, providing an underlying mechanism for defects in ZORI thymocyte development. This ZORI defect is similar to that observed in Omenn Syndrome.[16] ZORI mice and Omenn syndrome cases both have limited levels of V(D)J recombination due to either reduced *Rag* expression or the presence of hypomorphic mutations in *Rag* resulting in reduced Rag activity, respectively. This points to the fine balance of *Rag* expression and timing required for normal development and function of the immune system. A further manifestation of Omenn syndrome is development of autoimmune symptoms thought to result from the generation of a restricted T cell receptor or B cell receptor repertoire, indicating that reduced Rag function can produce additional immune phenotypes such as autoimmunity.

Our previous studies do not address whether Zfp608 represses *Rag* expression directly by, for example, targeting the Rag locus or indirectly by altering the expression or interfering with the function of transacting factors that directly regulate *Rag* expression. Based upon amino-acid sequence conservation, Zfp609 is the closest relative to Zfp608. This high level of shared identity supports the notion that *Zfp608* and *Zfp609* genes evolved from a common ancestral gene.[17–19] The expression and function of ancestrally related genes may change through evolution or may remain redundant. Therefore, we hypothesized that Zfp609 may also regulate *Rag* gene expression during T-cell development. Our results demonstrate that Zfp608 negatively regulates *Zfp609* expression and that Zfp609 expression is necessary for *Rag* expression by thymocytes.

RESULTS

Reciprocal expression of *Zfp608*, *Zfp609*, *Rag* and *Aire* in the thymus

Zinc-finger proteins are transcriptional regulators.[20] Previous studies have shown that *Zfp608* reduced *Rag* expression in thymocytes and impaired T-cell development. Based upon a total percent identity of 43.6% and a total sequence similarity of 57.6%, *Zfp609* is the closest relative to *Zfp608* (Supplementary Figure 1). A C2H2 zinc-finger and domain (D2) are conserved in each protein from vertebrates to deuterostomes.[17] This domain conservation, coupled with percent identity and percent similarity, supports the notion that *Zfp608* and *Zfp609* evolved from a common ancestral gene.[18,19] Expression and function of ancestrally related genes may change through evolution or may remain redundant with similar function. ZORI thymocytes constitutively express *Zfp608* and reduced levels of *Rag*. To address the functional relationships between *Zfp608*, *Zfp609* and *Rag* expression, we purified total thymocyte RNA from 6-week-old homozygous ZORI or BALB/c mice ($n = 3$). Using real-time PCR, we measured thymocyte expression of *Zfp608*, *Zfp609*, *Rag1* and *Rag2* relative to *Gapdh* (Figures 1a–d). As expected, ZORI thymocytes expressed higher levels of *Zfp608* than BALB/c thymocytes. In contrast, BALB/c thymocytes expressed higher levels of *Zfp609* than ZORI thymocytes. Thus, *Zfp608* expression negatively correlates with *Rag* expression, whereas *Zfp609* expression positively correlates with *Rag* expression.

Histone modification and remodeling is a dynamic strategy employed by all eukaryotes to regulate gene expression. *Brakeless*, the *drosophila* ortholog of *Zfp608*, is part of a corepressor complex that recruits histone deacetylases to suppress gene expression.[17] The conservation of function between *brakeless* and *Zfp608* has not been fully elucidated. We postulated that ZORI thymocytes might exhibit decreased histone acetylation at the *Rag* promoter relative to BALB/c thymocytes. To test this, we used chromatin immunoprecipitation to compare histone 4 acetylation at Rag promoters in ZORI and BALB/c thymocytes ($n = 3$). We found that levels of histone 4 acetylation were greater at both *Rag* promoters in BALB/c compared with ZORI thymocytes (Figures 1e and f). These results support the notion that Zfp608 promotes deacetylation of *Rag* promoters in ZORI thymocytes.

Rag mutations result in a loss of *Aire* expression by thymic epithelium. It is not known if decrease in Rag expression in the absence of Rag mutation, as seen in ZORI thymocytes, is sufficient to result in decreased *Aire* expression. To address this possibility, we determined levels of *Aire* expression in ZORI and BALB/c thymic epithelia. We found that levels of *Aire* expression were markedly reduced in ZORI thymic epithelia compared with BALB/c thymic epithelia (Figure 1g). These results indicate that decreased Rag expression in thymocytes is associated with a decrease in *Aire* expression in thymic epithelia. Whether *Aire* deficiency is associated with physical manifestations of the ZORI genotype is not known.

Zfp608 represses *Zfp609* expression

The VL3-3M2 thymocyte cell line constitutively expresses *Rag* genes and has been employed to investigate mechanisms of *Rag* regulation and thymocyte differentiation. We compared levels of *Zfp608* and *Zfp609* expression in VL3-3M2 cells with NIH-3T3 fibroblasts. We isolated total RNA from NIH-3T3 fibroblasts and VL3-3M2 thymocytes and used real-time PCR to compare levels of *Zfp608*, *Zfp609*, *Rag1* and *Rag2* relative to *Gapdh* ($n = 3$). NIH-3T3 fibroblasts expressed high levels of *Zfp608*, whereas it was undetectable in VL3-3M2 thymocytes (Figure 2a). In contrast, *Zfp609* transcript levels were higher in VL3-3M2 cells than in NIH-3T3 fibroblasts (Figure 2b). As reported, *Rag1* and *Rag2* were expressed in VL3-3M2 thymocytes but these transcripts were undetectable in NIH-3T3 fibroblasts (Figures 2c and d). As with thymocytes, these data demonstrate that expression of *Rag* is associated with high levels of *Zfp609* expression and low levels of *Zfp608* expression in VL3-3M2 cells.

From these data, we envision two possibilities: Zfp608 directly represses *Rag* gene expression or it directly represses expression of *Zfp609*, which, in turn, is required for Rag expression. To better distinguish between these two possibilities, we generated VL3-3M2 cells stably transfected with a vector constitutively expressing *Zfp608*, termed VL3-3M2-Zfp608. Total RNA was isolated from VL3-3M2 and VL3-3M2-Zfp608 cells and analyzed by real-time PCR ($n = 4$). VL3-3M2-Zfp608 cells had a higher level of *Zfp608* expression compared with VL3-3M2 cells (Figure 3a). *Rag1* and *Rag2* transcript levels were reduced in VL3-3M2-Zfp608 cells compared with VL3-3M2 cells (Figures 3b and c). There was also a marked reduction in *Zfp609* transcript levels in VL3-3M2-Zfp608 cells (Figure 3d). We observed a consistent relationship between expression levels of *Rag1*, *Rag2* and *Zfp609* relative to Zfp608 (Figure 3e). The changes in *Rag1*, *Rag2* and *Zfp609* expression levels were comparable relative to Zfp608. These results resemble the expression patterns observed in ZORI thymocytes and are consistent with the possibility that Zfp608 negatively regulates *Zfp609* expression in this thymocyte cell line. These results also suggest that there is a proportional relationship between expression levels of *Zfp609*, *Rag1* and *Rag2*.

Zfp609 is a positive regulator of *Rag* expression

Our data demonstrate that Zfp608 negatively regulates *Zfp609* expression in thymocytes, but do not address whether Zfp609 is a positive regulator of *Rag* expression. To test this possibility, we introduced a *Zfp609* expression plasmid or empty vector control into VL3-3M2-Zfp608 cells by transfection with diethylaminoethanol-dextran (DEAE-dextran) and monitored *Rag* expression ($n = 4$). Transfection of a *Zfp609* expression vector increased *Zfp609*, *Rag1* and *Rag2* expression in VL3-3M2-Zfp608 cells (Figures 4a–c). There was no difference in *Zfp608* expression in empty vector-transfected controls versus Zfp609 expression vector-transfected cells (Supplementary Figure 2). We compared the transcript levels of *Rag1* and *Rag2* relative to *Zfp609* and *Zfp608* (Figure 4d). We observed that expression levels of *Rag1* and *Rag2* relative to expression levels of *Zfp609* were consistent between controls and Zfp609-transfected cells. Thus, we conclude that Zfp609 is necessary for *Rag1* and *Rag2* expression in VL3-3M2-Zfp608 cells and that Zfp608 represses *Zfp609* and its downstream targets *Rag1* and *Rag2*.

These results prompted us to consider whether Zfp609 is a positive regulator of *Rag* expression. Accordingly, we determined if a reduction in Zfp609 altered *Rag* expression. To achieve this, we employed a small interfering RNA (siRNA) strategy for targeted knockdown of *Zfp609*. This was performed in both VL3-3M2 and BALB/c thymocytes (n = 5) using a scrambled/nonspecific siRNA and a *Zfp609*-specific siRNA. The knockdown proved to be effective based on reduced *Zfp609* expression in VL3-3M2 thymocytes transfected with the *Zfp609*-specific siRNA compared with scrambled/nonspecific controls (Figure 5a). siRNA-mediated knockdown of Zfp609 also produced a reduction in both *Rag1* and *Rag2* transcript levels in VL3-3M2 thymocytes (Figures 5b and c). Knockdown of Zfp609 similarly attenuated *Zfp609*, *Rag1* and *Rag2* transcripts in BALB/c thymocytes (Figures 5e–g). We observed an increase in *Rag1* and *Rag2* relative to *Zfp609* in siRNA-targeted VL3-3M2 cells and BALB/c thymocytes compared with scrambled controls (Figures 5d and h). This suggests that the knockdown was specific for *Zfp609* and that the corresponding reduction in *Rag1* and *Rag2* did not occur at the same magnitude as *Zfp609*. We conclude from these experiments that Zfp609 is a positive regulator of *Rag1* and *Rag2* gene expression in both VL3-3M2 and BALB/c thymocytes.

Zfp608 inhibits the *Zfp609* promoter and Zfp609 activates the *Rag* promoter

We have shown that *Zfp609* positively regulates *Rag* but is negatively regulated by *Zfp608*. We therefore hypothesized that Zfp608 directly represses the *Zfp609* promoter. The transcriptional start site and *Zfp609* promoter were identified using multispecies conservation.[19] The *Zfp609* promoter was cloned into a luciferase reporter vector and introduced into VL3-3M2 thymocytes by transient transfection with or without a *Zfp608* expression vector. Co-transfection of the *Zfp608* expression vector with the *Zfp609* promoter luciferase reporter led to a reduction of Zfp609 promoter activity (Figure 6a). These data demonstrated that Zfp608 represses *Zfp609* promoter activity, which most likely contributes to Zfp608-dependent reduction of *Zfp609* transcript levels.

Our data also show that Zfp609 is a positive regulator of *Rag* expression. Therefore, we sought to determine if Zfp609 positively regulated *Rag* promoter activity. To address this, we cotransfected VL3-3M2 cells with a *Rag1* or *Rag2* promoter luciferase vector with or without a vector constitutively expressing *Zfp609*.[21,22] Cells transfected with the *Zfp609* expression vector and the *Rag1* promoter construct exhibited increased *Rag1* promoter activity compared with cells transfected with the *Rag1* promoter luciferase reporter construct alone (Figure 6b). There was little change in cells transfected with *Zfp609* and the *Rag2* promoter luciferase reporter construct (not shown). To address specificity of effect, VL3-3M2 cells were co-transfected with a human interferon-γ (*IFNG*) luciferase reporter vector with or without Zfp608 and *Zfp609* expression vectors (Supplementary Figure 3). The presence of *Zfp608* or *Zfp609* had no effect on *IFNG* reporter activity. These results demonstrated that Zfp609 directly stimulates activity of the *Rag1* promoter.

DISCUSSION

Zfp608 is reciprocally expressed in thymocytes when compared with *Zfp609* and *Rag*. Conversely, *Zfp609* and *Rag* are coordinately expressed in all cell systems we examined. In

keeping with these expression patterns, we now show that Zfp608 is a negative regulator of *Zfp609* expression, whereas Zfp609 is a positive regulator of *Rag* expression. Promoter activity assays demonstrate that Zfp608 directly targets the *Zfp609* promoter and that Zfp609 directly targets the *Rag1* promoter. Ectopic expression of Zfp609 is sufficient to overcome Zfp608-mediated repression of *Rag1* and *Rag2* and siRNA-mediated knockdown of Zfp609 is sufficient to block *Rag* expression in both cell lines and developing thymocytes. We conclude from these studies that Zfp609 is necessary for *Rag* expression in developing T cells and that Zfp608 blocks *Rag* expression and T-cell development via targeting the *Zfp609* gene.

Rag1 and *Rag2* expression are coordinately regulated during T-cell differentiation; however, the underlying transcriptional mechanisms are incompletely understood. These genes are linked in the genome but each has a unique promoter that binds distinct transcription factors. A common transcription factor capable of activating or repressing both genes has not been identified. For example, Ikaros, E2A and NF-Y activate the *Rag1* promoter, and Pax5, Myb, Lef1 and GATA-3 activate the *Rag2* promoter.[23] Our results show that Zfp609 is necessary for both *Rag1* and *Rag2* expression in developing T cells and that Zfp609 directly activates the Rag1 promoter. *Zfp609* did not directly activate the Rag2 promoter. This result suggests that *Zfp609* stimulates *Rag2* expression through a regulatory sequence outside of the promoter region tested or through an unidentified interaction. This is plausible considering that several *cis*-regulatory sequences have been identified within the *Rag* locus. Interplay between *Zfp609* and these other transcription factors may be necessary to achieve the known oscillations of *Rag* gene expression during thymocyte differentiation as well as regulation of *Rag* during fetal and adult development.

Our results clearly demonstrate that Zfp609 is necessary for *Rag* expression by developing T cells and that Zfp608 represses *Zfp609* expression. Thus, a likely mechanism for Zfp608-mediated repression of *Rag* expression is via repression of *Zfp609* expression. However, our results cannot rule out the possibility that other mechanisms may contribute to Zfp608-mediated repression of *Rag* expression. For example, Zfp608 may also directly repress *Rag* expression or may compete with Zfp609 for access to the *Rag* locus. Alternatively, the *drosophila* ortholog of Zfp608, *brakeless*, is a transcriptional repressor by virtue of its ability to recruit histone deacetylase complexes to genomic loci.[17,24] Thus, one additional mode of action is that Zfp608 may recruit histone deacetylases to the *Zfp609* locus and/or the *Rag* locus. In a similar vein, Zfp609 may oppose the actions of Zfp608 by recruiting histone acetyl transferases to the *Rag* locus to activate *Rag* gene expression. Additional investigations will be necessary to distinguish among these alternate possibilities.

Complete Rag deficiency (Rag−/−) or murine hypomorphic *Rag* mutations identical to those seen in humans with Omenn syndrome result in markedly reduced expression of *Aire*. Low expression of the AIRE transcription factor contributes to the pathogenesis and autoimmune manifestations in this murine model of Omenn syndrome. ZORI mice, which exhibit reduced *Rag* expression to ~10% of normal levels, also have severely depressed levels of *Aire* expression similar to levels associated with either complete Rag deficiency (Rag−/− mice) or hypomorphic Rag mutations. Altered *Aire* expression in ZORI mice may produce additional autoimmune manifestations contributing to their poor health. In addition,

mutations affecting expression of function of ZNF608 or ZNF609 (human equivalents of murine Zfp608 or Zfp609) may also produce immunodeficiency syndromes or autoimmune syndromes in humans similar to Omenn syndrome.

MATERIALS AND METHODS

Mice

ZORI and BALB/c mice were housed in a nonsterile rodent facility at Vanderbilt University. Antibiotic treatment was administered with sulfamethoxazole (40 mg l^{-1}) and trimethoprim (8 mg ml^{-1}) (Hi-Tech Pharmacal, Amityville, NY, USA) in acidified drinking water. Research complied with all relevant federal guidelines and institutional policies and was approved by the institutional animal care and use committee of Vanderbilt University.

RNA isolation, complementary DNA synthesis and real-time PCR

Total RNA was purified from thymus or cell lines with Tri Reagent (Ambion, Grand Island, NY, USA) according to the manufacturer's instructions. The washed RNA pellet was allowed to air dry and dissolved in RNAse-free water. Complementary DNA was generated from total RNA using Superscript III First-Strand Synthesis System for RT–PCR (Life Technologies, Grand Island, NY, USA) according to the manufacturer's suggested protocol using oligo(dt) primers. Transcript levels of individual genes were determined by the standard ABI 7300 Real-time PCR Machine protocol using the following Taqman gene expression assays (Life Technologies) Zfp608: Mm00558913_m1, Zfp609: Mm00553138_m1, Rag1: Mm01270936_m1, Rag2: Mm01270938_m1 and Gapdh: Mm99999915_g1.

Thymus isolation

Thymocytes and thymic epithelial cells were isolated by mechanical disruption of the thymus and differential centrifugation.

Generation of VL3-3M2-Zfp608 cells

VL3-3M2 cells were transfected with a Zfp608 expression plasmid under the control of the CMV promoter using Lipofectamine 2000 (Life Technologies) according to the manufacturer's recommendations. The Zfp608 expression vector was previously generated and described by Zhang *et al*.[14] Stable transfectants were selected with G418 Sulfate (Mediatech Inc., Manassas, VA, USA).

Transient transfections

The DEAE-dextran method was used for transient transfections into VL3-3M2 or VL3-3M2-Zfp608 cells.[25] In brief, cells were incubated in 1 ml Tris-buffered saline containing 0.5 mg DEAE-dextran and 5 μg plasmid DNA for 10 min at room temperature. Cells were washed and incubated in complete culture media containing 100 μ_M chloroquine at 37 °C for 1 h. After incubation, cells were pelleted, washed and incubated in complete culture until RNA isolation. The plasmid used to transiently express Zfp609 was Clone ID:

3592524 (Open Biosystems, Lafayette, CO, USA). This plasmid contains *Zfp609* in the pCMV-SPORT6 vector.

Zfp609, *Rag1* and *Rag2* promoter cloning

The *Zfp609*, *Rag1* and *Rag2* promoters were cloned by conventional PCR using the following oligonucleotides:

Zfp609 Promoter Forward: 5′-GAGCTCTCCCCTTTCCTAGGTTCCT-3′

Zfp609 Promoter Reverse: 5′-GATATCCTTACCTGCGGCATAGGGTA-3′

Rag1 Promoter Forward: 5′-GAGCTCCATTCTCAGGAGATGAAATGACAGC-3′

Rag1 Promoter Reverse: 5′-GATATCGCAGCTTGGTTGTTAACACTC-3′

Rag2 Promoter Forward: 5′-GAGCTCAAGCTTAAGACAGTCATTTTTTGTGG-3′

Rag2 Promoter Reverse: 5′-GATATCATTGGACCTACCTGAAGGC-3′.

The above oligonucleotides were used as primers and genomic BALB/c DNA as template. The PCR product was cloned into the pGEMT-EASY vector for sequencing verification. The correct clone was excised using SacI and EcoRV and ligated into the pGL4.10 luciferase vector (Promega, Madison, WI, USA).

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were performed as previously described.[26] In brief, 10–20 × 10^6 cells were fixed in 1% formaldehyde for 12 min at room temperature on a platform rocker. After fixation, cells were then quenched with 135 mM glycine followed by two washed with ice-cold phosphate-buffered saline. Cells were resuspended in lysis buffer (50 mM Tris–HCl, 10 mM EDTA, 1% sodium dodecyl sulfate) containing protease inhibitors PMSF, Pepstatin and Aprotinin. Following sonication, chromatin fractions were precleared with protein A/G sepharose beads for 1 h at 4 °C. Chromatin was incubated overnight at 4 °C with experiment-specific primary antibodies. Immune complexes were bound to protein A/G sepharose beads for 2 h followed by washing with low-salt buffer, high-salt buffer, lithium chloride buffer and Tris–EDTA buffer. Bound complexes were isolated by incubation in elution buffer (1% sodium dodecyl sulfate and 0.1 M Na_2HCO_3) for 1 h at room temperature. Bound DNA was harvested by overnight incubation at 65 °C in proteinase K. DNA samples were purified by phenol/chloroform extraction followed by ethanol precipitation and resuspended in water. Samples were analyzed as previously described.[14] Oligonucleotide primers used were as follows:

Rag1: Forward, 5′-CATTCTCAGGAGATGAAATGACAGC-3′,

Reverse, 5′-GCAGCTTGGTTGTTAACACTC-3′,

Rag2: Forward, 5′-AAGCTTAAGACAGTCATTTTTTGTGG-3′,

Reverse, 5′-ATTGGACCTACCTGAAGGC-3′.

siRNA knockdowns

siRNAs specific for *Zfp609* were obtained from Ambion (ID: s103186, s103185, s103184). Lyophilized siRNAs were resuspended per manufacturer's recommendation to 100 m_M stock concentrations. siRNAs were introduced into thymocytes using Amaxa Nucleofactor electroporation (Lonza, Allendale, NJ, USA). In brief, 5×10^6 murine thymocytes or 1×10^6 VL3-3M2 cells were resuspended in 100 μl Nucleofector Solution. Then, 200 n_M of gene-specific siRNA was mixed with the cell solution. The siRNA-cell solution was transferred to an Amaxa cuvette and electroporated on Nucleofector program X-001. Cells were allowed to recover and cultured for 24 h before RNA was isolated for analysis. All siRNA knockdown experiments were done in triplicate.

Luciferase assay

Luciferase was measured using the Dual-Luciferase Reporter Assay (Promega). In brief, transfected cells were pelleted and lysed in 100 μl 5 × Passive lysis buffer after being cultured for 24 h. The lysate was then added to a luminometer tube containing 100 μl of LAR solution. The luciferase level was immediately read on the TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). All luciferase assay experiments were performed in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank John Tossberg, Chase Spurlock and Sarah Collier for scientific discussion and comments. Support was provided by grants from the National Institute of Allergy and Infectious Disease (AI 074945, AI 079732 and AI 044924) and the National Heart, Lung, and Blood Institute (T32HL094296).

REFERENCES

1. Godfrey DI, Zlotnik A. Control points in early T-cell development. Immunol Today. 1993; 14:547–553. [PubMed: 7903854]
2. Malissen B, Wegener AM, Hoeveler A, Marguet D. Molecular dissection of the T-cell receptor/CD3 complex. Immunol Ser. 1993; 59:29–40. [PubMed: 8461394]
3. Fehling HJ, Krotkova A, Saint-Ruf C, von Boehmer H. Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. Nature. 1995; 375:795–798. [PubMed: 7596413]
4. McBlane JF, van Gent DC, Ramsden DA, Romeo C, Cuomo CA, Gellert M, et al. Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. Cell. 1995; 83:387–395. [PubMed: 8521468]
5. Schatz DG, Oettinger MA, Baltimore D. The V(D)J recombination activating gene, RAG-1. Cell. 1989; 59:1035–1048. [PubMed: 2598259]
6. Oettinger MA, Schatz DG, Gorka C, Baltimore D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science. 1990; 248:1517–1523. [PubMed: 2360047]
7. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. Cell. 1992; 68:869–877. [PubMed: 1547488]
8. Hsu LY, Lauring J, Liang HE, Greenbaum S, Cado D, Zhuang Y, et al. A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. Immunity. 2003; 19:105–117. [PubMed: 12871643]

9. Kuo TC, Schlissel MS. Mechanisms controlling expression of the RAG locus during lymphocyte development. Curr Opin Immunol. 2009; 21:173–178. [PubMed: 19359154]
10. Piguet PF, Irle C, Kollatte E, Vassalli P. Post-thymic T lymphocyte maturation during ontogenesis. J Exp Med. 1981; 154:581–593. [PubMed: 6115885]
11. Spear PG, Wang AL, Rutishauser U, Edelman GM. Characterization of splenic lymphoid cells in fetal and newborn mice. J Exp Med. 1973; 138:557–573. [PubMed: 4580464]
12. Spear PG, Edelman GM. Maturation of the humoral immune response in mice. J Exp Med. 1974; 139:249–263. [PubMed: 4589987]
13. Amagai T, Itoi M, Kondo Y. Limited development capacity of the earliest embryonic murine thymus. Eur J Immunol. 1995; 25:757–762. [PubMed: 7705405]
14. Zhang F, Thomas LR, Oltz EM, Aune TM. Control of thymocyte development and recombination-activating gene expression by the zinc finger protein Zfp608. Nat Immunol. 2006; 7:1309–1316. [PubMed: 17057722]
15. Zhang F, Liang Z, Matsuki N, Van Kaer L, Joyce S, Wakeland EK, et al. A murine locus on chromosome 18 controls NKT cell homeostasis and Th cell differentiation. J Immunol. 2003; 171:4613–4620. [PubMed: 14568935]
16. Couedel C, Roman C, Jones A, Vezzoni P, Villa A, Cortes P. Analysis of mutations from SCID and Omenn syndrome patients reveals the central role of the Rag2 PHD domain in regulating V(D)J recombination. J Clin Invest. 2010; 120:1337–1344. [PubMed: 20234091]
17. Haecker A, Qi D, Lilja T, Moussian B, Andrioli LP, Luschnig S, et al. Drosophila brakeless interacts with atrophin and is required for tailless-mediated transcriptional repression in early embryos. PLoS Biol. 2007; 5:e145. [PubMed: 17503969]
18. Siepel A, Bejerano G, Pedersen JS, Hinrichs AS, Hou M, Rosenbloom K, et al. Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. Genome Res. 2005; 15:1034–1050. [PubMed: 16024819]
19. Reed NP, Mortlock DP. Identification of a distant cis-regulatory element controlling pharyngeal arch-specific expression of zebrafish gdf6a/radar. Dev Dyn. 2010; 239:1047–1060. [PubMed: 20201106]
20. Kitajima M, Iwamura C, Miki-Hosokawa T, Shinoda K, Endo Y, Watanabe Y, et al. Enhanced Th2 cell differentiation and allergen-induced airway inflammation in Zfp35-deficient mice. J Immunol. 2009; 183:5388–5396. [PubMed: 19783676]
21. Fuller K, Storb U. Identification and characterization of the murine Rag1 promoter. Mol Immunol. 1997; 34:939–954. [PubMed: 9464529]
22. Lauring J, Schlissel MS. Distinct factors regulate the murine RAG-2 promoter in B- and T-cell lines. Mol Cell Biol. 1999; 19:2601–2612. [PubMed: 10082526]
23. Chen Z, Xiao Y, Zhang J, Li J, Liu Y, Zhao Y, et al. Transcription factors E2A, FOXO1 and FOXP1 regulate recombination activating gene expression in cancer cells. PLoS One. 2011; 6:e20475. [PubMed: 21655267]
24. Wang L, Tsai CC. Atrophin proteins: an overview of a new class of nuclear receptor corepressors. Nucl Recept Signal. 2008; 6:e009. [PubMed: 19043594]
25. Cebrat M, Miazek A, Kisielow P. Identification of a third evolutionarily conserved gene within the RAG locus and its RAG1-dependent and -independent regulation. Eur J Immunol. 2005; 35:2230–2238. [PubMed: 15971274]
26. Su RC, Brown KE, Saaber S, Fisher AG, Merkenschlager M, Smale ST. Dynamic assembly of silent chromatin during thymocyte maturation. Nat Genet. 2004; 36:502–506. [PubMed: 15098035]

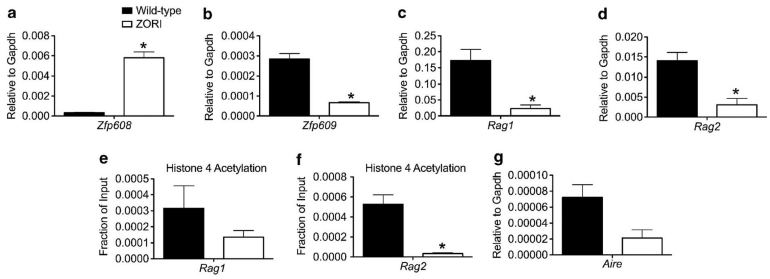

Figure 1.

Reciprocal expression of *Zfp608*, *Zfp609*, *Rag* and *Aire* in wild-type and ZORI thymocytes. Expression levels of the indicated genes (**a**, **b**, **c**, **d** and **g**) in wild-type and ZORI thymocytes (**a**, **b**, **c**, and **g**) and thymic epithelia (**d**), were determined by PCR using Taqman assays. Results are expressed relative to Gapdh levels: (**a**) *Zfp608*, (**b**) *Zfp609*, (**c**) *Rag1*, (**d**) *Rag2* and (**g**) *Aire*. (**e**) Acetylated histone 4 levels at *Rag1* promoter in wild-type and ZORI thymocytes as determined by chromatin immunoprecipitation (ChIP). Results are expressed as fraction of input. (**f**) Acetylated histone 4 levels at the *Rag2* promoter in wild-type and ZORI thymocytes. **P*<0.05.

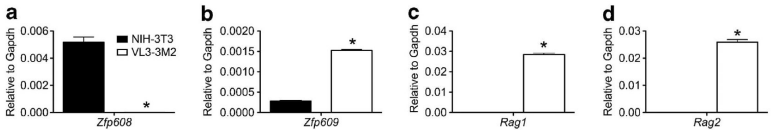

Figure 2.

mRNA expression levels of (**a**) *Zfp608*, (**b**) *Rag1*, (**c**) *Rag2* and (**d**) *Zfp609* in NIH-3T3 fibroblasts and VL3-3M2 thymocytes. Expression levels of the indicated genes were determined as in Figure 1. Results are normalized to expression levels of *Gapdh*. Error bars represent s.d. **P*<0.05.

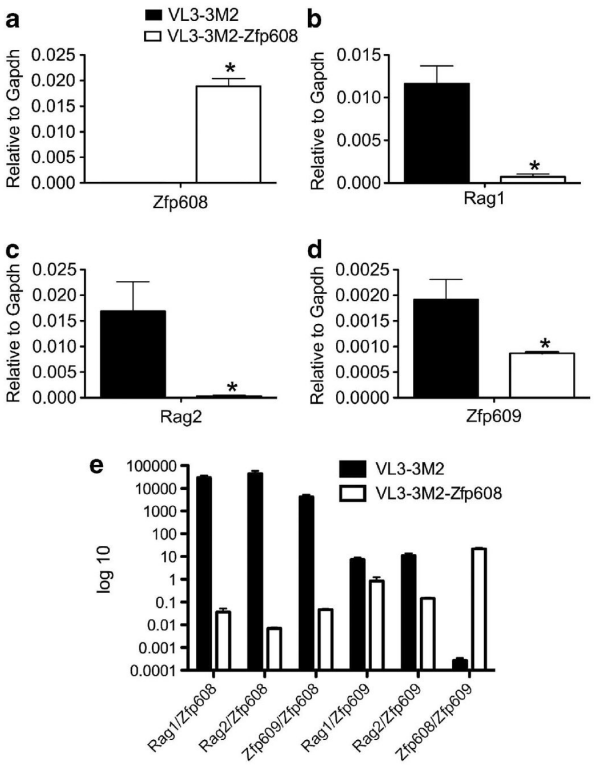

Figure 3.

Zfp608 decreases expression of Rag1, Rag2 and Zfp609 in VL3-3M2 cells. Expression levels of (**a**) *Zfp608*, (**b**) Rag1, (**c**) Rag2 and (**d**) Zfp609 (determined as in Figure 1) in VL3-3M2 and in VL3-3M2 cells after stable transfection of a Zfp608 expression plasmid, VL3-3M2-Zfp608 cells. Expression levels were normalized to Gapdh. (**e**) The levels of *Rag1* and *Rag2* relative to *Zfp608* and *Zfp609*. Error bars are s.d. **P*<0.05.

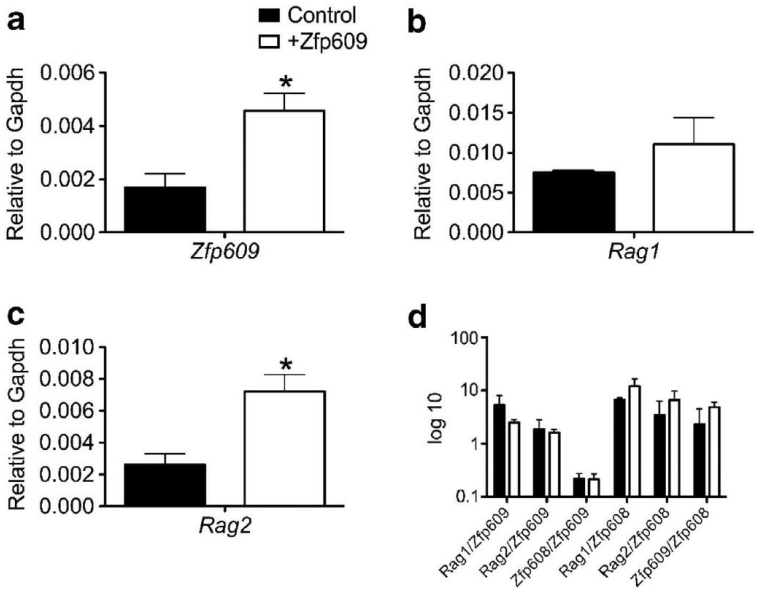

Figure 4.

Zfp609 is a positive regulator of *Rag1* and *Rag2* in VL3-3M2-Zfp608 cells. VL3-3M2-Zfp608 cells were transiently transfected with a Zfp609 expression plasmid or empty vector control. (**a**) *Zfp609*, (**b**) *Rag1* and (**c**) *Rag2* expression levels were determined as in Figure 1. Results are expressed relative to Gapdh. (**d**) *Rag1* and *Rag2* relative to *Zfp608* and *Zfp609*. Error bars are s.d. **P*<0.05.

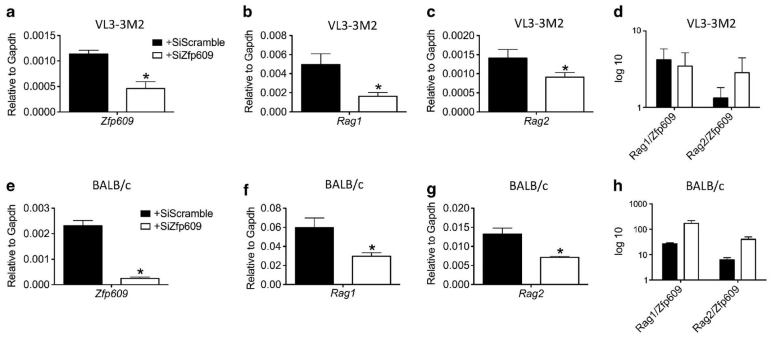

Figure 5.

siRNA-mediated knockdown of *Zfp609* in VL3-3M2 thymocytes and BALB/c thymocytes reduces *Rag* expression. (**a–c**) Gene expression levels of *Zfp609*, *Rag1* and *Rag2* in VL3-3M2 thymocytes after siRNA-mediated *Zfp609* knockdown relative to Gapdh. (**d**) *Rag1* and *Rag2* expression levels in VL3-3M2 thymocytes after siRNA-mediated knockdown relative to *Zfp609*. (**e–g**) *Zfp609*, *Rag1* and *Rag2* expression levels in BALB/c thymocytes after siRNA-mediated *Zfp609* knockdown. (**h**) *Rag1* and *Rag2* expression levels in BALB/c thymocytes after siRNA knockdown relative to *Zfp609*. **P*<0.05.

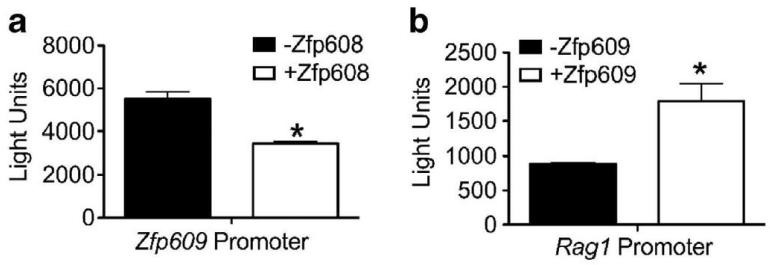

Figure 6.

Promoter activity of *Zfp609* and *Rag1* in VL3-3M2 thymocytes. (**a**) *Zfp609* promoter activity in the presence and absence of *Zfp608* in VL3-3M2 thymocytes. (**b**) *Rag1* promoter activity in the presence or absence of *Zfp609* in VL3-3M2 thymocytes. **P*<0.05.