Published OnlineFirst April 5, 2016; DOI: 10.1158/0008-5472.CAN-15-2698

Tumor and Stem Cell Biology

Cancer Research

Ribosomal Protein Rpl22 Controls the Dissemination of T-cell Lymphoma

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Abstract

Mutations in ribosomal proteins cause bone marrow failure syndromes associated with increased cancer risk, but the basis by which they do so remains unclear. We reported previously that the ribosomal protein Rpl22 is a tumor suppressor in T-cell acute lymphoblastic leukemia/lymphoma (T-ALL), and that loss of just one *Rpl22* allele accelerates T-cell lymphomagenesis by activating NF-κB and inducing the stem cell factor Lin28B. Here, we show that, paradoxically, loss of both alleles of *Rpl22* restricts lymphoma progression through a distinct effect on migration of malignant cells out of the thymus. Lymphoma-prone AKT2-transgenic or PTEN-deficient mice on an $Rpl22^{-/-}$ background developed significantly larger and markedly more vascularized thymic tumors than those observed in $Rpl22^{+/+}$ control mice. But, unlike $Rpl22^{+/+}$ or $Rpl22^{+/-}$ tumors, $Rpl22^{-/-}$ lymphomas did not disseminate to the periphery and were retained in the thymus. We traced the defect in *the* $Rpl22^{-/-}$ lymphoma migratory capacity to downregulation of the KLF2 transcription factor and its targets, including the key migratory factor sphingosine 1-phosphate receptor 1 (S1PR1). Indeed, reexpression of S1PR1 in Rpl22-deficient tumor cells restores their migratory capacity *in vitro*. The regulation of KLF2 and S1PR1 by Rpl22 appears to be proximal as Rpl22 reexpression in Rpl22-deficient lymphoma cells restores expression of KLF2 and S1P1R, while Rpl22 knockdown in Rpl22-sufficient lymphomas attenuates their expression. Collectively, these data reveal that, while loss of one copy of *Rpl22* promotes lymphomagenesis and disseminated disease, loss of both copies impairs responsiveness to migratory cues and restricts malignant cells to the thymus. *Cancer Res; 76(11); 3387–96. ©2016 AACR.*

Introduction

Evidence is emerging suggesting that some ribosomal proteins (RP) play critical roles in regulating development and disease (1–3) that are likely to be exerted outside of the ribosome. One such RP, Rpl22, is an RNA-binding component of the 60S ribosomal subunit, whose normal physiologic function remains poorly understood. Rpl22 is dispensable for ribosome biogenesis and global, cap-dependent translation; however, Rpl22 does play a critical, lineage-restricted role in supporting hematopoiesis (4, 5). Indeed, despite the ubiquitous expression of Rpl22, Rpl22-null mice are viable and fertile, with the only obvious defect being an exquisitely specific p53-dependent block in certain lymphoid subsets, including αβ lineage T cells (5, 6). The tissue-restricted nature of the developmental abnormalities caused by Rpl22 deficiency, clearly distinguish Rpl22 from other RPs, whose loss usually results in early lethality (3, 7). Because of its important role in normal T-cell development, we evaluated whether Rpl22 might regulate the development of T-cell malignancies. We determined that Rpl22 is capable of functioning as a haploinsufficient tumor suppressor in T-cell acute lymphoblastic leukemia/lymphoma (T-ALL). Indeed, loss of one copy of *Rpl22*, which does not affect normal T-cell development, markedly accelerated the development of thymic lymphoma in a mouse T-ALL model that uses a myristoylated, oncogenic Akt2 (*MyrAkt2*) transgene (Tg). Monoallelic inactivation of *Rpl22* in this *MyrAkt2 Tg* background enhances development of thymic lymphoma by activation of NF-κB and its target, Lin28B (2). Notably, *RPL22* inactivation is observed in ∼10% of human T-ALL (2), where its loss correlates with poor survival. Mono-allelic inactivation or deletion of *RPL22* has also been observed in endometrial and colorectal cancer (8, 9).

While monoallelic inactivation of *RPL22* has been reported in human T-ALL and other solid tumors (8, 9), to date biallelic mutations and/or deletions of *RPL22* have only been reported in solid tumors (9). Given that mono- and biallelic inactivation of tumor suppressor genes sometimes results in distinct prognostic and phenotypic characteristics in the resulting cancers (10, 11), we wished to assess the impact of eliminating both *Rpl22* alleles on T-ALL development and progression, as this has not previously been investigated. This was also of particular interest because biallelic inactivation of *Rpl22* in the germline results in the arrest of T-cell development (5), suggesting that complete loss of Rpl22 ($Rpl22^{-/-}$) might actually delay the development of disease relative to the acceleration observed under conditions of haploinsufficiency ($Rpl22^{+/-}$). To test this hypothesis, we used two different T-cell lymphoma models with constitutive activation of the Akt pathway, which is frequently observed in human T-ALL. Indeed, Gutierrez and colleagues reported alterations of the PTEN–PI3K–Akt pathway in nearly 50% of pediatric T-ALL (12). The two models used were a *MyrAkt2 Tg* mouse, in which MyrAkt2 is expressed in T-lineage progenitors under the control of

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-15-2698

Published OnlineFirst April 5, 2016; DOI: 10.1158/0008-5472.CAN-15-2698

a proximal Lck promoter (13) and a conditional knockout mouse where the *Pten* tumor suppressor gene is ablated in T-cell precursors using pre-Tα-Cre (14). Interestingly, both enforced expression of *MyrAkt2* and biallelic loss of *Pten* resulted in a partial rescue of the block in T-cell development caused by Rpl22 deficiency. Moreover, to our surprise, Rpl22-deficiency resulted in a thymic lymphoma phenotype distinct from that observed in Rpl22 heterozygous ($Rpl22^{+/-}$) or wild-type ($Rpl22^{+/+}$) mice. Indeed, in $Rpl22^{+/+}$ and $Rpl22^{+/-}$ mice, thymic lymphoma rapidly disseminated from the thymus to peripheral organs, including lymph nodes, spleen, and liver. In contrast, in $Rpl22^{-/-}$ mice, the thymic lymphoma did not disseminate or migrate to peripheral organs, was retained mediastinally, and exhibited markedly enhanced angiogenesis. The absence of dissemination was associated with a marked reduction in expression of the KLF2 transcription factor, which controls the expression of some critical regulators of T-cell migration, including S1PR1, CD62L, and other chemokine receptors (15–17). Together, these data reveal a new role for Rpl22 in regulating the progression and dissemination of T-cell malignancies.

Materials and Methods

Mouse lymphoma models and animal care

Mice were maintained in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited Laboratory Animal Facility at the Fox Chase Cancer Center and were handled in compliance with guidelines established by the Institutional Animal Care and Use Committee. *MyrAkt2 Tg* were generated as previously described using a proximal Lck promoter to restrict expression to T-lineage progenitors (18). Conditional ablation of the *Pten* locus in T-lineage progenitors was accomplished by crossing $Pten^{flox/flox}$ mice (19) with those expressing Cre in T-lineage progenitors only, under control of the pre-Tα promoter (14). Upon crossing *MyrAkt2 Tg* or PTEN-deficient mice to $Rpl22^{-/-}$ mice, littermates on a mixed 129-C57BL/6 background were monitored weekly for signs of disease, as described (2). Upon manifesting signs of disease, mice were sacrificed and the thymic lymphomas were excised for further analysis. All analysis of premalignant phenotypes was performed on mice at 4 to 6 weeks of age.

IHC analysis

Tissues including thymus, spleen, liver, lymph nodes, and bone marrow were collected and fixed with 10% formaldehyde. After fixation, tissues were paraffin embedded, sectioned, and stained with H&E, Ki67, anti-CD31, anti-CD3, and anti-CD45R (B220). For senescence-associated-β-galactosidase (β-Gal) staining, tissue sections were fixed for 5 minutes in 2% formaldehyde and 0.2% glutaraldehyde in PBS at room temperature, followed by overnight staining at 37°C in staining solution (40 mmol/L Na_2HPO_4, 150 mmol/L NaCl, 2 mmol/L $MgCl_2$, 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6$, and 1 mg/mL X-gal (purchased from Sigma).

Flow cytometry

Single-cell suspensions were prepared from thymic lymphomas, lymph nodes, spleen and liver, and stained with optimal amounts of the following fluorochrome-conjugated antibodies: anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-Ter119 (Ter-119), anti-TCRβ (H57-597), anti-CD69 (H1.2F3), anti-CD62L (MEL-14), anti-CCR7 (4B12), anti-CD3 (500A2), and anti-B220 (RA3-6B2). For S1PR1, cells were stained with a monoclonal anti-S1PR1 antibody (FAB7089A; R&D Systems) followed by PE-conjugated rat IgG_{2A} antibody (R2a-21B2). The antibodies were purchased from eBioscience, BD Biosciences, or Biolegend. Flow cytometry was performed on stained cells using an LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Plasmids, cell culture, and viral production

The S1PR1/EDG1 cDNA (RDC0053; R&D Systems) was subcloned into the bicistronic retroviral vector pMSCV-IRES-YFP (pMIY; ref. 20) using standard methods. Lentiviral shRNA constructs targeting murine S1PR1 (pLKO.1-puro) were purchased from Sigma-Aldrich. Explanted thymic lymphomas were adapted to growth *in vitro* by serial passage, and their Rpl22 status was assessed by immunoblotting, as previously described (2) and in this article. Mouse lymphoma cells were maintained in DMEM with standard supplements including 10% FBS (Hyclone). To ectopically express S1PR1, lymphoma cells were spin infected in the presence of 8 μg/mL polybrene with retrovirus (pMIY-S1PR1) produced by transient transfection of phoenix-ecotropic packaging cells, as described (2, 20). The infected YFP-expressing cells were electronically sorted using a FACSAria II (BD Biosciences). For knockdown experiments, lentivirus (pLKO.1-shS1PR1) was produced by transfection of HEK293T with both packaging (deltaR8.2 and VSV-G) and pLKO.1 shRNA vectors using FuGENE 6 (Roche). Virus-infected lymphoma cells were puromycin selected for at least 5 days prior to use (2).

Chemotactic migration assay

Migration assays were performed in triplicate using 24-well Transwell chambers with 5 μm polycarbonate membranes (Corning; ref. 21). The lower chamber was filled with 600 μL of 1% FBS DMEM supplemented with chemoattractants sphingosine 1-phosphate (S1P) or chemokine ligand 21 (CCL21; R&D Systems). Thymic lymphoma cells (1×10^5) were placed in the upper chamber and incubated for 4 hours at 37°C in 5% CO_2. Cells that migrated to the lower chamber were collected, pelleted, resuspended in FACS buffer (PBS with 1% BSA), and counted using a cytometer (LSRII; BD Biosciences) or by light microscopy.

RNA isolation and real-time PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's specifications. cDNA was synthesized using the Super Script II kit (Invitrogen) with Random Primers (Invitrogen), prior to RT-PCR quantification on an ABI Prism 7700 RT-PCR machine using TaqMan FAM-probes from ABI. Analysis was performed in triplicate and normalized to *Gapdh or Actin*. Primers and probes were from Applied Biosystems: *Gapdh*, Mm99999915_g1; *Actin*, Mm00607939_s1; *Vegfa*, Mm01281449_m1; *Hif1α*, Mm00468869_m1; *S1p1*, Mm00514644_m1; *Klf2*, Mm01244979_g1; *Sell (CD62L)*, *Mm00441291_m1*.

Results

Rpl22 deficiency promotes development of thymic lymphoma in *MyrAkt2 Tg* mice

Monoallelic inactivation of *Rpl22* has been shown to accelerate the development of thymic lymphoma in mice expressing an oncogenic *MyrAkt2 Tg* (2). Accordingly, we wished to determine how loss of the remaining *Rpl22* allele affected the development

Published OnlineFirst April 5, 2016; DOI: 10.1158/0008-5472.CAN-15-2698

and subsequent behavior of thymic lymphoma in mouse models. Rpl22 is required for the generation of T cells (2), as *Rpl22*-null mice exhibit a severe, p53-dependent defect in the development of αβ lineage T cells in the thymus (Fig. 1A and B; ref. 5). Interestingly, enforced expression of the *MyrAkt2 Tg* antagonized this p53-dependent arrest and rescued development of thymocytes from the $CD4^-CD8^-$ (double negative or DN) stage to the $CD4^+CD8^+$ (DP) stage (Fig. 1A; refs. 2, 5) and increased thymic cellularity (Supplementary Fig. S1). The *MyrAkt2 Tg* also caused Rpl22-null mice to die faster due to the development of thymic lymphoma, which was already well developed in *MyrAkt2 Tg Rpl22*$^{+/-}$ and *MyrAkt2 Tg Rpl22*$^{-}$/$^{-}$ mice, prior to the onset of any sign of disease in *MyrAkt2 Tg Rpl22*$^{+/+}$ mice (Fig. 1B). *MyrAkt2 Tg Rpl22*$^{-/-}$ mice had a median survival of 94 days versus 150 days for *MyrAkt2 Tg Rpl22*$^{+/+}$ mice (*, $P < 0.05$), as well as earlier disease onset (67 days vs. 113 days, respectively; Fig. 1C). Compared with *MyrAkt2 Tg Rpl22*$^{+/+}$ mice, *MyrAkt2 Tg Rpl22*$^{-/-}$ mice also exhibited severe weight loss beginning at 2 months of age (Fig. 1D and E), which correlated with their poor survival (Fig. 1C).

Biallelic inactivation of *Rpl22* in *MyrAkt2 Tg* mice promotes the growth of large mediastinal lymphoma masses exhibiting increased angiogenesis

Histopatholgic analysis demonstrated that the large mediastinal masses that developed in Rpl22-deficient mice exhibited increased proliferation, as indicated by Ki67 staining (Fig. 2A) and decreased senescence indicated by senescence-associated-β-gal staining (Fig. 2B). Interestingly, while *Rpl22*$^{+/+}$ and *Rpl22*$^{+-}$ tumors exhibited little evidence of angiogenesis, the large mediastinal lymphoma masses that developed in *Rpl22*$^{-/-}$ mice exhibited substantial angiogenesis as evidenced by increased anti-CD31 staining (Fig. 2A) and by the accumulation of $Ter119^+$ erythroid cells (Fig. 2C). As this has been reported for solid tumors and certain non-Hodgkin lymphomas, including T-cell

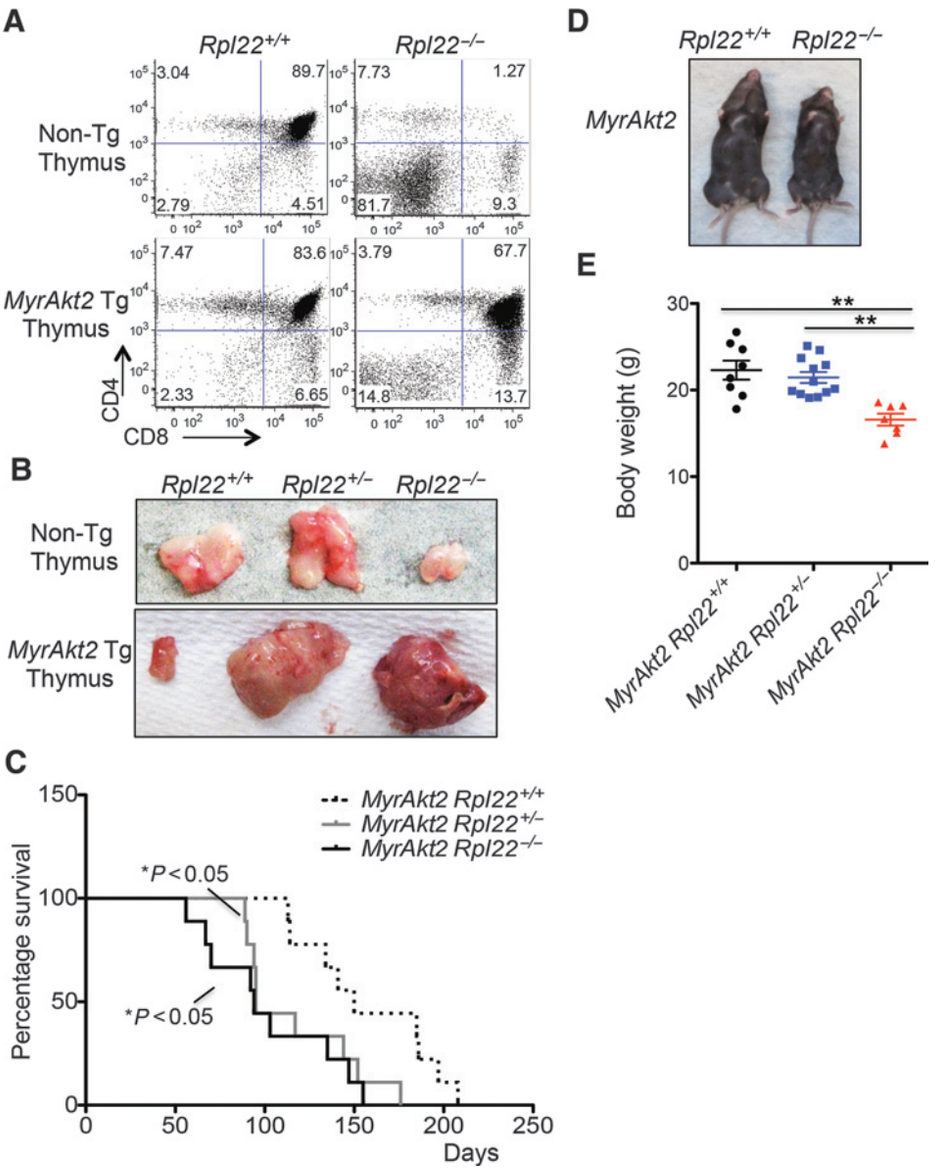

Figure 1.
Constitutively active Akt2 (MyrAkt2) rescues the developmental arrest of Rpl22-deficient thymocytes and induces thymic lymphoma. A, flow cytometry analysis of T-cell development in non-*Tg* and *MyrAkt2 Tg* mice of the indicated genotypes. B, representative images of the thymi of non-*MyrAkt2 Tg* littermates at the age of 6 weeks and *MyrAkt2 Tg* mice at 12 weeks of age. C, Kaplan–Meier curves depicting percent survival of *MyrAkt2 Tg* mice with the indicated genotypes ($n = 9$ for each group). The statistical significance was analyzed with the Mantel–Cox log-rank test (*MyrAkt2 Tg Rpl22*$^{-/-}$ versus *MyrAkt2 Tg Rpl22*$^{+/+}$, **, $P < 0.005$; *MyrAkt2 Tg Rpl22*$^{+/-}$ versus *MyrAkt2 Tg Rpl22*$^{+/+}$, **, $P < 0.005$). D, representative images of *MyrAkt2 Tg Rpl22*$^{+/+}$ and *MyrAkt2 Tg Rpl22*$^{-/-}$ mice. E, body weight of *MyrAkt2 Tg* mice with the indicated genotypes: *MyrAkt2 Tg Rpl22*$^{+/+}$, $n = 8$; *MyrAkt2 Tg Rpl22*$^{+/-}$, $n = 12$; and *MyrAkt2 Tg Rpl22*$^{-/-}$, $n = 7$. Analysis was performed using the unpaired *t* test (**, $P < 0.05$).

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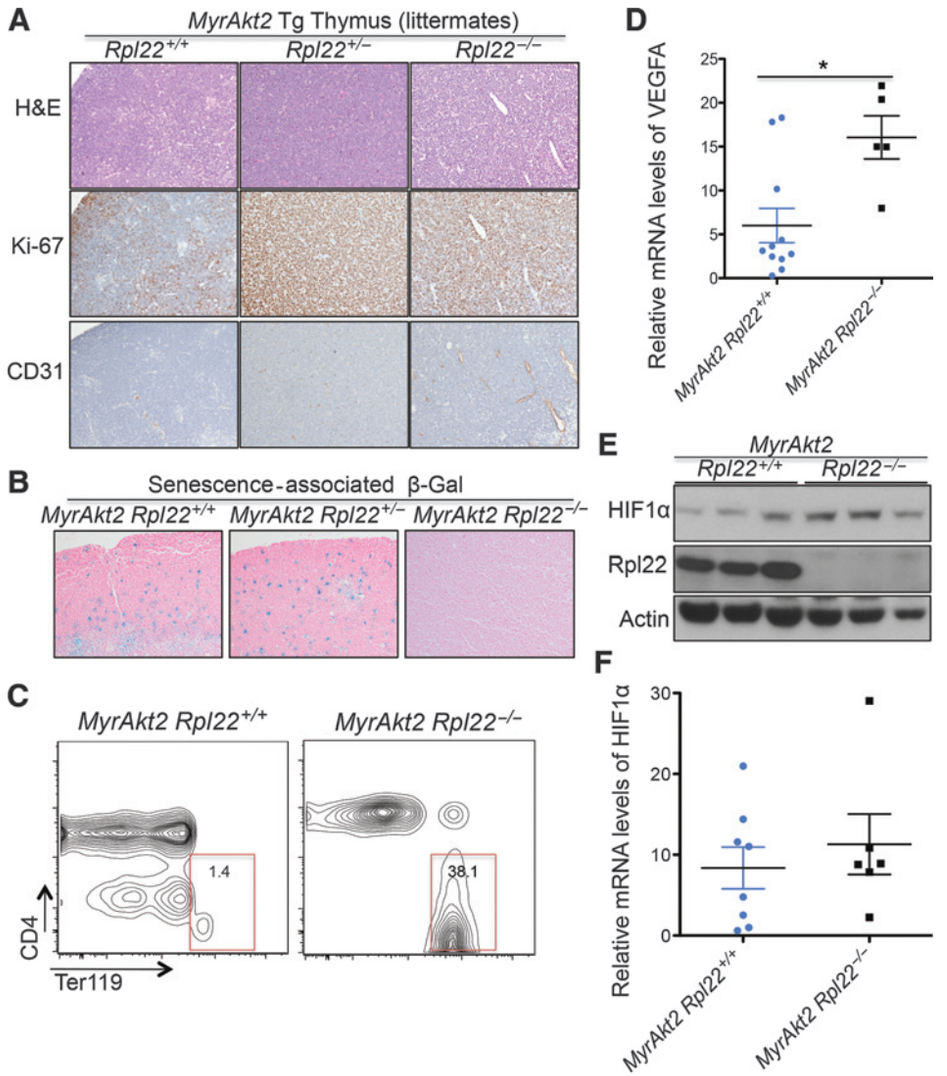

Figure 2.
Thymic lymphomas in *Rpl22*-deficient mice showed enhanced proliferation and angiogenesis. A, representative images of H&E staining and IHC for Ki67 and CD31 in thymic lymphomas from *MyrAkt2 Tg* mice with the indicated *Rpl22* genotypes. B, representative images of senescence-associated β-Gal staining of thymic lymphomas from *MyrAkt2 Tg* mice with the indicated genotypes. C, flow cytometry analysis of erythroid cells (Ter119$^+$) in single-cell suspensions prepared from thymic lymphomas of *MyrAk2t Tg* mice. D, *Vegfa* mRNA levels in thymic lymphomas from *MyrAkt2 Tg* mice were quantified by real-time PCR (*MyrAkt2 Tg;Rpl22*$^{+/+}$, $n = 11$; *MyrAkt2 Tg;Rpl22*$^{-/-}$, $n = 6$). The statistical significance was assessed using an unpaired *t* test (*, $P < 0.05$). E, Western blotting revealed alterations in Hif1α expression in lymphomas from *MyrAkt2 Tg Rpl22*$^{+/+}$ and *MyrAkt2 Tg Rpl22*$^{-/-}$ mice. F, *Hif1* mRNA levels quantified by real-time PCR in thymic lymphomas from *MyrAkt2 Tg Rpl22*$^{+/+}$ ($n = 8$) and *MyrAkt2 Tg Rpl22*$^{-/-}$ ($n = 6$) mice.

lymphoma (22, 23), we asked if this was associated with increased expression of proangiogenic factors, such as VEGF. Indeed, VEGF mRNA levels were significantly enhanced in *Rpl22*$^{-/-}$ lymphomas (Fig. 2D). The increased VEGF mRNA was associated with increased expression of the master regulator of VEGF transcription, HIF1α, whose protein but not mRNA expression was increased in *Rpl22*$^{-/-}$ lymphomas (Fig. 2E and F), suggesting the change in expression was posttranscriptional, and resulted from hypoxia.

Rpl22 deficiency impairs tumor dissemination

Both mono- (*Rpl22*$^{+/-}$) and biallelic (*Rpl22*$^{-/-}$) inactivation of *Rpl22* accelerates lymphomagenesis in MyrAkt2 Tg mice; however, the lymphomas that develop exhibit distinctive behaviors. While *Rpl22*$^{+/+}$ and *Rpl22*$^{+/-}$ mice developed lymphomas that presented as relatively modest thymic masses with extensive dissemination to peripheral organs (2) (data not shown), *Rpl22*-deficient lymphomas accumulated mediastinally and did not disseminate to the periphery, with minimal involvement of lymph nodes, liver, and spleen (Fig. 3A and B). The hepatomegaly and splenomegaly observed in *MyrAkt2 Tg Rpl22*$^{+/+}$ and *MyrAkt2 Tg Rpl22*$^{+/-}$ mice resulted from the accumulation of thymic lymphoma cells, as evidenced by increased representation of CD3$^+$ cells measured by flow cytometry and IHC (data not shown). IHC analysis indicated that along with more extensive dissemination, *Rpl22*$^{+/+}$ lymphomas also displayed increased lymphoma cell proliferation in both the spleen (Fig. 3C) and liver (data not shown).

Rpl22-deficient lymphomas also fail to disseminate in a PTEN-deficient lymphoma model

To determine if the mediastinal retention of *Rpl22*$^{-/-}$ thymic lymphomas in the *MyrAkt2 Tg* model was also observed in other models of thymic lymphoma, we next used mice in which the *Pten* tumor suppressor gene was conditionally ablated in T-lineage progenitors using pTα-Cre (19). As with the *MyrAkt2 Tg*, ablation of the *Pten* tumor suppressor also partially rescued the defect in T-cell development caused by Rpl22 deficiency (Fig. 4A). Moreover, as we observed in *MyrAkt2 Tg* mice, the thymic lymphomas that developed in *Rpl22*-deficient mice lacking PTEN also failed to disseminate to liver, spleen, and lymph nodes and were instead retained mediastinally (Fig. 4B–D). Finally, similar to the

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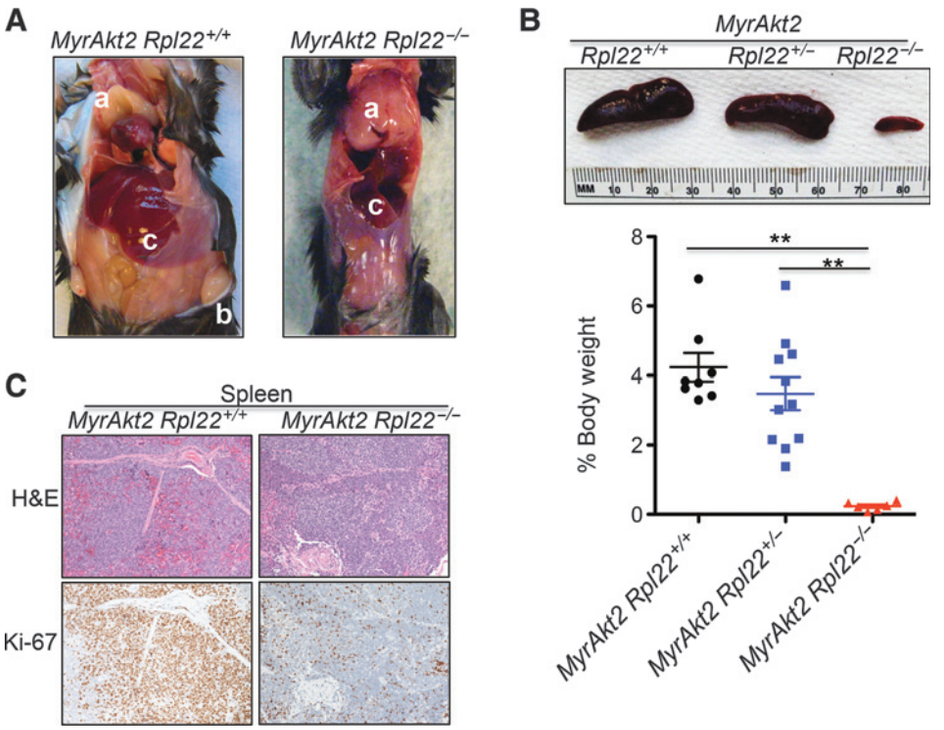

Figure 3.
Rpl22 deficiency blocks thymic lymphoma dissemination to peripheral tissues in a *MyrAkt2 Tg* mouse model. A, representative images of thymic lymphomas (a) and their dissemination to peripheral tissues, including lymph nodes (b), spleen, and liver (c) in *MyrAkt2 Tg;Rpl22*$^{+/+}$ transgenic mice. B, splenomegaly in *MyrAkt2 Tg Rpl22*$^{+/+}$ and *MyrAkt2 Tg Rpl22*$^{+/-}$ mice compared with *MyrAkt2 Tg Rpl22*$^{-/-}$ mice, with representative images on top. Statistical analysis was performed using the unpaired *t* test (*MyrAkt2 Rpl22*$^{+/+}$, $n = 8$; *MyrAkt2 Tg Rpl22*$^{+/-}$, $n = 11$; *MyrAkt2 Tg Rpl22*$^{-/-}$, $n = 6$; **, $P < 0.05$). C, representative H&E and Ki67 staining of spleens from *MyrAkt2 Tg Rpl22*$^{+/+}$ and *MyrAkt2 Tg Rpl22*$^{-/-}$ mice.

lymphomas in *MyrAkt2 Tg* mice, thymic lymphomas that developed in *Pten*$^{-/-}$*Rpl22*$^{-/-}$ mice also exhibited enhanced proliferation, as demonstrated by Ki67 staining (Fig. 4E), and angiogenesis, as evidenced by the increased presence of blood vessels marked by CD31 expression (Fig. 4F). Collectively, our data suggest that Rpl22 plays a critical role in regulating lymphoma dissemination.

Rpl22-deficient lymphomas that fail to disseminate are unresponsive to S1P ligand

Because the thymic lymphomas that develop in *Rpl22*-deficient mice are retained mediastinally and fail to disseminate, we hypothesized that this might result from a failure to respond to migratory cues. We were particularly interested in their response to S1P ligand, as it is responsible for emigration of normal mature thymocytes to the periphery (15). Indeed, Boyden chamber migration analysis revealed that while *Rpl22*$^{+/+}$ tumor cells exhibited migration in response to S1P that was comparable with that of normal single positive (SP) T cells, *Rpl22*-deficient lymphomas failed to migrate in response to S1P ligand (Fig. 5A; refs. 24, 25). We reasoned that this might occur because Rpl22 deficiency attenuated the cellular pathways required for S1P responsiveness. Alternatively, Rpl22 deficiency might impair S1P responsiveness indirectly by facilitating transformation of Rpl22-deficient progenitors at an earlier stage than occurs for those expressing Rpl22, perhaps prior to the developmental acquisition of S1P responsiveness. To distinguish these possibilities, we assessed the phenotype of the thymic lymphomas arising in *Rpl22*$^{+/+}$ and *Rpl22*$^{-/-}$ mice. Interestingly, the thymic lymphomas arising in Rpl22$^{+/+}$ and Rpl22$^{-/-}$ mice were both phenotypically identical, with virtually all cells being CD4^{+}CD8^{+} DP (Fig. 5B), suggesting that altered development was not the explanation for the failure to respond to S1P migratory cues. This is consistent with our observation that both ectopic expression of the *MyrAkt2 Tg* and PTEN deficiency rescued the developmental arrest of Rpl22-deficient progenitors, leading to a similar distribution of thymic subsets (Fig. 1A and 4A).

To determine if Rpl22 is regulating S1P responsiveness in a more proximal manner, we investigated expression levels of S1PR1 in *Rpl22*$^{+/+}$ and *Rpl22*$^{-/-}$ lymphomas. Indeed, *Rpl22*-deficient thymic lymphomas exhibited reduced expression of S1PR1 mRNA (Fig. 5C). Because the expression levels of S1PR1 are highest in cells that have matured beyond the DP stage (15, 26), we asked if transformation resulted in aberrant expression of S1PR1. Indeed, we found that S1PR1 expression is elevated in DP *Rpl22*$^{+/+}$ thymic lymphoma cells, relative to undetectable levels in their non-transformed DP counterparts (Fig. 5D). Moreover, the increased expression of S1PR1 by Rpl22-expressing DP lymphoma cells, and their capacity to migrate in response to S1P ligand *in vitro*, are correlated with the ability of these DP lymphomas to disseminate to the periphery, including into the spleen and liver (Supplementary Fig. S2A and data not shown). Conversely, DP thymic lymphomas from Rpl22-deficient mice expressed substantially reduced levels of S1PR1, failed to migrate in response to S1P ligand, and failed to emigrate to the periphery (Fig. 5A and D; Supplementary Fig. S2A). To test whether the reduced expression of S1PR1 might be responsible for the failure of Rpl22-deficient thymic lymphomas to emigrate to the periphery, we performed gain-of-function and loss-of-function analyses for S1PR1 *in vitro*. Indeed, ectopic expression of S1PR1 in Rpl22-deficient thymic lymphoma cells restored their ability to migrate in response to S1P exposure (Fig. 5E; Supplementary Fig. S2B). Moreover, knockdown of S1PR1 in Rpl22-expressing thymic lymphoma cells attenuated their ability to migrate in response to S1P (Fig. 5F). Together, these data suggest that attenuation of S1PR1 expression

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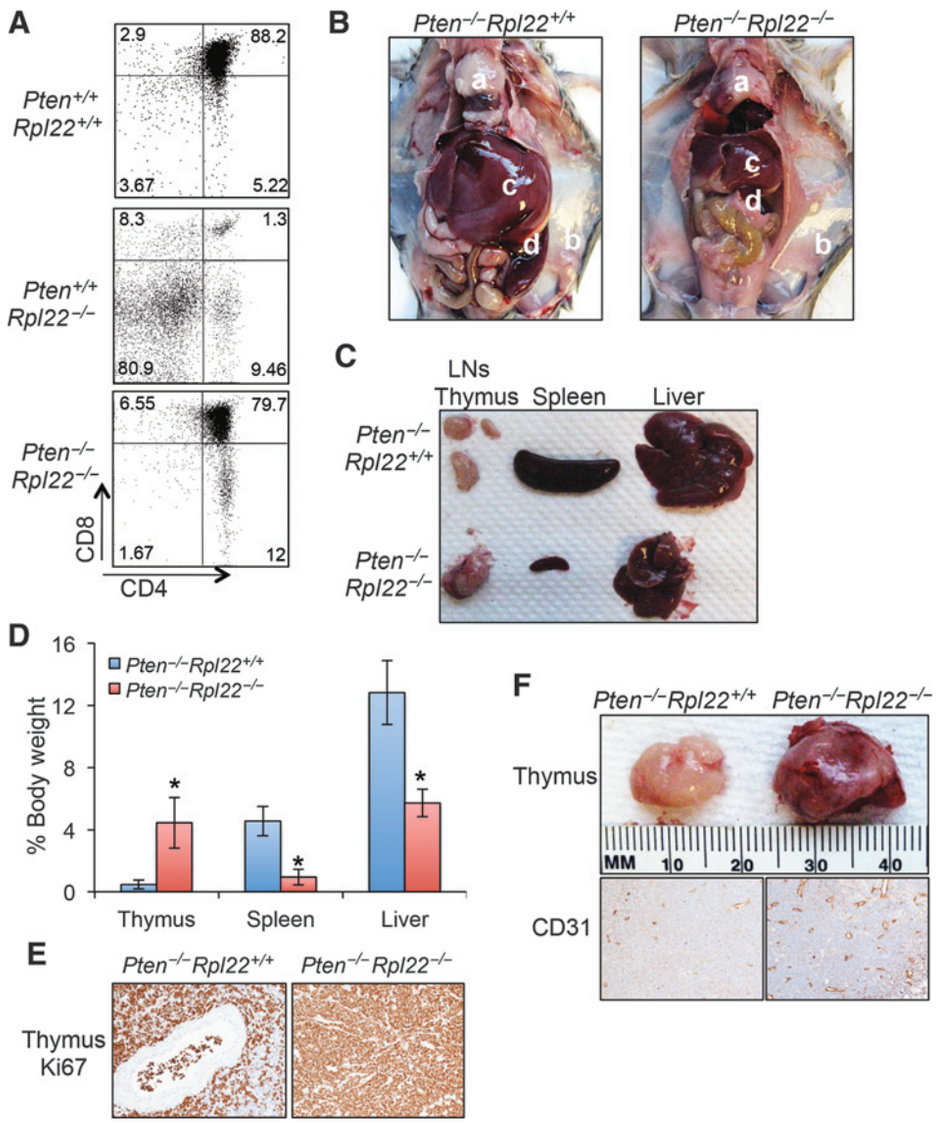

Figure 4.
Rpl22 deficiency blocks thymic lymphoma dissemination to peripheral tissues in a PTEN-deficient lymphoma model. A, flow cytometry analysis of T-cell development in mice with the indicated genotypes. B, representative images of thymic lymphomas (a) and their dissemination to peripheral tissues, including lymph node (b), liver (c), and spleen (d) in *Pten*$^{-/-}$ mice. C, representative images of thymus, lymph node, spleen, and liver from mice in B. D, quantification of organ weights as a proportion of the mouse body weight. Statistical significance was assessed by the Student *t* test (*, $P < 0.05$, $n = 3$ in each group). E, representative IHC of Ki67 staining of the thymi of *Pten*$^{-/-}$*Rpl22*$^{+/+}$ and *Pten*$^{-/-}$*Rpl22*$^{-/-}$ mice. F, visualization of angiogenesis in the thymi of representative PTEN-deficient mice and corresponding IHC staining for CD31.

by Rpl22 loss contributes to the migration defect observed in Rpl22-deficient lymphomas.

Rpl22 loss attenuates expression of S1PR1 by impairing expression of its transcriptional activator Klf2

To determine how Rpl22-deficiency impairs S1PR1 expression, we investigated the expression of Klf2, a transcriptional regulator of S1PR1 expression (16, 27). Klf2 mRNA and protein levels were markedly reduced in Rpl22-deficient thymic lymphomas (Fig. 6A and B). While Rpl22-sufficient DP lymphoma cells showed expression levels of Klf2 similar to normal SP cells, Rpl22 loss significantly reduced Klf2 expression in DP lymphoma cells from both *MyrAkt2 Tg* mice and PTEN-deficient mice (Fig. 6B; and data not shown), as well as in more mature thymic populations undergoing positive selection (Supplementary Fig. S3A and B). S1PR1 was not the only Klf2 target reduced by Rpl22 deficiency, as another critical Klf2 target, CD62L, was also found to be downregulated in both DP cells (data not shown) and mature thymic progenitors from *MyrAkt2 Tg mice* (Supplementary Fig. S3C and D). Klf2 expression is responsive to manipulation of Rpl22 expression in transformed cells, as ectopic expression of Rpl22 in *Rpl22*-deficient lymphomas restored the expression of both Klf2 and its direct target, S1PR1 (Fig. 6C). The role of Rpl22 in regulating Klf2 and S1PR1 expression is not restricted to mouse models of T-cell lymphoma. In fact, an analysis of human T-ALL cell lines harboring intact and mutant *RPL22* alleles revealed that in human T-ALL cell lines bearing *RPL22* mutations, the expression of both KLF2 and S1PR1 was reduced (Fig. 6D; Supplementary Fig. S4). Furthermore, knockdown of Rpl22 in human T-ALL cells downregulated both KLF2 and S1PR1 expression (Fig. 6E), supporting the notion that Rpl22 is regulating S1PR1 expression through effects on Klf2.

Discussion

Here we report, using two different murine models of T-ALL, that Rpl22, in addition to regulating the development of thymic lymphoma, also regulates its dissemination. This very unique

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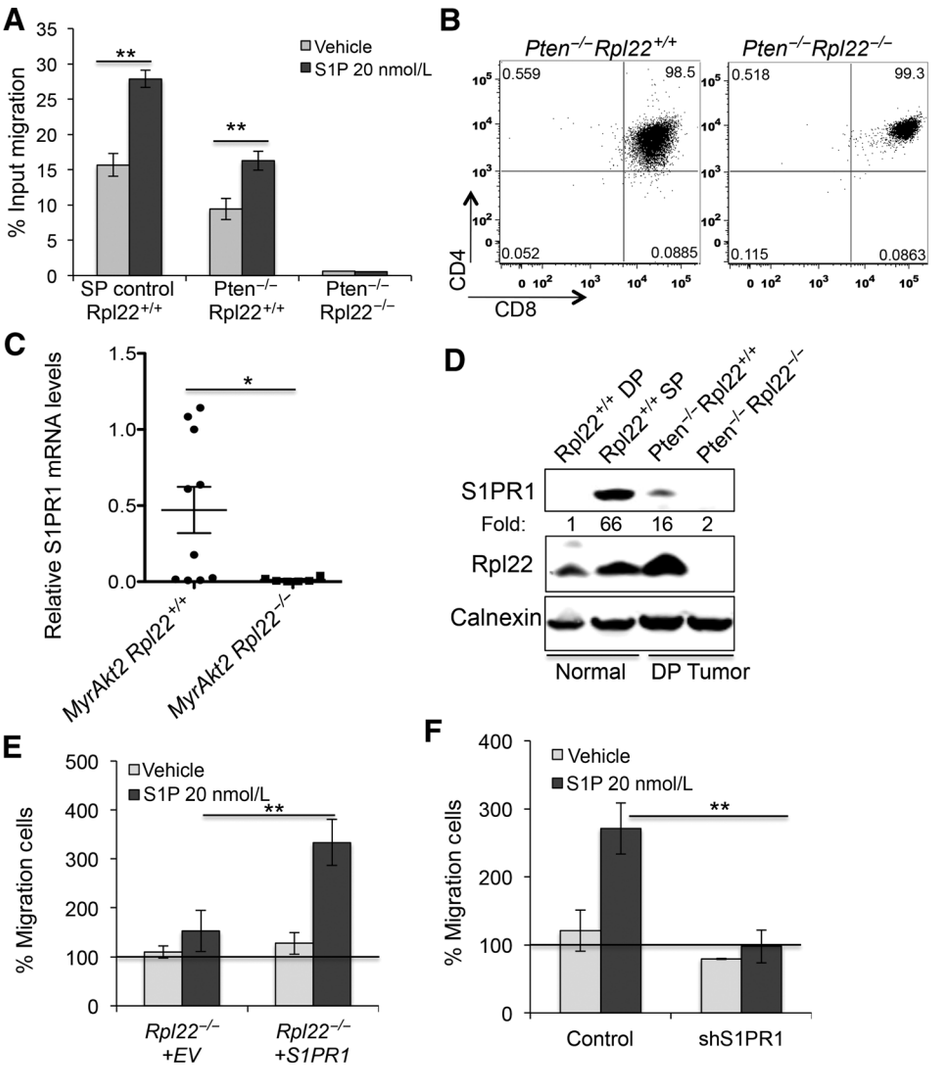

Figure 5.
Rpl22 deficiency blocks lymphoma migration through downregulation of surface S1PR1. A, analysis of migration of PTEN-deficient lymphoma cells *in vitro* in response to 24-hour exposure to S1P (20 nmol/L). Data are plotted as a percentage of input, and sorted SP thymocytes are used as positive control. B, flow cytometry analysis of thymic lymphomas that developed in PTEN-deficient mice with the indicated genotypes. C, S1PR1 mRNA levels measured by RT-PCR in mouse thymic lymphoma cells from *MyrAkt2 Tg* mice of the indicated genotypes (*MyrAkt2 Tg Rpl22*$^{+/+}$, $n = 10$; *MyrAkt2 Tg Rpl22*$^{-/-}$, $n = 6$). Significance was determined with unpaired *t* test (*, $P < 0.05$). D, immunoblots analysis of S1PR1 protein levels in DP lymphoma cells isolated from PTEN-deficient mice with indicated genotypes. SP and DP populations sorted from normal mice were used as positive and negative controls, respectively. S1PR1 expression levels were quantified by ImageJ, normalized to calnexin, and presented as fold change over the DP control. E, analysis of the migration of Rpl22-deficient lymphoma cells ectopically expressing S1PR1. Values were normalized to vehicle-treated, control-transduced *Rpl22*$^{-/-}$ lymphomas. F, analysis of the migration of Rpl22-sufficient lymphoma cells in which S1PR1 has been knocked down. All migration assays were performed at least twice in triplicate and data are plotted after normalization to the control group (mean ± SD). Significance was determined by the Student *t* test (**, $P < 0.05$).

phenotype was observed only in Rpl22-deficient mice, as biallelic inactivation of *Rpl22* restricts thymic lymphoma development and progression within the primary site in the thymus, blocking dissemination in T-ALL mouse models driven by *MyrAkt2 Tg* and PTEN deficiency. The mediastinal lymphoma masses that develop in *Rpl22*$^{-/-}$ *MyrAkt2 Tg* mice accelerate death, as is observed in *Rpl22*$^{+/-}$ mice, but appear to do so by a very different mechanism. While *Rpl22*$^{+/-}$ mice succumb to disseminated disease, the *Rpl22*$^{-/-}$ mice succumb to mediastinal masses that likely induce death by suffocation, due to lung compression. Thus, we conclude that Rpl22 plays a critical role in controlling lymphoma dissemination.

We also revealed that the mediastinal retention of Rpl22-deficient lymphomas is associated with cell-autonomous alterations in the expression of key regulators of migration. Indeed, Rpl22 loss reduces the expression of the transcription factor Klf2, a master regulator that controls T-cell trafficking through its targets, the chemotactic receptor for S1PR1, which is required for thymic emigration (15), as well as other regulators of cell trafficking, including CD62L and additional chemokine receptors (16, 17, 27). Interestingly, the ability of Rpl22 to regulate Klf2 and its targets appears to be linked to transformation, as we only observed effects of Rpl22 deficiency on expression of Klf2 and its targets in our mouse transformation models. This observation may suggest that the ability of Rpl22 to regulate the Klf2–S1PR1 axis depends on contributions from the Akt signaling pathway, which is activated in both of our lymphoma models. In agreement, a previous report had implicated the PI3K/Akt pathway in regulating Klf2 expression (28). Moreover, we found that while Klf2 expression in normal DP thymocytes was quite low, its expression in Rpl22-expressing DP thymic lymphomas was significantly increased and was comparable to that in normal SP thymocytes. Importantly, KLF2 expression in thymic lymphomas was sharply attenuated by Rpl22 deficiency. The basis by which Rpl22 controls Klf2 expression remains unclear at present. Klf2 mRNA levels are reduced in *Rpl22*-deficient lymphomas, suggesting that Klf2 expression is controlled either transcriptionally or through effects on mRNA stability. Rpl22 may control Klf2 expression indirectly, by acting to facilitate the expression of a transcription factor that transactivates the *Klf2* gene. Alternatively,

Published OnlineFirst April 5, 2016; DOI: 10.1158/0008-5472.CAN-15-2698

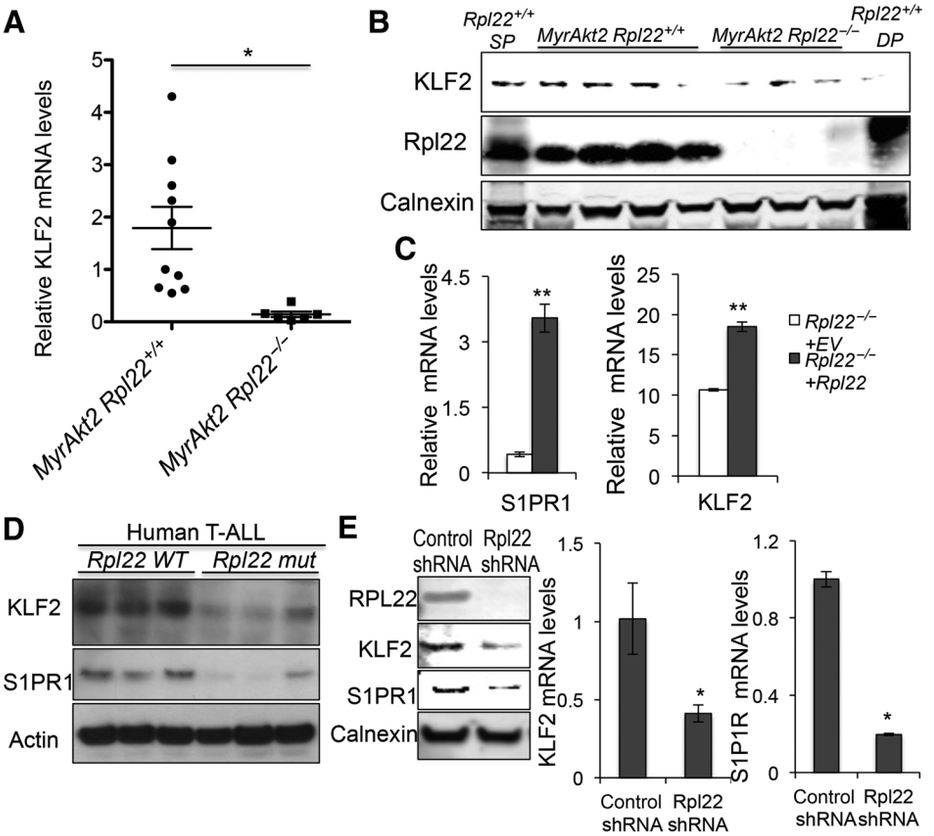

Figure 6.
Rpl22 regulates S1PR1 through effects on Klf2. A, Klf2 mRNA levels in mouse thymic lymphomas from *MyrAkt2 Tg* mice (*MyrAkt2 Rpl22*$^{+/+}$, $n = 10$; *MyrAkt2 Rpl22*$^{-/-}$, $n = 6$) were quantified by RT-PCR. Statistical analysis was performed using an unpaired *t* test (*, $P < 0.05$). B, Western blot to assess Klf2 protein levels in lymphomas from *MyrAkt2 Tg Rpl22*$^{+/+}$ and *MyrAkt2 Tg Rpl22*$^{-/-}$ mice. SP and DP populations sorted from normal mice were used as positive and negative controls, respectively. C, S1PR1 and Klf2 mRNA levels were quantified in triplicate by RT-PCR in Rpl22-deficient lymphoma cells in which Rpl22 was reintroduced by retroviral transduction. Data are representative of three independent experiments. Significance was assessed by the Student *t* test (**, $P < 0.005$). D, immunoblot analysis of S1PR1 and KLF2 expression in six human T-ALL cell lines with either wild-type or mutant *RPL22* alleles. E, analysis of S1PR1 and KLF2 protein levels by immunoblotting (left) and RNA levels (right) by RT-PCR in human T-ALL cell lines transduced with control or Rpl22 shRNA. Data are representative of two independent experiments. Significance was assessed by the Student *t* test (*, $P < 0.05$).

the control could be direct, as has been noted for other RNA-binding proteins (29), and involve Rpl22 binding to Klf2 mRNA and controlling its stability. Finally, while there is no evidence to suggest that Rpl22 is able to directly influence transcription, a recent report indicated that another RNA-binding protein, nucleolin, is not only able to bind mRNAs and regulate their translation (30), but can also bind to the KLF2 promoter in response to fluid shear stress (28). Thus, it is possible that Rpl22 is acting to regulate Klf2 expression in a similar manner. The basis by which Rpl22 regulates KLF2 expression is currently under investigation.

In both of the thymic lymphoma models we examined, Rpl22 deficiency substantially increased the mediastinal tumor burden, which was accompanied by a marked enhancement in angiogenesis. Whether the increased angiogenesis enables the expansion of the mediastinal mass or is induced by it remains unclear. The increased HIF-1/VEGF signaling we observed in the Rpl22-deficient mediastinal masses could have been induced indirectly as a result of the hypoxic conditions caused by rapid expansion of the mediastinal lymphoma mass. Consistent with this, Hif1α mRNA levels were unchanged in Rpl22-deficient thymic lymphomas indicating a posttranscriptional mode of regulation, which usually accompanies hypoxia (31). Alternatively, we did not observe obvious necrosis in the center of the large thymic tumors from Rpl22-deficent mice, suggesting Rpl22-deficiency may directly increase angiogenesis, thereby enabling the more robust blood supply to support the development of larger tumor masses. Consistent with this possibility, we observed increased NF-κB signaling and IL6 production in Rpl22-deficient tumors, which could contribute to VEGF transactivation (32, 33). Efforts are in progress to distinguish these possibilities. Irrespective of the outcome of these studies, because we observe enhanced angiogenesis in Rpl22-deficient tumors, perhaps *RPL22* status could be used to identify a molecularly defined subset against which to apply antiangiogenic therapy, which has not proven effective when used generally in peripheral T-cell lymphoma (34), diffuse large B-cell lymphoma (35), or mantle cell lymphoma (36).

In addition to enhancing angiogenesis, both mouse models showed that Rpl22 deficiency alters the migratory behavior of the lymphomas, causing them to be retained mediastinally, in the thymus. Mediastinal retention is one of the chief characteristics that distinguish T-lymphoblastic lymphoma (T-LBL) from T-ALL. A recent study provided some insights into the basis for this difference in localization, making the counterintuitive observation that mediastinally retained T-LBL exhibited markedly elevated expression of S1PR1, which might otherwise be expected to facilitate dissemination (37). However, these investigators reported that elevated S1PR1 expression blocked dissemination by activating ICAM1 signaling and homotypic adhesion. Accordingly, the T-LBL cases examined in this study exhibited altered migration resulting from excessive S1PR1 signaling. In contrast, mediastinal retention of Rpl22-deficient thymic lymphomas is accompanied by attenuated S1PR1 expression, and is not associated with increased ICAM1 expression (data not shown). KLF2 and its target S1PR1 are critical regulators of thymic egress that are most highly expressed in mature SP thymocytes (15, 26). Importantly, the DP thymic lymphomas that arise in *MyrAkt2 Tg* and

Published OnlineFirst April 5, 2016; DOI: 10.1158/0008-5472.CAN-15-2698

PTEN-deficient mice express far higher levels of KLF2 and S1PR1 than their nontransformed counterparts. Moreover, the migration of these DP thymic lymphomas in response to S1P ligand is comparable with that of mature SP thymocytes. In contrast, Rpl22-deficient thymic lymphomas that are retained mediastinally express markedly reduced levels of KLF2 and S1PR1 and are unable to migrate in response to S1P ligand. Importantly, their migratory capacity can be restored by ectopic expression of S1PR1, indicating that the alteration of S1PR1 is causally linked to the observed migration defect. Taken together, the cell-autonomous defects in the KLF2–S1PR1 migratory axis in Rpl22-deficient thymic lymphomas provide a plausible explanation for the failure of Rpl22-deficient thymic lymphomas to disseminate; however, it is important to note that additional factors may play a role. Indeed, KLF2 regulates the expression of other molecules that control migration, such as CD62L, which is repressed in Rpl22-deficient thymic lymphomas, and CCR7, which is induced (data not shown; refs. 38, 39). Accordingly, the failure of *Rpl22*-deficient lymphomas to disseminate *in vivo* may involve alterations in responsiveness to these or other chemokines. Finally, it is also possible that cell-extrinsic factors might be involved. For example, Rpl22-deficient thymic stroma may express an altered complement of chemokines, cytokines, or adhesion molecules that might fail to promote migration or, alternatively, actively retain the Rpl22-deficient lymphomas. Efforts are currently ongoing to identify other molecular effectors of migration whose expression is altered in Rpl22-deficient lymphomas or in Rpl22-deficient thymic stroma.

Collectively, we have found that Rpl22 haploinsufficiency and deficiency both facilitate development of T-lymphoid tumors that kill their hosts, but by fundamentally distinct mechanisms, with haploinsufficiency killing through dissemination and Rpl22-deficiency killing through mediastinal retention. The mediastinal mass is one of the key characteristics of T-LBL, along with no or minimal marrow involvement. While we have previously identified RPL22 haploinsufficiency in about 10% of T-ALL, the extent of *RPL22* inactivation in T-LBL has not been assessed. Nevertheless, our data also suggest that Rpl22 may play an important role in the dissemination of human leukemias/lymphomas. This is of particular interest in T-cell leukemia, where chemokines and chemokine receptors like CCR9/CCL19 and CXCR4/CXCL12 influence T-cell leukemia maintenance and progression (39–41). Given that S1PR1 signaling has also been linked to the dissemination of solid tumors (42, 43), Rpl22 loss may affect the metastasis of solid tumors as well. Indeed, biallelic inactivation of *RPL22* has been reported in colorectal cancer (8, 9); however, its association with metastatic spread and disease course has not been evaluated. Future efforts will be directed toward evaluating the link between *RPL22* inactivation and dissemination in both hematologic and solid malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Rao, J.E. Stadanlick, D.L. Wiest
Development of methodology: S. Rao, K.Q. Cai, S.-Y. Lee
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Rao, K.Q. Cai, J.E. Stadanlick, N. Solanki-Patel, J.R. Testa
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Rao, K.Q. Cai, S.P. Fahl, D.L. Wiest
Writing, review, and/or revision of the manuscript: S. Rao, J.R. Testa, D.L. Wiest
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Rao, N. Greenberg-Kushnir
Study supervision: D.L. Wiest

Acknowledgments

The authors thank Dr. Siddharth Balachandran for critical review of the manuscript, Dr. Hans-Jorg Fehling for providing pTα-Cre mice, and Dr. Antonio Di Cristofano for $Pten^{flox/flox}$ mice. They gratefully acknowledge the assistance of the following core facilities of the Fox Chase Cancer Center: Cell Culture, DNA Sequencing, Flow Cytometry, and Laboratory Animal.

Grant Support

This work was supported by NIH grants R01AI110985 and core grant P30CA006927, Leukemia and Lymphoma Society grant 6057-14, and an appropriation from the Commonwealth of Pennsylvania. J.R. Testa and D.L. Wiest also received support from NIH grant R01CA077429 for the development and characterization of the MyrAkt2 Tg mouse model. S. Rao was supported by a Department of Defense Bone Marrow Failure Postdoctoral Fellowship W81XWH-13-1-0403.

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Received September 30, 2015; revised March 22, 2016; accepted March 25, 2016; published OnlineFirst April 5, 2016.

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Published OnlineFirst April 5, 2016; DOI: 10.1158/0008-5472.CAN-15-2698

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Published OnlineFirst April 5, 2016; DOI: 10.1158/0008-5472.CAN-15-2698

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Cancer Res 2016;76:3387-3396. Published OnlineFirst April 5, 2016.