Increasing evidence from our lab and others suggests that ribosomal proteins play a critical regulatory role in development and disease, including bone marrow disease, that can be mediated independently of their functions in ribosomal biosynthesis. Previously I have determined that ribosomal protein Rpl22 functions as a haploinsufficient tumor suppressor by activating NFκB and its target Lin28B in a mouse T-cell lymphoma model driven by Myr-Akt2. Recently we also found that Rpl22 knockout mice exhibit an MDS-like phenotype associated with anemia and abnormal bone marrow (BM) hematopoiesis. Consistently, our collaborator found that RPL22 indeed was mutated or deleted in some MDS and AML patients. Further we found that loss of Rpl22 but not other ribosomal proteins induces Lin28B, and the activation of NFκB and Lin28B mainly depends on ER stress signaling through PERK. Rpl22 has a homolog Rpl22-Like1 (Like1) that is induced upon Rpl22 loss. Interestingly, Rpl22 functions through the regulation of Like1. Like1 overexpression is both necessary and sufficient for both transformation and NFκB-mediated Lin28B induction. Mechanistic studies revealed that Rpl22 directly bind to Like1 mRNA and inhibit its translation. Through MLL-AF9 AML BM transplant mouse model, I found that Rpl22 inactivation accelerates AML progression and correlated with poor survival. We are still in the progress of investigate Rpl22 and its paralog Like1 in MDS/AML and hopefully can find out new therapeutic target through these efforts.
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INTRODUCTION:
Ribosomal proteins (RPs) have been well known to be essential for protein synthesis. Increasing evidence from our lab and others suggests that RP play a critical but poorly understood role in development as well as disease. Mutations in RPs cause a group of diseases collectively termed ribosomopathies, which are mainly bone marrow failure diseases and are often associated with increased risk for development of myeloid neoplasms. We are interested in Rpl22, an RNA-binding component of the 60S ribosomal subunit. Rpl22 is dispensable for protein biosynthesis but plays a role in regulating transformation and hematopoiesis (1, 3). Previously I have determined that Rpl22 functions as a haploinsufficient tumor suppressor, whose mono-allelic inactivation can accelerate the development of T-cell lymphoma in a mouse model where disease is driven by an MyrAkt2-transgene. Rpl22 inactivation predisposes to transformation by activating the NFκB and its direct target, the stem cell factor Lin28B (3). In addition, we also found that Rpl22 knockout mice exhibit an MDS-like phenotype associated with anemia and abnormal bone marrow (BM) hematopoiesis. Consistent with what we observed in our mouse model, our collaborator found that Rpl22 was mutated or deleted in some MDS and AML patients. Based on these data, I intend to investigate the role of Rpl22 in MDS and its predisposition of AML and hopefully can find out new therapeutic target through these efforts. Rpl22 has a paralog Rpl22-like1 (Like1) that shares 70% sequence identity with Rpl22 (1, 4), and I found that Like1 is induced by Rpl22 inactivation both in vivo and in vitro. These results led me to further explore the role of Rpl22 and its paralog Like1 in the predisposition of blood malignancies as well.

KEYWORDS: Rpl22, MDS, AML, MLL-AF9, ER stress, Rpl22-Like1

OVERALL PROJECT SUMMARY:

1. To explore the molecular basis for Lin28B induction by inactivation of Rpl22

![Figure 1. NFκB/Lin28B induction depends on ER stress PERK/eIF2α pathway. A, Schematic to show the activation of NFκB through ER stress PERK signaling. B, western blot to show that Rpl22 loss increases PERK-eIF2α activation as displayed by phospho-PERK and phospho-eIF2α. C, western blot to show that Lin28B induction is dependent on PERK.](image)
**1A**: Test whether Rpl22 loss activates NFκB through endoplasmic reticulum stress pathway
PERK-phospho-eIF2α-IκBα or IRE1α-TRAF-IKKβ

Recent publications have showed ER stress activation of NFκB signaling (5) and we observed increased ER stress response in Rpl22-deficient cells as demonstrated here (Fig.1A,B) by increased phospho-PERK and its target phospho-EIF2α. Therefore, we utilized retroviral-mediated shRNA targeting different ER stress components including PERK, IRE1α, and ATF6 to determine which signaling pathway is required for Lin28B induction. As shown in Fig.1C, knockdown of PERK significantly reduced NFκB activation as displayed by phospho-NFκB and Lin28B induction. We did not see significant differences upon ATF6 knockdown. These data support that PERK signaling is responsible the activation NFκB activation and Lin28B induction.

**1B**: NFκB/Lin28B induction caused by Rpl22 inactivation also relies on Rpl22 paralog Rpl22-Like1

I also made an interesting finding that links NFκB/Lin28B induction upon Rpl22 loss with Rpl22 paralog, Rpl22-Like1, (Like1) expression. I have found that Like1 is induced upon Rpl22 inactivation (Fig.2A). Knockdown of Like1 inhibits Lin28B induction (Fig.2B,C) whereas...
overexpression of Like1 increases Lin28B levels as well as NFκB signaling as demonstrated by phosphorylation of p65 (Fig.2D). We don’t know whether PERK activation depends on Like1 induction yet. We are now investigating whether Like1 is required for ER stress to activate NFκB signaling and Lin28B induction. Furthermore, I also observed Like1 induction is both necessary and sufficient to promote transformation in colony formation assay (Fig.2D-F). Like1 is also critical for disease progression in vivo (Fig.2G). All these data suggest that Like1 induction occurs before Lin28B induction and plays critical in transformation. We are now focusing on the role of Like1 in the progression of blood malignancies (6, 7) and other solid tumors (8) because Like1 amplification was found in these diseases. I also further explored the molecular mechanism associated with the post-transcriptional regulation of Like1 by Rpl22 and found out that Rpl22 directly binds to Like1 mRNA and inhibits its translation (Fig.3) but not protein degradation (data not shown).

1C. To determine whether inactivation of other ribosomal proteins increases Lin28B

By knockdown other ribosomal proteins with lentiviral mediated shRNA targeting Rps7, Rpl36, Rpl36a, Rps19 and Rpl14, we failed to observe significant induction of Lin28B as determined by realtime PCR (Fig.4). These data suggest that regulation of Lin28B is specific with Rpl22.
2. To assess the role of p53 induction in the perturbation of hematopoiesis observed in Rpl22-/- mice

Ribosomal protein defects frequently induce p53 (9, 10). To assess the role of p53 in perturbation of hematopoiesis in Rpl22-/- mice, p53 null mice were crossed with Rpl22-/- mice. We examined p53 levels in the bone marrow from Rpl22 wild type (WT) and knockout (KO) mice and found out p53 is not dramatically altered in bone marrow cells, particularly upon enriching for stem/progenitor cells by depleting cells expressing lineage markers (Fig.5). We also analyzed the phenotype in Rpl22-/-p53-/- mice and found that the mice exhibit similar phenotype with Rpl22-/. We also did RNA-seq analysis and real-time PCR in the bone marrow cells from Rpl22 WT and Rpl22 KO and found out the p53 targets were not altered significantly except p21. Based on this, we assume that p53 may not significantly influence hematopoiesis in Rpl22 KO mice.

3. To explore whether Rpl22 loss accelerates Myelodysplastic Syndrome /Acute Myelogenous Leukemia progression

We used MLL-AF9, a frequent translocation in AML with oncogenic activity in murine models (11, 12). Mice transplanted with MLL-AF9 expressing cells displayed leukemia phenotype as early as 6 weeks (13). So we transduced BM cells from Rpl22-/- and Rpl22+/+ mice with MLL-AF9 and transplanted the cells into the recipient mice. As we expected, mice receiving Rpl22 KO cells display AML phenotype as shown in Fig.6. Rpl22 loss seems to be predisposed to AML.

Figure 5. Western blot to show p53 expression levels in bone marrow cells (BM) isolated from Rpl22 wild type (WT) or knockout mice (KO) with or without lineage depletion (Lin Dep.) and 2 days or 5 days 5-Fu treatment. Actin is used as loading control.

Figure 6. A. Schematic to show the transplant mouse model. BM cells were harvested, transduced with MLL-AF9 oncogene and then transplanted to irradiate recipient mice. B. Spleen weight of the recipient mice receiving transformed BM cells with MLL-AF9. C, Hematocrit of recipient mice receiving BM cells transduced with MLL-AF9.
We also generated MLL-AF9 transgenic mice with Rpl22 KO to study whether Rpl22 deletion will accelerate AML development in vivo. We found Rpl22 knockout MLL-AF9 mice displayed poor survival compared with wild type counterpart (Rpl22-/- ;MLL-AF9 VS Rpl22+/+ ;MLL-AF9, p<0.05) (Fig.7).

**KEY PROJECT ACCOMPLISHMENTS:**
1. Linked Rpl22 defects with ER stress and PERK signaling and induction of oncogenic targets.
2. Revealed that Rpl22 functions through its paralog Like1 in transformation and Lin28B induction.
3. Found Rp22 paralog Like1 is a proto-oncogene that is sufficient and necessary for transformation mediated by Rpl22 defects.
4. Revealed that Rpl22 directly binds to Like1 mRNA for translational repression.
5. Although p53 induction occurs upon ribosomal defects and is usually linked with bone marrow disease, our data did not support p53 as the key player in MDS/AML caused by Rpl22 defect.
6. Uncovered a pathological role of Rpl22 defect, in promoting AML progression.

**CONCLUSION:**
Together, my data provide insights into the mechanism underlying the basis by which Rpl22 defects promote MDS/AML and have linked Rpl22 loss to exacerbation of ER stress PERK signaling. Our survival data in MLL-AF9 transgenic mice strongly suggest that Rpl22 inactivation or deletion might be a poor prognostic marker in AML. Interestingly, I also made the novel observation that the tumor suppressor function of Rpl22 is mediated through repression of its paralog Like1. Like1 is induced upon Rpl22 inactivation and its expression is required and sufficient to promote transformation and Lin28B induction, suggesting Like1 might be a proto-oncogene in tumorigenesis. We are investigating the role of Rpl22 inactivation as well as Like1 induction in the development and progression of blood malignancies as well as solid tumors. We

![Figure 7. Percent survival of MLL-AF9 transgenic mice and Rpl22 knockout (Rpl22/-) mice showed poor survival compared with Rpl22 wild type mice (Rpl22+/+).](image-url)
are also exploring prognostic value of Rpl22 inactivation and Like1 overexpression in both blood malignancies and solid tumors.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. Publications:

b. Abstract presented in conference
- Rao S, Stadanlick JE, Cai KQ, Wiest DL. 2014. Loss of Rpl22 promotes tumor progression through regulation of angiogenesis and dissemination. AACR-Hematologic Malignancies Conference, Philadelphia, USA (Travel Award)

INVENTIONS, PATENTS AND LICENSES: Nothing to report.

REPORTABLE OUTCOMES:
Developed Like1 antibody and tested for the application of western blot, immunoprecipitation, immunofluorescence and immunohistochemistry (IHC) in tissue microarray.

OTHER ACHIEVEMENTS: Nothing to report.

TRAINING AND PROFESSIONAL DEVELOPMENT ACTIVITIES:
I joined Dr. David Wiest’s lab for postdoctoral training in 2010 to study the mechanism by which inactivation of the ribosomal protein Rpl22 predisposes blood malignancies. Dr. Wiest’s expertise in preclinical animal models of T cell malignancy as well as in the manipulation of development of primary hematopoietic stem cells in vitro and in vivo, has enabled me to significantly expand my technical repertoire. In my pursuit of these studies, I have developed a strong interest in hematologic malignancies. Under Dr. Wiest’s sponsorship I have gone through the following trainings, which is necessary for an independent position in academia: 1) Writing Skills – I have enrolled in the interactive 6wk grant writing course at Fox Chase and improved my writing skills. Additionally, I am preparing two manuscripts (one submitted to *Cancer Research*). This entails several rounds of review and revision with Dr. Wiest, which also helps to improve my writing ability. 2) Oral Presentations – I have won awards for my oral presentations; I also attended weekly Wiest lab meetings, the postdoctoral seminar series, weekly departmental journal club and Fox Chase Research Festival to further improve my speaking ability 3) Research Networking – our weekly departmental seminar series where I am able to meet with the outstanding speakers that visit Fox Chase Cancer Center and scientific meetings. I attended the 2014 AACR-Hematologic Malignancies Conference and 2015 AACR Annual Conference in
Philadelphia. All these experiences ensure that I received a well-rounded training experience and will be fully prepared me for independence as a researcher in blood malignancies. I will also re-submit a K22 grant application this coming year from George Washington, with strong support from Dr. Wiest and the two more manuscripts in progress, I am on my way towards research independence.

REFERENCES:


APPENDICES:

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-kB 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. Lympoma-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 (SIP1R). Indeed, reexpression of SIP1R in Rpl22-deficient tumor cells restores their migratory capacity in vitro. The regulation of KLF2 and SIP1R by Rpl22 appears to be proximal as Rpl22 reexpression in Rpl22-deficient lymphoma cells restores expression of KLF2 and SIP1R, 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.

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 γδ 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-kB 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
a proximal Lck promoter (13) and a conditional knockout mouse where the Pten tumor suppressor gene is ablated in T-cell precursors using pre-Tre-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 mice 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 Ptenlox/lox mice (19) with those expressing Cre in T-lineage progenitors only, under control of the pre-Tre 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 Na2HPO4, 150 mmol/L NaCl, 2 mmol/L MgCl2, 5 mmol/L K2Fe(CN)6, 5 mmol/L K4Fe(CN)6, and 1 mg/mL X-gal (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 (ME-14), anti-CCR7 (4B12), anti-CD3 (500A2), and anti-B220 (RA3-6B2). For IHC analysis, tissues were parafixed for 5 minutes in 2% formaldehyde and 0.2% glutaraldehyde in staining solution (40 mmol/L Na2HPO4, 150 mmol/L NaCl, 2 mmol/L MgCl2, 5 mmol/L K2Fe(CN)6, 5 mmol/L K4Fe(CN)6, and 1 mg/mL X-gal (Sigma)).

Plasmids, cell culture, and viral production

The S1PR1/EDG1 cDNA (RDC0053; R&D Systems) was subcloned into the bicistronic retroviral vector pMSCV-IRE5-FYP (pMY; ref. 20) using standard methods. Lentiviral shRNA constructs targeting murine S1PR1 (pLKO.1-puro) were purchased from Sigma-Aldrich. Exemplified 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 (pMY-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 (delta8.2 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).

Chemoattract migration assay

Migration assays were performed in triplicate using 24-well Transwell chambers with 5 μm polycarbonate membranes (Corning; refs. 21). The lower chamber was filled with 600 μL of 1% FBS DMEM supplemented with chemotactants sphingosine 1-phosphate (S1P) or chemokine ligand 21 (CCL21; R&D Systems). Thymic lymphoma cells (1 × 105) were placed in the upper chamber and incubated for 4 hours at 37°C in 5% CO2. 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, Mm00180668_m1; Actin, Mm00607939_s1; Vegfa, Mm01281449_m1; Hif1a, Mm00443261_m1; S1pr1, Mm00443261_m1; Slp1, Mm00443261_m1; Klf2, Mm00443261_m1; Ccr7, Mm00443261_m1.

Results

Rpl22 deficiency promotes development of thymic lymphoma in MyrAkt2 Tg mice

Monoaclonal 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
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

Histopathologic 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 lymphomas, this suggests that Rpl22 may play a role in the dissemination of thymic lymphoma.

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 = 6; MyrAkt2 Tg Rpl22+/−, n = 12; and MyrAkt2 Tg Rpl22+/−, n = 7. Analysis was performed using the unpaired t test (**, P < 0.05).
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 failed 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
Role for Rpl22 in T-cell Lymphoma Dissemination

Rao, Shuyun

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 = 1; 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.

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

Because the thymic lymphomas that develop in Rpl22−/− mice are retained mediastinally and fail to disseminate, we hypothesized that this result might come 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 in S1PR1−/− mice. 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...
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
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/splint mice with the indicated genotypes (MyrAkt2 Tg Rpl22<sup>−/−</sup>, n = 10; MyrAkt2 Tg Rpl22<sup>−/−</sup>, n = 6). Significance was determined with unpaired t test (*, P < 0.05). D, immunoblot analysis of S1PR1 protein levels in DP lymphomas 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<sup>−/−</sup> 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).
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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 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).

The increased angiogenesis 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
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Role for Rpl22 in T-cell Lymphoma Dissemination

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 lymphomas (40, 41), 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 CCR5/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 (4, 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

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