The Ras GTPase Activating Protein p120 RasGAP as a regulator of cardiovascular system development, lymphatic system maintenance, and a T cell lineage tumor suppressor

by

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## Abstract

Ras signaling activates multiple pathways that drive cell growth, proliferation, survival, and differentiation. Ras is activated downstream of multiple receptors by the recruitment and interaction with Ras guanine nucleotide exchange factors (RasGEFs). Efficient regulation of this signaling is essential for the avoidance of developmental defects and cancer, and cessation of Ras signaling is dependent on the recruitment of Ras GTPase activating proteins (RasGAPs) for juxtaposition with Ras. RasGAPs physically interact with Ras to allow for Ras hydrolization of bound GTP. There are multiple RasGAPs, which have varied tissue expression patterns. The RasGAP p120 RasGAP (RASA1) has been shown to be important in the mouse embryonic cardiovasculature and adult lymphatic system. However, RASA1 has other roles than regulation of Ras, including interactions with p190 RhoGAP for directed cell migration. To address this, we generated a mouse carrying a point mutation in its GAP domain that ablates its GAP activity while retaining all other functions. With the use of these mice we show that it is the loss of RASA1's ability to regulate Ras that results in failed cardiovascular development in embryos and the instability and outgrowth of lymphatic vessels in adult mice.

Although RASA1 plays an important role in many tissues, it has a minor role in the regulation of Ras during T cell development. Another RasGAP, Neurofibromin 1 (NF1) also appears to have a largely dispensable function in the T cell compartment. Since loss of both RASA1 and NF1 in developing embryos results in earlier embryonic lethality than loss of either

RasGAP alone, we hypothesized that these RasGAPs may have overlapping functions in T cells. Indeed, concurrent deletion of RASA1 and NF1 in the T cell compartment resulted in in a randomly occurring T cell acute lymphoblastic leukemia (T-ALL) in mice associated with acquisition of somatic activating mutations in the Notch1 receptor previously implicated in T-ALL pathogenesis.

# Chapter 1
# Introduction

## 1.1 Ras and Ras Signaling

Ras is a small, inner membrane-bound G protein that plays a vital role in many important cellular functions, including cell growth, differentiation, proliferation, and survival [1]. Ras functions downstream of many cell surface receptors including cytokine receptors, growth factor receptors, and the T cell receptor (TCR). Ras acts as a molecular 'on-off' switch, converting between active and inactive states. Until it is activated, Ras exists in a GDP-bound inactive state. During receptor signaling, Ras guanine nucleotide exchange factors (RasGEFs) are recruited to membranes where they become juxtaposed to Ras [2]. RasGEFs interact with Ras to allow for the ejection of the GDP. Since GTP exists at a higher concentration within the cell cytosol than GDP, Ras then preferentially binds GTP and switches to an active conformation that triggers multiple downstream signaling pathways.

One of the signal transduction pathways Ras activates is the mitogen-activated protein kinase (MAPK) pathway (Figure 1). GTP-bound Ras binds and activates the serine/threonine kinase Raf1, which phosphorylates two MAPK kinases, MEK1 and 2 [3]. MEK1/2 phosphorylate the MAPK extracellular signal-regulated kinases 1 and 2 (ERK1/2), resulting in their activation and translocation to the cell nucleus. Within the nucleus ERK1/2 then phosphorylate members of the ternary complex factor (TCF) family of transcription factors, including Elk1, Sap1a and Sap1b, and Net [4]. The TCFs interact with serum response factor

(SFC) to bind specific sites in promoters of early response genes. This results in increased transcriptions of these genes, which include *c-Fos*. C-Fos and c-Jun are transcription factors that heterodimerize to form the transcription factor complex activation protein 1 (AP-1). ERK1/2 also phosphorylate both c-Fos and c-Jun, resulting in a stabilized c-Fos and increased transcriptional activity [5]. Through late activating genes, the MAPK pathway drives cell proliferation, along with other interactions with proteins that drive DNA synthesis and cell cycle progression [6].

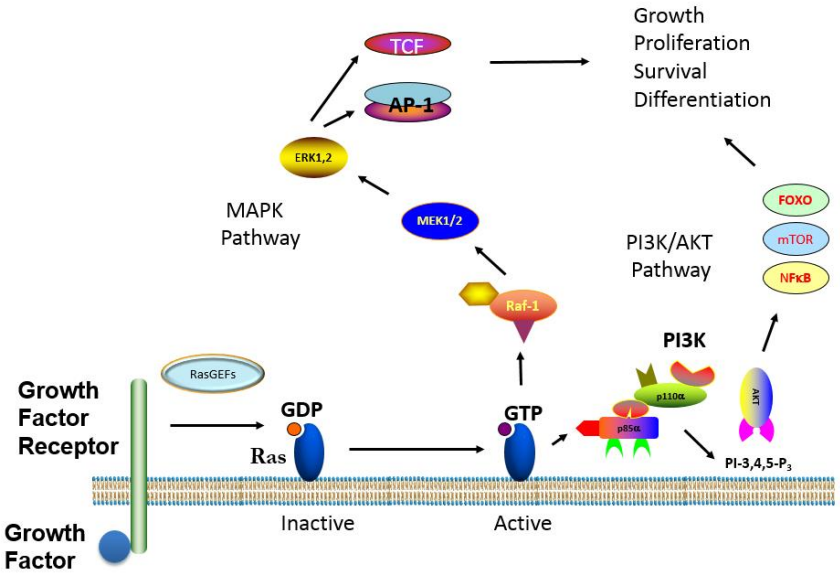



**Figure 1. Activation and regulation of intracellular Ras signaling.** Ras signaling occurs downstream of various receptors, including growth factor receptors. Activation of the receptors by their binding to specific ligands results tyrosine phosphorylation of the receptor leading to the recruitment of RasGEFs. RasGEF allows inactive GDP-bound Ras to eject the GDP and bind GTP and initiate signaling through multiple pathways, including the MAPK and PI3K/AKT pathways. RasGAPs are then recruited for efficient cessation of signaling by aiding in the hydrolyzation of Ras-bound GTP.

Ras also signals through the phosphatidylinositol 3-kinase (PI3K) pathway (Figure 1). GTP-bound Ras activates the catalytic subunit of PI3K (p110) independently from the p85 subunit and phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [7, 8]. PIP3 binds AKT and phosphoinositide-

dependent kinase-1 (PDK1) by their pleckstrin homology (PH) domains, and brings both proteins to the cellular membrane. PDK1 phosphorylates AKT on Thr308, resulting in activation of AKT that leads to inhibition of the pro-apoptotic BAX and BAD along with negative regulation of NF-κB. AKT can also phosphorylate the tuberous sclerosis complex 2 (TSC2) complex, which allows for mammalian target of rapamycin complex 1 (mTORC1) signaling. mTORC1 signaling leads to increased p70S6 kinase activity, which stimulates protein biosynthesis [7, 8]. Mammalian target of rapamycin complex 2 (mTORC2) phosphorylates AKT on Ser473, and this phosphorylation is required for full activation of the protein [9].

### 1.2 Regulation of Ras Signaling

Although Ras has some intrinsic GTPase activity, Ras GTPase activating proteins (RasGAPs) are required for efficient GTP hydrolysis. RasGAPs increase Ras GTP hydrolysis by several orders of magnitude [2]. This occurs by the insertion of the RasGAP arginine finger (Arginine 789 in humans, 780 in mice) into the active site of Ras [10]. RasGAPs stabilize the switch II region of Ras, which allows Glutamine 61 (in humans) to carry out catalysis [11]. Also important in maintaining the interaction between Ras and RasGAPs are Glycines 12 and 13, mutations of which lead to impaired interactions between RasGAP Arg789 and Ras Glu61.

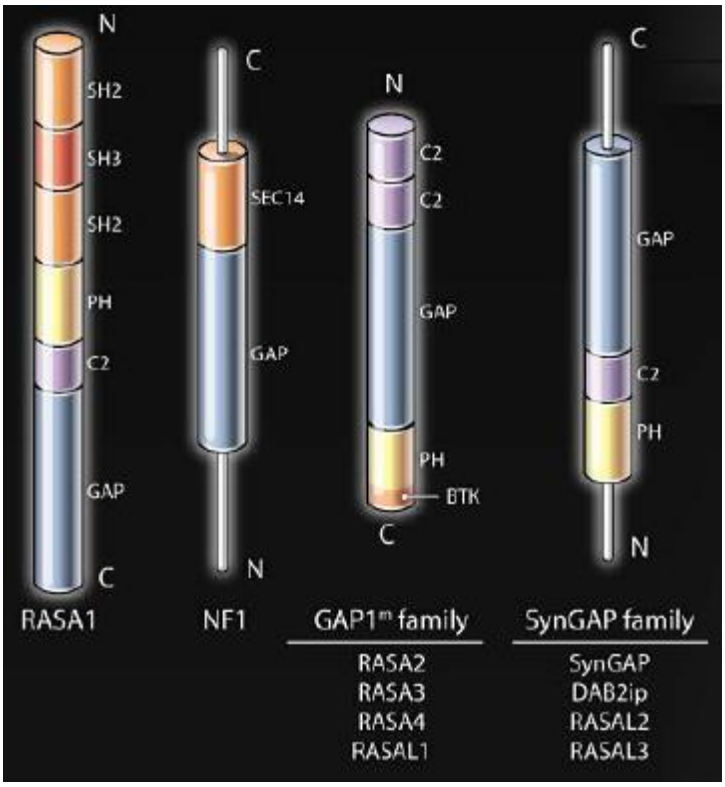



**Figure 2. The domain structures of RasGAPs [12].**

The RasGAP family currently has ten known members, each of which contain a GAP domain that is required for interaction with and inactivation of Ras (Figure 2) [12]. This approximately 330 amino acid catalytic domain is the only common domain shared between all family members [10]. The RasGAPs contain other domains, including Src homology 2 (SH2) and SH3, PH, protein kinase C2, Bruton tyrosine kinase (BTK), and SEC14-PH domains [12]. These domains are often involved in the recruitment of the RasGAPs to membranes for juxtaposition with Ras or can act as the basis for the organization of protein complexes [2]. While identified as RasGAPs, these proteins may also have Ras-independent functions [10]. RasGAPs have variable expression patterns. Some RasGAPs, including p120 RasGAP, Neurofibromin 1, RASA2, DAB2ip, and RASAL2 are expressed ubiquitously, while other RasGAPs are more limited in expression [12]. For example, SynGAP is expressed in neurons

while RASA3 is localized to the brain and spleen. Thus it is likely that any given tissue expresses multiple RasGAPs.

### 1.3 Cancer and Rasopathies

Because of the breadth of cellular processes that are under the control of Ras, Ras activation is tightly regulated by RasGAPs. However, mutations in Ras occur that affect the structure of Ras such that RasGAPs are unable to stabilize the switch regions. This includes mutations of Gly12 and 13 and Glu61[13]. These mutations result in constitutively active Ras signaling [11]. Ras mutations occur in approximately 30% of all human cancers, with a higher incidence in pancreatic, colon, lung and thyroid cancers, and in hematological malignancies such as myeloid leukemias and T and B cell lymphoma/leukemias [13]. In order to study the role of Ras mutations in cancer, mouse models have been generated to express constitutively active Ras carrying similar mutations to those found in cancer patients through induction of Cre transgene expression [14]. When induced in the hematopoietic compartment, the activating *K-ras*$^{G12D}$ mutation results in all mice developing a lethal myeloproliferative disorder [15]. Transferring *K-ras*$^{G12D}$ expressing bone marrow into wild type mice results in the majority of recipients developing a T-cell leukemia/lymphoma carrying mutations in additional genes [16].

Germline mutations that cause perturbations in Ras signaling, particularly through the MAPK pathway, can result in a variety of disorders collectively called Rasopathies. These include Noonan syndrome, Costello syndrome, Noonan syndrome with multiple lentigines (NSML, formerly known as LEOPARD syndrome), cardio-facial-cutaneous syndrome, Legius syndrome, neurofibromatosis type 1, and cardiovascular malformation-arteriovenous

malformation (CM-AVM) [17]. These developmental disorders share some characteristic phenotypes including cardiac malformations, aberrant craniofacial morphology, varying levels of cognitive impairment and delay, along with malformations in other systems [18]. Not surprisingly, some of these disorders are associated with an increased risk of cancer.

| Syndrome | Ras/MAPK pathway gene | Protein | Protein function |
|---|---|---|---|
| **Capillary malformation-arteriovenous malformation** | *RASA1* | p120 RasGAP (RASA1) | RasGAP |
| **Cardio-facial-cutaneous Syndrome** | *BRAF*<br>*MAP2K1*<br>*MAP2K2*<br>*KRAS* | BRAF<br>MEK1<br>MEK2<br>KRAS | Kinase<br>Kinase<br>Kinase<br>GTPase |
| **Costello Syndrome** | *HRAS* | HRAS | GTPase |
| **Legius Syndrome** | *SPRED1* | SPRED1 | SPROUTY-related, EVH1 domain-containing protein |
| **Neurofibromatosis 1** | *NF1* | Neurofibromin (NF1) | RasGAP |
| **Noonan Syndrome** | *PTPN11*<br>*SOS1*<br>*RAF1*<br>*KRAS*<br>*NRAS*<br>*SHOC2*<br>*CBL* | SHP2<br>SOS1<br>CRAF<br>KRAS<br>NRAS<br>SHOC2<br>CBL | Phosphatase<br>RasGEF<br>Kinase<br>GTPase<br>GTPase<br>Scaffolding<br>E3 ubiquitin ligase |
| **Noonan Syndrome with multiple lentigines** | *PTPN11*<br>*RAF1* | SHP2<br>RAF1/CRAF | Phosphatase<br>Kinase |

**Table 1. List of Rasopathies with associated genes, their encoded proteins, and protein functions [17].**

### 1.4 T Cell Development

T cells undergo a multistep developmental process, originating from hematopoietic stem cells in the bone marrow which traffic to the thymus [19]. The thymus consists of multiple lobes, each containing medullary and cortical regions, through which thymocytes pass as they develop. Early thymocyte progenitors (ETPs) are the first cells to seed the thymus [20]. Although ETPs are generally considered to be equivalent to double negative 1 (DN) cells, which do not yet express the T cell co-receptors CD4 or CD8, ETPs often have low CD4 expression [21, 22]. DN cells continue from stages 1 to 4, identified by changing expression of CD44 and CD25 (DN1 CD44+CD25-; DN2 CD44+CD25+; DN3 CD44-CD25+; DN4 CD44-CD25-) [23]. At the DN2-DN3 transition, the TCRβ chain gene undergoes rearrangement, resulting in the expression of the pre-TCR at DN3. At this point, signaling through the pre-TCR is essential for thymocyte survival and progression to DN4 [24].

In response to pre-TCR signaling, thymocytes undergo a proliferative burst and start expressing the CD4 and CD8 co-receptors (double positive, DP). DP cells undergo TCRα gene rearrangement which if successful results in expression of the TCRαβ receptor at the cell surface. At this stage, the TCR expressing DP cells undergo a process known as positive selection, in which the newly formed TCRαβ are tested for their ability to recognize self-MHC when bound to self-peptide. TCR recognition of the MHC-bound peptide complex at this stage results in cell survival. The strength of the interaction determines whether the cells commit to either the CD4 or CD8 lineage, and results in the loss of the other co-receptor [25].

After cells that can recognize self MHC stop proliferating, they go through a process known as negative selection. Signal strength from interaction with MCH with self-peptides determines the fate of the developing thymocyte [26]. Cells that do not receive enough signal die by neglect [27]. However, cells that have a strong affinity for self-antigen cannot be released

into the periphery as this could result in autoimmunity. Thus, those that react strongly to various self-antigens are deleted [28].

Ras signaling has been shown to be essential for thymocyte development. ERK downstream of MEK1 is required for the differentiation of DN3 thymocytes [29]. ERK1/2 downstream of Ras is also important for positive selection. TCRβ development in mice deficient in both ERK1 and 2 is nearly completely blocked at this stage [30, 31] Further on in development, constitutive expression of ERK2 leads to a preferential development of DP thymocytes to CD4 cells [32]. Ras signaling during positive selection may be driven by the RasGEF RasGRP1 [33, 34], however negative regulation of Ras throughout thymocyte development is not well understood. Similarly, Ras activation downstream of the TCR is known to be driven by the RasGEFs Sos and RasGRP1 [35, 36], while the RasGAP or GAPs that act to regulate this signaling are unknown.

### 1.5 Physiological Functions of p120 RasGAP

The first described RasGAP family member was p120 RasGAP (RASA1), which along with a GAP domain contains SH2, SH3, PH, and protein kinase C2 domains [2]. Mice that are homozygous null for *Rasa1* die *in utero* at embryonic day 10.5 (E10.5) due to aberrant blood vessel development [37]. At E9.5 RASA1 null embryos showed failed vascular organization in the yolk sac along with a thinner dorsal aorta with aberrant intersegmental artery branching in the embryo proper. Interestingly, although RASA1 is ubiquitously expressed, adult mice that have induced loss of RASA1 in all tissues have no identifiable spontaneous cardiovascular phenotype [38], although loss of RASA1 may result in increased angiogenesis under certain experimental conditions [39]. Specific deletion of RASA1 in the T cell compartment during T

cell development leads to a slight increase in thymocyte positive selection in the thymus and reduced numbers of naïve cells in the periphery [40]. However, the increase in positive selection is apparent only on TCR transgene backgrounds. This suggests that other RasGAPs are more important in regulation of T cell development and function and cardiovascular maintenance. Alternatively, other RasGAPs have overlapping functions with RASA1 in these systems.

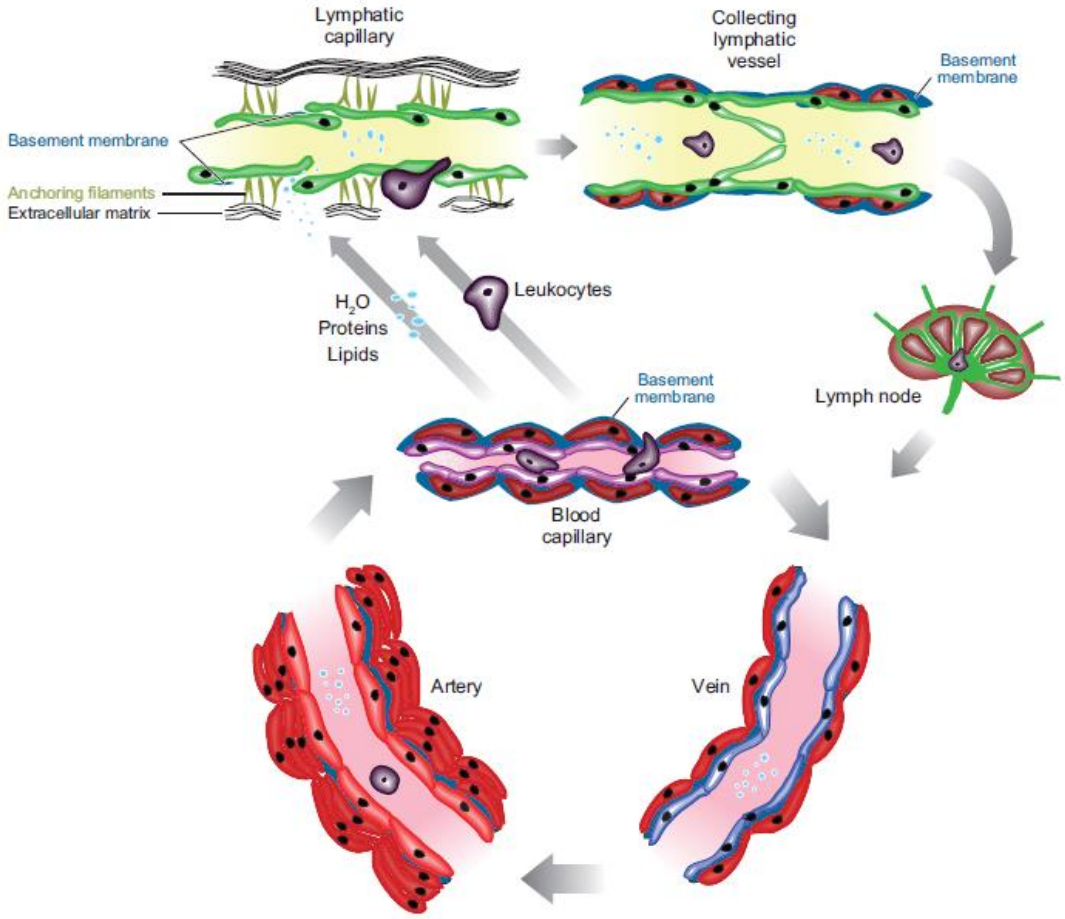



**Figure 3. Lymphatic and cardiovascular systems [41].**

Although RASA1 is not necessary for cardiovascular maintenance, adult mice with induced loss of RASA1 develop a striking lymphatic system hyperplasia and leakage [38]. The lymphatic system is essential for the uptake of interstitial fluid, macromolecules and trafficking of immune cells, and returns them to the bloodstream [42]. Lymphatic capillaries are composed of oak leaf shaped endothelial cells (LEC) that form button-like junctions with overlapping edges that result in valve-like openings (Figure 3) [41]. Entrance of fluid is possible as lymphatic capillaries lack basement membrane and maintain structural integrity via anchoring filaments [43]. These capillaries feed into larger pre-collecting lymphatics, which have basement membrane and have characteristics of both capillaries and collecting vessels. Collecting vessels also have basement membrane, and are surrounded by vascular smooth muscle cells (SMC) [41]. SMC cells have contractile activity that drives lymph flow, and precollecting and collecting lymphatic vessels have valve cells to maintain the unidirectional flow of lymph. LEC in collecting vessels form zipper-like junctions, composed of the same tight and adherens junction associated proteins (such as VE-cadherin) as the button-like junctions of lymphatic vessels, but in a different arrangement (Figure 4) [44].

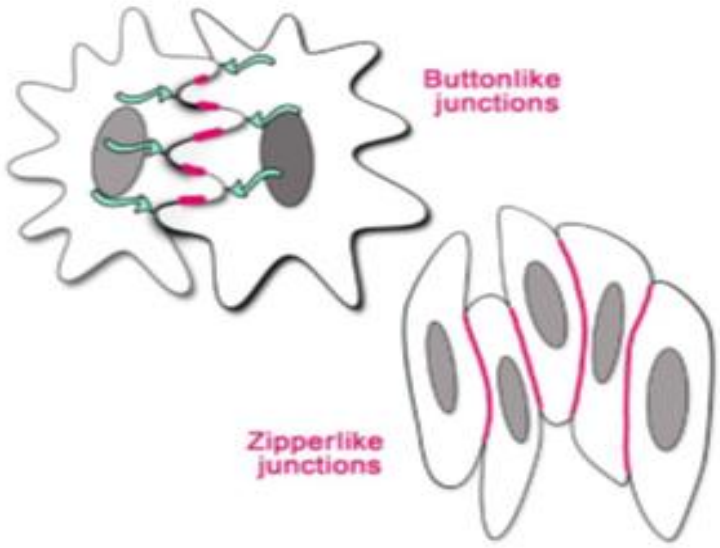



**Figure 4. Junctional organization of LEC in lymphatic capillaries and collecting vessels [43].** Both types of junctions are composed of tight- and adherens junction associated proteins, but have different organization.

Lymph sacs containing LEC develop off of the cardinal and iliac veins, and these cells are the progenitors of the entire lymphatic vasculature [45]. The homeobox transcription factor *Prox1* that specifies LEC fate is detectable in mice at starting at E9.5. Lymphatic endothelial hyaluronan receptor-1 (LYVE-1) is expressed at E9, making it the first identifier of lymphatic cell fate, although it does not seem essential for lymphatic vessel development [41]. Vascular endothelial growth factor C (VEGF-C) signaling through vascular endothelial growth factor receptor 3 (VEGFR3) is essential for lymphatic development and maintenance, as loss of *Vegfc* in embryos results in complete lack of lymphatic vasculature [46]. VEGFR3 signaling is also important in mature lymphatic vessels, as blockade of VEGFR3 results in impaired lymphatic vessel regeneration [47]. Similarly, overexpression of VEGF-C in embryos results in them developing chylothorax, where lymphatic fluid fills the thoracic cavity [48].

Perturbations in lymphatic vessel maintenance and structure in humans can give rise to different disorders. Loss of uptake by the lymphatic vessels results in accumulation of interstitial fluid and proteins, eventually resulting in lymphedema, which is a chronic progressive swelling [41]. Primary lymphedema is caused by genetic mutations, including some of the Rasopathies [49]. Secondary lymphedema arises after some trauma to the lymphatic vasculature, such as after mastectomy [41]. The most common form of secondary lymphedema is filariasis, which is a nematode infection of the lymphatic system that results in lymphatic vessel dilation and blockage. Treatment options for lymphedema patients are mostly limited to ameliorating the symptoms, and current options are not very effective.

In RASA1 deficient adult mice, the lymphatic system undergoes extensive growth and dilation, as the lymphatic endothelial cells (LEC) have significantly increased proliferation [38]. The lymphatic system is also leaky, resulting in chylous ascites and chylothorax, from which the mice succumb. This phenotype is recapitulated when RASA1 is specifically deleted in LEC, suggesting that the lymphatic disorder is intrinsic to these cells. Stimulation of RASA1 deficient LEC with the growth factor VEGF-C results in aberrant Ras signaling through the LEC expressed receptor VEGFR-3, which is the major pathway involved in the development of the lymphatic phenotype [38].

Mutations in RASA1 in humans also affect the cardiovascular and lymphatic systems. Humans with heterozygous, germline mutations in RASA1 develop the disease capillary malformation-arteriovenous malformation (CM-AVM) with a high percentage of penetrance [50]. CM-AVM is characterized by multifocal, randomly distributed skin CM, with approximately one third of patients also displaying fast flow vascular lesions [51]. These fast flow lesions can include AVM and arteriovenous fistulas which can occur intracranially, and

Parkes Weber syndrome, which often presents with an outgrowth of the affected extremity [52]. It is believed that complete loss of protein occurs through second hit mutations, which is consistent with the multifocal nature and range in severity of the disorder. Due to the lymphatic phenotype developed by the *Rasa1* deficient adult mouse, human CM-AVM patients were screened and were shown to present with lymphatic system malformations [53]. Treatment options for these patients are limited and generally focus on alleviating symptoms, thus a better understanding of the downstream effects of loss of RASA1 would be beneficial in elucidating the most effective course of treatment.

### 1.6 Ras Independent Functions of RASA1

While originally identified as a RasGAP, RASA1 has been shown to interact with other proteins during cellular signal transduction, in addition to Ras. RASA1 interacts with p190 RhoGAP, a RhoGTPase activating protein (RhoGAP), via its SH2 domains [54]. Rho, a member of the Ras GTPase superfamily, is a molecular switch protein that is known to regulate the actin cytoskeleton [55]. Loss of RASA1 in murine embryonic fibroblasts (MEF) results in reduced levels of tyrosine phosphorylation of p190 RhoGAP both in resting cells and after stimulation with platelet-derived growth factor (PDGF) [56]. RASA1 interactions with p190 RhoGap have been shown to be involved in regulating stress fiber and actin filament remodeling in directed cell migration [55]. Loss of RASA1 results in lack of cell polarization during wound healing which can be rescued by a RASA1 protein lacking the GAP domain. RASA1 also interacts with deleted in liver cancer 1 (DLC1), a RhoGAP via its SH3 domain [57]. Overexpression of RASA1 in a RasGAP-insensitive cancer cell line results in increased RhoA activity, and adenovirus driven expression of RASA1 in carcinoma cells expressing DLC1 results in increased

cell proliferation. Clearly RASA1 has multiple different roles, and loss of the entire protein can disrupt multiple different pathways controlling various cellular processes.

### 1.7 Neurofibromin 1

Along with RASA1, other RasGAPs are also ubiquitously expressed. The overlap in expression of multiple RasGAPs suggests that there may be redundancy in RasGAP function. However, RasGAPs can have non-redundant roles in the same tissues. Another widely expressed RasGAP is Neurofibromin 1 (NF1), which contains a glycerophospholipid-binding SEC14-PH domain along with its GAP domain [12]. NF1 deficient mice die *in utero* around E13.5 due to abnormal heart development [58, 59]. Conditional deletion of NF1 in different tissues in mice results in the development of multiple phenotypes, including myeloid proliferation disorders [60-62]. Mice that are deficient of NF1 in Schwann cells with NF1 haploinsufficient bone marrow develop complex tumors known as neurofibromas [61]. Loss of NF1 specifically in the T cell compartment results in a subtle phenotype similar to that of RASA1 [63]. Loss of NF1 results in a decrease in naïve peripheral T cells, and impaired positive selection in the thymus on a TCR transgenic background. The concurrent loss of both RASA1 and NF1 in mice results in arrested development earlier than loss of either RasGAP alone, as E9.0 *Rasa1* and *Nf1* deficient embryos have reduced somite numbers, lack of limb buds, and impaired heart and anterior structure formation [37].

Germline NF1 mutations in humans result in the disease neurofibromatosis, which is characterized by plexiform neurofibromas on peripheral nerves [64]. Along with neurofibromatosis, children with mutations in NF1 are at risk of developing various myeloid

disorders including juvenile myelomonocytic leukemia (JMML) [65]. This myeloid disorder is less aggressive than that developed by induction of the activating Ras mutation in the hematopoietic compartment, but both types of mutations are found in patients with JMML, suggesting this cancer is due to aberrant Ras activation [15].

### 1.8 T Cell Acute Lymphoblastic Leukemia/Lymphoma

Wild type mice that receive bone marrow transplants from $Ras^{G12D}$ mice succumb to T cell acute lymphoblastic leukemia/lymphoma (T-ALL) [16]. T-ALL accounts for 15% and 25% of childhood and adult ALLs, and requires aggressive treatment regimens that can be detrimental to the patients [66]. Ras mutations in human T-ALLs are relatively rare, however increased Ras signaling can be detected, suggesting that there are perturbations in the Ras signaling pathway [67]. Along with chromosomal translocations that result in gene fusions or deletions of tumor suppressing genes, the most common mutations in human T-ALL are activating gene mutations [68]. One of the most commonly mutated genes identified in T-ALL patients is *NOTCH1*, which is mutated in over 50% of cases [69]. These mutations result in gain of function of the NOTCH1 protein. The T-ALL that develops in mice that receive *K-*$ras^{G12D}$ expressing bone marrow also carries Notch1 mutations [16].

### 1.9 Notch1 Mutations in T-ALL

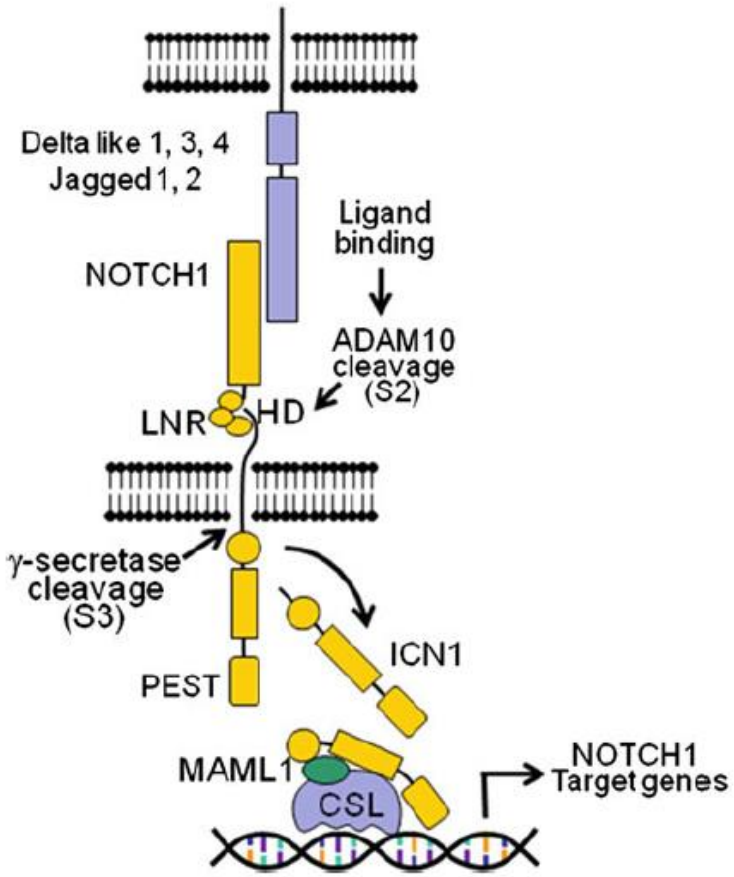



**Figure 5. Notch1 signaling pathway [70].** Notch1 recognition of ligands (Delta-like and Jagged) results in ADAM10 cleavage at the S2 site, followed by cleavage at the S3 site by γ-secretase. The intercellular portion (ICN1) is released and translocates into the nucleus where it forms a complex with MAML1 and CSL to drive transcription of Notch1 target genes.

Notch1 is member of the Notch protein family, which consists of four transmembrane receptors that are involved in various aspects of cellular differentiation [71]. Notch1 is a heterodimer that has an extracellular domain which consists of epidermal growth factor (EGF) repeats which bind ligand, and LIN12/Notch repeats which block ligand-independent signaling [71, 72]. When Notch1 binds a ligand, which include Jagged and Delta-like ligands, it undergoes a metalloprotease-dependent cleavage at the S2 site near the transmembrane domain (Figure 5). Secondary cleavage within the transmembrane domain at S3 occurs by a multi-protein complex

with γ-secretase activity, resulting in an intracellular form of Notch1 (ICN). The ICN consists of a RAM domain, nuclear localization sequences, ankyrin repeats, a transactivation domain, and a proline-glutamic acid-serine-threonine (PEST) domain. The ICN translocates into the nucleus, where it interacts with the DNA-binding factor CSL and Mastermind-like (MAML) to form a complex that results in the expression of Notch1 target genes.

Notch1 signaling is required for normal development of T cells, as loss of Notch1 in common lymphoid progenitors results in the absence of T cells and the production of B cells in the thymus [73]. Loss of Notch1 during the DN2/DN3 stage of thymocyte development by use of conditional knockouts driven by *pLCK-Cre* results in aberrant DN3 and DN4 populations that do not undergo correct TCR rearrangement but still progress, although with reduced numbers of DP and a small thymus overall [74]. Interestingly, constitutive Notch1 signaling permits cells without TCRβ rearrangements to progress on to the DN4 stage, suggesting Notch1 signaling acts through the same pathways as the pre-TCR [75]. Clearly Notch1 signaling is important for correct T cell development and its expression must be tightly regulated.

Gain of function mutations in Notch1 can occur at multiple sites. In human T-ALL, mutations often occur in exons 26 and 27, which encode the heterodimerization domain (HD) [69]. These mutations result in ligand-independent cleavage at S2, allowing for γ-secretase cleavage and release of ICN to drive constitutively active Notch1 signaling. Mutations also occur in exon 34, which encodes the PEST domain. Mutations in the PEST domain result in premature stop codons and the loss of a recognition sequence that prevents ICN from being targeted for ubiquitylation and thus extends the timing of Notch1 signaling [76]. These mutations can occur in the same T-ALL, often on the same chromosome [69]. However, these PEST mutations vary in strength, and are generally not sufficient to initiate T-ALL [77]. Instead, it has

been shown that these mutations are collaborative with other oncogenic mutations, such as Ras mutations.

PEST domain mutations also occur in mouse models of T-ALL, whereas mutations in the HD domain are rare [78]. Interestingly, mouse models of T-ALL often carry different types of mutations that result in ligand-independent cleavage of Notch1. Type 1 mutations are recombination activating gene (RAG)-mediated recombinations that result in the excision of exons 1 and 2, while Type 2 mutations are RAG-independent deletions that lead to the loss of exon 2 through 25 or 26 [79, 80]. In both cases, translation starts at methionine 1727, which results in a membrane-bound form of Notch1 that can be cleaved by γ-secretase, resulting in constitutive ICN that is sufficient to drive T-ALL [79]. In all cases, these Notch1 mutations were identified in T-ALL that developed in mice carrying known genetic alterations of other genes, including *K-ras*$^{G12D}$ [78-80].

### 1.10 Generation of Genetically Altered Mice

Genetically altered mice provide opportunity to explore protein function in the context of the whole animal. Traditionally, genetic targeting is achieved by homologous recombination in embryonic stem (ES) cells. Removal or replacement of exons is carried out by generating a targeting construct carrying the mutations, with homologous sequences flanking each end, along with an antibiotic resistance cassette such as Neomycin. This construct is injected into ES cells, and homologous recombination results in the mutation integrating into the genome such that it replaces the endogenous exons, and does not insert randomly. ES are grown on antibiotic-containing media for selection of those expressing the targeted allele, and those are injected into

blastocysts and implanted into pseudo-pregnant females to generate chimeric mice. The chimeras are crossed to wild type mice for germline expression of the targeted allele, resulting in mice with heterozygous expression of the mutated protein. These mice are then crossed to generate homozygous mice that only express the targeted allele [81].

Since even partial loss of protein expression can often result in embryonic lethality, conditional knockout mice can be generated to allow the mouse to develop into adulthood, as well as to study the role of a protein in specific tissues. Similar to the knockout mouse, a targeting vector is constructed, but exon(s) of interest are flanked by recombination site, such as *loxP* sites (floxed). *LoxP* sites are specific sequences that are recognized by Cre recombinase, originally identified in bacteriophage P1 [82]. Cre recognition of *loxP* sites results in the recombination of the DNA between the sites, splicing out the floxed exons. Because *loxP* sites are only 34 bp, their insertion into introns does not usually affect normal gene expression, so floxed alleles typically express normal levels of functioning protein until Cre-mediated deletion occurs. Mice carrying a floxed allele are generated through the same process as the knockout mice, with the additional step of being crossed to transgenic mice carrying the Cre recombinase driven by tissue-specific and/or inducible promoters. Thus, the role of the protein in adult mice or in specific organ systems may be more easily elucidated.

RASA1 knockout mice were generated by the replacement of the exons encoding the N-terminal SH2 domain with a neomycin resistance gene [37]. RASA1 conditional knockouts have been generated by placing *loxP* sites around exon 18, which encodes the part of GAP domain containing the arginine finger [83]. Loss of exon 18 results in the complete loss of the RASA1 protein, presumably through nonsense mediated RNA decay. Thus, it is unknown whether the phenotypes developed by either the null or the conditional knockout mice are due to loss of

RASA1's regulation of Ras or some Ras-independent function. Point mutations of RASA1's arginine finger to a glutamine abrogates RASA1's GAP activity while retaining the tertiary structure of the protein. This amino acid substitution knocked in to a mouse would allow for the study of the role of RASA1's Ras-targeted catalytic activity.

### 1.11 Scope of the Thesis

There are two main questions addressed in this thesis. The first is to elucidate the role of RASA1's GAP domain in cardiovascular development and lymphatic vessel maintenance. By generating a knock-in mouse carrying an R780Q point mutation, we remove RASA1's ability to regulate Ras while retaining all other functions of the protein. In Chapter 2, generation of the *Rasa1 R780Q* knock-in mouse is detailed. In Chapter 3 this mouse is used to study the influence of the mutation upon the cardiovascular and lymphatic systems. The second question addressed in this thesis is the possibility that multiple RasGAPs are involved in regulation of Ras in T cell development. In Chapter 4, using mice that are floxed for both RASA1 and NF1 with the T cell specific LCK promoter driven Cre, we analyze the effects of the loss of these RasGAPs in the T cell compartment.

# Chapter 2

## Generation of RASA1 R780Q knock-in mice

### 2.1 Abstract

p120 RasGAP (RASA1) is a prototypical member of the RasGAP family that regulates Ras signaling downstream of various cell surface receptors. Along with its GAP domain, RASA1 has multiple other domains through which it carries out Ras-independent functions. Aside from its function as a Ras regulator, RASA1 has been shown to interact with p190 RhoGAP through its SH2 domain and DLC1 via its SH3 domain to regulate directed cell migration and Rho activation. Mutations in humans result in the disease capillary malformation-arteriovenous malformation (CM-AVM), which is characterized by small multifocal skin lesions as well as fast flow lesions along with lymphatic vessel issues. Similarly, RASA1 deficient mice die *in utero* due to failed cardiovascular system development whereas deletion of *Rasa1* in adult mice leads to a lymphatic hyperplasia and leakage.

Since the RASA1 protein is entirely lost in these mouse models and is also thought to be lost in CM-AVM, it is unknown whether the phenotypes are due to loss of RASA1's regulation of Ras or loss of a Ras-independent function. This knowledge would be beneficial for identifying possible treatments for CM-AVM. To address this, we have generated a knock-in mouse carrying a point mutation in its GAP domain that abrogates its ability to successfully

promote Ras hydrolysis of GTP. This point mutation does not affect the tertiary structure of RASA1, such that the protein is competent to carry out other functions. This RASA1 R780Q mouse can be used to address the importance of RASA1's Ras directed catalytic activity in both the cardiovascular and lymphatic systems.

## 2.2 Introduction

Ras is a small GTP binding protein that activates signaling downstream of a number of receptors, including growth factor receptors, cytokine receptors, and the TCR [1]. Binding of the receptor to its ligand results in the recruitment of RasGEFs, which allow Ras to bind GTP and become active. Ras signaling is important in multiple cellular processes, including growth, differentiation, and survival. Regulation of this signaling is critical for normal cell growth, proliferation and survival. For efficient cessation of Ras signaling RasGAPs are recruited to interact with Ras, increasing its ability to hydrolyze GTP by several orders of magnitude [2].

There are ten known RasGAP family members [12]. The first RasGAP identified was p120 RasGAP (RASA1). Along with its GAP domain, through which it is able to stabilize Ras for efficient GTP hydrolysis, RASA1 has two SH2 domains as well as SH3, PH, and C2 domains. While these domains may be involved in recruitment of RASA1 to the membrane for juxtaposition with Ras, RASA1 has other known Ras-independent functions. Via its SH2 domain, RASA1 interacts with p190 RhoGAP [56]. This interaction is essential for directed cell movement of murine embryonic fibroblasts (MEFs) *in vitro* [55]. The loss of cell polarity in RASA1 deficient cells can be rescued by a RASA1 protein construct with no GAP domain, suggesting this interaction is Ras independent. RASA1 has also been shown to interact with DLC1, a RhoGAP, resulting in increased RhoA activation in a RasGAP resistant cell line and

increased cell proliferation [57]. This data indicates that through its modular binding domains, RASA1 has Ras independent functions.

The complete loss of RASA1 in mice results in an embryonic lethal phenotype at mid-gestation [37]. The yolk sacs of these embryos fail to develop a correctly organized blood vessel network, instead the endothelial cells maintain a honeycomb like structure with no reorganization into vessels. At the twelve somite stage the embryo proper has a thinner dorsal aorta than either wild type or heterozygous knockout littermates that are indistinguishable from one another in all respects. The dorsal aorta also shows aberrant ventral branching, and there is often pericardial edema. Interestingly, ubiquitous loss of RASA1 in adult mice does not result in the development of any spontaneous cardiovascular phenotypes [38]. Instead, the mice develop a severe lymphatic vessel hyperplasia, with an increased proliferation of LEC. The lymphatic vasculature is also leaky, resulting in chylous ascites and lethal chylothorax.

Patients with the disorder CM-AVM have recently been found to carry RASA1 mutations [51]. CM-AVM is characterized by small, multifocal skin CM. One third of patients also have fast flow lesions, including AVM, arteriovenous fistulas, and Parkes Weber syndrome. Patients may also present with lymphatic abnormalities [53]. The mutations in humans span the length of the gene, and are believed to result in the loss of the entire protein [50, 52]. It is hypothesized that second hit mutations occur during development that result in the complete loss of RASA1. This theory is supported by the multifocal nature of the disorder as well as the range in severity of the disorder, which could be due to the timing of the mutation during development.

Since in both mouse and humans RASA1 phenotypes are consequent to complete loss of RASA1, it is uncertain whether these phenotypes are due to loss of Ras or Ras independent functions. It has been shown previously that a point mutation in the GAP domain (R786Q)

would abrogate RASA1's GAP activity by removing the arginine finger while not affecting the tertiary structure of the protein [84]. Therefore, we generated a R780Q knock-in mouse to address the importance of RASA1's regulation of Ras in cardiovascular development and lymphatic system maintenance.

### 2.3 Targeting Strategy and Construction of Knock-in Allele

The arginine finger of RASA1 is essential for the stabilization of Ras for efficient GTP hydrolysis. Mutation of the arginine in the arginine finger of RASA1 to a glutamine results in complete loss of GAP activity with no disruption of protein folding [84]. We designed a *Rasa1* targeting construct with a CGA to CAA codon change to generate the R780Q point mutation (Figure 6). The targeting construct carried a *FRT*-flanked Neomycin resistance cassette in intron 18 for selection purposes. The *FRT* site is a recognized by the FLP recombinase originally identified in *Saccharomyces cerevisiae* and adapted for use in mammalian systems [85]. This construct was electroporated into W4 ES cells, which were cultured in neomycin. DNA was isolated from growing cells and a quantitative PCR (qPCR) screen was used to distinguish correctly targeted ES cells from random integrants.

In order to identify correctly targeted ES cells, primer probe sets were designed which would allow identification of clones with one copy of the targeting construct that had been correctly integrated. Taking advantage of the insertion of the Neomycin resistance cassette (*NeoR*), a primer probe set was generated that spanned the insertion site. This results in the loss of amplification and probe binding in cells that have the *NeoR* cassette containing allele. We calculated fold change and looked for a two-fold difference as compared to wild type genomic

DNA [86, 87]. The clones that had the correct fold change underwent a second qPCR where the primer probe set was located in exon 14, which was contained in the targeting construct, to eliminate clones with additional random integration on the targeting construct (Figure 7).

Correctly targeted clones were sequenced to confirm the presence of all exons and that the point mutation was present. Two of the clones were injected into C57BL/6J X (C57BL/6J X DBA/2) blastocysts to generate chimeric mice. Germline transmission was achieved by breeding to C57BL/6 actin promoter-driven *Flp* transgenic mice which removed the *NeoR* cassette[88]. The resulting germline transmitted mice were crossed to C57BL/6J mice to confirm that deletion of the *NeoR* cassette was germline and not due to continued presence and activity of the *Flp* transgene. Resulting heterozygous RASA1 R780Q mice were normal with no obvious phenotypes, similar to the RASA1 heterozygous null mice. Heterozygous RASA1 R780Q mice were crossed with mice carrying conditional alleles of *Rasa1* (fl) to generate $Rasa1^{fl/R780Q}$ mice.

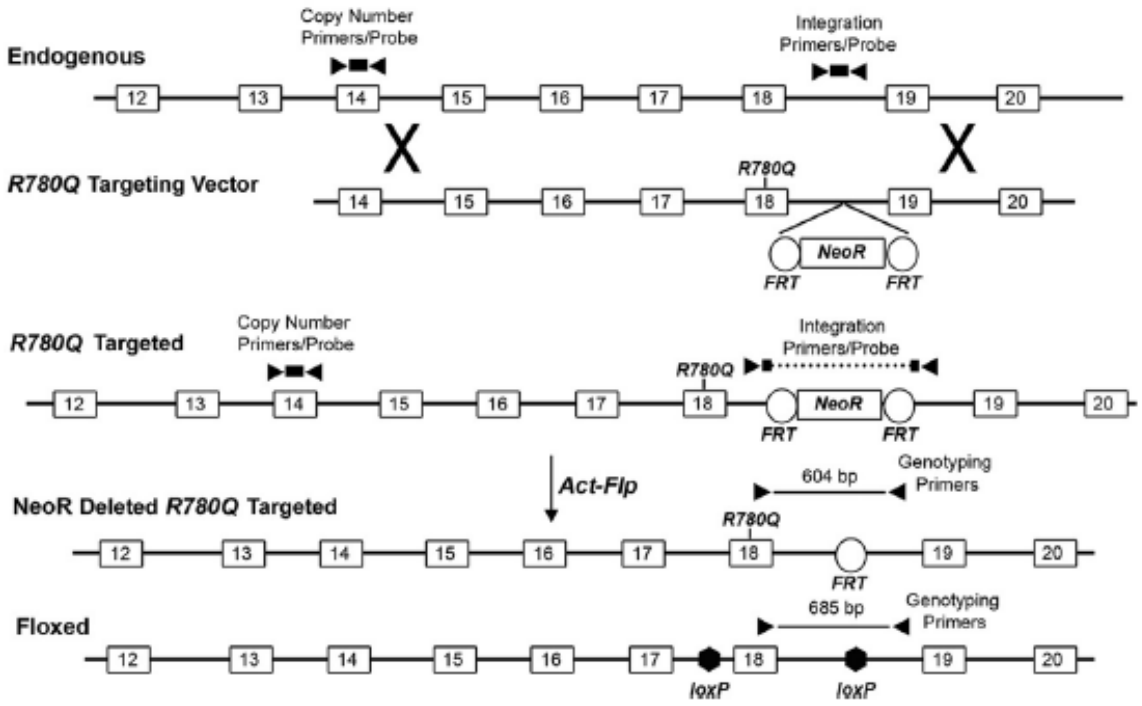



**Figure 6. Targeting vector and homologously recombined correctly targeted allele.** An *R780Q* targeting vector with an *R780Q* mutation in exon 18 and a *FRT*-flanked *NeoR* cassette in intron 18 is shown below the endogenous allele. In the middle is shown the *R780Q* targeted allele before and after excision of the *NeoR* cassette achieved with an actin promoter-driven *Flp* transgene (*Act-Flp*). At bottom is depicted a *Rasa1* floxed allele in which exon 18 is flanked by *loxP* sites. Positions of real-time PCR primer/probe pairs used in the detection of ES cell homologous recombinants and PCR primer pairs used in mouse genotyping are indicated. C2, protein kinase C2 homology; GAP, GTPase-activating protein domain; PH, pleckstrin homology; SH2, Src homology 2; SH3, Src homology 3

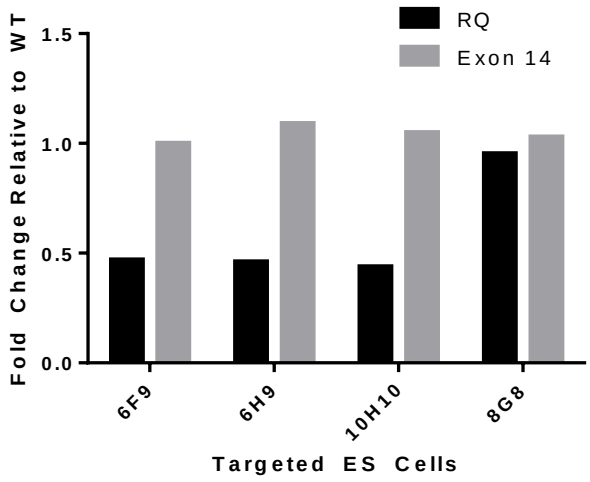



**Figure 7. qPCR screening of targeted ES cells.** DNA was isolated from ES cells and qPCR was carried out using two primer/probe sets. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method as compared to wild type DNA. In correctly targeted ES cells (6F9, 6H9, 10H10) the primer/probe set located on the *NeoR* insertion site (RQ) is expected to have a 50% fold reduction compared to wild type, while the exon 14 primer/probe set should have no fold change. 8G8 shows ES cells with no integration.

### 2.4 Confirmation of RASA1 R780Q protein expression

*Rasa1*$^{fl/R780Q}$ mice were intercrossed in order to generate homozygous RASA1 R780Q embryos. At E9.5, embryos were harvested, and DNA was isolated from yolk sacs for genotyping. Genotyping was carried out using the same PCR primers used to determine *NeoR* cassette deletion, as the presence of the *loxP* site generates products of a longer length than that of the *Rasa1 R780Q* allele with the *FRT* site (685 vs 604) (Figure 8). Embryos were minced and digested to isolate murine embryonic fibroblasts (MEF), which were grown in culture in fibroblast media until confluent. Protein was isolated from an equivalent number of cells and Western blotting was done to determine level of RASA1 protein present in embryos of all genotypes (Figure 8). Blots were reprobed with an anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH) as a loading control. Homozygous RASA1 R780Q embryos

expressed the same levels of RASA1 protein as *Rasa1*$^{fl/fl}$ littermates, which would be consistent with the fact that the mutation does not affect the tertiary structure of RASA1 in a manner that that might lead to increased degradation.


**Figure 8. Genotyping *Rasa1 R780Q* embryos and expression of RASA1 R780Q protein in homozygous embryos.** Left, DNA from embryos was isolated and PCR amplified using primers described above. Top band (685bp) is the floxed allele, bottom band (604bp) is the *R780Q* allele. Right, MEFs derived from E9.5 *Rasa1 R780Q/R780Q* (*rq/rq*) and *Rasa1 fl/fl* (*fl/fl*) embryos were analyzed for RASA1 protein levels using Western blot analysis. Blots were reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to show equivalent protein loading. Shown are RASA1 amounts in MEF derived from two different embryos of each genotype, representative of n=3.

## 2.5 Materials and Methods

### 2.5.1 Generation of R780Q knock-in allele

A *Rasa1 R780Q* targeting construct was assembled in *p-loxP-2FRT-PGKneo*. A 5' arm that contained introns 13 through 17 of the *Rasa1* gene was generated by PCR from a C57BL/6 genomic Rasa1 BAC clone and inserted into the EcoRI/KpnI sites of the vector. The primers used were: 5' arm forward, 5'-GCGCGAATTCGCGGCCGCTTAGTCTTTCAGGCATTT-TATAGC-3'; and 5' arm reverse, 5'-GCGCGGTACCGAATGCTTATTTACCAGGAGTGAC-3'. A middle segment spanning intron 17 through 18 was generated by PCR from the same *Rasa1* BAC clone that contained a CGA to CAA codon change resulting in a R780Q mutation in

exon 18 that was generated by homologous recombination in *Escherichia coli*. The middle fragment was inserted into the KpnI and 3' BglII sites of the vector. The primers used were as follows: M forward, 5'-GCGCGGTACCAGATCTAAATATTTGAGCCTATGAGGACC-ATTC-3'; and M reverse, 5'-GCGCGGATCCCATATCCAACTTCACATGATGTGC-3'. A 3' arm that spanned introns 18 through 20 was generated by PCR from the wild-type *Rasa1* BAC clone and was inserted into the XhoI site of the vector. The primers used were as follows: 3' arm forward, 5'-GCGCCTCGAGGAATTTCCCACATGGA-3'; and 3' arm reverse, 5'-GCGCCTCGAGATATGTTGTCATGTAA-3'. The targeting construct was sequenced to verify the absence of any PCR-induced errors. The construct was electroporated into W4 embryonic stem (ES) cells that were subsequently cultured in neomycin.

Genomic DNA from neomycin-resistant ES cell clones was analyzed by qPCR using an integration primer/probe set in which the 5' and 3' primers flanked the *FRT-NeoR-FRT* insertion site and the probe was complimentary to regions both 5' and 3' to the point of insertion [87]. Fold differences in the amount of exon 18 target relative to that in genomic DNA prepared from wild-type W4 ES cells were calculated as described [86]. Clones that demonstrated a twofold reduction in the intron 18 target were analyzed further by qPCR using a copy number primer/probe set based within exon 14 of the *Rasa1* gene (Life Technologies). Of those clones that contained diploid amounts of the exon 14 target, a long-range PCR was performed using a *Rasa1* forward primer based on the 5' of the 5' end of the *Rasa1* targeting vector insert and a reverse primer based in intron 18. A similar long-range PCR was performed using a forward primer based in intron 18 and a reverse primer located 3' of the 3' end of the *Rasa1* targeting vector insert. Both PCR products were sequenced to confirm the presence of the R780Q mutation within the endogenous *Rasa1* locus.

Two of several correctly targeted *Rasa1 R780Q* clones were injected into C57BL/6J X (C57BL/6J X DBA/2) blastocysts to generate chimeric mice that were then bred with C57BL/6 actin promoter-driven *Flp* transgenic mice to achieve germline transmission of the targeted *Rasa1 R780Q* allele and deletion of the *NeoR* cassette in intron 18 [88]. Germline transmission and *NeoR* deletion were determined by PCR of tail genomic DNA prepared from agouti coat-colored progeny using a forward primer based in exon 18 and a reverse primer based 3' to the 3' *FRT* site in intron 18. Germline transmitted mice were then crossed with C57BL/6 mice and the same PCR was performed on tail DNA of progeny that had not inherited the actin-Flp transgene, also determined by PCR. This step was undertaken to obtain mice with confirmed germline deletion of the *NeoR* cassette within the *Rasa1 R780Q* locus.

### 2.5.2 Western Blotting for RASA1 expression

Individual E9.5 embryos were minced and digested in trypsin/EDTA (Life Technologies) at 37°C for 15 minutes. MEFs were generated by culture of released cells in fibroblast media (Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine). At confluence, MEFs were harvested and 3 x $10^6$ cells were lysed in 1% NP-40 lysis buffer. RASA1 protein abundance was determined by using Western blot analysis of cell lysates using an anti-RASA1 antibody (B4F8; Santa Cruz, Dallas, Q6 TX). Blots were stripped and reprobed with an anti-GAPDH antibody (Santa Cruz) to ascertain equivalent protein loading.

## 2.6 Discussion

We have successfully designed and implemented a targeting scheme which resulted in the generation of a mouse carrying a *Rasa1 R780Q* point mutation, which was confirmed by

sequencing. R780Q RASA1 protein is expressed at normal levels, as there is no difference in protein level between homozygous R780Q MEF and wild type MEF. The generation of the *Rasa1 R780Q* knock-in mouse will allow for further exploration of the function of RASA1 in the development of the mouse cardiovascular system and maintenance of the lymphatic vasculature. This work has translational implications as it will permit identification of which pathways disrupted as a consequence of loss of RASA1 are responsible for cardiovascular and lymphatic abnormalities in CM-AVM. In turn this could inform as to potential new therapies for CM-AVM patients.

# Chapter 3

# The GAP domain of RASA1 is required for normal cardiovascular development and lymphatic vessel maintenance

## 3.1 Abstract

p120 RasGAP (RASA1) is the prototypical Ras GTPase activating protein, identified as a regulator of Ras signaling. It has since been shown to regulate distinct pathways in a Ras-independent manner. These interactions are mediated by domains other than the GAP domain that is essential for stabilization of Ras and efficient GTP hydrolysis. Previously reported non-conditional and tissue specific *Rasa1* gene disrupted mice completely lack RASA1 protein in all or targeted tissues, respectively. Absence of *Rasa1* in mice causes embryonic lethality due to failed cardiovascular development, while loss of *Rasa1* in adult mice results in lymphatic hyperplasia and leakage. In humans with the disorder capillary malformation-arteriovenous malformation (CM-AVM), mutations in *RASA1* span the length of the gene, and along with second hit mutations of the wild type *RASA1* allele are also believed to result in complete loss of protein. In all of these cases, the loss of RASA1 could result in the perturbation of multiple different pathways and cell processes. In order to determine if phenotypes result from loss of an ability of RASA1 to regulate Ras or not, a knock-in mouse carrying a point mutation that ablates RASA1's GAP activity while maintaining other potential functions of the protein was generated. In its homozygous form, the *Rasa1* knock-in allele is embryonic lethal due to impaired

cardiovascular development. Embryonic lethality occurs at the same time as observed in RASA1 null embryos and is associated with failed yolk sac vascularization and aberrant vascularization in the embryo proper. Generation of mice that are heterozygous for a *Rasa1* knock-in allele and a conditional floxed *Rasa1* allele and which also carry an inducible ubiquitin promoter-driven ert2Cre transgene were generated. These mice allow for examination of the importance of loss of RASA1's activity to regulate Ras to the development of the lymphatic disease that results upon loss of *Rasa1* in adults. Mice that were induced to express RASA1 R780Q alone develop striking lymphatic hyperplasia and leakage before succumbing to chylothorax. The severity and time of onset of lymphatic disease was similar to that observed in the induced *Rasa1* deficient mice. Induced loss of an ability of RASA1 to regulate Ras is responsible for the development of both the cardiovascular and lymphatic phenotypes in *Rasa1* deficient mice. This has important implications for the treatment of patients with CM-AVM, as it suggests that inhibitors of downstream effectors of Ras may be effective in the treatment of this disease.

### 3.2 Introduction

Ras is a small membrane bound GTP-binding protein that is activated downstream of multiple different cell surface receptors [1]. Ras guanine nucleotide exchange factors (RasGEF) eject GDP from inactive Ras and allow Ras to bind GTP, resulting in activation of multiple different pathways, including the MAPK and PI3K/AKT pathways [3, 6, 7]. Activation of these pathways results in cell survival, proliferation, and differentiation. Cessation of this signaling is dependent on the hydrolysis of Ras-GTP to Ras-GDP. While Ras has some innate GTPase activity, Ras GTPase activating proteins increase GTP hydrolysis by several orders of magnitude [2]. Originally identified as a RasGAP, p120 RasGAP (RASA1) interacts with Ras via its GAP

domain. The GAP domain contains an arginine finger that is essential for stabilizing Ras in the correct confirmation for Ras to efficiently hydrolyze GTP.

Along with Ras, RASA1 has been shown to interact with multiple other proteins through its modular binding domains. One such protein is p190 RhoGAP, which interacts with RASA1's N-terminal SH2 domain [56]. RASA1 interactions with p190 RhoGAP have been shown to be important in directed cell movement, as disruption of this complex impairs actin filament rearrangement and orientation [55]. RASA1 also interacts with DLC1, a RhoGAP, via its SH3 domain [57]. RASA1 inhibits DLC1 activity, leading to the increase in RhoA activation which drives increased cell proliferation.

By replacing the SH2 domain of RASA1 with a Neomycin resistance cassette, RASA1 protein expression was ablated [37]. Heterozygous RASA1 deficient embryos develop normally and have no obvious phenotype, and can be intercrossed to generate homozygous RASA1 null embryos. Mice deficient of RASA1 are not viable, and die *in utero* around E10.5. The yolk sac of these embryos fail to develop vascular networks, with the endothelial cells failing to rearrange from a honeycomb pattern. Within the embryo proper, the dorsal aortas are thinner than either wild type or heterozygous RASA1 deficient littermates, and they have aberrant branching of intersegmental arteries.

In order to bypass the embryonic lethality, mice with a conditional allele of RASA1 were generated [83]. These mice have *loxP* sites up- and downstream of exon 18, allowing for Cre recombination that results in nonsense mediated RNA decay and complete loss of protein. Ubiquitous deletion of RASA1 in adult mice by tamoxifen injection in *Rasa1*$^{fl/fl}$ *ub-ert2cre* mice results in a striking lymphatic hyperplasia [38]. The lymphatic vasculature is also extremely dilated and leaky, and mice develop chylous ascites and chylothorax, to which the mice

succumb. The lymphatic endothelial cells (LEC) are highly proliferative, with aberrant biphasic pERK and pAKT signaling through vascular endothelial growth factor receptor 3 (VEGFR3). Mice with loss of RASA1 specifically in LEC develop the same phenotype. Interestingly, ubiquitous loss of RASA1 in adults does not result in any spontaneous cardiovascular phenotype, although there is some data that suggests RASA1 inhibits angiogenesis in tumors [39].

Humans with mutations in RASA1 develop capillary malformation-arteriovenous malformation (CM-AVM) with high penetrance [51]. CM-AVM is characterized by small multifocal dermal CM, with at least one third of patients also presenting with fast flow lesions including AVM, arteriovenous fistula, as well as Parkes Weber syndrome [50]. Recently it has been identified that patients with CM-AVM also have lymphatic malformations [53]. Mutations in CM-AVM patients occur throughout the gene, and are believed to result in complete loss of expression from the mutated allele. Due to the variable nature of the disease, it is believed that second hit mutations occur to completely delete RASA1 protein from certain subsets of cells during development [51]. However, due to the predicted loss of the entire RASA1 protein, it is unknown what particular signaling pathways are responsible for the development of CM-AVM.

RASA1 promotes Ras GTP hydrolysis via a conserved arginine finger. Mutation of the arginine in the arginine finger to a glutamine abrogates GAP activity while retaining the tertiary structure of the protein and allowing it to carry out putative other functions [84]. We have generated a knock-in mouse carrying a RASA1 R780Q point mutation in order to specifically study the role of loss of RASA1's Ras directed catalytic activity in both the development of embryonic cardiovascular abnormalities and adult lymphatic vascular abnormalities. We show here that both phenotypes result from the loss of RASA1's ability to regulate Ras, resulting in

aberrant Ras activation. This work will have important translational implications in identifying potential treatments for patients with CM-AVM.

### 3.3 Embryonic Lethality of Homozygous RASA1 R780Q mice

*Rasa1* $^{fl/R780Q}$ mice were generated and intercrossed to generate *Rasa1*$^{R780Q/R780Q}$ mice. Multiple litters were genotyped at 3 weeks and no homozygous RASA1 R780Q pups were identified, which suggested that *Rasa1*$^{R780Q/R780Q}$ mice die *in utero* (Figure 9). In order to determine the time of embryonic lethality, timed matings were carried out and embryos were harvested and genotyped at multiple time points. *Rasa1*$^{R780Q/R780Q}$ embryos were identified in expected Mendelian ratios at E9.5, but were present in a less than expected number at E11 and absent at E13.5. This is consistent with embryonic lethality around E10.5, i.e. at the same time of embryonic lethality as in RASA1 null mice.

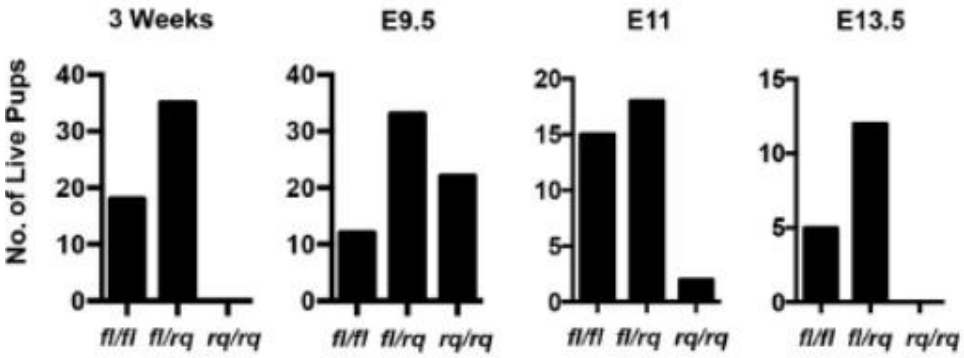



**Figure 9. *Rasa1*$^{R780Q/R780Q}$ embryos are embryonic lethal mid-gestation.** Graphs show the total number of pups of the indicated genotypes derived from intercrosses of *Rasa1 R780Q/fl* mice determined at 3 weeks after birth and at E9.5, E11, and E13.5 of development. At E9.5, genotype frequencies are consistent with mendelian inheritance (c2 test). At E11, E13.5, and 3 weeks of age, genotype frequencies are not consistent with mendelian inheritance ($P < 0.025$, $P < 0.05$, and $P < 0.005$, respectively).

At E9.5, homozygous RASA1 R780Q embryos are easily distinguishable from control littermates (Figure 10). *Rasa1*$^{R780Q/R780Q}$ embryos are grossly smaller in size with a distended pericardial sac. The yolk sac of these embryos when removed is wrinkled in appearance. Of the few E11 *Rasa1*$^{R780Q/R780Q}$ embryos that were identified the same phenotype was noted. All of these features were also present in the complete RASA1 knockout E9.5 embryos [37].

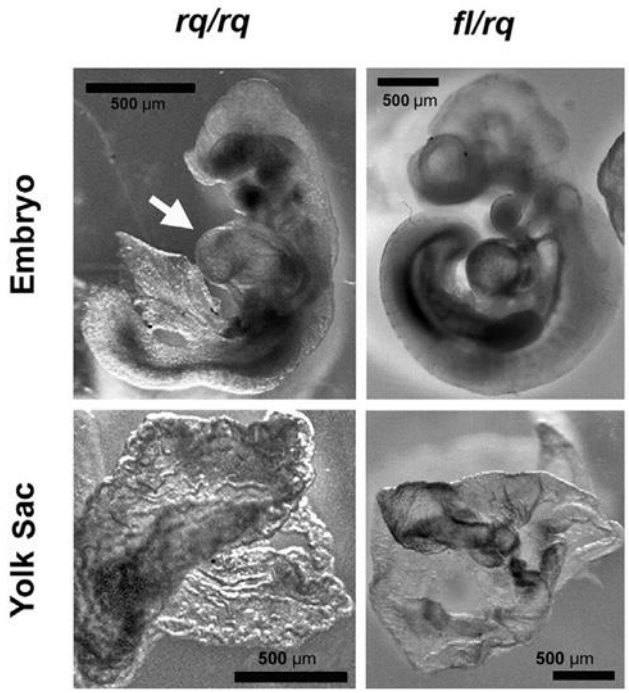



**Figure 10. *Rasa1*$^{R780Q/R780Q}$ embryos are identifiably different from littermate controls.** Light microscopic appearance of *Rasa1 R780Q/R780Q* and *Rasa1 R780Q/fl* embryos and yolk sacs at E9.5. *Rasa1 R780Q/R780Q* embryos are reduced in size, and have a distended pericardial sac (arrow) and wrinkled appearance of the yolk sac.

To more closely analyze the vascular abnormalities in the E9.5 embryos, whole mount immunofluorescence was carried out on the embryos and yolk sacs using an anti-CD31 antibody to detect blood endothelial cells (BEC). Both *Rasa1*$^{fl/fl}$ and *Rasa1*$^{fl/R780Q}$ littermate controls show normal blood vasculature development both in the yolk sac and the embryo proper (Figure 11). Yolk sacs are correctly vascularized, with vessels connecting to the embryo. The embryos have

dorsal aortas with intersegmental arteries branching dorsally between somites, and the dorsal aorta is continuous and extends from the heart to posterior of the embryo. Whole mount staining with the BEC-specific anti-CD31 antibody shows that in contrast to wild type littermates, *Rasa1*$^{R780Q/R780Q}$ yolk sacs fail to develop a blood vascular network, with the endothelial cells arresting in a honeycomb pattern of fused blood islands (Figure 11). The embryo proper has a thin, malformed dorsal aorta with irregular intersegmental arteries. Further analysis by immunohistochemistry using anti-CD31 in sections shows that the dorsal aorta is discontinuous in these embryos compared to control littermates. The yolk sac and dorsal aorta phenotypes are the same as those of *Rasa1* null E9.5 embryos [37].

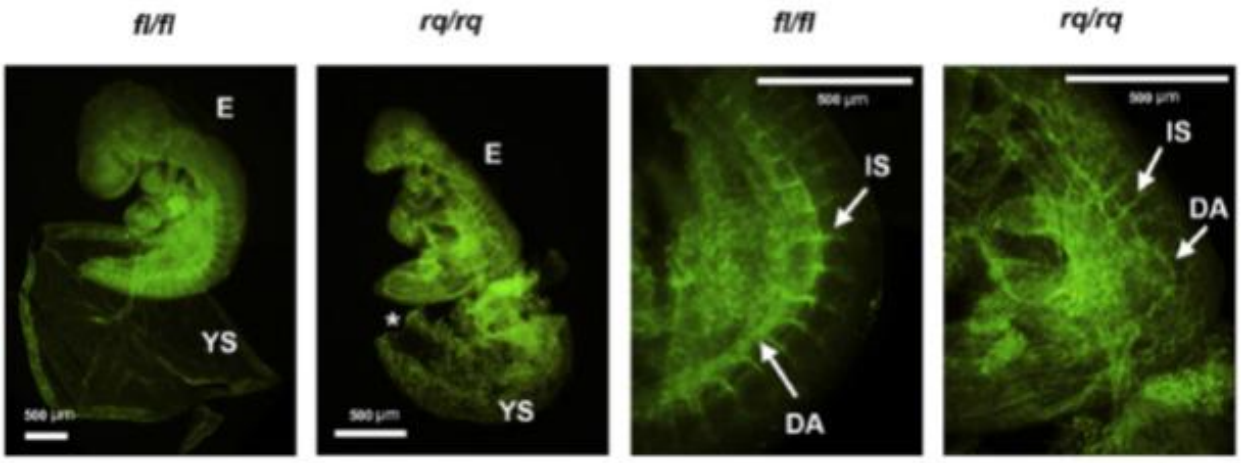



**Figure 11. The blood vascular system of *Rasa1*$^{R780Q/R780Q}$ embryos does not develop correctly.** Whole mount *Rasa1* $^{R780Q/R780Q}$ and *Rasa1* $^{fl/fl}$ embryos were stained with an anti-CD31 antibody to label blood vasculature. *Rasa1* $^{fl/fl}$ yolk sacs have organized blood vessel networks while *Rasa1*$^{R780Q/R780Q}$ yolk sac blood vasculature is honeycombed in appearance. The homozygous R780Q embryo proper also had irregular blood vasculature. E, embryo; YS, yolk sac; IS, intersegmental artery; DA, dorsal aorta.

Predictably the R780Q RASA1 protein would not be able to promote Ras hydrolysis of GTP, which suggests that the cardiovascular phenotypes develop as a result of loss of control of Ras signaling. In order to confirm that Ras was dysregulated in *Rasa1*$^{R780Q/R780Q}$ embryos, E9.5

embryos were sectioned and stained with antibodies against the phosphorylated activated form of ERK MAPK (pERK) along with anti-CD31 to identify BEC. In both *Rasa1* $^{fl/fl}$ and *Rasa1*$^{fl/R780Q}$ littermate control, pERK positive BEC were infrequently identified (Figure 12). However in *Rasa1*$^{R780Q/R780Q}$ embryos most BEC expressed pERK. Thus, as expected, the Ras MAPK is aberrantly activated in BEC of homozygous R780Q mice.

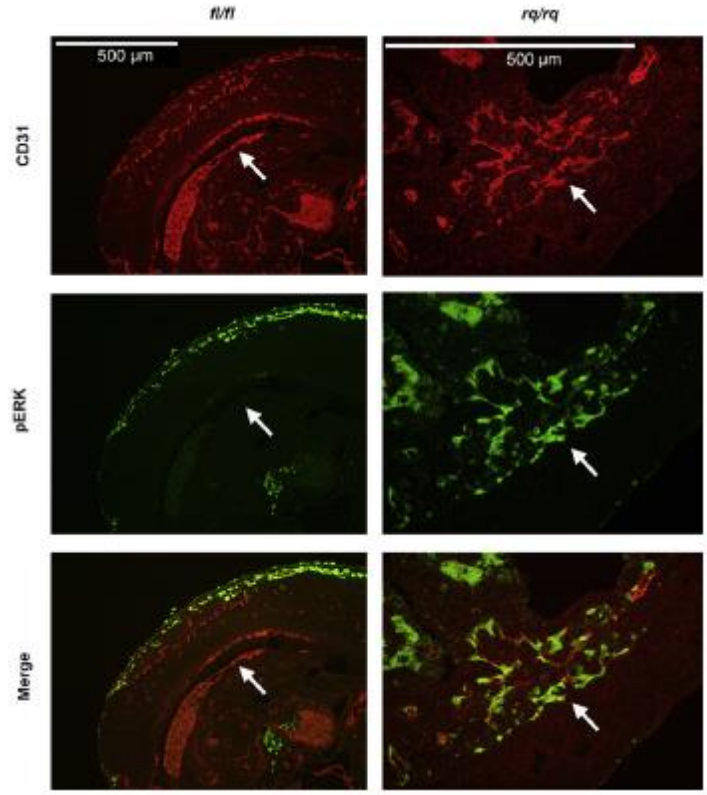



**Figure 12.** ***Rasa1***$^{R780Q/R780Q}$ **E9.5 embryo blood endothelial cells have activated Ras MAPK signaling.** E9.5 embryos were sectioned and stained with anti-CD31 (red) in conjunction with anti-pERK (green) antibodies. In control embryos, the majority of BEC do not express pERK, while in most Rasa1 BEC pERK is present.

### 3.4 Loss of RASA1 regulation of Ras in adult mice

In order to address whether the lymphatic disease in adults originated from loss of regulation of Ras or loss of a Ras-independent function, *Rasa1*$^{R780Q/fl}$ *ub-ert2Cre* mice were generated. Injection of mice with tamoxifen induces expression of the Cre recombinase

ubiquitously throughout the mouse, resulting in the deletion of the floxed allele and expression of only the RASA1 R780Q protein. Mice were injected at 3 weeks of age, along with littermate *Rasa1*$^{fl/fl}$ *ub-ert2Cre*, *Rasa1*$^{R780Q/fl}$ and *Rasa1*$^{fl/fl}$ mice as positive and negative controls.

Approximately 10 weeks after tamoxifen injections, *Rasa1*$^{R780Q/fl}$ *ub-ert2Cre* mice developed labored breathing and were euthanized. Analysis of the euthanized mice showed that the entire thoracic cavity was filled with a pleural effusion (Figure 13). Flow cytometry was carried out on the thoracic fluid to identify the cellular constituents. The majority of non-erythroid cells in the effusion were T- and B-cells, confirming that the fluid is chyle.

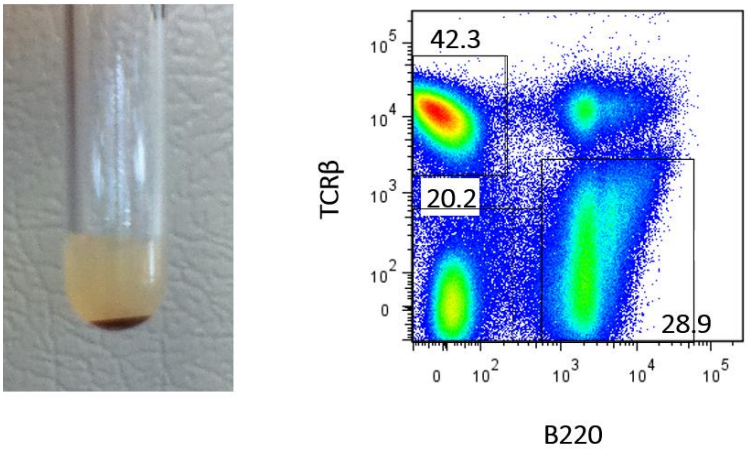
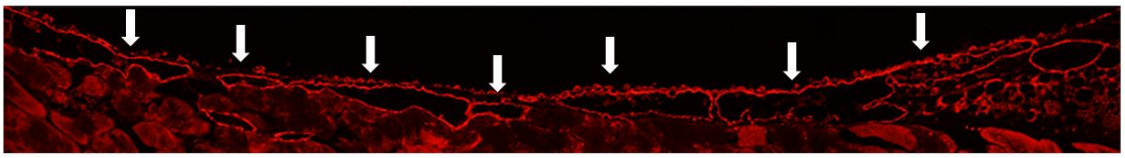



**Figure 13. *Rasa1*$^{R780Q/fl}$ *ub-ert2Cre* develop chylothorax.** The chest cavities of *Rasa1*$^{R780Q/fl}$ *ub-ert2Cre* mice fill with a milky fluid (left). Right, flow cytometry of pleural effusion. The majority of non-erythroid cells (Ter119-) are lymphocytes.

Organs were harvested from euthanized *Rasa1*$^{R780Q/fl}$ *ub-ert2Cre* mice for whole mount staining with anti-LYVE1 antibodies to identify LEC. Diaphragms, ears, and trachea showed an increased density of lymphatic vessels as compared to littermate controls, similarly to *Rasa1*$^{fl/fl}$ *ub-ert2Cre* mice. Ribcages were sectioned and stained with anti-LYVE1. Lymphatic vessels are rarely found lining the pleural cavity of wild type littermate controls. In contrast, *Rasa1*$^{R780Q/fl}$ *ub-ert2Cre* mice, the entire pleural face of the ribcage is lined with lymphatic vessels, similar to

the *Rasa1*$^{fl/fl}$ *ub-ert2Cre* mouse (Figure 14). *Rasa1*$^{R780Q/fl}$ *ub-ert2Cre* mice do not show a spontaneous cardiovascular phenotype, again similar to the *Rasa1*$^{fl/fl}$ *ub-ert2Cre* mouse [38].



*Rasa1*$^{R780Q/fl}$ *ub-ert2cre*

**Figure 14. The pleural face of *Rasa1*$^{R780Q/fl}$ *ub-ert2Cre* ribcages are lined with lymphatic vessels.** Sections of ribcages were stained with anti-LYVE1 to identify lymphatic vessels. Control ribcages rarely have lymphatic vessels on the pleural face, while *Rasa1*$^{R780Q/fl}$ *ub-ert2Cre* mice have multiple vessels lining the entire pleural cavity.

As loss of the RASA1's GAP domain results in the same lymphatic hyperplasia and leakage as the complete conditional knockout, we hypothesized that there would be aberrant Ras signaling in LEC. Serial sections of ribcage were stained with anti-LYVE1, anti-CD31, and anti-pERK antibodies in order to identify LEC and BEC where active Ras signaling was present. Interestingly, the majority of lymphatic vessels do not express high levels pERK, while the majority of blood vessels have pERK present (Figure 15). Despite the active Ras MAPK signaling in BEC, these mice do not develop any spontaneous cardiovascular phenotypes.

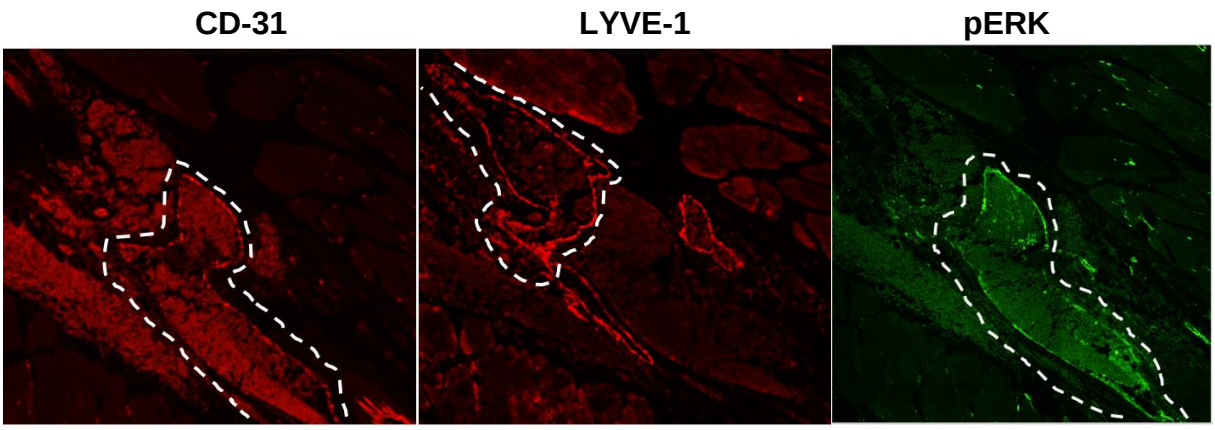



**Figure 15. *Rasa1*$^{R780Q/fl}$ *ub-ert2Cre* mice have active Ras MAPK signaling in BEC but not in LEC.** Serial sections of *Rasa1*$^{R780Q/fl}$ *ub-ert2Cre* ribcages were stained to identify blood and lymphatic vessels along with pERK. Left, anti-CD31 staining, middle, anti-LYVE1 staining, and right, anti-pERK staining. Dotted lines identify specific vessels.

## 3.5 Materials and Methods

### 3.5.1 Whole Mount Staining

E9.5 embryos were fixed in 1% paraformaldehyde in phosphate-buffered saline. Fixed embryos were stained with a rat anti-CD31 antibody (BD Biosciences), followed by a secondary goat anti-rat Ig Alexa Fluor 488 antibody (Invitrogen). Images of embryos were acquired on an Olympus BX60 upright fluorescence microscope equipped with a digital camera (Nikon).

### 3.5.2 Immunohistology

E9.5 embryos were fixed in formalin and embedded in paraffin. Sections (5 mm thick) were stained with a rabbit anti-phospho-extracellular signal regulated kinase (ERK) antibody (D13.14.4E; Cell Signaling), followed by biotinylated anti-rabbit Ig (Jackson Immunoresearch) and streptavidin-horseradish peroxidase with tyramide signal amplification (Perkin Elmer, Waltham, MA). Sections were then stained with a rat anti-CD31 antibody (sz31; Dianova), followed by secondary goat anti-rat Alexa Fluor 594 (Invitrogen). Some sections were stained with CD31 and secondary antibody alone or with hematoxylin and eosin. Images of sections

were acquired as described in the Whole Mount Staining section. Rib cage sections were stained with rabbit anti-LYVE1 (AbCam) followed by goat anti-rabbit Alexa Fluor 488 (Invitrogen), as well as CD31 and pERK as described.

### 3.5.3 Flow Cytometry

Cells isolated from *Rasa1*$^{R780Q/fl}$ *ert2Cre* pleural effusion were stained with fluorochrome-coupled anti-CD4, anti-CD8, anti-TCR, anti-BB20, anti-GR1, and anti-CD11b. Cell staining was analyzed by use of a FACS Canto.

## 3.6 Discussion

In its homozygous form, the *Rasa1 R780Q* allele results in embryonic lethality of embryos mid-gestation, the timing of which is similar to that of RASA1 null embryos. Embryos have thin, discontinuous dorsal aortas with aberrant projects, and failed blood vessel reorganization in the yolk sac. BEC in these mice express increased levels of pERK as compared to wild type littermate controls. Thus, this blood vessel disorder is due to aberrant Ras activation through loss of RASA1's ability to promote Ras GTP hydrolysis. This has important implications for treatment options for patients with CM-AVM. Inhibitors of Ras downstream effectors, including ERK1/2, are already in clinical use. It is possible that treatment of these patients with ERK inhibitors during development may alleviate the severity of disease. The efficacy of these inhibitors as treatments for these patients can be tested in *Rasa1*$^{R780Q/R780Q}$ embryonic development to see if they rescue blood vessel organization in these embryos.

Interestingly, overexpression of Ras in embryos results in lymphatic hyperplasia, with no impairment of cardiovascular development [89]. It is possible that this occurs because the

lymphatic system is more sensitive to aberrant Ras activation than the blood vascular system. As this model of Ras overexpression is not refractory to RasGAPs, it is possible that this level of Ras signaling can be controlled effectively enough by RASA1 in BEC to maintain normal development and function. However, in lymphatic vessels, it may be that this level of overexpressed Ras is not efficiently controlled enough, especially downstream of VEGFR3.

Loss of the complete RASA1 protein in adult mice results in severe lymphatic hyperplasia and leakage, resulting in death by chylothorax. In order to investigate the role of RASA1's promotion of Ras GTP hydrolysis in the development of the lymphatic phenotype, mice were generated that have an allele with a point mutation that abrogates RASA1's GAP activity in conjunction with a floxed allele of RASA1 and a ubiquitously expressed Cre recombinase transgene. Induction of deletion of the conditional allele in adult mice 3 weeks of age results in death by chylothorax around 10 weeks post injection. These mice have increased lymphatic vessel density in multiple organ systems, including diaphragm and ribcage. However, no aberrant blood vascular phenotype was identified. This phenotype is consistent with mouse models of overexpression and knockouts of Ras, in which embryos develop lymphatic hyper- and hypoplasia, respectively [89].

Since the phenotype develops through loss of correct Ras regulation, lymphatic vessels were screened for aberrant activation of Ras downstream effectors. Interestingly, high Ras MAPK activation levels were identified in BEC, but is comparatively rare in LEC composed vessels. This is consistent with the expression of active pERK in the BEC of the *Rasa1*$^{R780Q/R780Q}$ embryos. However, in the Ras overexpression lymphatic hyperplasia model and the conditional *Rasa1* model, the LEC have aberrant Ras MAPK signaling through VEGFR3 [38, 89]. It is possible that detection of pERK in sections is not a sensitive enough form of readout for aberrant

Ras signaling in lymphatic vessels. Further work would include isolating LEC from *Rasa1*$^{R780Q/fl}$ *ert2Cre* mice and stimulating them with growth factors, then using Western blotting to follow activation of Ras downstream effectors.

As the lymphatic vessel hyperplasia and leakage develops specifically due to loss of RASA1's ability to promote Ras GTP hydrolysis, this suggests that lymphatic malformations in CM-AVM patients are due to aberrant Ras activation. Again, inhibitors of Ras downstream effectors may be a potential treatment for CM-AVM patients. However, recent attempts to use these inhibitors in *Rasa1*$^{fl/fl}$ *ert2Cre* mice had not been successful at delaying or preventing the lymphatic vessel hyperplasia. Further work in this area will be to use mice with mutations in genes in the Ras MAPK and PI3K pathways and cross them with *Rasa1*$^{R780Q/fl}$ *ert2Cre* or *Rasa1*$^{fl/fl}$ *ert2Cre* mice to see if impairment of downstream effector pathways can rescue the lymphatic phenotype.

# Chapter 4

# RasGAPs p120 RasGAP and Neurofibromin1 act in concert as tumor suppressors during T cell development

## 4.1 Abstract

Ras GTPase activating proteins (RasGAPs) are essential for regulation of Ras signaling downstream of multiple different receptors. Activation of Ras is dependent on the recruitment of Ras guanine nucleotide exchange proteins (RasGEFs). The RasGEFs essential for Ras activation downstream of the TCR are well characterized. However, the RasGAP that is essential for cessation of Ras signaling downstream of the TCR is unknown. Singular loss of the RasGAPs p120 RasGAP (RASA1) and Neurofibromin 1 (NF1) in the T cell compartment have failed to identify the required RasGAP, as these mice develop subtle T cell phenotypes. Loss of both RASA1 and NF1 in embryos results in earlier embryonic lethality, suggesting they have overlapping function in the same pathway. To explore the possibility that these RasGAPs work in concert in T cells, RASA1 and NF1 were concurrently deleted in developing thymocytes. While no defects were found in number or percentages of thymocyte subpopulations, over half the double deficient mice develop lethal T cell acute lymphoblastic leukemia/lymphoma (T-ALL). The T-ALL develop early in T cell development and are dependent upon strongly activating mutations in *Notch1*. Analysis of freshly isolated T-ALL cells shows increased basal

Ras signaling through the MAPK and PI3K/AKT signaling pathways compared to wild type cells, suggesting that Ras signaling acts downstream of Notch1 mutations in tumor formation.

## 4.2 Introduction

Ras signaling has a wide range of functions, from driving cell proliferation to differentiation and survival [1]. Ras acts as a molecular switch, as it is inactive when bound to GDP but becomes active upon binding to GTP. Inactive Ras requires Ras guanine nucleotide exchange factors (RasGEFs) for ejecting the bound GDP and allowing binding of GTP, which is in higher concentration than GDP within the cell cytosol. Ras signals through multiple downstream pathways including MAPK and PI3K [3, 6, 7]. This signaling requires careful regulation, as aberrant Ras signaling often results in the development of cancer [13]. Although Ras has some intrinsic GTPase activity, efficient cessation of signaling requires Ras interaction with Ras GTPase activating proteins (RasGAPs) which increase Ras GTPase activity by several orders of magnitude [2].

Ras signaling is essential for correct T cell development and activation. ERK1/2 activation through MEK1 downstream of Ras are essential for positive selection, and ERK1 and 2 have individual roles in DN stage progression [29-31]. Once past positive selection, DP thymocytes undergo negative selection to avoid the generation of autoreactive T cells. Ras signaling is also involved in TCR signaling, where it is well characterized that the RasGEFs RasGRP1 and Sos are recruited after TCR signaling to activate Ras [35, 36]. However, the negative regulation of Ras both in T cell development and downstream of the TCR is not understood.

Failure to regulate Ras can result in the development of multiple different cancers. Mutations of Ras that make them refractory to RasGAP activation or otherwise constitutively active are found in 30% of all cancers [11]. Murine models of Ras overexpression have been generated utilizing a *K-ras*$^{G12D}$ allele, and these mice develop various cancers dependent on the tissue of expression [14, 15]. Transfer of *K-ras*$^{G12D}$ bone marrow into lethally irradiated wild type recipients results in the mice developing an aggressive T cell acute lymphoblastic leukemia [16].

Along with activating Ras mutations, murine models of T-ALL often carry secondary mutations that are required for the genesis of the cancer [16, 78]. The Notch1 protein undergoes gain of function mutations in over 50% of human T-ALL cases [69]. In murine models of T-ALL HD mutations are rare, but PEST domain mutations are still common [78]. More recently two new types of murine Notch1 mutations were described [79, 80]. Type 1 mutations (NT1) are recombination activating gene (RAG)-mediated recombinations that splice out exons 1 and 2, while Type 2 mutations (NT2) are RAG-independent recombinations that result in loss of exons 2 through 25 or 26. In both cases, this results in translation from an internal methionine and generation of a transmembrane protein that undergoes ligand-independent cleavage.

While correct regulation of Ras signaling in T cells is clearly essential, how Ras is regulated either in developing cells or downstream of TCR activation is yet unknown. Single loss of the RasGAP p120 RasGAP (RASA1) specifically in developing thymocytes results in a slight increase in positive selection in the thymus along with reduced numbers of naïve cells in the periphery only when on a transgenic TCR background [40]. Similarly, thymocyte specific deletion of the RasGAP Neurofibromin 1 (NF1) on a TCR transgenic background results in

impaired positive selection in the thymus and a decrease in naïve peripheral T cells [63]. Thus, the RasGAP or GAPs essential for efficient Ras GTP hydrolysis in T cells are still unknown.

It has been previously shown that RASA1 and NF1 have overlapping functions in the same pathway during embryonic development, as embryos deficient for both RasGAPs have earlier timing of embryonic lethality than the lethality due to loss of either RasGAP alone [37]. We hypothesized that these RasGAPs may play a cooperative role in regulating Ras in T cells. To address this, mice with conditional alleles of both RASA1 and NF1 with and without a thymocyte specific Cre transgene were generated. We show that while T cell development and function in these mice are unaffected, mice with concurrent deletion of RASA1 and NF1 in the T cell compartment undergo early lethality due to the development of T-ALL. We further show that the T-ALLs carry activating Notch1 mutations, and that aberrant Ras signaling is most likely important downstream of these mutations for the generation of T-ALL.

### 4.3 Generation of Double Knockout Mice and Analysis of T cell development

In order to address the possibility of overlapping Ras regulation by RASA1 and NF1 in T cells, *Rasa1*$^{fl/fl}$ and *Nf1*$^{fl/fl}$ mice were crossed to generate *Rasa1*$^{fl/fl}$*Nf1*$^{fl/fl}$ mice with and without *pLck-Cre*. These mice develop normally into adulthood. At 5 weeks of age, thymi were isolated from *Rasa1*$^{fl/fl}$*Nf1*$^{fl/fl}$ *pLck-Cre* (pLCK-DKO) mice and *Rasa1*$^{fl/fl}$*Nf1*$^{fl/fl}$ littermate controls. Flow cytometry was carried out to identity developmental populations of thymocytes and determine if there were any aberrations in T cell development in the double deficient mice. There were no significant differences in either the number or percentages of DN subsets in pLCK-DKO mice compare to control mice. A small decrease in the numbers of some DP subpopulations were observed, however these decreases were similar to those found in *Nf1*$^{fl/fl}$ *pLck-Cre* mice and did

not result from compound loss of NF1 and RASA1 [63]. Small decreases in single positive thymocytes were also noted but were similar to the reduction found in both *Nf1$^{fl/fl}$ pLck-Cre* and *Rasa1$^{fl/fl}$ pLck-Cre* mice [40, 63]. Mature T cells were isolated from 5 week old mice and were stimulated through the TCR and CD28 costimulation receptor. There was no apparent defect in cytokine production from pLCK-DKO T cells (unpublished data, Philip Lapinski).

### 4.4 Development of T cell Acute Lymphoblastic Leukemia/Lymphoma

While the thymocytes and T cells of pLCK-DKO mice appear to develop and function normally, these mice succumb early, starting at around 3 months of age and affecting 60% of mice by 20 months of age (Figure 16). Gross analysis of these mice shows a greatly enlarged thymus that fills the entire thoracic cavity, resulting in labored breathing and eventual suffocation due to impaired lung function. Other lymphoid organs and liver are also grossly enlarged.

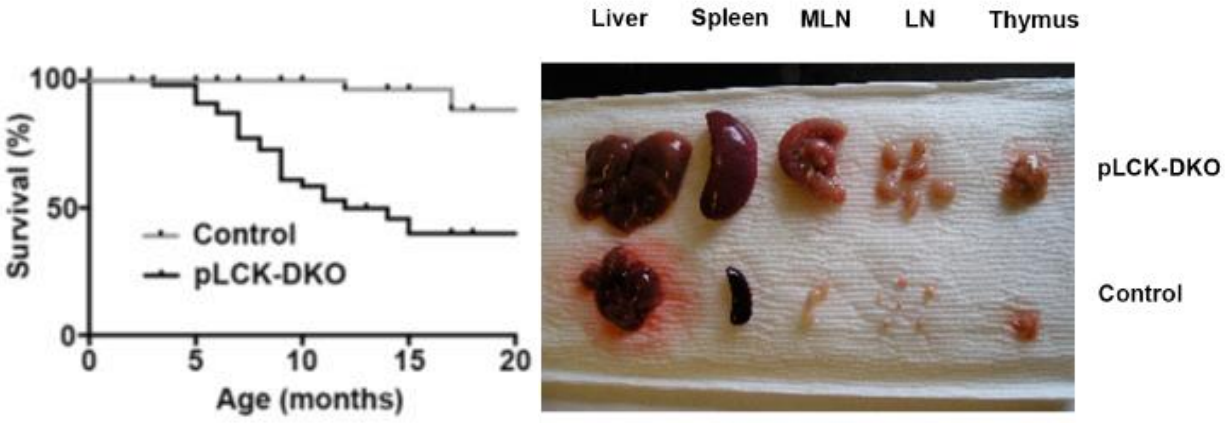



**Figure 16. Lymphoid organs from euthanized pLCK-DKO mice are grossly enlarged.** Left, survival curve of pLCK-DKO mice compared to littermates. Right, Lymphoid organs isolated from an 8 month old moribund pLCK-DKO mouse are much larger than the same organs from a littermate control. MLN, mesenteric lymph node; LN, lymph nodes.

Histological analysis was carried out on the hematopoietic organs and shows a loss of normal organ structure (Figure 17). Within the thymus, there is a loss of differentiation between the cortex and the medulla. This breakdown in structure is also found in the spleen, which is comprised entirely of lymphocytes with loss of red and white pulp. The liver also shows signs of infiltration with large numbers of perivascular lymphocytes. Higher power analysis shows a starry sky appearance in spleen and lymph nodes, which is due to the presence of macrophages engulfing cellular debris. This is a classic feature of lymphomas.

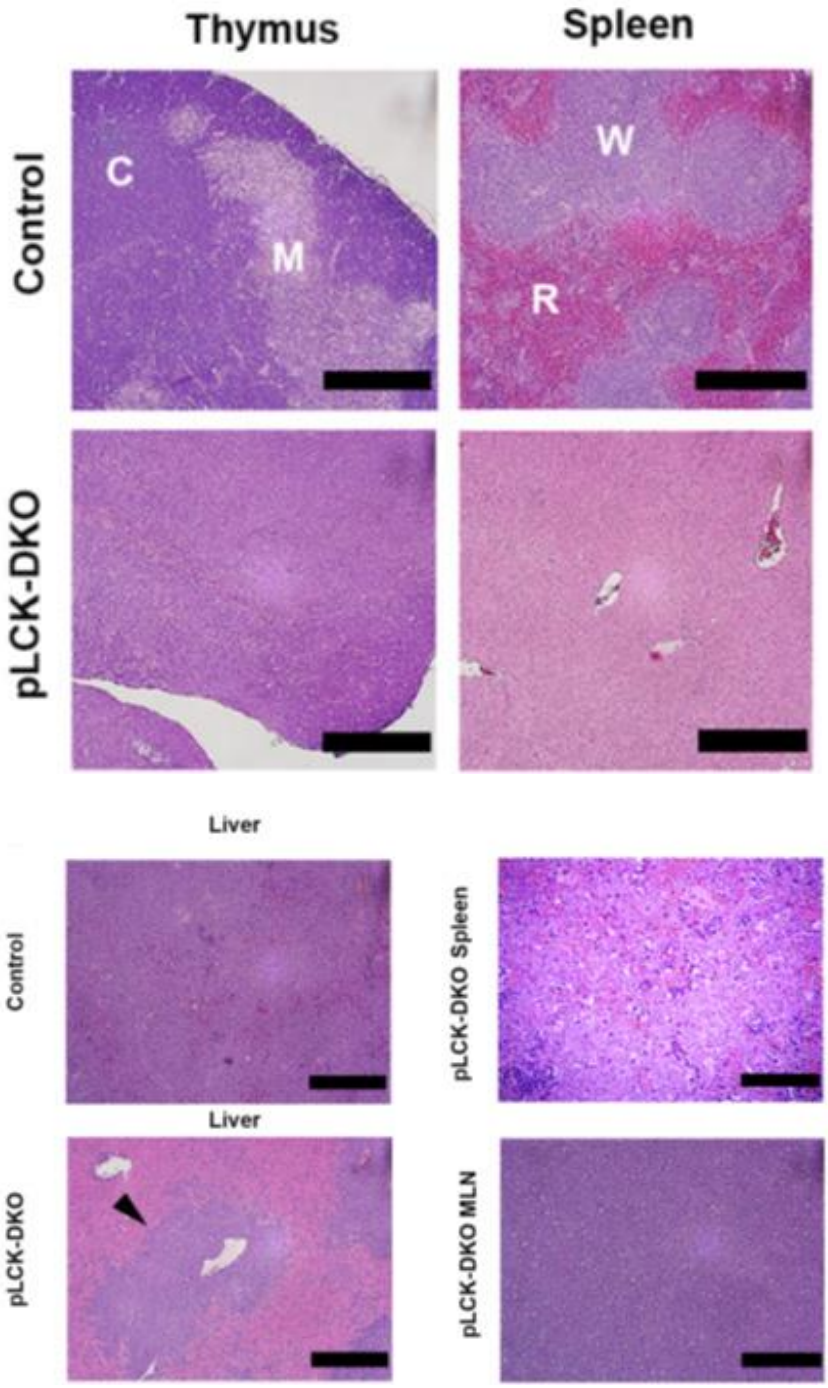



**Figure 17. Lymphoid organs and liver from moribund pLCK-DKO mice.** Top, 8 month old moribund pLCK-DKO and litter mate control thymus and spleen. C, cortex; M, medulla; W, white pulp; R, red pulp. Bar represents 400µm. Bottom left, liver of moribund pLCK-DKO and control. Arrowhead points to perivascular infiltration of lymphocytes. Bar represents 400µm. Bottom right, higher magnification of moribund pLCK-DKO spleen and mesenteric lymph node (MLN) to highlight starry sky appearance. Bar represents 100µm.

Flow cytometric analysis was carried out on the thymocytes of moribund pLCK-DKO mice. Thymocytes from sick mice were consistently CD8+ with variable CD4 expression, and these cells were the majority of cells identified in spleen, lymph node, and blood (Figure 18). Further analysis of the cells showed that they expressed lower levels of TCRβ and had increased expression of CD25. DNA was isolated from these cells and copy number quantitative PCR (qPCR) was carried out to confirm deletion of *Rasa1* and *Nf1* using primer/probe sets located in regions deleted by Cre recombination (Figure 19). All mice showed near complete deletion of both genes, confirming that this phenotype is due to combined loss of both RasGAPs.

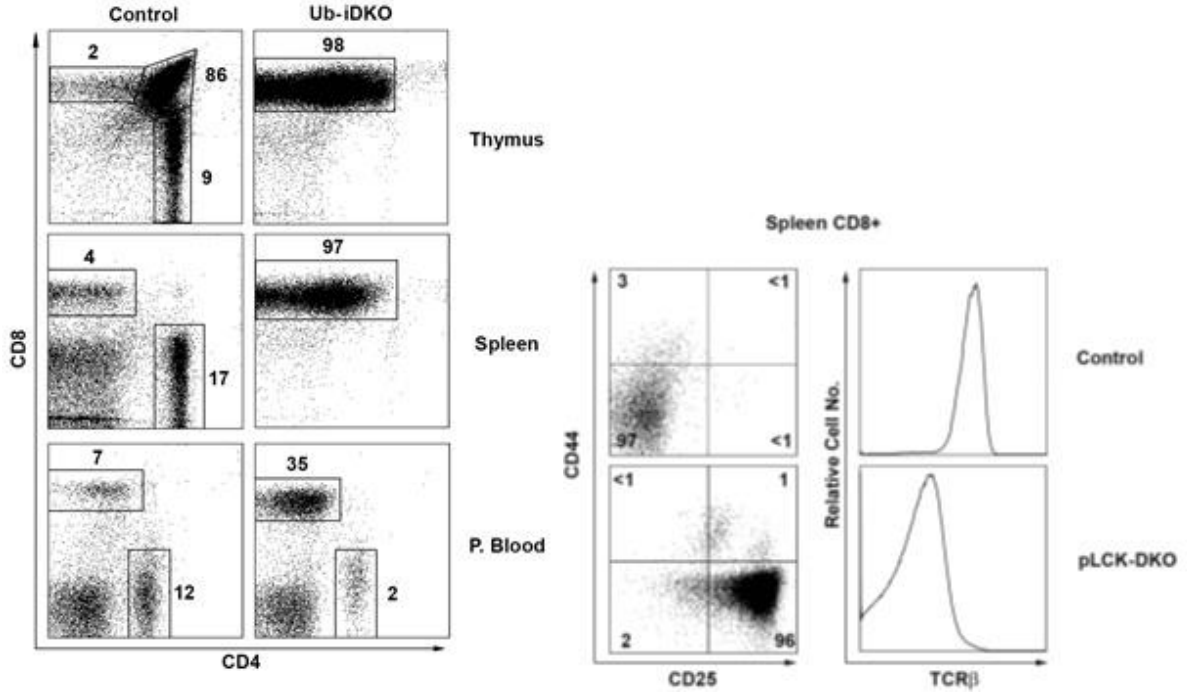



**Figure 18. Cell surface phenotype of pLCK-DKO T-ALL cells.** Left, cells isolated from thymus, spleen, and peripheral blood (P. Blood) of moribund 6 month old pLCK-DKO mouse and littermate control and stained with anti-CD8 and CD4 antibodies. Right, splenocytes from moribund 6 month old pLCK-DKO mouse and littermate control were gated on CD8+ cells. CD25 vs CD44 plots show pLCK-DKO CD8+ cells express CD25. Histograms of TCRβ expression show pLCK-DKO CD8+ cells have lower TCRβ expression than littermate control CD8+ cells.

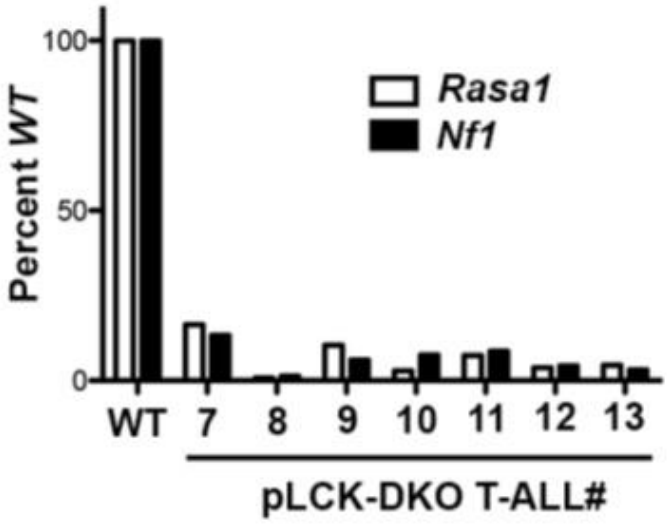



**Figure 19. RASA1 and NF1 gene disruption in T-ALL.** Genomic DNA was prepared from thymocytes from wild type (WT) mice and seven different moribund pLCK-DKO mice. Relative amounts of intact *Nf1* and *Rasa1* genes were determined by qPCR. Values are expressed as a percent of gene abundance in WT mice.

The T cell phenotype developed by pLCK-DKO mice is very similar to other reported mouse models of T-ALL. In order to confirm T-ALL, thymocytes were taken from a moribund mouse and injected into sublethally irradiated wild type recipients. Within two months, recipients became moribund. Isolation of spleens and lymph nodes showed the same large population of CD8+CD4variable cells found in the donor mouse (Figure 20). All these data taken together suggested that these mice develop a spontaneously occurring T-ALL dependent on combined loss of both *Rasa1* and *Nf1* in the T cell compartment.

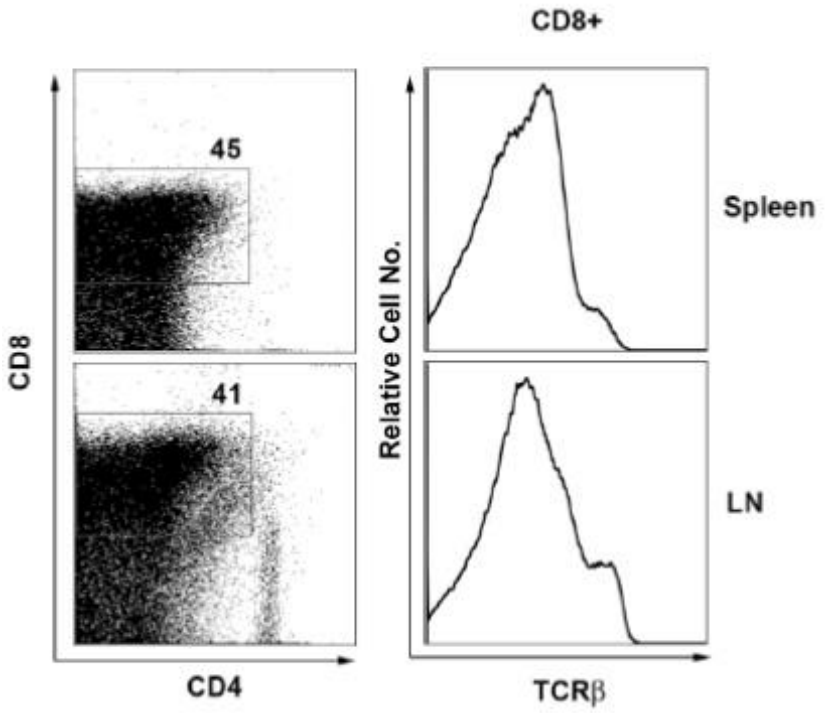



**Figure 20. pLCK-DKO T-ALL is transferrable to sublethally irradiated recipient mice.** Left, CD8+CD4variable cells infiltrate peripheral lymphoid organs such as spleen and lymph nodes in sublethally irradiated mice (LN). Right, the T-ALL cells retain their TCRβ low phenotype.

### 4.5 T-ALL cells carry strongly activating Notch1 mutations

Notch1 mutations are common in the majority of both human and murine T-ALL, although the types of mutations vary. Common mutations in murine T-ALL models include PEST domain mutations, which are not sufficient to drive T-ALL, along with Type 1 (NT1) and Type 2 mutations (NT2), which are strongly activating mutations. Human patients often have mutations in Notch1's HD domain, but this is uncommon in mouse models. DNA was isolated from pLCK-DKO T-ALL cells. HD and PEST domain mutations were screened for by amplifying and sequencing exons 26 and 27, and exon 34, respectively (Figure 21). NT1 mutations were identified by utilizing a PCR primer set that only results in a product when rearrangement has occurred. NT2 mutations were detected by qPCR by determining the ratio between spliced out and unaffected exons.

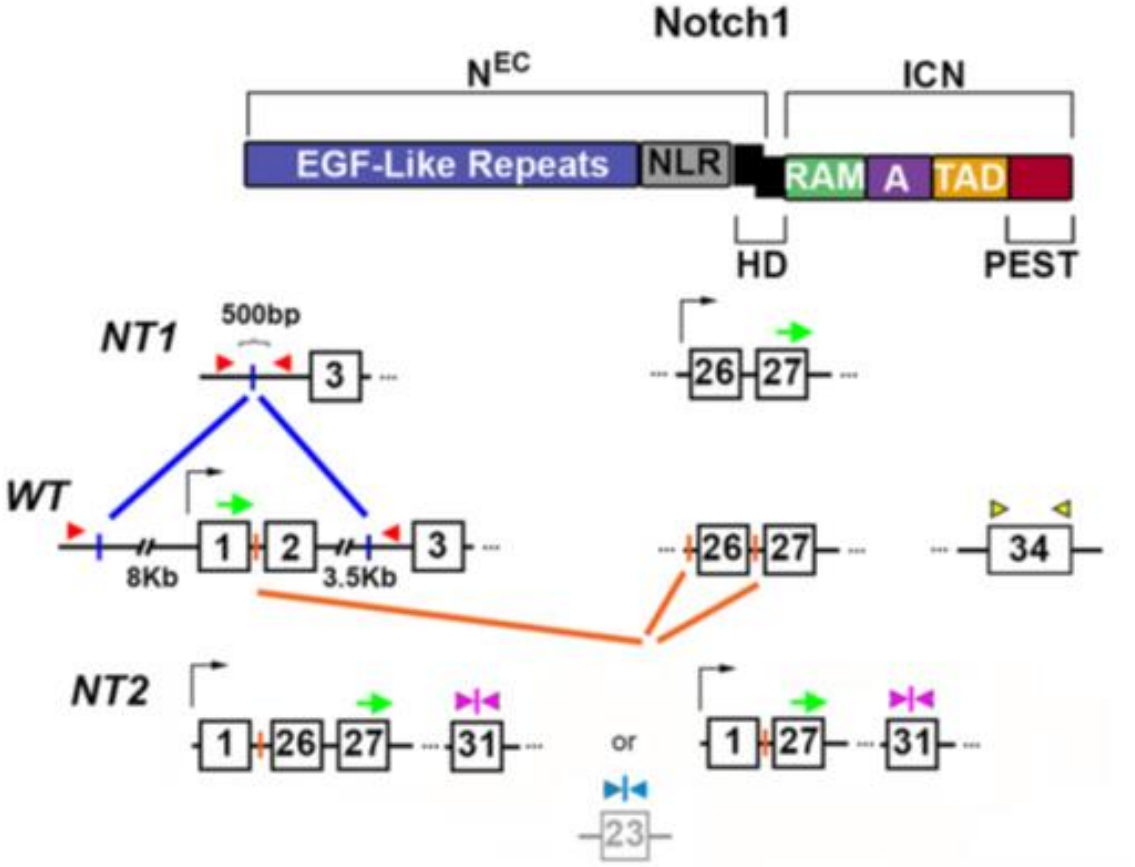



**Figure 21. Notch1 protein domains and associated mutations and screening protocols.** Top, schematic of Notch 1 protein. EGF, epidermal growth factor; NLR, Notch Lin repeat; HD, heterodimerization domain; RAM, RBJ kappa-associated module; A, Ankyrin repeat; TAD, transcriptional activation domain; PEST, proline/glutamic acid/serine/threonine rich domain; NEC, Notch extracellular domain; ICN, intracellular Notch. Below, schematic depicting different types of possible *Notch1* gene mutations. The wild type *Notch1* gene is shown at center. Numbered boxes indicate exons and are aligned approximately with the protein domains that they encode. Primers used to detect *PEST* domain mutations in genomic DNA are shown as yellow arrowheads. In *Notch1* type 1 (*NT1*) mutations (top), the region between the vertical blue bars that includes the 5' promoter and exons 1 and 2 of *Notch1* is deleted and transcription is initiated from a cryptic promoter upstream of exon 26. Translation is initiated from within exon 27 (green arrow) to yield a form of Notch1 from which ICN is generated independently of Notch ligand. Red arrowheads indicate positions of PCR primers used to detect a 500 bp product from genomic DNA upon *NT1* mutation. In *Notch1* type 2 (*NT2*) mutations (bottom), DNA downstream of exon 1 is cut and spliced to DNA upstream of exon 26 or exon 27 (vertical orange bars) to yield transcripts from which translation is also initiated from within exon 27 (green arrows), thereby resulting in a constitutively active form of Notch1. The presence of *NT2* mutations in samples was determined by qPCR using a primer/probe set (turquoise) to detect loss of exon 23 relative to WT thymocytes. Similar qPCRs were performed using a primer/probe set based in exon 31 (purple) as an additional control.

No pLCK-DKO mice had mutations in their HD domain, but 10 out of 11 T-ALLs had mutations in their PEST domain that resulted in truncated forms of the protein (Figure 22, Table 2). Mutations were short insertion, deletion, or a combination of the two, leading to a frame shift and a premature stop codon. These mutations occurred on one chromosome and were present in all the cells, suggesting that the T-ALLs arise from one precursor cell and are thus monoclonal. All T-ALLs also had Type 1 mutations, although it is unknown if Type 1 and PEST mutations occurred on the same chromosome (Figure 22, Table 2). Three T-ALLs carried Type 2 mutations. The T-ALLs with Type 2 mutations also had both Type 1 and PEST domain mutations (Table 2). Because of the recombinations involved in both Type 1 and Type 2, these rearrangements are mutually exclusive and must be on homologous chromosomes.

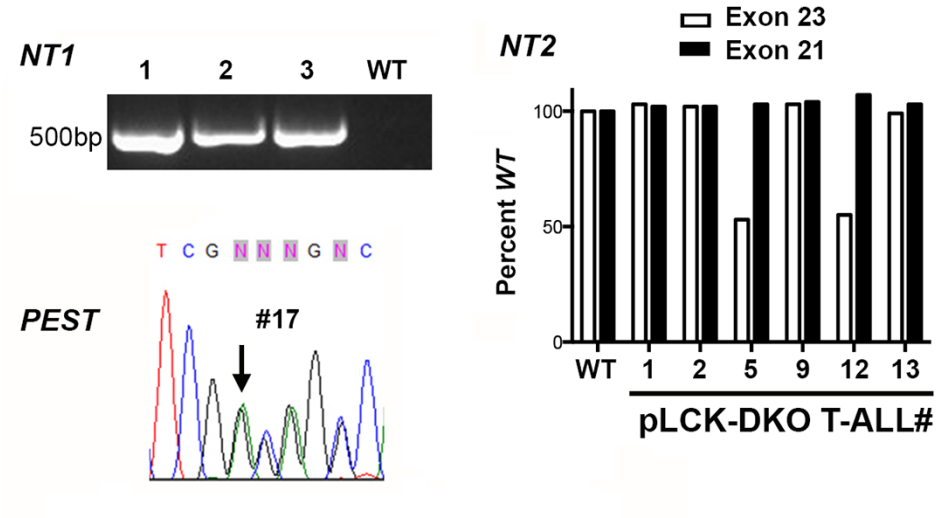



**Figure 22. All T-ALL carry activating *Notch1* mutations.** Shown are examples of *NT1*, *PEST* and *NT2 Notch1* mutations in freshly isolated thymocytes from numbered pLCK-DKO mice with T-ALL, analyzed as described in Figure 21. The arrow on the *PEST* DNA sequence trace indicates the point of insertion of an AGGG sequence with resulting frameshift. Note equivalent height of base peaks at "N" positions on DNA sequence trace. Note also the 50% reduction in exon 23 abundance for T-ALL samples #5 and #12 compared to WT.

| pLCK DKO T-ALL # | NT1 | NT2 | PEST Mutation | PEST Amino Acid |
|---|---|---|---|---|
| 1 | Y | N | c.7193_7194insAGAATAATA | p.Ser2397fsX3 |
| 2 | Y | N | c.7194_7195insAGGG | p.Ala2399ArgfsX11 |
| 3 | Y | N | c.7080_7081insGG<br>c.7115_7118delCAGG | p.Leu2362GlyfsX128 |
| 4 | Y | N | ND | ND |
| 5 | Y | Y | c.7051_7052insAA | p.Pro2351fsX39 |
| 6 | Y | ND | ND | ND |
| 7 | Y | ND | c.7081_7082delG,insACC | p.Arg2361HisfsX35 |
| 8 | Y | N | ND | ND |
| 9 | Y | N | ND | ND |
| 10 | Y | N | ND | ND |
| 11 | Y | N | c.7081_7082insAGGGGCCC | p.Arg2361GlnfsX129 |
| 12 | Y | Y | c.7193_7194_insCC | p.Ser2397fsX12 |
| 13 | Y | N | ND | ND |
| 14 | Y | ND | ND | ND |
| 15 | Y | Y | c.7081_7082delG,insCCC | p.Arg2361ProfsX36 |
| 16 | Y | N | N | N/A |
| 17 | Y | N | c.7193_7194_insAGGG | p.Ala2399ArgfsX92 |
| 18 | Y | N | c.7081_7082insCC | p.Arg2361ProfsX36 |

**Table 2. List of pLCK-DKO T-ALLs and their associated *Notch1* mutations.**

### 4.6 Ras signaling in pLCK-DKO thymocytes and T-ALL cells

As all the pLCK-DKO T-ALLs have NT1 mutations, these mutations must be an initiating event. Since NT1 mutations are RAG mediated, they must occur during stages of T cell development while RAG is active. This limits the possible precursor populations to late DN2/early DN3 to pre-positive selection DP stages [90]. Also limiting the populations is the expression of the *pLck-Cre* transgene, which does not induce deletion of *Rasa1* and *Nf1* until late DN2/early DN3 [91]. Since concurrent deletion of both RasGAPs is necessary for T-ALL development, it is possible that the loss of both RasGAPs results in aberrant Ras signaling that renders cells more permissive to *Notch1* mutations. In order to address this theory, thymocytes were isolated from 5 week old pLCK-DKO mice and littermate controls, since pLCK-DKO mice do not develop T-ALL prior to approximately 12 weeks. Basal and stimulated levels of MAPK signaling were analyzed in pre-leukemic pLCK-DKO mice and littermate controls by phosphor-

flow cytomtery. No significant changes in strength or duration of MAPK signaling were identified in pLCK-DKO DN3, DN4, DP, CD4+ or CD8+ populations (Figure 23). As Ras signaling can result in increased survival, it is also possible that pLCK-DKO cells that develop a mutation are more likely to escape apoptosis. However, pre-leukemic pLCK-DKO thymocyte survival was normal compared to littermate controls after undergoing multiple forms of apoptotic stimuli, and no increase in survival was identified in any of the developmental subpopulations (Figure 23).

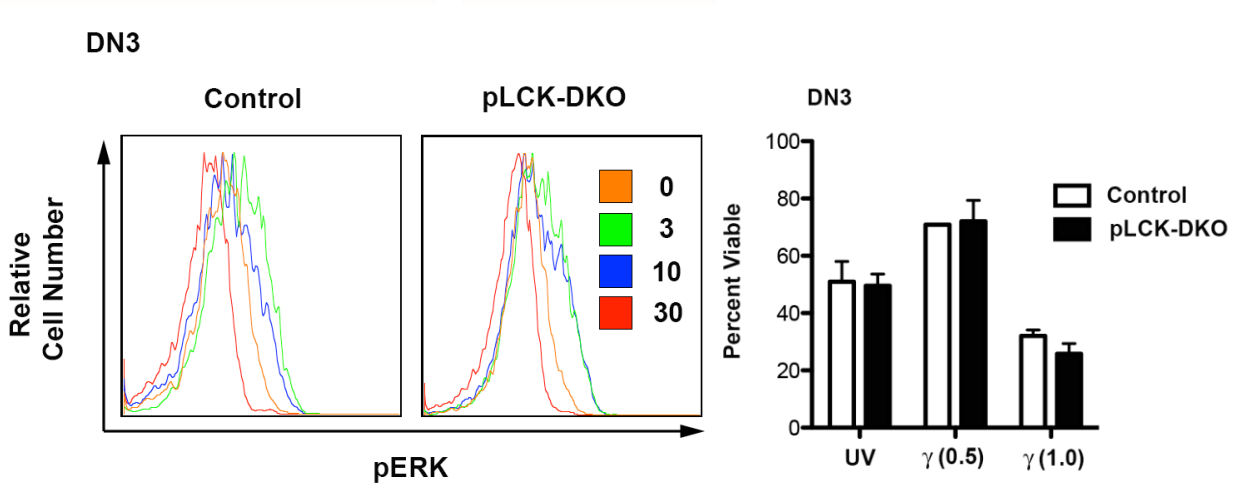



**Figure 23. Pre-leukemic thymocyte populations have no change in MAPK signaling kinetics or survival after apoptotic stimuli.** Left, representative plots from subpopulation analysis show neither increased strength nor duration of pERK signaling. DN3 cells from 5 week old pLCK-DKO mice and littermate controls were either unstimulated (0) or stimulated with 50 ng/ml of PMA for 3, 10, or 30 minutes. Activation of Ras signaling was determined by flow cytometry using a phospho-specific ERK antibody. Right, pre-leukemic pLCK-DKO DN3 cells were treated with UV and gamma irradiation (.5 and 1.0 Gy), then cultured for 12 hours. Survival was determined by flow cytometry, with viable cells defined as Annexin V and 7AAD negative. Percent viability was calculated by taking the percent viable cells and normalizing it to the percentage of viable untreated thymocytes assigned a value of 100. Shown are the mean percentage + 1 SE (n= 3 mice of each genotype).

These data suggest that loss of RasGAP regulation of Ras may be more important downstream of *Notch1* mutations. To test this, freshly isolated pLCK-DKO T-ALL cells were

analyzed using phospho-flow cytometry to analyze basal levels of MAPK and PI3K activity. Interestingly, both pathways showed slight but consistent increases in basal levels of activation compared to levels in littermate controls (Figure 24). This supports the hypothesis that Ras signaling acts downstream of *Notch1* mutations for genesis of T-ALL.

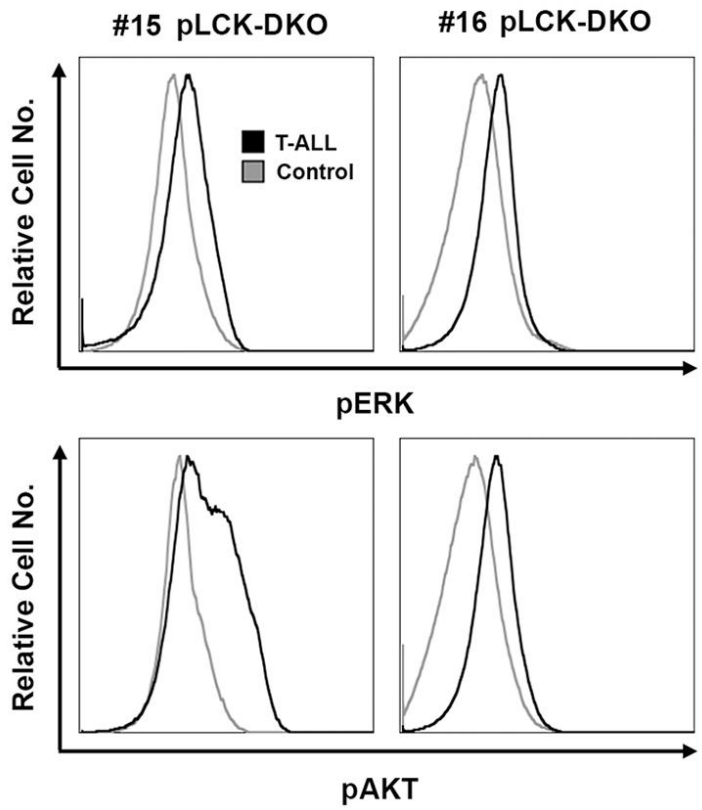



**Figure 24. Freshly isolated T-ALL cells have increased basal levels of Ras downstream effector activation.** Flow cytometry plots showing phospho-ERK (pERK) and phospho-AKT (pAKT) staining in freshly isolated DP thymocytes from two independent sick pLCK-DKO mice compared to DP thymocytes from age-matched littermate controls.

## 4.7 Materials and Methods

### 4.7.1 Mice

*Rasa1*$^{fl/fl}$ and *Nf1*$^{fl/fl}$*pLck-Cre* mice have been described previously [40, 63]. These mice were crossed to generate *Rasa1*$^{fl/fl}$*Nf1*$^{fl/fl}$ mice with and without *pLck-Cre*. Genotypes were

determined by PCR of tail genomic DNA using the same PCR primers as described [40, 63]. Mice were on a mixed129S6/Sv X C57BL/6 background. Moribund mice were euthanized and were recorded as end points in survival studies. All experiments were performed in compliance with University of Michigan guidelines and were approved by the University Committee on the Use and Care of Animals.

### 4.7.2 Flow Cytometry

Single cell suspensions were prepared from thymus, spleen and lymph nodes. Suspensions were stained with fluorochrome-labeled CD4 (GK1.5), CD8 (53-6.7), CD44 (IM7), CD25 (PC61), TCRβ (H57-597) (all BD Biosciences) and phospho-ERK1/2 (D13.14.4E) and phospho-AKT S473 (D9E) (both Cell Signaling) mAb as described previously [40, 63]. Apoptosis staining was done using Annexin V and 7AAD. Cell staining was analyzed by flow cytometry on a FACSCanto (BD Biosciences).

### 4.7.3 Tissue staining

Thymus, spleen, lymph nodes, and liver were fixed in 10% buffered formalin and then embedded in paraffin. Five micrometer sections of tissues were stained with H&E. Sections were imaged with an Olympus IX70 fluorescence microscope.

### 4.7.4 Quantitative PCR

Genomic DNA was isolated from thymus tissue of mice with T-ALL (Qiagen). Efficiency of *Rasa1* and *Nf1* gene disruption was determined by qPCR using Taqman primer/probe sets based in deleted exons (Mm00404879_cn and Mm00351296_cn; Life

Technologies) and a 7500 Fast PCR machine (Applied Biosystems). A transferrin receptor primer/probe set was used as an internal control for all samples. The amount of intact wild type *Rasa1* and *Nf1* in T-ALL samples relative to thymi from *Rasa1*$^{fl/fl}$*Nf1*$^{fl/fl}$ littermates was calculated as described [86].

### 4.7.5 Notch mutation analysis

To identify *Notch1* PEST domain mutations, genomic DNA from thymus was used as a template for PCR amplification of the entire exon of the *Notch1* gene. PCR products were then analyzed by Sanger sequencing. To identify *Notch1* Type 1 mutations, thymus genomic DNA was PCR-amplified using forward and reverse primers that flank exon 1 and 2 of the *Notch1* gene [79]. A 500bp product is only generated from a recombined *Notch1* allele (Figure 21). To identify *Notch1* Type 2 mutations, thymus genomic DNA was screened by qPCR using primer/probe sets located in exons 23 and 31 (Mm00539165_cn, Mm00539178_cn, Life Technologies) (Figure 21). A transferrin receptor primer/probe set was used as an internal control.

## 4.8 Discussion

Ras activation in T cell development has been well characterized, from its activation to the role of specific downstream effectors [29-31, 34]. However, the negative regulation of Ras signaling during T cell development is not well understood. Attempts to identify the RasGAP responsible for cessation of Ras signaling either downstream of the TCR or during thymocyte development have been unsuccessful, as loss of single RasGAPs results only in subtle thymocyte and T cell phenotypes [40, 63]. This suggests that either other RasGAPs are required for these

processes, or that multiple RasGAPs may have overlapping functions, resulting in normal T cell development and activation when only one RasGAP is lost.

Concurrent deletion of *Rasa1* and *Nf1* in the T cell compartment of mice resulted spontaneous lethal T-ALL. Careful examination of the pre-leukemic thymocyte populations shows no gross changes in number or percentages between wild type and pLCK-DKO populations, other than those that were previously shown in the single T cell specific *Rasa1* or *Nf1* deleted mice. This suggests that concurrent loss of both RasGAPs does not impair normal T cell development for the majority of cells. Thus, the RasGAP – or RasGAPs – essential for thymocyte development, as well as those required for regulation of signaling downstream of MHC-TCR interactions, are still unknown.

However, over half of the pLCK-DKO mice develop T-ALL, indicating that RASA1 and NF1 act as co-regulators of Ras in tumor suppression in the T cell compartment. Deletion of either *Rasa1* or *Nf1* specifically in T cells never resulted in T-ALL in those mice. Loss of NF1 in the hematopoetic compartment in mice results in a myeloid proliferative disorder, while deletion of *Nf1* in the Schwann cell linage along with *Nf1* heterozygosity in the bone marrow results in plexiform neurofibromas, which are benign tumors [61, 62]. Germline mutations in *NF1* in human patients lead to the disease Neurofibromatosis, which is characterized by multiple plexiform neurofibromas, and result in a high susceptibility for other neoplasms such as juvenile myelomonocitic leukemia (JMML) [64]. Thus, while there is precedent for NF1 as a tumor suppressor, loss of RASA1 has not been shown to result in any form of cancer in either mice or humans. Thus, the development of T-ALL after loss of RASA1 and NF1 is the first known role of RASA1 as a tumor suppressor.

Along with deletions of *Rasa1* and *Nf1*, the T-ALL in pLCK-DKO mice harbor activating mutations in *Notch1*. *Notch1* mutations are extremely common in both human T-ALL and mouse models, although the types of mutations may vary [16, 78, 92, 93]. Gain of function Notch1 mutations arise in mice that already have other genetic alterations. While Notch1 mutations in mice are well characterized, the interplay between the mutations in not fully understood. Clearly in the $Ras^{G12D}$ bone marrow transfer models of T-ALL, *Notch1* mutations must occur after the activating Ras mutation. But just how this occurs is unknown.

In the pLCK-DKO mice, the potential population of origin is narrowed down to DN2/DN3 to DP based on the RAG-mediated recombination required for the NT1 mutation [79] and the activity of *pLCK-Cre* [91]. Our attempts to identify aberrant Ras signaling or increased survival after apoptotic stimuli failed to identify a particular population where these mutations may be arising, suggesting that the mechanism may not be due to aberrant Ras activation permitting Notch1 mutated cells to survive and/or expand. Downstream of the Notch1 mutations, freshly isolated T-ALL cells shows consistent, slight increases in Ras signaling, which suggests that Notch1 mutations occur randomly and loss of Ras regulation in these cells promotes tumor formation. This hypothesis has also been put forth to explain the T-ALL developed in mice that overexpress the RasGEF RasGRP1 [94]. We conclude that loss of both RasGAPs are essential for the development of T-ALL, while single loss of either RasGAP either results in the other RasGAP working in a compensatory manner, or the dysregulation of Ras fails to reach a threshold required for T-ALL to arise. Further work is required to fully understand how Ras signaling works downstream of NT1 mutations to drive T-ALL development.

# Chapter 5

## Discussion and Future Directions

Ras signaling is involved in multiple cellular processes, and negative regulation of that signaling requires the activity of different RasGAPs. The RasGAP RASA1 has been shown to play an essential role in mouse cardiovascular development and lymphatic vessel maintenance [37, 38]. Humans with germline mutations of RASA1 are believed to gain second hit mutations of the wild type allele and develop CM-AVM due to complete loss of protein. This disease is characterized by cardiovascular abnormalities, with some patients presenting with lymphatic abnormalities [50, 53]. As both of these disorders are believed to be due to the complete loss of RASA1 protein, it is unknown whether the resulting cardiovascular and lymphatic phenotypes are due to loss of RASA1's ability to promote Ras hydrolysis of GTP or loss of a Ras-independent function. In order to address this question, we generated a knock-in mouse with a GAP-deficient RASA1 protein.

In Chapter 2 the construction of the knock-in mouse is described. The arginine finger that is required to promote Ras GTP hydrolysis is mutated to a glutamine by changing a base pair in the codon (R780Q). This point mutation does not disrupt the overall tertiary structure of the protein, allowing RASA1 to carry out putative other functions. We confirm that *Rasa1*$^{R780Q/R780Q}$ embryos express the same level of RASA1 protein as both heterozygote R780Q and floxed littermate controls. This knock-in mouse allows us to investigate the influence of RASA1's GAP function on the development of cardiovascular and lymphatic phenotypes.

In Chapter 3, we show that the homozygous *Rasa1 R780Q* embryos die *in utero* mid-gestation due to failed cardiovascular development similar to that of the RASA1 null mice [37]. The *Rasa1*$^{R780Q/R780Q}$ embryos have thin, disrupted dorsal aortas with aberrant branching and failed blood vessel organization in yolk sacs as compared to littermate controls. Blood vessels in homozygous R780Q embryos all have increased Ras MAPK signaling compared to littermate controls, which confirms that it is aberrant Ras activation that is essential for the cardiovascular abnormalities. This has important implications for patients with CM-AVM, as it suggests that this disorder may be treatable with Ras MAPK pathway inhibitors that are already clinically available. It is possible that treatment with these inhibitors during development may ameliorate cardiovascular defects in patients. To test this, different approaches can be used. The inhibitors could be used on *Rasa1*$^{R780Q/R780Q}$ embryos to see if they rescue or at least improve the cardiovascular formation defects in these embryos. This could also be accomplished by intercrossing heterozygous RASA1 R780Q mice with mice with mutations in the MAPK pathway, to generate *Rasa1*$^{R780Q/R780Q}$ embryos with heterozygous loss of MAPK signaling. The embryos could then be analyzed to determine if a reduction in MAPK signaling allows for correct generation of the cardiovascular system.

Overexpression and knockouts of Ras result in lymphatic hyper- and hypoplasia, respectively, with no apparent cardiovascular defects [89]. This could possibly be explained by recent work showing that RASA1's regulation of VEGFR3 signaling is dependent on localization through interactions with Ephrin receptors [95, 96]. Ephrin signaling is known to regulate both angiogenesis and lymphangiogenesis via VEGF signaling. One hypothesis could be that the lymphatic system is more sensitive to aberrant Ras signaling than the blood vascular system. In the overexpressed Ras model, the Ras is not refractory to RasGAP promoted GTP

hydrolysis, so it is possible that cardiovascular system development is correctly regulated by Ephrin receptor recruitment of RASA1, while the lymphatic system is not. This would fit with the fact that in RASA1 null or homozygous R780Q mice, the lack of RASA1 to promote Ras GTP hydrolysis results in uncontrolled Ras signaling in BEC leading to cardiovascular phenotype. This could be tested by impairing RASA1 recruitment by Ephrin receptors in mice overexpressing Ras, in an attempt to induce impaired cardiovascular development.

Expression of only *Rasa1 R780Q* in adult mice results in lymphatic hyperplasia and leakage, similar to that of the *Rasa1*$^{fl/fl}$ *ert2Cre* mice [38]. Interestingly, while some lymphatic vessels expressed elevated pERK, the majority of blood vessels in these mice expressed pERK, despite the fact that there was no spontaneous cardiovascular phenotype noted. This is consistent with the activated MAPK seen in both RASA1 null and homozygous R780Q embryos. It is possible that in adult mice, the blood vessels are stable and the increased Ras signaling is not detrimental to vessel structure and function. However, in *Rasa1*$^{fl/fl}$ *ert2Cre* mice, isolated LEC have aberrant Ras signaling responses downstream of VEGFR3, including a second wave of activation that is not seen in LEC isolated from littermate controls. This suggests that the lymphatic vessels are experiencing dysregulated Ras signaling through the MAPK pathway. While it is difficult to find LEC in *Rasa1*$^{R780Q/fl}$ *ert2Cre* mice expressing pERK, Ras signaling could be analyzed by Western blot. Isolation of *Rasa1*$^{R780Q/fl}$ *ert2Cre* LEC, stimulation with VEGF-C, and probing for pERK, such as was done with the *Rasa1*$^{fl/fl}$ *ert2Cre* mice, would show if the loss of RASA1's ability to promote Ras GTP hydrolysis results in the same aberrant pattern of activation.

Recent work in the laboratory has resulted in a hypothesis for the lack of obvious pERK signaling in the entirety of lymphatic vessels. Close inspection of the structure of the lymphatic

vessels in both *Rasa1$^{fl/fl}$ ert2Cre* and *Rasa1$^{R780Q/fl}$ ert2Cre* mice shows that the lymphatic vessel valves in these mice are disorganized. Thus, we hypothesize that loss of RASA1 in these valve cells results in aberrant Ras signaling that results in loss of valve function and development. Valve cells express high levels of VEGFR3, and we have shown that mice without RASA1 have aberrant Ras activation downstream of VEGFR3. Overexpression of the VEGFR3 ligand VEGF-C in mice results in chylothorax, suggesting these mice have impaired valve function [48]. We predict that RASA1 regulation of Ras signaling through VEGFR3 is essential for valve maintenance. In order to elucidate the function of RASA1 in valve cell maintenance, inhibitors of Ras effector molecules can be utilized to rescue the valve disruption phenotype in either *Rasa1$^{fl/fl}$ ert2Cre* of *Rasa1$^{R780Q/fl}$ ert2Cre* mice. These mice could also be intercrossed with mice with impairments in MAPK and PI3K pathways, to limit Ras signaling in an attempt to rescue valve maintenance. The importance of regulation of Ras signaling specifically in valve cells could explain why lymphatic vessels in adult mice are seemingly more sensitive to aberrant Ras signaling than blood vessels in RASA1 and RASA1 GAP deficient mice.

Recent attempts in the laboratory to inhibit the lymphatic phenotype in induced *Rasa1$^{fl/fl}$ ert2Cre* mice with inhibitors of AKT and Erk have been unsuccessful (PEL, personal communication). This is possibly due to the dosage used in the experiment, which can be avoided by use of mice with loss of these Ras effectors. Another possibility is that there is a different Ras effector that is essential for the development of the lymphatic hyperplasia and leakage. Other potential Ras downstream effectors include the Ras-like (Ral) GTPase proteins RalA and RalB [97]. RalA and RalB are activated by RalGEFs, which contain Ras-association domains by which Ras and other Ras-family members can bind and activate them. Ral activation is involved in cell proliferation, including anchorage-independent proliferation in cancers, along

with vesicle transport and cytoskeletal organization [98]. In order to investigate the role of Ral activation in the *Rasa1*$^{fl/fl}$ *ert2Cre* lymphatic vasculature, lymphatic endothelial cells can be isolated and stimulated with VEGF-C, and GTP-bound Ral can be detected by pull-down and Western blot [99]. Further work could then include inhibition of Ral or Ral downstream effectors to determine effect on the lymphatic phenotype, or generation of RalA and RalB conditional mice could be used to remove Ral signaling in the lymphatic system.

In Chapter 4, we show that while single loss of the RasGAPs RASA1 and NF1 in mice result in subtle T cell phenotypes, concurrent loss of both RasGAPs specifically in the T cell compartment results in T cell acute lymphoblastic leukemia/lymphoma (T-ALL). Any perturbations in thymocyte subpopulation cell number or percentages is the same as the single knockout of one RasGAP, so any changes are not due to the additional loss of another RasGAP. Instead, over half the mice spontaneously develop T-ALL, which is not seen in either single knockout mouse.

The T-ALL that occur in these mice all have mutations in Notch1. These mutations vary, but all the T-ALL have at least one strongly activating mutation that would be sufficient to drive T-ALL. Due to the nature of the PEST domain mutations, we can conclude that these T-ALL are clonal, as the short insertions and/or deletions occur within all cells isolated. This suggests that these T-ALL arise from one precursor cell that undergoes mutations of Notch1. However, how these mutations lead to the generation of T-ALL is unclear. As the NT1 mutations are RAG mediated, and *pLCK Cre* transgene activation is required for *Rasa1* and *Nf1* deletion, the T-ALL precursor population must be within the late DN2/early DN3 to DP thymocyte populations.

Analysis of these populations for aberrant Ras signaling showed no differences in strength or duration of signal compared to wild type littermate controls. This could be due to the

level of detection possible from phospho-flow cytometry, or that the subset of cells with overactive Ras signaling is so small that it would be lost within the subpopulations analyzed. In an attempt to address this, thymocyte populations were subjected to apoptotic stresses, in an attempt to identify populations where Ras activation suppresses apoptosis. Again, no significant differences were found between pLCK-DKO and wild type littermate control cell survival. Further work into identifying the precursor population entails screening thymocyte populations for NT1 mutations, to determine if there is a difference in the rate of occurrence in pLCK-DKO and wild type cells, and if so, if this is thymocyte population specific.

As there does not appear to be aberrant Ras activation prior to Notch1 mutations, freshly isolated T-ALL cells were analyzed using phospho-flow cytometry on activated forms of Ras downstream effectors. Interestingly, there was a slight but consistent increase in the strength of Ras signaling through both MAPK and PI3K pathways in these cells compared to littermate controls. However, further work in cell lines generated from pLCK-DKO T-ALL results in variable levels of Ras signaling both at basal levels and after stimulation, whether analyzed by phosphor-flow cytometry or Western blotting. This is consistent with signaling seen in cell lines generated from T-ALL resulting from overexpression of the RasGEF RasGRP1, where different cell lines have various levels of pERK levels [94]. We propose a model where aberrant Ras activation works downstream of the Notch1 mutations in the formation of T-ALL. Further work is needed to elucidate how this signaling synergizes with activated Notch1 to result in clonal expansion of cells. Preliminary experiments to address T-ALL cell line sensitivity to MAPK and PI3K inhibitors are underway. These experiments will also address the question of whether the loss of the RasGAPs is essential for maintenance of the T-ALL or just in the initiation. Inhibition of Ras downstream effectors, singularly or in conjunction, may impair the cell line survival or

proliferation to a greater extent in Ras-dependent T-ALL cell lines as compared to those with no involvement of Ras signaling.

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