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P-21 Activated Kinase 2: Signal Transduction in Mast Cells, Megakaryocytes and in Vivo Homeostasis

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Abstract
Upon immune cell activation with antigen, growth factors, or other stimuli, the cytoskeleton undergoes extensive reorganization to elicit a cellular response. The cytoskeleton, consisting of microtubules and actin, is a highly organized network regulated by various signal transduction pathways. Specifically, Rho GTPases (RhoA, Rac1 and Cdc42) regulate the cytoskeleton, albeit through different pathways. p21-activated kinases (Pak) are serine/threonine kinases directly bound and activated by Rac1 and Cdc42. There are 6 Pak isoforms separated into 2 groups (groups I&II) in this family of kinases, and only recently have isoform specificities been identified by the use of genetically-engineered mouse models deleted for individual isoforms. In this dissertation we sought to identify if differences exist between Pak1 and Pak2 in immune function, in particular how they differ in regulation of the cytoskeleton reorganization required for immune cell function. Using primary bone marrow derived mast cells, an immune cell type responsible for anaphylaxis and allergic responses, we identified that Pak1 and Pak2 function in opposing manners with regard to antigen-induced degranulation. We identified key mechanisms involved in Pak2's negative regulation of mast cell degranulation. These findings identify potential therapeutic side effects with the use of recently developed pan-Pak inhibitors in the clinic. Pak2 deletion was additionally investigated in an in vivo mouse model. We discovered that Pak2 is critical for homeostasis and survival in an adult animal. We identified macrothrombocytopenia, cause by an increase in circulating platelet half-life and clearance, as well as other defects in Pak2-deleted adult mice. Therefore, we evaluated the maturation process of the platelet-producing megakaryocyte and found that Pak2-null megakaryocytes have altered microtubules, proplatelet extensions and polyploidization. Various signaling pathways that regulate these functions were also suppressed with Pak2 deletion. Together, our findings identify Pak2 as the predominant isoform in hematopoietic compartment and immune cells, and suggest further analysis of critical immune cell side effects, which could occur in the patient with the use of pan-Pak inhibitors in the treatment of various cancers.

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Dedication

To my father, who taught me the wonders and joy of science exploration.

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ABSTRACT

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Upon immune cell activation with antigen, growth factors, or other stimuli, the cytoskeleton undergoes extensive reorganization to elicit a cellular response. The cytoskeleton, consisting of microtubules and actin, is a highly organized network regulated by various signal transduction pathways. Specifically, Rho GTPases (RhoA, Rac1 and Cdc42) regulate the cytoskeleton, albeit through different pathways. p21-activated kinases (Pak) are serine/threonine kinases directly bound and activated by Rac1 and Cdc42. There are 6 Pak isoforms separated into 2 groups (groups I&II) in this family of kinases, and only recently have isoform specificities been identified by the use of genetically-engineered mouse models deleted for individual isoforms. In this dissertation we sought to identify if differences exist between Pak1 and Pak2 in immune function, in particular how they differ in regulation of the cytoskeleton reorganization required for immune cell function. Using primary bone marrow derived mast cells, an immune cell type responsible for anaphylaxis and allergic responses, we identified that Pak1 and Pak2 function in opposing manners with regard to antigen-induced degranulation. We identified key mechanisms involved in *Pak2*'s negative regulation of mast cell degranulation. These findings identify potential therapeutic side effects with the use of recently developed pan-Pak inhibitors in the clinic. *Pak2* deletion was additionally

investigated in an *in vivo* mouse model. We discovered that *Pak2* is critical for homeostasis and survival in an adult animal. We identified macrothrombocytopenia, cause by an increase in circulating platelet half-life and clearance, as well as other defects in *Pak2*-deleted adult mice. Therefore, we evaluated the maturation process of the platelet-producing megakaryocyte and found that *Pak2*-null megakaryocytes have altered microtubules, proplatelet extensions and polyploidization. Various signaling pathways that regulate these functions were also suppressed with *Pak2* deletion. Together, our findings identify Pak2 as the predominant isoform in hematopoietic compartment and immune cells, and suggest further analysis of critical immune cell side effects, which could occur in the patient with the use of pan-Pak inhibitors in the treatment of various cancers.

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1.1 p21-activated kinases 1 and 2

Protein kinases function by phosphorylating effector proteins at specific sites containing serine, threonine and/or tyrosine amino acid residues. This post-translational modification functions to control subcellular protein localization, tag proteins for degradation or stabilization, assemble multi-protein complexes, and regulate biochemical activities (e.g. activation or repression of an enzyme and transcription factor). Through these processes, kinases regulate a wide array of fundamental cellular activities, including proliferation, migration, differentiation and survival. These processes are frequently dysregulated in cancer. Kinases are among the most common drivers of tumorigenesis, making them suitable targets for cancer therapies. In addition to oncology, kinases are targets of therapeutics in inflammation, diabetes, and neurodegenerative diseases. Knowledge of a kinase's physiological cellular function in disease and normal states is important when choosing a kinase as a therapeutic target.

Challenges facing researchers in this field include knowledge of temporal and spatial requirements for kinase function, the in vivo conditions required for kinase functionality and how that differs among cell types. Additionally critical is the identification of downstream effectors to drive the myriad of physiological changes induced by kinases.

In this dissertation, I focus on the role of two isoforms of p21-activated kinase (Pak), Pak1 and Pak2, in mast cell function, megakaryocyte biology and describe differences observed in a genetically-deficient animal model. Pak proteins are serine/threonine kinases that are activated by small 21-kD GTPases, Rac1 and Cdc42.

This family of kinases has a diverse set of cellular activities, including proliferation, survival and apoptosis and cytoskeleton-related functions such as focal adhesions, contractility, directional motility and polarity.[1] Pak1 and Pak4 are drivers of many common cancer types, and therefore therapeutics to inhibit kinase function are under development.

Until the widespread use of silencing techniques with isoform-specific siRNAs, most assays to identify Pak substrates utilized kinase-dead, mutant, or loss-of-function Pak proteins. These techniques may not be appropriate to identify isoform-specific functions since kinase-dead mutants act in a dominant-negative fashion by associating nonproductively with Pak substrates. Pak1 and Pak2 share virtually identical *in vitro* substrate specificity, as assessed by peptide arrays to identify consensus phosphorylation motifs.[2] The amino acid sequence surrounding the phosphoacceptor amino acid is one critical determinant of kinase specificity. The consensus substrate amino acid motif for Group I Paks is RRRRRSWYFG, where the serine (S) is the kinase target site. Group I Paks prefer large hydrophobic residues after the phosphoacceptor serine (+1- to +3-positions). Interestingly, Group II Paks consensus sequence is RRRRRSWASP, similar to Group I with the series of arginines (R) prior to serine, but differ after the phosphorylated serine. Group II Paks prefer alanine at +2 and another serine at +3 position, as opposed to the hydrophobic residues preferred by Group I. Identification of these phosphorylation motifs is important for identification of novel substrates. Additionally, factors that influence substrate phosphorylation include surface accessibility of the target site, other interaction sites between the kinase and substrate,

proper kinase docking sites on the substrate, and kinase and substrate localization and expression within the cell.

Since substrate sequence motifs for Pak1 and Pak2 are identical, it is likely the use of dominant-negative mutants in cell-based assays make isoform substrate specificity difficult to identify. Furthermore, protein overexpression vectors can override endogenous isoform specificity.[3,4] The optimal tool to study isoform specificity is the use of primary cells from genetically engineered mouse models. In this dissertation, I provide novel evidence of isoform specificity of Pak1 and Pak2 in hematopoietic-derived primary cell culture of mast cells and megakaryocytes.

Pak1, Pak2 and Pak3 make up Group I Pak kinases. These proteins are highly homologous (Appendix 1), but differ in their transcriptional regulation and tissue distribution.[5] Pak1 is expressed mainly in cardiac tissue, brain and spleen, whereas Pak2 is ubiquitously expressed and Pak3 is expressed in brain tissue.[6] *Pak1*- and *Pak3*-deficient mice are viable adults; however a double-knockout of *Pak1/3* demonstrates a strong neurological phenotype, providing evidence that these isoforms are functionally redundant in some tissue types.[7] Soon after birth these DKO mice succumb to major brain volume loss, despite normal brain organization, implicating a role for *Pak1/3* in coordinating neuronal complexity and neuronal synapses. These DKO *Pak1/3* mice also display learning and memory defects, along with hyperactivity behavior, possibly due to shortened dendrite/axon length and limited branching. *Pak2* deletion *in vivo* results in embryonic lethality by day 9.5, due to improper formation and disruption to the vasculature. (Radu, M and Chernoff, J manuscript in preparation, 2014)

In this dissertation, I elucidate the function of Pak2, relative to the highly homologous isoform Pak1, in order to identify differences among bone marrow derived cells, mast cells and megakaryocytes (Chapter 2 and 3, respectively). Finally, in chapter 4, I will describe the phenotype observed upon *in vivo* deletion of Pak2 in an adult mouse model.

1.2 Pak1

Pak1 kinase is located on chromosome 11q13.5, and is amplified or overexpressed in a variety of tumor types, leading to anchorage-independent growth and tumor progression.[1] *Pak1*-deficient mice are viable and fertile with a normal lifespan, lacking any obvert phenotype.[6] Recent reports have identified a subtle cardiac phenotype under pressure overload conditions, and we found a mast cell phenotype downstream of neurofibromin and Ras activation.[8-10]

Pak1 protein is highly expressed in all regions of the heart, whereas Pak2 and Pak3 are expressed at low levels.[11] Pak1 regulates SERCA2, the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase type 2, through transcriptional regulation.[12] Impaired SERCA2 expression is associated with heart failure caused by ventricular arrhythmias. A cardiomyocyte-specific Cre-recombinase to drive $Pak1^{f/f}$ deletion, causes cardiac hypertrophy during either acute or chronic β-adrenergic stress. In culture, *Pak1*-null cardiomyocytes demonstrated aberrant Ca^{2+}-homeostasis.

Another observed phenotype in adult *Pak1*-deficient mice is decreased mast cell degranulation in response to allergen stimulation. Mast cell studies evaluating signal transduction downstream of the c-Kit receptor and FcεRI receptor demonstrate that Pak1 functions as a positive regulator. *Pak1*-null mice administered stem cell factor (SCF) to

stimulate the c-kit receptor recruited fewer mast cells to the dermis. Additionally, *Pak1*-null mice evaluated for systemic and cutaneous anaphylaxis with allergen mounted a suppressed mast cell response.[9,10,13] Consistent with *in vivo* results, *in vitro* culture of *Pak1*-null mast cells derived from bone marrow had decreased antigen induced-degranulation and decreased SCF-mediated migration. Reduced degranulation in *Pak1*-deficient mast cells was attributed to impaired extracellular calcium mobilization and persistent cortical F-actin polymerization, leading to reduced antigen-induced degranulation. Pak1 was further demonstrated as a positive regulator of mast cell secretion by its phosphorylation and activation of the phosphatase PP2A. Activated PP2A dephosphorylates the ERM protein complex (Ezrin/Radixin/Moesin), leading to an uncoupling of the actin network from the cytoskeleton, a key event in mast cell degranulation.[13] The persistence of cortical F-actin in *Pak1*$^{-/-}$ mast cells partially explains the reduced antigen-induced calcium influx. Actin depolymerization is required for sensing of calcium depletion in the ER, and localization of the ER to the plasma membrane.[14,15] Considering what is known about Pak1 in mast cells, we were curious what role, if any, Pak2 played in mast cell degranulation. These data would help delineate if pan-Pak inhibition with Group I specific inhibitors could be a viable therapeutic for anaphylaxis and other mast cell hyperactivity disorders.

1.3 Pak2

Unlike Pak1, studies involving Pak2 function *in vivo* have been challenging due to embryonic lethality at day 9.5 caused by severe cardiovascular abnormalities.[16] Pak2 is ubiquitiously expressed, unlike the tissue specific expression of Pak1. Our lab generated a *Pak2*-floxed animal, in which Pak2 is conditionally deleted by crossing these

mice with an inducible cre-recombinase, such as the hematopoietic-specific Mx1-cre, in order to study changes to the bone marrow upon Pak2 deletion in an adult. Chapter 3 and Chapter 4 of this dissertation address observations in this animal model.

Although *in vitro* Pak1 and Pak2 share many properties and sequence identity, deeper examination of these isoforms is beginning to reveal differences in their function. The research in this dissertation delves into isoform differences in primary bone marrow-derived mast cells and megakaryocytes, as well as describes changes in adult animals. Unlike Pak1, Pak2 has no clear role in tumorigenesis, but instead is critical for embryonic development, vascular integrity and barrier function.[1,17] Pak1 and Pak2 share 93% sequence identity in their kinase domain and 97% sequence identity in their regulatory domain. Despite these similarities, recent findings suggest that they may function through different signaling cascades.[18,19]

A primary function of Pak kinases is regulation of cell migration and invasion, properties essential to cancer metastasis. The first study to comprehensively look at both Pak1 and Pak2 isoform differences measured differences in invasion of breast cancer cells and found their signaling pathways differed widely.[18] Silencing of Pak1 resulted in reduction of lamellipodia formation, necessary for migration and invasion of cancer cells. Pak1 regulates lamellipodia formation by inhibition of actin-severing function of cofilin. Silencing of Pak2 had no effect on lamellipodia formation. The authors continued on to discover an opposing role for Pak1 and Pak2 in phosphorylating myosin light chain 2 (MLC2), a protein downstream of RhoA known to mediate myosin II assembly and actomyosin contractility, both required for focal adhesion formation. Downregulation of Pak1 resulted in reduced MLC2 phosphorylation and reduced focal adhesions, whereas

inhibition of Pak2 resulted in elevated MLC2 phosphorylation and increased focal adhesions.

An independent study found that silencing Pak2 in hepatocellular carincoma (HCC) cell lines caused increased focal adhesion formation, and decreased migration in response to TGF-beta activation.[20] Clinical HCC samples had elevated phosphorylated (activated) Pak2, which correlated with increased tumor progression, metastasis and reoccurrence. Elevated Pak2 activity decreased focal adhesions and increased migratory properties. The precise mechanism by which Paks regulate focal adhesion formation in a precise temporal-spatial fashion during cell motility, however, is poorly understood.

In the above-mentioned study by Coniglio et al., they additionally found that Pak2, but not Pak1 regulates RhoA GTPase activity, by an unidentified mechanism.[18] These data begin to describe signaling cascade differences between Pak1 and Pak2. This dissertation will evaluate if these signaling differences are found in primary bone-marrow derived immune cells from genetically-deleted animals. Since the work published by Coniglio et al., other groups have identified substrate differences between Pak1 and Pak2, including a study by Chu et al. evaluating Pak's function in intestinal smooth muscle contractility.[21] Under physiological conditions, Pak1, but not Pak2 phosphorylated and inactivated the phosphatase MYPT1, promoting MLC2 phosphorylation to control smooth muscle contractility. However, under pathological conditions, Pak1 levels were elevated, but no longer able to phosphorylate and inactivate MYPT1, resulting in inhibiting MLC2, reduced contractility and exacerbated disease.

These studies together demonstrate that Pak1 and Pak2 signal through different mechanisms to effect MLC2 phosphorylation and subsequent action on cellular

contractility. These pathways are described in figure 1.3. This thesis will investigate some of these differences in primary immune cell function, rather than adherent cell lines.

Since the construction of a *Pak2*-floxed animal, several groups, in collaboration with the Chernoff laboratory, have studied the effect of this kinase on immune and hematopoietic function. Dorrance et al. was the first to study Pak2 inhibition in hematopoietic stem cell engraftment.[22] They found that Pak2, but not Pak1 or Pak3, was required for proper stem cell proliferation and homing to the bone marrow niche, through the ERK signaling cascade. Phee et al. found that Pak2, but not Pak1, was required for T-cell development and maturation, as well as actin remodeling required for T-cell receptor activation.[23] As more researchers investigate primary cells from *Pak2*-floxed animals, we will continue to find unique features of the Pak2 isoform. These findings are critical to understanding the effects of inhibition of each Group I Pak isoform, as current small molecular inhibitors inhibit the entire group without discretion to isoform specificity.

1.4 Mechanisms of Activation of Pak 1-3 (Group I)

Activation of Group I Paks is primarily achieved through binding of small GTPases, Rac1 and Cdc42, which relieves *trans* autoinhibition and induce conformation changes to activate the catalytic domain. There are alternative mechanisms of activation including the binding of sphingolipids, phosphoinositides, SH3-domain containing proteins NCK, GRB2, and transphosphorylation by other kinases (i.e. PDK-1, PKA, CK2, Akt).[24-31] These can function to activate Paks in the absence of active small GTPases. Additionally, Pak1 can be activated by exposure to EspG, a virulence effector

protein from enterohaemorrhagic *E coli* O157:H7. EspG was shown to bind to an alternative region of the regulatory domain, releasing the homodimers, resulting in monomeric activation of Pak.[32,33]

The N-terminus of Group I Pak proteins contain an autoregulatory fragment, consisting of Rac1/Cdc42 binding domain (PBD), dimerization fragment (DI), inhibitor switch domain (IS), and the kinase inhibitory fragment (KI). These fragments all interact to stabilize Pak in the inactive confirmation. Following binding of Rac1 and Cdc42, Pak undergoes conformational changes resulting in activation of the catalytic domain, uncoupling of the homodimers, (auto)phosphorylation of Pak (Thr-423), and stabilization of the monomeric active conformation (Figure 1.1).

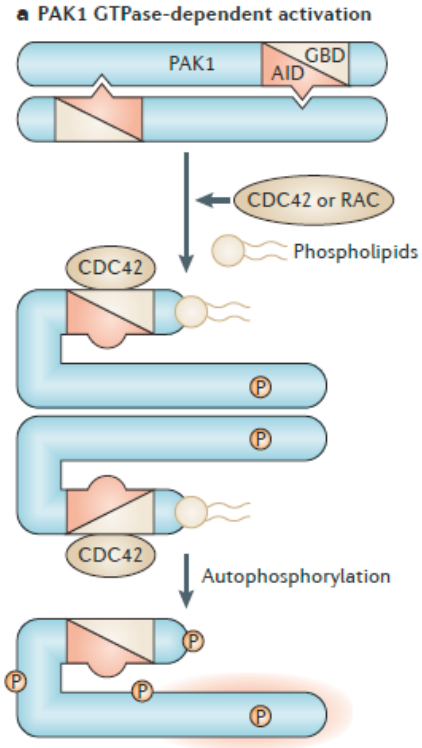

Figure 1.1 : Schematic representation of Pak1-3 activation upon Rac1 and Cdc42 binding. Binding of Rac1/Cdc42, along with other mechanisms described in the text, relieves Pak autoinhibition. Pak is fully functional upon (auto) phosphorylation at multiple sites, which stabilizes the catalytic domain, resulting in an active monomer.
Figure adapted from Radu et al. [1]

1.5 Downstream Effectors of Pak

Catalytically active Pak activates or inactivates numerous signaling pathways critical for proper cellular function. These include proliferation, apoptosis, survival, as well as cytoskeletal-intense pathways to drive migration, adhesion, and contractility. Of all the signaling pathways identified, the most well established cascade to regulate proliferation and survival involves direct phosphorylation of Mek-1 and Raf-1 by Pak1 to drive the ERK/MAPK signaling pathway.[34,35] Additionally, Pak drives substrate phosphorylation of both actin- and microtubule-associated proteins to regulate actin and microtubule dynamics. Pak regulates actin polymerization by directly phosphorylating LIMK, which inhibits the actin-severing protein, cofilin, leading to actin stabilization.[36-39] Additional mechanisms of actin regulation include phosphorylation of myosin light chain (MLC), dynein light chain 1 (DLC1), and filamin A.[36,40]

Regulation of microtubule (MT) dynamics is also a prominent feature of Pak kinases. There are multiple mechanisms by which Paks affect MT dynamics. These include phosphorylation and inactivation of the microtubule destabilizing protein, Op18/stathmin, to promote MT polymerization[41] and phosphorylation of Aurora kinase at key threonine and serine sites required for mitosis and bipolar spindle assembly.[42] Aurora A regulates MT assembly through numerous signaling cascades leading to polarized MTs for mitosis, cell motility and other cellular functions. Aurora kinases regulate MT by phosphorylating and inhibiting Op18/stathmin, leading to inhibition of microtubule depolymerization and by downregulating MCAK (mitotic centromere-associated

kinesin), a microtubule associated protein which functions to promote MT disassembly.[43,44]

This dissertation will identify downstream signaling cascades regulated by Pak2 to elicit cellular responses from mast cells and megakaryocytes. Specifically, I evaluated FcεRI-mediated signaling in mast cells, and TPO-driven signaling cascades in megakaryocytes required for maturation and polyploidization. Our findings indicate that Pak2 functions in these two disparate cell types through different pathways to elicit a response. It is likely that Pak2 is active in most immune cells, based on our research and the research of our collaborators.

1.6 Pak activation in inflammation

An inflammatory response in most immune cells requires remodeling of the cytoskeleton to respond to stimuli; therefore, it is of no surprise that Pak isoforms are required for immune cell function and inflammation. A number of reviews have addressed this in recent years.[45,46] In this section I will address mechanisms by which Paks regulate inflammation, several key signaling pathways involved, and how Paks regulate T-cells and mast cells.

A number of inflammatory extracellular stimuli activate Paks, these include chemokines such as CXCL1, interleukins such as IL-1β and T cell receptor ligation.[47-49] For these stimuli to elicit an immune response, signaling cascades leading to the MAPK pathway, along with JAK pathway are often elicited.[50] The use of MAPK inhibitors are emerging as an attractive strategy against chronic inflammation as they are capable of reducing both the synthesis of pro-inflammatory cytokines and their signaling cascades.[50] Considering that key functions of PAKs are to regulate the MAP kinases ERK, JNK,

and/or p38 pathways in response to inflammatory stimuli, Pak inhibition is being considered for regulation of inflammatory disorders.[51]

Pak kinases are key nodes in inflammatory signaling, by regulating reactive oxygen species, chemokine signaling, NADPH oxidase activity and the cytoskeleton. Pak kinase regulation of the actin cytoskeleton plays a key role in inflammatory signaling among most immune cell types. Maintaining control of Pak kinases are critical for immune regulation. Therefore, therapeutic intervention of Paks is most likely a critical component of controlling inflammation, through Paks control and regulation of multiple signaling pathways.

A well-known inflammatory signaling regulator, NF-κB, has been linked to Pak in several studies.[52-55] In both canonical (TNFα -activated) and non-canonical (LPS-activated) NF-κB signaling, Pak1 is activated and signals to stimulate nuclear translocation of the p65 subunit of NF-κB, but does not activate IKKα or IKKβ.[54] In canonical NF-κB signaling, Pak activates c-jun kinase (JNK) and in non-canonical signaling, Pak phosphorylates NF-κB -interacting kinase (NIK). Non-canonical *H. pylori*-induced LPS activation of NF-κB signaling demonstrates the connection between Pak kinases and activation of innate immunity.[52] Activation of this pathway, through Pak, results in release of proinflammatory cytokines, such as IL-1β, TNFα and IL-6 from macrophages.[52] Pak1 also drives M1 macrophage polarization and activation in response to endotoxin shock with LPS.[56] LPS is a potent inducer of Pak1 in macrophages. Inhibition of Pak1 in LPS-stimulated macrophages leads to impaired NF-κB activation and TNFα transcription, as well as blunting of M1 macrophage polarization to protect cells from endotoxin shock.[54,56] Interestingly, deletion of Pak1 solely in myeloid-derived

macrophages ($Pak1^{Lyz2cre}$), as well as pharmacologic inhibition of group I Paks protected mice from LPS-induced sepsis. This suggests that therapeutically targeting Pak in the inflammatory pathway has clinical potential.

1.7 Pak function in T-cell receptor activation

T lymphocytes develop in the thymus and undergo selection to generate mature T cells that express functional and self-tolerant T cell receptors (TCR). The most immature double negative thymocytes mature into double positive CD4/CD8 T cell. Following TCR alpha-chain rearrangement, the double positive cell undergoes positive selection to recognize self peptide-major histocompatibility complex (pMHC) proteins. Some double positive thymocytes undergo positive selection to mature into single positive $CD4^+$ and $CD8^+$ cells.

T cell activation, following engagement of the TCR, involves the coordinated activities of a diverse set of intracellular signaling pathways, including mobilization of calcium, and activation of Ras and Rho family GTPases, which result in the triggering of the MAPK pathway. Activated T cells express a variety of surface activation markers and produce cytokines, such as IL-2, to elicit a particular immune response.

Pak1 and Pak2 are activated upon TCR engagement, which leads to T-cell proliferation and differentiation.[48,57,58] The T-cell immunological synapse is the site where the T-cell engages with antigen on the antigen-presenting cell (APC). Many kinases and adapter molecules are quickly recruited to the synapse to initiate a signaling cascade. One of these kinases is Pak1, which becomes catalytically activated upon TCR engagement.[48,49] TCR engagement creates a signaling complex involving phosphorylated membrane protein LAT, adapter protein SLP-76, Rac1-GTPase activator Vav, and

adapter protein Nck which recruits actin remodeling proteins. However, there is evidence that activation of Pak is independent of this complex. Pak1 is activated in *LAT*-deficient T cells, providing evidence of alternative activation pathway.[49] Downstream of TCR activation, Pak1 was reported to be activated, and dependent on the tyrosine kinases ZAP70, and Syk, but not the cytoplasmic adaptor SLP-76.[48,49] Pak is recruited to the site of TCR stimulation by formation of a trimolecular complex (Pak-PIX-GIT) consisting of PIX (GEF for Rac1 and Pak binding partner) and GIT (ADP-ribosylation factor GTPase-activating protein (Arf-GAP)) to drive a cellular response to TCR engagement.[59]

The functions of Pak2 in T-cell signaling are now being investigated, with the creation of an inducible *Pak2*-floxed mouse model recently developed by the Chernoff lab. Prior to development of this mouse model, studies were only done with dominant-negative or kinase-dead forms of Pak2. A screen for novel effectors downstream of TCR activation found Pak2 activated upon TCR stimulation.[58] Expression of a kinase-inactive form of Pak2 decreased upregulation of CD69 and impaired NFAT activation in Jurkat T-cells. In primary T cells transfected with a dominant-negative form of Pak2, cells were impaired in anti-CD3/CD28-induced production of IL-2. Additionally, inhibition of Pak2 impaired TCR-induced CD40 ligand expression. These two functions are key to the activation of the T cell receptor, and were the first to demonstrate that Pak2 is a positive regulator of T cell activation.[58]

In addition to Pak2's involvement in TCR activation, Phee et al., in collaboration with the Chernoff lab, recently discovered that Pak2 was essential to thymocyte development and maturation.[23] Utilizing a mouse model with T-cell specific deletion for Pak2 at two different developmental stages (*Lck*-Cre and *CD4*-Cre), the authors

discovered that Pak2 deletion, driven by different Cre-recombinases, resulted in severe T-cell lymphopenia, however for different reasons. *Pak2*$^{F/F}$*;Lck*-Cre resulted in severe T-cell lymphopenia, accompanied by T cells which failed to undergo pre-TCR β-selection and positive selection in order to mature into double positive and single positive, CD4 and/or CD8 cells. *Pak2*$^{F/F}$*;CD4*-Cre mice contained a full T-cell repertoire, however CD4^{+} cells from these mice were arrested in a semi-mature stage, unable to increase expression of CD62L and integrin β7. CD4^{+} T cells from the *Pak2*$^{F/F}$*;CD4*-Cre also failed to proliferate upon CD3 stimulation, and instead apoptosed upon stimulation.[23]

In the absence of Pak2, TCR-stimulated T cells failed to activate ERK1/2 and PLCγ1 (phospholipase gamma-1), and failed to remodel the actin cytoskeleton and spread.[23] These data suggest that without Pak2, T-cells fail to remodel the cytoskeleton, which is essential to transduce signals upon TCR-stimulation. Inefficient activation of ERK1/2 and PLCγ1 can also lead to impaired positive selection, by disrupting the avidity and affinity of the TCR/MHC interaction.[60]

Adaptive and innate immune cells respond to stimuli through a variety of receptor types, and these receptors signal through broadly similar downstream pathways to elicit immune function. T cells respond through the TCR and mast cells through the Fc-epsilon receptor (FcεRI). Mast cell receptor stimulation depends on bound antibody (IgE) to elicit a response. Mast cells are responsible for the immediate hypersensitivity response when the cognate antigen is presented to bound IgE molecules. Antigen receptors on both T-cells and mast cells signal through a similar mechanism, in which an initial tyrosine kinase phosphorylation cascade forms (heavily phosphorylated transmembrane adapter LAT) to phosphorylate and activate PLCγ1 (phospholipase gamma-1). T cells and mast

cells share common adapter proteins, LAT, Gads, and SLP-76 to form a heterotrimeric complex in order to phosphorylate PLCγ1. Considering the common features of mast cells and T-cell receptor signaling and the extensive work done on the role of Paks in T-cells, this dissertation embarks on discovering the distinct roles for Pak1 and Pak2 in mast cells (Chapter 2).

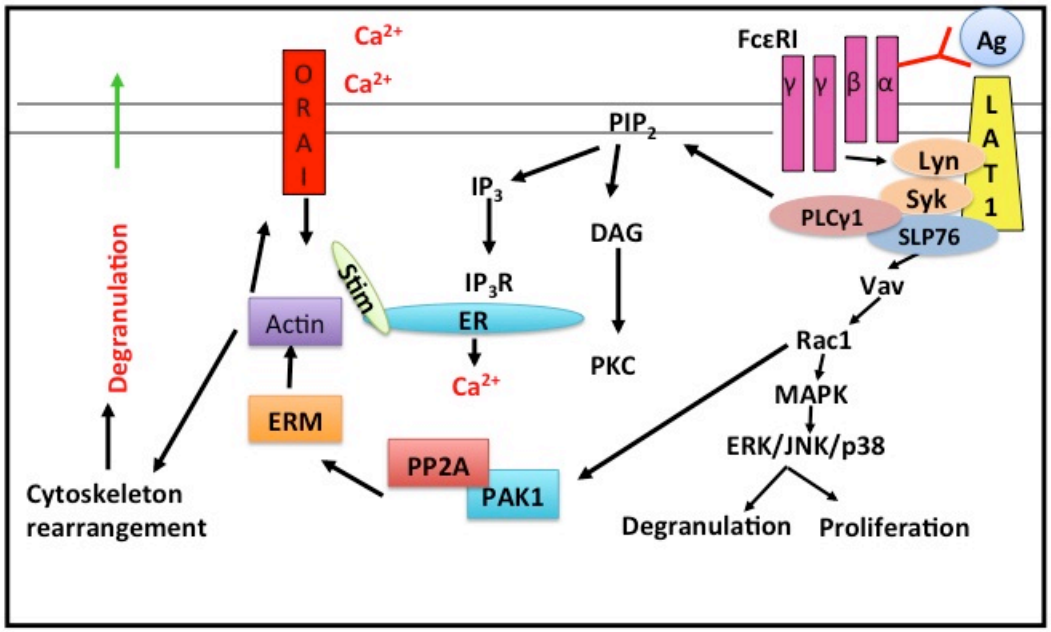

Figure 1.2 Mast cell FcεRI signaling cascade

Antigen bound IgE crosslinks the FcεRI to initiate a signaling cascade in mast cells to drive secretion of pre-formed granules and de novo synthesized cytokines. The cascade is described in the text below.

1.8 Mast cells

Mast cells are long-lived, tissue-resident innate immune cells, derived from hematopoietic precursors, which complete their differentiation in almost all vascularized tissues. Predominantly, mast cells reside in tissues most exposed to the environment, such as lung, skin, and intestines. These cells initiate inflammation upon antigen stimulation

by secreting proinflammatory mediators, such as histamine and hydrolytic enzymes. They also signal to other innate immune cells, and help shape adaptive immunity through MHC-I, CD28/80, complement receptors, and FcγII/III receptors. Mast cells respond to antigen via their FcεRI receptor bound to antigen specific IgE. As diagrammed in Figure 1.2, upon recognition of cognate antigen, FcεRI aggregate and crosslink, eliciting a signaling cascade downstream of the ITAM (immunoreceptor tyrosine-based activation motif).[61] Multiple tyrosine phosphorylation events on ITAM recruit in key signaling molecules to assemble the LAT adapter-signaling complex. This is followed by activation of PLCγ1, cleavage of membrane bound PIP2 and production of two secondary messengers, IP3 (inositol triphosphate) and DAG (diacyl glycerol). IP3 binds its receptor on the endoplasmic reticulum (ER), releasing internal calcium stores. Upon depletion of internal calcium stores, STIM1 on the ER senses this depletion and translocates to the cell periphery, associates with the $Ca2^{+}$ release–activated $Ca2^{+}$ (CRAC) channel and opens up the calcium channel to allow influx of extracellular calcium.[14] It was recently discovered that *Pak1*$^{-/-}$ mast cells were deficient in their ability to mobilize extracellular calcium in response to antigen stimulation, possibly due to the persistence of cortical F-actin, preventing movement of the ER to the plasma membrane.[10] *Pak1*$^{-/-}$ mast cells are also defective in mast cell antigen-induced degranulation, *in vitro* and *in vivo*.

Cytoskeleton remodeling is required for both phases of calcium mobilization (internal release, and influx of extracellular calcium).[60] Calcium influx drives secretion of biologically active products implicated in allergic reactions. The inflammatory response includes secretion of vasoactive amines (histamine and serotonin), proteases, proteoglycans, cytokines and prostaglandins. It is important to note, that mast cells can be

activated by not only IgE and allergen, but also by physical agents, pathogen products, danger signals, chemokines, cytokines and products of complement activation.[62] These alternative mechanisms of activation function in innate and adaptive immune responses against bacteria and pathogens[63], autoimmunity[64], tolerance induction[65], angiogenesis, and malignant diseases[66]. Considering the extensive immunological role of mast cells, it is imperative to identify complete signal transduction pathways upon mast cell stimulation. The impact of Pak kinases on mast cell signaling cascades has the potential to control myriad of diseases.

1.9 Cytoskeleton remodeling to elicit mast cell degranulation

Regulated exocytosis induced by antigen stimulation requires restructuring of the cortical acto-myosin barrier to allow vesicles to fuse with the plasma membrane and release inflammatory mediators.[67] This barrier prevents granule release in the absence of external stimuli. Remodeling of the acto-myosin cortex is a prerequisite for regulated exocytosis. Disruption of the barrier with the inhibitor, Jasplakinolide, inhibited secretion.[68] Another regulator of the acto-myosin complex is myosin light chain (MLC). Increased p-MLC levels inside the cell enhanced acto-myosin contractile tension and ultimately induced formation of actin stress fibers. The MLCK inhibitors KT5926 and ML-7 impair mast cell degranulation.[69,70] MLC2 regulation by Pak was found to have a significant role in MLC phosphorylation, primarily through indirect regulation. These data are summarized below in Figure 1.3. Considering the prominent role of RhoA GTPase in mast cell secretion, we hypothesize in Chapter 2 that Pak2 negatively regulates MLC2 phosphorylation via RhoA regulation in mast cells to control secretion.

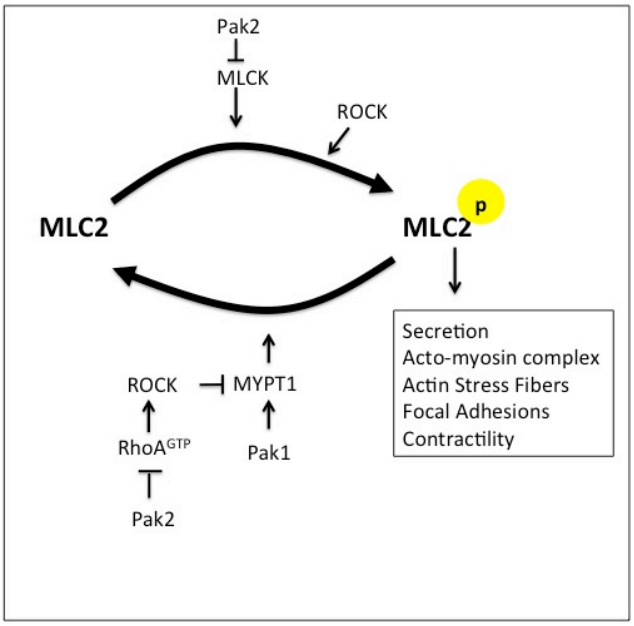

Figure 1.3 Pak1 and Pak2 are negative regulators of MLC2 phosphorylation through multiple mechanisms. Pak inhibits MLC2 phosphorylation by inhibition of an activating kinase (MLCK)[71-73], inhibition of RhoA[18] and activation of the inhibitory phosphatase MYPT1[21,74]. This schematic describes the multiple mechanisms by which Pak kinases are known to negatively regulate MLC2 phosphorylation.

Microtubule formation and actin depolymerization are required for mast cell degranulation, as shown by the use of cytoskeleton specific pharmacologic inhibitors. Inhibition of microtubule polymerization with nocadazole impaired degranulation, and stabilization of microtubules with taxol also impaired degranulation.[75] Actin cytoskeleton dynamics also function to regulate mast cell degranulation. Disassembly of F-actin rings with Latrunculin B promoted mast cell degranulation, where as stabilization of F-actin with Jasplakinolide decreased mast cell degranulation.[75] Cortical F-actin disassembly may function in mast cell degranulation by permitting granules greater access to the plasma membrane. Additionally, cortical F-actin may act as a barrier between the granule reserves and the release-ready granule pool. F-actin disassembly disrupts the actin barrier. In addition to cytoskeletal regulation of mast cell granule release, the entire process of degranulation requires adhesion and spreading onto a substratum, assembly of

F-actin, formation of focal adhesions, and actin- and myosin-containing plaques and filaments on the basal surface.[67]

In summary, cytoskeletal remodeling (disassembly and assembly) is critical for proper mast cell exocytosis. Cortical actin disassembly is required for movement of granules to the plasma membrane (hence a negative regulator, by impeding translocation), and simultaneously F-actin assembly at the plasma membrane is required for proper granule-plasma membrane fusion and release.[76]

These data provide insight into the cytoskeletal regulation, which must occur for proper mast cell degranulation. It is well documented that Pak1 and Pak2 signal to regulate the actin and microtubule network, however, isoform differences using primary cell culture from genetic knockout animals, instead of RNAi and dominant-negative mutants, have not been identified. This dissertation will evaluate isoform redundancies and/or differences and identify how they pertain to physiological responses to stimuli in mast cells.

1.10 Small GTPases and mast cell antigen signaling

Small RhoGTPases specifically Rac, RhoA and Cdc42, regulate various vesicle trafficking and exocytosis events, including calcium mobilization and cytoskeletal (actin) remodeling, both integral to FcεRI-mediated mast cell degranulation.[77-80] In addition to mast cell signaling, Rho GTPases act as central regulators of several vital immune cells functions, including migration, ROS production, cytokine production, and exocytosis. Previous work found that expression of constitutively active Rac1 and Cdc42 stimulated granule exocytosis.[77,78] These GTPases are predominant activators of Pak kinase.

Given that degranulation involves complex cytoskeletal rearrangements, and that Paks regulate filamentous actin (F-actin) dynamics, we investigated a role for Pak2 in allergen-stimulated mast cell degranulation. Previous work established that Pak1 is a positive regulator of mast cell degranulation, driven through association with PP2A phosphatase and dephosphorylation of ERM (Ezrin/ Radixin/Moesin) to depolymerize F-actin. This signaling cascade also regulates the influx of extracellular calcium.[9,10,13] Mice reconstituted with *Pak1*$^{-/-}$ mast cells demonstrated decreased allergen-induced vascular permeability and decreased *in vivo* degranulation in a systemic anaphylaxis experiment.[13] The signaling cascade downstream of Pak1 facilitates F-actin rearrangement, which precedes antigen-mediated degranulation. Considering what is known about the function of Pak1 in mast cell secretion, we sought to determine the role of another Pak isoform, Pak2.

RhoA and mast cells

RhoA activity drives mast cell degranulation, as documented by various researchers nearly 20 years ago.[75,81-83] Constitutively-active (CA) forms of RhoA promote degranulation in mast cell lines, and dominant-negative (DN) RhoA mutants impair secretion. The mechanism by which RhoA functions to regulate secretion is multifaceted. For one, RhoA controls microtubule formation independent of calcium signaling, allowing vesicle translocation and membrane fusion. DN-RhoA inhibited FcεRI-induced microtubule formation, resulting in reduced degranulation without affecting F-actin polymerization. However, the mechanism by which RhoA signals to control microtubule dynamics is not well investigated. In Chapter 2 of this dissertation I

will describe findings detailing the mechanism by which RhoA signals to regulate mast cell degranulation.

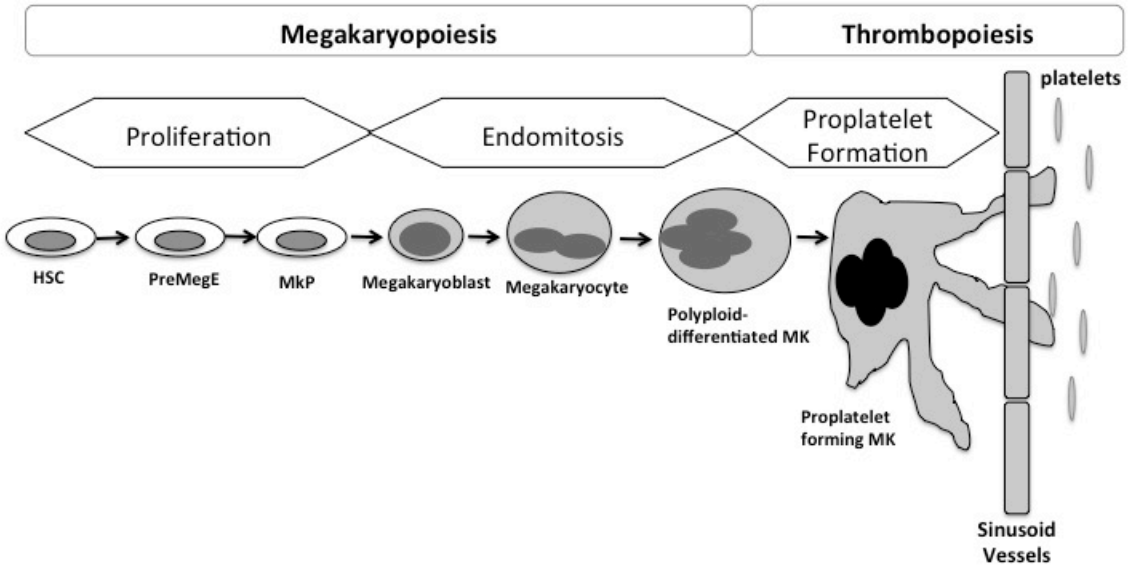

Figure 1.4. Megakaryocyte maturation and endomitosis to produce functioning platelets in circulation. Modified from Severin et al. 2010.[84]

1.11 Megakaryocytes

The megakaryocyte is the least abundant cell type in the bone marrow, yet is challenged with the task to replace platelets in circulation, which are removed at a rate of 1/10 per day. Few other cells encounter this intense biosynthetic requirement. This process requires enormous reserves of membranes, organelles, cytoskeleton and protein synthesis. For this reason, megakaryocytes become hundreds of times larger than the average cell. Megakaryocytes undergo significant increase in their DNA content to provide enough material to synthesize platelets, whereas a typical 2N diploid cell is inadequate to have enough gene expression for the synthesis of thousands of platelets per

megakaryocyte. Considering the function of cytoskeletal proteins in megakaryocytes[85], we sought to investigate Pak2 as a therapeutic target in megakaryocyte disorders. We evaluated the impact of Pak2-deletion in megakaryocyte maturation, endomitosis and platelet biosynthesis using an in vitro and in vivo model system of Pak2-deletion.

Megakaryopoiesis is a complex process whereby committed bone marrow megakaryocyte progenitors undergo terminal differentiation, with thrombopoietin (TPO) and SDF-1α, to form nuclear polyploid megakaryocytes identified by expression of cell surface markers (CD41 and CD61) (Figure 1.4).[86,87] Mature megakaryocytes undergo differentiation by a process termed endomitosis. Endomitosis refers to the process of chromosomal duplication that proceeds without nuclear envelope rupture. As with mitosis, endomitosis begins with duplication of the centrosomes, enters prophase with development of mitotic spindles, chromatin condensation, and rupture of the nuclear membrane, metaphase alignment of the chromosomes, and finally separation of sister chromatids during anaphase. In a polyploid megakaryocyte, the spindle is distinct from a mitotic spindle, as it is multipolar, with the number of poles corresponding to ploidy level. It was recently discovered, through time-lapse microscopy, that endomitosis corresponds to a failure of late cytokinesis.[88,89]

Endomitosis is tightly regulated, such that various mutations can lead to diseases of megakaryocyte development (i.e. acute megakaryocytic leukemia (AMKL)). AMKL consists of bone marrow megakaryoblasts in a hyperproliferative immature state, failing to undergo endomitosis. This disruption in normal megakaryocyte development is deadly, due to myelofibrosis and blast crisis (expansion of immature cells). Therapeutics to drive megakaryocyte differentiation and polyploidization are vital to increasing survival rates

from this disease.[90] Recent work using an extensive screen to identify proteins involved in megakaryocyte differentiation, found that Aurora A and B kinases were negative regulators of leukemic cell expansion, such that inhibition of these kinases led to megakaryocyte differentiation, a possible mechanism for leukemic therapy.[90] The work in this dissertation looks at the multi-functional role that Pak2 has in megakaryocyte development through the endomitotic stage, and its potential as a target for AMKL. After cells complete endomitosis, the mature megakaryocyte enter a terminal differentiation phase, where the cell membrane undergoes extensive remodeling of the cytoskeleton to form cytoskeleton-rich proplatelet extensions that transverse the sinusoid vessel to release platelets into circulation.[85] This process is extremely regulated, and any perturbation to the system can result in thrombocytopenia, bleeding disorders and various types of leukemia. Understanding the pathways involved in this process is critical to identifying therapeutics for types of platelet disorders and megakaryocytic leukemia.

1.12 Cytoskeleton regulation of polyploidization and proplatelet formation

Actin and polyploidization

Megakaryocyte polyploidization is a cytoskeleton intensive cellular process. Identification of this was first done by incubation with cytoskeleton inhibitors. Incubation of cell lines and primary megakaryocytes with an inhibitor of actin polymerization (Cytochalasin B) caused elevated polyploidization without stimulation of expression of CD41 and CD61, mature megakaryocyte markers.[91-93] Therefore, actin polymerization is a negative regulator of megakaryocyte polyploidization. Cytochalasin B inhibits actin polymerization by blocking elongation of polymerizing actin filaments. This blockade induces polyploidization in mammalian cultured cells, Xenopus eggs and yeast.[93] At the

end of mitosis during normal cell division, cytoplasmic separation requires the integrity of cytoskeleton-associated proteins for the formation of the tubulin spindle and actin contractile ring. Inhibition of actin polymerization with cytochalasin B therefore inhibits normal separation of cytoplasm and induces polyploidization.

Microtubules and polyploidization

Microtubules are also involved in the process of polyploidization. This was first identified by the use of pharmacologic inhibitors of MT polymerization. Enhanced microtubule depolymerization in cells treated with colchicine, nocodazole and vincristine had increased levels of polyploidization.[92,94,95] Megakaryocyte cell lines (DAMI and HEL) treated with colchicine, a tubulin spindle inhibitor, increased their endomitotic index and were larger in diameter than untreated cells. Ploidy measurements increased from the predominantly diploid (2N) state to the 32N state with the addition of colchicine. Colchicine treatment also affected actin polymerization by decreasing the monomeric G-actin and polymerized F-actin content significantly, and thereby increased polyploidization by preventing the actin constriction ring during telophase.[94] Nocodazole treatment on megakaryocytes led to progressive accumulation in pseudo-metaphase, without spontaneous escape from this blockade. Nocodazole treatment on a human megakaryocytic leukemia cell line (UT-7) also induced polyploidization by depolymerizing microtubules.[96]

These data indicate that actin and microtubule dynamics are critical to megakaryocyte polyploidization and are potential therapeutic targets for various types of megakaryocytic leukemias to drive differentiation and polyploidization. This dissertation will evaluate the role of the cytoskeletal regulatory protein, Pak2 on megakaryocyte

polyploidization, as a potential therapeutic target for acute megakaryoblastic leukemia, as well as hyperproliferative diseases involving megakaryocytes, such as essential thrombocytosis (ET) and primary myelofibrosis.

1.13 Small GTPases in megakaryocyte development

RhoA and megakaryocyte polyploidization

Nearly 20 years ago, the role of small GTPase proteins in polyploidization was identified in human megakaryocytic cell line, CMK by Takada et al.[91] By using C3 exoenzyme (*Clostridium botulinum*) a RhoA inactivating enzyme, they discovered that cells expressed higher levels of CD41 and CD61, markers of mature megakaryocytes. Further work identified RhoA specifically, and not Rac1 or Cdc42, as the predominant RhoGTPase required for megakaryocyte polyploidization, a process required for maturation and platelet production.

RhoA functions during normal mitosis to drive cytokinesis.[97] RhoA is concentrated at the midzone during anaphase and at the cleavage furrow during telophase of diploid cells. RhoA inhibition prevents cytokinesis since localization of RhoA at the midzone is required for furrow ingression and spindle elongation during cytokinesis.[89] As megakaryocytes become multipolar during endomitosis, RhoA-GTP levels decrease along with decreased F-actin accumulation. RhoA functions in polyploidy megakaryocytes to regulate actin polymerization and myosin activation at the midzone through different effectors. As cells undergo endomitosis (>8N) F-actin accumulation decreases at the cleavage furrow, and RhoA activity is inhibited so that cells do not undergo cytokinesis after DNA replication.

RhoA and F-actin accumulate at the cleavage furrow to drive cytokinesis. RhoA signaling is required to establish the actomyosin ring at the cleavage furrow, generating the contraction forces needed for completion of cytokinesis. The mechanism by which RhoA is inhibited for megakaryocytes to undergo endomitosis was unknown until recent work by Gao et al.[97] They discovered, through various models using RNAi, that RhoA is active during the early development at the 2N stage, but is suppressed at 8N by inhibition of the RhoA specific guanine-nucleotide exchange factors (GEFs), GEF-H1 and ECT2 during later stages of development.[97] RhoA suppression through dominant-negative RhoA models in megakaryocyte cell lines, as well as genetic models of megakaryocyte-specific inhibition of RhoA, demonstrated increased ploidy levels with RhoA inhibition.[97-99] Together, these data demonstrate that therapeutics to inhibit the RhoA pathway in megakaryocytic leukemias could be a viable differentiation therapy to promote polyploidization and escape blast crisis.

RhoA also regulates proplatelet formation, necessary for platelet formation.[100,101] Bortezomib, a proteasome inhibitor used in the treatment of multiple myeloma, caused cyclic thrombocytopenia in patients and mouse models.[100] *In vitro* studies evaluating the mechanism by which Bortezomib induced thrombocytopenia found an accumulation of active RhoA in megakaryocytes. This caused impaired proplatelet formation, which could be rescued by inhibition of the downstream effector, ROCK. Several other studies, using genetic deletion of RhoA in *in vitro* culture and *in vivo* mouse models demonstrated that RhoA is a negative regulator of proplatelet formation.[89,98,101] Dominant-negative (DN) mutants of RhoA displayed increased proplatelet formation, and constitutively-active (CA) forms of RhoA had decreased proplatelet formation. Additionally, CA-

RhoA had increased stress fiber formation, whereas DN-RhoA had decreased stress fiber formation. The mechanism by which RhoA negatively regulates proplatelet formation is by regulating myosin contractility through phosphorylation of myosin light chain 2 (MLC). In the presence of active RhoA, MLC2 is phosphorylated, driving myosin contractility and stress fiber formation, leading to decreased proplatelet extensions in megakaryocytes.[101]

Rac1 and Cdc42 in megakaryocytes

Research on other RhoGTPases (Rac1 and Cdc42) in megakaryocytes recently elucidated a distinct function in megakaryocyte development. Pleines et al. demonstrated for the first time that a megakaryocyte and platelet specific deletion of both Rac1/Cdc42 results in severe macrothrombocytopenia due to aberrant tubulin organization in megakaryocytes and platelets and virtual abrogation of proplatelet formation rather than from a failure of megakaryopoiesis.[102] Remaining platelets from these mice have abnormal ultrastructure, ~50% of remaining platelets are overloaded with granules and ~30% are virtually devoid of granules, indicating disrupted granule formation and/or trafficking. Due to defective platelets in double-knockout Rac1/Cdc42 mice, they were cleared more rapidly from circulation than wild-type mice.

In our studies, we investigated if Rac1 and Cdc42 signal through Pak to regulate proplatelet formation and platelet release into circulation. We also evaluated if Pak deletion effects megakaryocyte maturation and development in an alternative pathway to Rac1 and Cdc42. Rac1/Cdc42 are the principle activators of p21-activated kinases (Group I). Therefore, it is interesting to follow up on the phenotype observed in the *Rac1/Cdc42* double deletion mouse models, to confirm or reject that signaling

downstream of Rac1 and Cdc42 is through Pak1 or Pak2 leading to this megakaryocyte phenotype. Additionally, if the Pak2 megakaryocyte phenotype differs from *Rac1/Cdc42* deletion in megakaryocytes, it would suggest that an alternative pathway activates Pak2 in megakaryocytes. Since these GTPases regulate many other signaling pathways, it is important to delineate if Pak has a function downstream in megakaryocytes.

Purpose

The function of Pak2 in immune cells is a novel field of study, with limited data. Here I discussed what is known about how the cytoskeleton regulates mast cells degranulation and megakaryocyte polyploidization and proplatetet formation, and what is known about the role of p21-activated kinases in these diverse processes. I endeavored to understand the role of Pak2 on mast cells and megakaryocytes signaling pathways leading to antigen-induced mast cell degranulation and megakaryocyte maturation into polyploid cells and platelet production. First, I used primary mast cells from *Pak2-floxed* animals to study FcεRI signaling in vitro, and to identify differences with Pak1 in mast cells. Secondly, using *in vivo* and *in vitro* techniques, I studied the effects of Pak2 in megakaryocyte proliferation, polyploidization and proplatelet formation during megakaryopoiesis. Thirdly, I worked to identify key changes in the Pak2-deficient adult mouse that would lead to rapid lethality. Collectively these studies highlight a novel role for Pak2 in mast cells and megakaryocytes, as well as in adult homeostasis.

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Chapter 2:

Pak2 restrains mast cell FcεRI-signaling through modulation of Rho GEF activity

Introduction

Mast cells are immune cells that reside in nearly all vascularized tissues, particularly in tissues in close contact with the outside environment. Mast cells are one of the first immune cells to interact with foreign allergens and it is therefore critical that these cells respond immediately to antigen by secreting biologically active mediators to promote or downregulate inflammation.[1] Secretion or degranulation is regulated by antigenic challenge to the high affinity IgE receptor (FcεRI). Upon antigen challenge, a signaling cascade of phosphatases and kinases is activated that coordinates dynamic changes in the cytoskeleton to promote immediate release of preformed granules via vesicle translocation and fusion to the plasma membrane.[1,2] Despite significant knowledge of mast cell biology and allergen response, the downstream effectors that link the complete signaling cascade from FcεRI to degranulation remain poorly understood.

p21-activated kinases (Paks) are serine/threonine protein kinases that are activated by small GTPases, Rac1 and Cdc42. Paks regulate a variety of important cellular processes, including control of the cytoskeleton and proliferation.[3,4] Paks comprise a family of six enzymes that are categorized into two subgroups: Group A (Pak1-3) and Group B (Pak4-6). Group A Paks, in particular Pak1, have been much studied due to their role in processes that affect neoplastic growth.[5-8] Given that degranulation requires complete cytoskeleton rearrangement, we examined the roles of Group A Pak isoforms 1 and 2 in mast cell secretion.

Currently, there is limited research on the biological differences among the Pak Group A isoforms. Pak1 and Pak2 are broadly expressed and share many substrates in common, including c-Raf and Mek1.[3] However, despite extensive structural similarities, particularly in the protein kinase domain, hints are beginning to emerge that these two isoforms have distinct functions. Gene knock-outs in mice reveal that loss of *Pak1* is well-tolerated, with notable defects only in subsets of immune cells, such as mast cells and macrophages, whereas loss of *Pak2* results in early embryonic lethality.[9-12] In a breast carcinoma cell line (T47D), Pak1 and Pak2 regulate invasion by distinct signaling mechanisms: Pak1 via regulation of cofilin phosphorylation, and Pak2 via regulation of RhoA GTPase activity.[13] Similar results were reported by Bright *et al.,* who showed that, in DU145 prostate carcinoma cells, Pak1 promotes the loss of cell-cell E-cadherin junctions resulting in enhanced migration, whereas Pak2 does not affect migration but instead regulates lamellipodia extensions.[8]

We previously reported that *Pak1* loss in mouse bone-marrow derived mast cells (BMMCs) was associated with reduced MAPK phosphorylation (Erk1/2 and p38), resulting in impaired stem cell factor-mediated migration *in vitro* and *in vivo.*[11] *Pak1* was found to positively regulate IgE-mediated degranulation via regulation of extracellular calcium influx through modulation of F-actin rearrangement.[10] Recent data indicate that Pak1 regulates mast cell cytoskeleton rearrangement and degranulation through a kinase-dependent interaction with the phosphatase PP2A, which regulates Ezrin/Radixin/Moesin (ERM) proteins that uncouple the plasma membrane from actin prior to degranulation.[14]

These studies suggested that Group A Paks play a positive role in mast cell secretion and would be beneficial targets in asthma related diseases.

In this study, we generated a conditional *Pak2* knockout animal to investigate the function of other group A Paks in allergen-mediated secretion. Surprisingly, we found that Pak1 and Pak2 play distinct and, in some cases, opposing roles in mast cell secretion. In contrast to Pak1, we found that Pak2 is a negative regulator of secretion via phosphorylation and inactivation of GEF-H1, leading to RhoA GTPase inhibition. These studies establish vital, but distinct roles for Pak1 and Pak2 in mast cell secretion.

EXPERIMENTAL PROCEDURES

Mice

Syngeneic *Pak2*$^{fl/fl}$ mice on mixed background (sv129/C57Bl/6) were used for experimentation. Additionally, a colony of polyI:C (polyinosinic:polycytidylic acid) inducible cre-recombinase mice, *Mx1-cre*$^{+}$*;Pak2*$^{fl/fl}$, along with Mx1-cre^{-} controls were generated for in vivo studies. Mice were administrated 400ug of polyI:C dissolved in PBS, every other day for 5 days (3 intraperitoneal injections total). Animal care and experimental procedures were conducted on a protocol approved by the Fox Chase Cancer Center Institutional Animal Care and Use Committee.

Genotyping by PCR

Tail DNA was digested with DirectPCR lysis buffer (Viagen, Los Angeles, CA) for tails with proteinase K and used for polymerase chain reaction (PCR) designed to amplify DNA fragments from the WT and targeted *Pak1* and *Pak2* alleles. For *Pak1* genotyping, a common forward primer (5-GCC CTT CAC AGG AGC TTA ATG A-3)

was used with a *Pak1*-specific reverse primer (5-GAA AGG ACT GAA TCT AAT AGC A-3) to amplify a 240-bp product from the WT allele; and with a *neo*-specific reverse primer (5-CAT TTG TCACGT CCT GCACGA-3) to amplify a 360-bp product from the targeted allele. For $Pak2^{fl/fl}$ genotyping, forward primer: 5-ATCTTCCCAGGCTCCTGACA-3 and reverse primer: 5-TGAAGCTGCATCAATCTATTCTG-3. WT mice demonstrate a 306-bp band and floxed mice a 391-bp band. For Mx1-cre genotyping, standard generic cre genotyping was done, according to Jackson Laboratory protocols.

In vitro Cre activation

A retroviral vector for Cre recombinase (MSCV-CRE-ERT2) under tamoxifen control, was used to excise Pak2 (Addgene plasmid 22776).[15] Cre-ERT2 contains Cre-recombinase fused to the ligand-binding domain of a mutated estrogen receptor, which recognizes tamoxifen or its derivative 4-hydroxytamoxifen (4-HT). This construct permits tamoxifen-dependent Cre activity. Recombinant virus was produced by retroviral packaging into 293-FT cells, co-transfected using lipofectamine 2000 with vectors pVPack gag-pol and pVPack eco (Stratagene, La Jolla, CA). Viral supernatant was collected 48 and 72 hours post transfection. Transduction of bone marrow was performed within one week of extraction, and performed by spin-infection with 4 μg/ml polybrene. At least two rounds of transduction were performed prior to addition of puromycin for drug selection. 250nM 4-hydroxytamoxifen (4-HT) was added to mature mast cells 4 days prior to experimentation. Western blot analysis confirms deletion of Pak2 by 4 days.

Western Blotting

Whole-cell protein extracts, after antigen stimulation, were prepared by addition of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA pH 8.0, 1% Triton X-100, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 10% glycerol, and complete protease inhibitor (Sigma, St. Louis MO), clarified by centrifugation and denatured with 1X SDS sample buffer. Samples were separated by SDS–polyacrylamide gel electrophoresis (PAGE) on a 4% to 20% gradient gel (BioRad, Carlsbad, CA) and transferred to PVDF membrane. Blots were probed with anti-Pak1, anti-Pak2, anti–phospho-phospholipase Cγ1, anti–total phospholipase Cɣ1, anti-phospho-myosin light chain 2, anti-total myosin light chain 2, anti-phospho-stathmin, anti-phospho-p38, anti-p38, anti-phospho-ERK1/2, anti-ERK1/2 and anti-GEF-H1 (all 1:1000, Cell Signaling, Danvers, MA). Anti-phospho-GEF-H1 (S885) was a gift from Celine Dermardirossian. All blots were then visualized with HRP-conjugated goat anti–rabbit or anti-mouse IgG antibody (1:10000; Jackson Immunoresearch, West Grove, PA). Films were developed using the enhanced chemiluminescence (ECL) (EMD Millipore, Billerica, MA). Phosphorylated proteins were quantified by subjecting autoradiographs to densitometry (NIH Image software; National Institutes of Health, Bethesda, MD) and calculated relative to total protein. Bands were normalized to background and the ratio of background-corrected raw intensities of protein of interest / total protein was calculated.

Cell Culture and activation

BMMCs were cultured in RPMI (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 1% glutamine (Lonza Walkersville, Walkersville, MD), 1.5% 1 M N-2-hydroxyethylpiperazine-N-2-

ethanesulfonic acid (LonzaWalkersville), 2% penicillin/streptomycin (Lonza Walkersville), and 5 ng/mL recombinant murine interleukin-3 (IL-3) and 10 ng/mL SCF (PeproTech, Rocky Hill, NJ). All cellular and biochemical assays used BMMCs that had been in culture between 4 and 10 weeks. All experiments were conducted using at least 3 independent lines from each genotype. BMMCs sensitized in media with 0.5 μg/mL anti-DNP IgE monoclonal antibody (clone SPE-7; Sigma-Aldrich, St Louis, MO) overnight, and stimulated with 10 ng/mL and 30 ng/mL dinitrophenyl conjugated to human serum albumin (DNP-HSA, 30-40 mol DNP/mol HSA; Sigma-Aldrich, St. Louis, MO) for degranulation assays, and 100 ng/mL in phosphorylation and pulldown assays.

Detection of c-kit/FcεRI receptors

C-Kit and FcεRI expression were analyzed by fluorescence cytometry as described.[16] Cells were blocked with unconjugated anti-FcγRII/III (BD Pharmingen, San Diego, CA) and stained with anti-DNP monoclonal antibody IgE clone SPE-7 (Sigma-Aldrich, St. Louis, MO), anti–mouse CD 117 (c-kit) PE-conjugated antibody, and FITC-conjugated anti–mouse IgE (both BD Pharmingen) secondary antibody. Cells were washed and resuspended in 0.5% FBS/PBS buffer. Cells were analyzed on a fluorescence-activated cell sorting (FACS) Calibur (Becton Dickinson, San Jose, CA).

Degranulation assays

BMMC degranulation was determined by β-hexosaminidase release as previously described[17] with minor modification. IgE-primed (see "Cell culture and activation") BMMCs were suspended at $5x10^6$ cells/mL in Hepes-BSA buffer (10 mM HEPES buffer, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 0.05% BSA,

pH 7.4) then stimulated with 10 or 30 ng/mL DNP-HSA (Sigma-Aldrich) for 45 minutes at 37°C. For receptor-independent stimulation, unsensitized cells were incubated in Hepes-BSA buffer and stimulated with 1 μM ionomycin for 45 minutes. The cell pellets were solubilized in Hepes-BSA buffer, 0.5% Triton X-100. β-hexosaminidase release was measured in both the supernatants and the cell pellets by incubating with 4-nitrophenyl *N*-acetyl-beta-D-glucosaminide (Sigma-Aldrich) in sodium citrate (pH 4.5) for 1.5 hours at 37°C. Sodium carbonate/sodium bicarbonate buffer (0.1 M, pH 10) was used to stop the reaction and absorbance was read at 405 nm. Degranulation was expressed as a percentage of β-hexosaminidase released = supernatant activity/total (supernatant plus pellet) activity x 100. Samples were assayed in triplicate.

ELISA

BMMCs cytokine secretion was determined using ELISA for TNFα and IL-6 (Ebioscience, San Diego, CA). Cells were sensitized overnight in cytokine free media with 0.5 μg/mL anti-DNP IgE. Cells were washed and stimulated with 30 ng/mL DNP-HSA for 6 hours. Supernatants were collected and cytokine release was measured. Cytokine release was normalized to phorbol 12-myristate 13-acetate (PMA) and ionomycin treatment.

GTPase pulldown assay

Recombinant glutathione S transferase (GST) conjugated PAK CRIB domain was expressed from pGEX-CRIB and Rhotekin RBD-GST beads were acquired from Millipore. Cells were sensitized overnight in anti-DNP IgE (Sigma) without cytokines and stimulated with 100ng/mL DNP-HSA for 10 minutes at 37ºC. Lysates were

incubated with CRIB-GST or RBD-GST beads for 45 minutes rotating at 4ºC, washed and run out on a 4-20% SDS-PAGE gel (BioRad, Carlsbad, CA). Immunoblots of lysates incubated with beads and input were probed for anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Rac1 (Transduction Laboratory, BD Biosciences, San Jose, CA) or anti-Cdc42 (Cell Signaling, Danvers, MA). GTP-bound lane intensity was normalized to input lane intensity and calculated relative to unstimulated levels in wildtype cells using ImageJ (NIH).

BMMC Adhesion Assay

Bone marrow derived mast cells (BMMCs) were starved of cytokines and sensitized overnight with anti-DNP-IgE (0.5 μg/ml). Cells were washed in HEPES-BSA buffer and placed in wells of a 96-well black microtiter plate (5 x10^4 cells/well). Wells were precoated with 0.2% gelatin for 1 hour, washed, and serum was added for 1 hour at room temperature. This procedure resulted in a fibronectin coating of the wells. After 30 minutes of rest, cells were stimulated with DNP-HSA (1 ng/ml or 10 ng/ml) for 45 minutes. 10 minutes prior to the end of the assay, the membrane permeable viability dye Calcein-AM (2 μg/mL) (Ebioscience) was loaded onto the cells. Perkin Elmer Envision plate reader (Waltham, Massachusetts) was used to measure fluorescence at 530 nm. First, wells were read to obtain a total fluorescence, after which they were washed and bound cells were measured. Background fluorescence was subtracted and percent adherence calculated by taking adherent cell fluorescence as a percent of total well calcein-AM fluorescence. At least 3 individual mice were used per group, and assays run in triplicate.

Calcium mobilization

IgE-primed BMMCs were resuspended at 10^6 cells/mL in HEPES-BSA buffer containing Indo-1-AM (Ebioscience) and probenecid at 37°C for 1 hour. Cells were washed and resuspended at 10^6 cells/mL in HEPES-BSA buffer. Samples were warmed to 37°C and baseline fluorescence was measured for 1 minute. 30 ng/ml DNP-HSA was added to the cells, and the change in fluorescence (420 nm Ca^{2+}-bound, 510 nm Ca^{2+}-free) was monitored using the JSAN flow cytometer for 6 minutes. Positive controls were measured by stimulation with 1 μM thapsigargin. Data were graphed using Flowjo software (TreeStar, Ashland, Oregon) and also used to analyze peak calcium flux normalized to baseline levels.

In vitro kinase assay

HEK293 were transfected with pCDNA3-GEF-H1-GFP (gift from Celine DerMardirossian) using lipofectamine 2000. Protein was purified using anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and conjugated to Protein A/G sepharose beads (GE). Protein was treated with lambda phosphatase (NEB) prior to use in the kinase assay. Immunoprecipitated GEF-H1 was incubated with recombinant Pak2 (ProQinase, Freiburg, Germany) and ATP in kinase buffer (40 mM HEPES pH 7.5, 10 mM $MgCl_2$, 20 μM ATP) for 30 minutes at 30ºC. The reaction was fractionated on a 4-20% SDS-PAGE gel (BioRad, Carlsbad, CA) and transferred to PVDF membrane. Membranes were probed for phospho-Ser 885 GEF-H1 (a gift from Celine DerMardirossian) and total GEF-H1 (Cell Signaling, Danvers, MA).

Passive systemic anaphylaxis

Mx1-cre^{+}*;Pak2*$^{f/f}$ mice along with littermate controls were injected 3 times with

400ug of poly I:C every other day. 11 days after the first injection, mice were retroorbitally injected with 50ug of DNP-specific mouse IgE (SPE-7 clone, Sigma). 16 hours later, mice were injected with 10 ug of DNP-human serum albumin (DNP-HSA). 90 seconds after injection, mice were bled retro-orbitally, serum was isolated and frozen at -80C. ELISA for serum histamine levels was performed at a 1:250 dilution and concentration was calculated from a standard curve (Beckman Coulter).

RESULTS

Genetic disruption of murine Pak2

Previously, our laboratory with colleagues reported that Pak1 was a positive regulator of antigen-mediated degranulation in BMMCs via regulation of extracellular calcium mobilization.[10] A closely related isoform, Pak2, is expressed at much higher levels than Pak1 (Figure 2.1A) in mast cells; thus we assessed the function of this kinase in antigen-mediated degranulation. Since the deletion of the *Pak2* gene is associated with embryonic lethality at approximately E9.5[9], we made a conditional knockout of this gene (Figure 4.1A). A targeting vector was designed to flank exon 2 of *Pak2* with *loxP* sites.

To confirm that the *Pak2* floxed alleles indeed resulted in deletion of Pak2, bone marrow from *Pak2*$^{flox/flox}$ mice was infected with a tamoxifen-regulated Cre retrovirus (MSCV-Cre-ERT2-puromycin). After maturation to mast cells and selection with puromycin, the cells were treated with 250 nM 4-hydroxytamoxifen (4-HT) for 4 days to excise *Pak2*. Protein extracts from these cells were immunoblotted and probed with anti-Pak2 antibodies (Figure 2.1B). As expected, 4-HT-treated Cre-ERT2 infected cells showed near total loss of Pak2 protein. For comparison, immunoblots from *Pak1*$^{-/-}$ BMMCs are also shown.

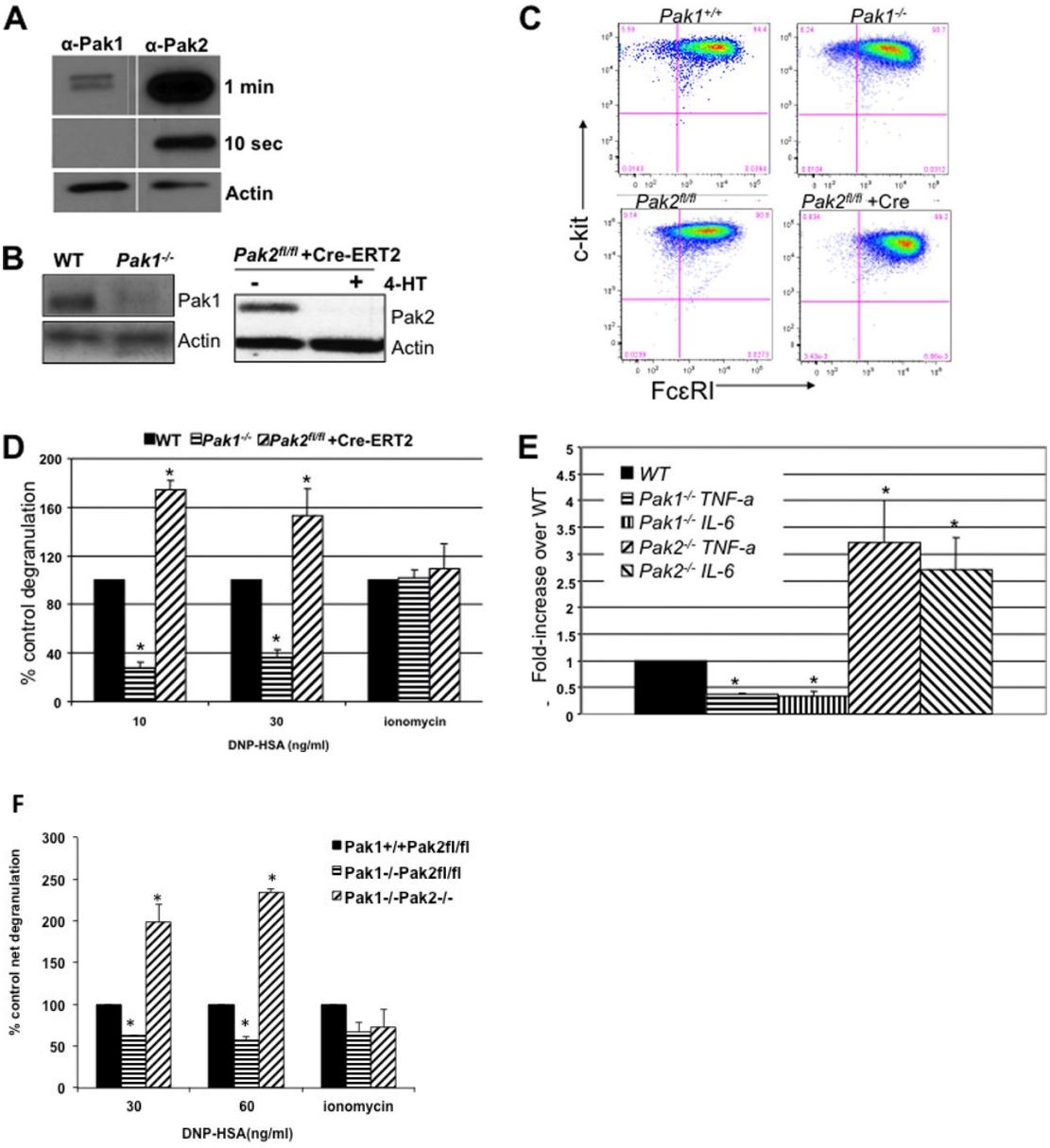

FIGURE 2.1. Effect of Pak1 and Pak2 on mast cell maturation and antigen-mediated secretion. *A*, WT MC-9 mouse mast cell line lysates were subjected to immunoblotting and probed with either anti-Pak1 or anti-Pak2 antibodies. Membranes were exposed together on the same film for the same amount of time for semi-quantitative analysis. 10 second and 1 minute exposures are shown, with actin for a loading control. *B,* BMMC lysates from *Pak1* knockout mice and *Pak2*$^{fl/fl}$ infected with MSCV-Cre-ERT2 and treated with 250 nM 4-HT. Actin loading control. *C,* Loss of *Pak1* or *Pak2* does not affect expression of mast cell maturation markers. Data shown are representative of 6 independent lines from each genotype. *D,* β-hexosaminidase release was measured in IgE-primed *Pak1* and *Pak2* knockout BMMCs stimulated with 10 or 30

Figure 2.1 (Continued) ng/ml DNP-HSA for 45 minutes. FcεRI-independent degranulation is shown with ionomycin treatment. In all conditions, the extent of degranulation is represented as percent of total β-hexosaminidase activity in cells. p-value<0.05, Wilcoxon signed-rank test. *E*, ELISA was performed for tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) secreted by *Pak1*-/- and *Pak2*-/- BMMCs in response to 30 ng/ml DNP-HSA. *F,* BMMCs cultured from Pak1-/-Pak2f/f mice, and infected with MSCV-Cre-ERT2. Cre induced with 250nM 4-OHT, and amount of degranulation measured by B-hexosaminidase release. All data mean±SD, at least n=3, *p*-value<0.05, Wilcoxon signed-rank test.

Mast cell maturation from Pak1 and Pak2-deficient mice

Bone marrow was cultured for 5 weeks with interleukin-3 (IL-3) and stem cell factor (SCF) to derive mature mast cells. The development and maturation of these cells were measured using flow cytometry analysis of the cell surface receptors FcεRI and c-kit (CD117). Flow cytometry plots demonstrated over 90% $FcεRI^{+}$ $c\text{-}kit^{+}$ mature mast cells after 5 weeks of culture (Figure 2.1C), irrespective of the presence or absence of Pak1 or Pak2 protein. Thus, neither *Pak1* nor *Pak2* is essential for full maturation of bone marrow into mast cells *in vitro*.

Pak2 is a negative regulator of mast cell secretion

To examine the physiological responses of Pak2-deficient mast cells *in vitro*, we measured antigen-mediated degranulation and cytokine secretion. IgE sensitization leads to FcεRI aggregation and calcium flux, triggering degranulation. Previously, Pak1 was identified to regulate antigen-mediated degranulation in vitro.[10] To determine if Pak2 shares these properties, deletion was done by Cre-mediated gene excision in mature mast cells and sensitized with 0.5μg/ml anti-DNP IgE overnight. Aggregation of FcεRI with antigen DNP-HSA for 45 minutes in *Pak2*-null cells led to increased degranulation. The level of degranulation was measured by the amount of β-hexosaminidase release (an enzyme present in mast cell preformed granules). Surprisingly, *Pak2*-deleted mast cells showed a significant increase in degranulation (*p-value*<0.05, Wilcoxon signed-rank test) compared with cells from the same mouse without Cre activation (infected with MSCV-Cre-ERT2, however not given 4-HT) (Figure 2.1D). Total enzyme content measured in the cell pellet were similar between $Pak2^{fl/fl}$ and $Pak2^{-/-}$ cells, indicating *Pak2* deletion had no effect on amount of β-hexosaminidase present, only on the release of granules.

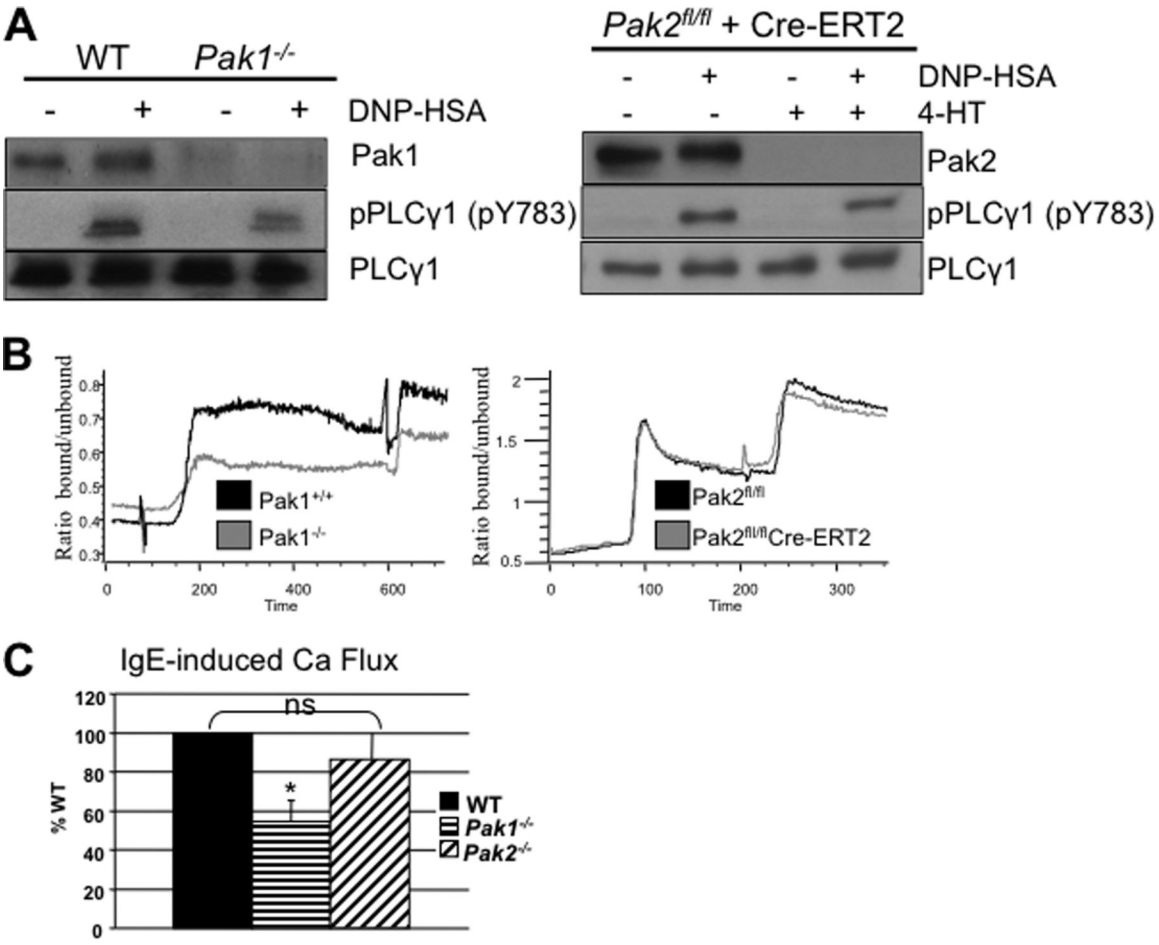

FIGURE 2.2. Effect of Pak1 and Pak2 on signaling and PLCγ1. *A,* Representative immunoblots for activated phospholipase C-gamma 1 (PLCγ1) in *Pak1* and *Pak2* knockout BMMCs. IgE-primed WT and knockout BMMCs were stimulated with DNP-HSA (100 ng/mL) for 3 minutes and lysates were subjected to immunoblotting with anti-phospho-PLCγ1 pY783 (middle panel) or anti-total PLCγ1 (bottom panel). Experiments were done on 3 different mouse BMMCs. *Pak1* and *Pak2* status is shown for each blot (top panel). *B,* IgE-primed WT, *Pak1* knockout and *Pak2* knock-out BMMCs were loaded with Ca^{2+}-sensitive dye (Indo-1-AM) and suspended in Ca^{2+}-containing medium. After baseline collection on JSAN flow cytometer, FcεRI was activated by addition of 30 ng/ml DNP-HSA. Second stimulation peak is from thapsigargin treatment. Representative experiments of WT (black) *vs Pak1* knockout (gray) (left panel) and WT (black) *vs Pak2* knockout (gray) (right panel) BMMCs is shown. *C*, Mean (± SD) stimulation (peak of ratio minus baseline as percent of WT) of at least 3 independent experiments for *Pak1* knockout and *Pak2* knockout BMMCs. *Pak1* knockout p-value<0.05, Wilcoxon signed-rank test. Pak2 is not statistically different from WT.

This finding is in stark contrast to *Pak1*-deleted mast cells, which had reduced degranulation (*p-value*<0.05, Wilcoxon signed-rank test), as reported previously.[10] (Figure 2.1D) To rule out non-specific effects of Cre activation, Cre was activated in WT BMMCs and measured for degranulation. In these cells, no enhanced degranulation was observed.

We also tested the response to ionomycin, a calcium ionophore used to activate store-operated calcium channels and initiate degranulation independent of antigen, and demonstrated no differences between genotypes. (Figure 2.1D). Thus, our data show that Pak1 and Pak2 play opposing roles in IgE-mediated degranulation in mast cells: Pak1 positively regulates and Pak2 negatively regulates degranulation.

Activated mast cells also synthesize and secrete cytokines in response to antigen. We measured the release of IL-6 and TNF-α in FcεRI-activated mast cells by ELISA and found that $Pak1^{-/-}$ BMMCs were defective in secretion of IL-6 (*p-value*<0.05, Wilcoxon signed-rank test) and TNF-α (*p-value*<0.05, Wilcoxon signed-rank test), consistent with the defect in degranulation (Figure 2.1E). Alternatively, $Pak2^{-/-}$ BMMCs demonstrated significantly enhanced cytokine secretion for IL-6 (*p-value*<0.05, Wilcoxon signed-rank test) and TNF-α (*p-value*<0.05, Wilcoxon signed-rank test) (Figure 2.1E). These results show that, as with degranulation, Pak1 and Pak2 play opposing roles in IgE-mediated secretion of cytokines from mast cells.

To determine if Pak1 and Pak2 equally effect mast cell degranulation, we developed a double knockout mouse model (DKO), $Pak1^{-/-};Pak2^{f/f}$. Bone marrow from these animals was transfected with MSCV-Cre-ERT2 to conditionally drive deletion of

Pak2 in an *in vitro* culture of mast cells. This control allowed us to compare bone marrow from the same animals. We discovered that deletion of both Pak1 and Pak2 results in increased mast cell degranulation, as opposed to a rescue of this phenotype (Figure 2.1F). These data provide the first evidence that Pak2 is the predominant isoform in mast cells, and contributes the most to regulate degranulation.

FcεRI-dependent calcium mobilization is dependent on Pak1 but not Pak2

The profound differences in regulation of degranulation between Pak1 and Pak2 in mast cells led us to examine differences along the signaling pathway leading to IgE-mediated degranulation. Antigen-induced PLCγ1 (phospholipase-Cγ1) activation leads to IP3-dependent release of calcium from the endoplasmic reticulum (ER), resulting in influx of extracellular calcium. Since neither loss of Pak1 or Pak2 was associated with alterations in phosphorylation of PLCγ1 at Tyr783 (Figure 2.2A), leading us to examine downstream events such as calcium influx. FcεRI signaling upon antigen stimulation results in calcium release from ER internal stores (first stage of Ca^{2+} mobilization) and subsequent prolonged influx of extracellular calcium (second stage of Ca^{2+} mobilization) through store-operated calcium release-activated calcium (CRAC) channels in the plasma membrane. Cells from all genotypes were sensitized with anti-DNP IgE, and loaded with calcium binding dye Indo-AM, and influx of extracellular calcium was measured by flow cytometry analysis by measuring Ca^{2+}-bound versus unbound Indo-AM after stimulation with antigen. Compared with their appropriate controls, *Pak1*-deleted BMMCs were significantly impaired for second stage calcium influx (Figure 2.2B,C; *p-value*<0.05, Wilcoxon signed-rank test), but had normal first stage release from the ER with antigen stimulation.[10] *Pak2*-deleted BMMCs, however, were not defective in calcium influx from

antigen stimulation (Figure 2.2B,C). These differences identify a key distinction in mast cell regulation between Pak1 and Pak2 in FcεRI stimulated mast cells. These results collectively indicate that Pak1 and Pak2 play distinct roles in regulating calcium influx in FcεRI-stimulated BMMCs.

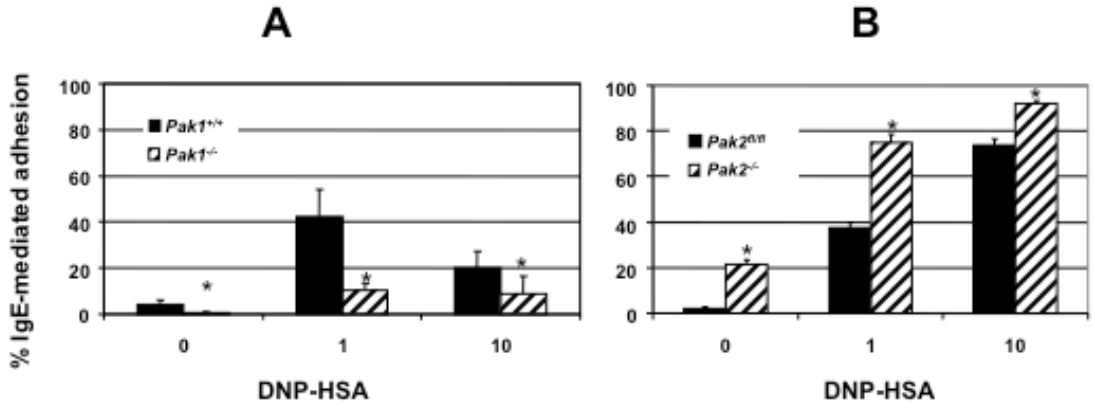

FIGURE 2.3. Effect of Pak1 and Pak2 on adhesion. *A*, Percent IgE-mediated adhesion of *Pak1* knockout BMMCs on fibronectin-coated plates with 1 ng/ml and 10 ng/ml DNP-HSA (p-value<0.05, Student *t* test). *B*, Percent IgE-mediated adhesion of *Pak2* knockout BMMCs (p-value <0.05, Student *t* test). Data are shown as an average and standard deviation of at least 3 independent experiments run in triplicate. Data calculated as percent adhesion relative to total cells in each well.

Pak1 and Pak2 play distinct roles in IgE-mediated adhesion

FcεRI receptor activation in mast cells not only leads to changes in secretion of inflammatory mediators, but also leads to increased adhesion.[18] Mast cell activation of FcεRI receptor stimulates an increase in cell adhesion and this adherence is required to facilitate localized synthesis of cytokines.[19,20] Given the role of Paks in cell migration, adhesion and cytoskeleton regulation, we sought to discover a role for Paks in mast cell adhesion. Based on our data demonstrating altered secretion between the two genotypes,

we hypothesized that Pak1 and Pak2 would differ in their effects on cell adhesion in response to antigen. Our findings demonstrated a significant reduction in antigen-mediated adhesion in *Pak1*$^{-/-}$ BMMCs (Figure 2.3A, p-value<0.05, Student t test) and a significant increase in adhesion in *Pak2*$^{-/-}$ BMMCs (Figure 2.3B, p-value<0.05 Student t test). We conclude from these data that differences observed between Pak1 and Pak2 in antigen-mediated secretion stem primarily from their opposing roles in adhesion.

Pak2 regulates BMMC secretion by regulating RhoA GTPase activity

RhoA GTPase activation is required for mast cell adhesion and secretion in response to IgE stimulation.[21-23] Since Pak1 and Pak2 are thought to regulate RhoA by opposing mechanisms,[13] we proceeded to investigate activation differences of RhoA in *Pak2*-deficient mast cells as a potential cause of enhanced secretion and adhesion. Using pull-down assays with the RhoA binding domain, Rhotekin, we found that deletion of *Pak2* significantly increased the level of active GTP-bound RhoA in antigen-stimulated cells (p-*value*<0.05 Student t test) (Figure 2.4A).

RhoA, through its effector ROCK, phosphorylates and inactivates myosin phosphatase, leading to phosphorylation and activation of myosin light chain 2 (MLC2).[24] The active form of MLC2 was significantly elevated in *Pak2*$^{-/-}$ cells (p-*value*<0.05, Student t test) (Figure 2.4B). This finding is consistent with previous studies in breast cancer cells that showed elevated MLC2 phosphorylation when *Pak2* was silenced with siRNA.[13]

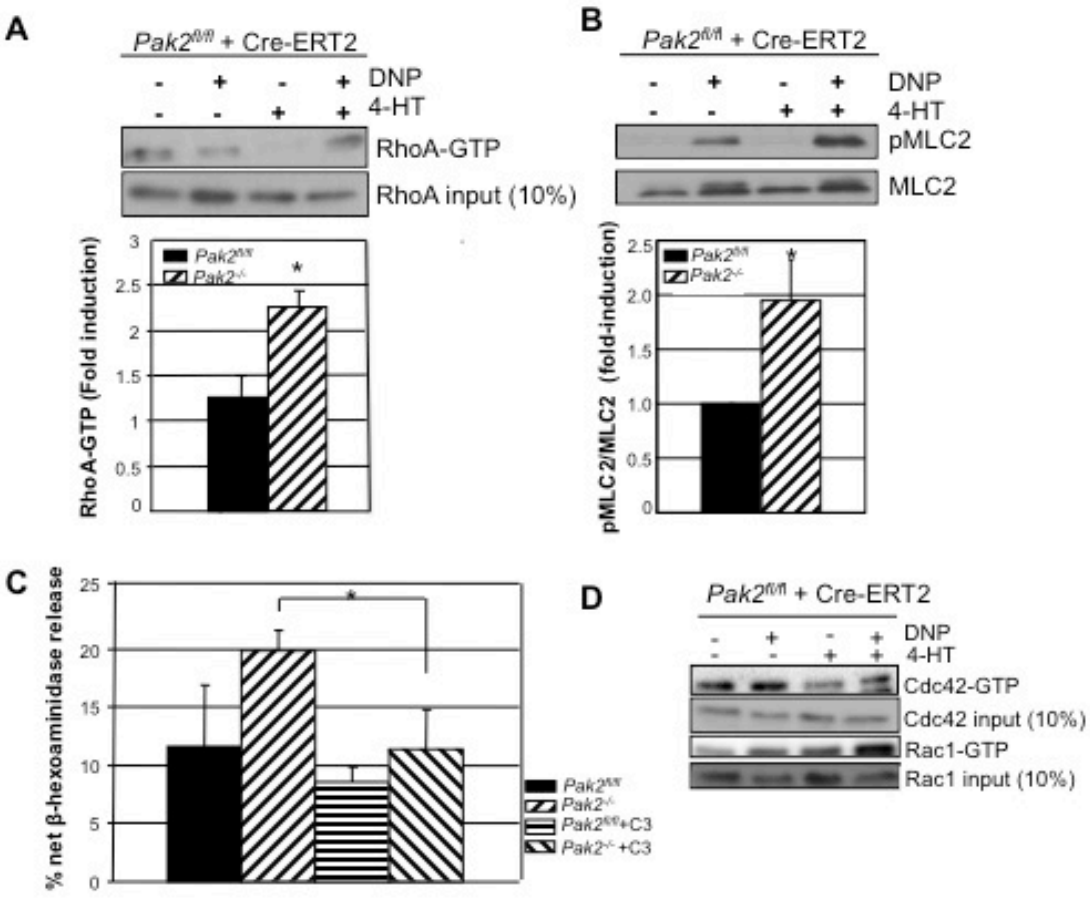

FIGURE 2.4. Effect of Pak2 on Rho GTPase activity. *A,* IgE-primed *Pak2*$^{fl/fl}$ and *Pak2*$^{-/-}$ BMMCs were stimulated with 100 ng/mL DNP-HSA for 10 minutes, lysates cleared and incubated with Rhotekin RBD-GST beads (Millipore). RhoA-GTP was detected by anti-RhoA antibody and 10% of input demonstrates equal loading. One representative Western blot of three experiments is shown, and densitometry of the fold induction relative to unstimulated cells (all normalized to input) of 3 independent experiments is shown (p-value<0.05, Student *t* test). *B,* Activation status of MLC2, a downstream RhoA effector, at pThr18/Ser19 in lysates from 10 minute DNP-HSA stimulation. Blot is representative of 3 separate experiments, and densitometry shows average fold (±st dev) change over stimulated wildtype cells normalized to total MLC2 for 3 separate experiments (p-value<0.05, Student *t* test). *C,* C3-exoenzyme, a RhoA inhibitor, was treated on *Pak2*$^{fl/fl}$ and *Pak2*$^{-/-}$ BMMCs for 6 hours without serum. A degranulation assay was performed with 30 ng/ml DNP-HSA. Graph is average of 3 independent experiments run in triplicate with standard deviation (p-value<0.05, *Pak2*$^{-/-}$ *vs Pak2*$^{-/-}$ +C3, Student *t* test). *D,* Rac1 and Cdc42 activity (GTP-bound isoforms) of *Pak2*$^{fl/fl}$ and *Pak2*$^{-/-}$ BMMCs after DNP-HSA stimulation for 10 minutes (representative blot of 3 independent BMMCs). Total Rac1 and Cdc42 protein for each sample before performing pull-down is detected with monoclonal anti-Rac1 and polyclonal anti-Cdc42.

If activated RhoA is responsible for the enhanced degranulation seen in *Pak2*$^{-/-}$ BMMCs, then pharmacologic inhibition of RhoA should block these effects. Inhibition of RhoA GTPase was achieved with *Clostridium botulinum* C3 exoenzyme, which selectively catalyzes the ADP-ribosylation and subsequent inactivation of RhoA, RhoB, and RhoC.[25] Treatment of *Pak2*$^{-/-}$ BMMCs with C3 blocked the enhanced secretory phenotype in Pak2-null cells (*p-value*<0.05, Student *t* test) (Figure 2.4C). The phenotype observed in *Pak2*$^{-/-}$ BMMCs was RhoA specific, and not due to elevated Rac1 or Cdc42, as deletion of *Pak2* did not affect Rac1-GTP or Cdc42-GTP levels as assessed by pull-down assay (Figure 2.4D,E). Therefore, we conclude that Pak2 is a specific negative regulator of RhoA-GTPase activity in BMMCs.

Pak2 negatively regulates p38MAPK through RhoA inhibition

RhoA activates p38^{MAPK}, and this MAP kinase is known to regulate degranulation in mast cells.[26] Pak1 was previously found to regulate p38^{MAPK} in stem cell factor-c-kit receptor signaling.[11] Based on this evidence, we asked if Pak2 loss affected p38 activation by IgE engagement. We observed that loss of Pak2 resulted in a pronounced activation of p38^{MAPK} in response to antigen, with ~1.5-fold induction over WT cells at 2 and 5 minutes post stimulation (Figure 2.5A, p-value<0.05, Student *t* test). Addition of the p38 inhibitor, SB203580, reduced the enhanced degranulation phenotype observed in *Pak2*$^{-/-}$ BMMCs (Figure 2.5B, p-value<0.05, Student *t* test). These results, along with the observed effects on RhoA shown in Figure 2.4A, suggest that Pak2 affects degranulation via a RhoA/p38 specific pathway, as Pak2 loss had no effect on Erk1/2 activity (Figure

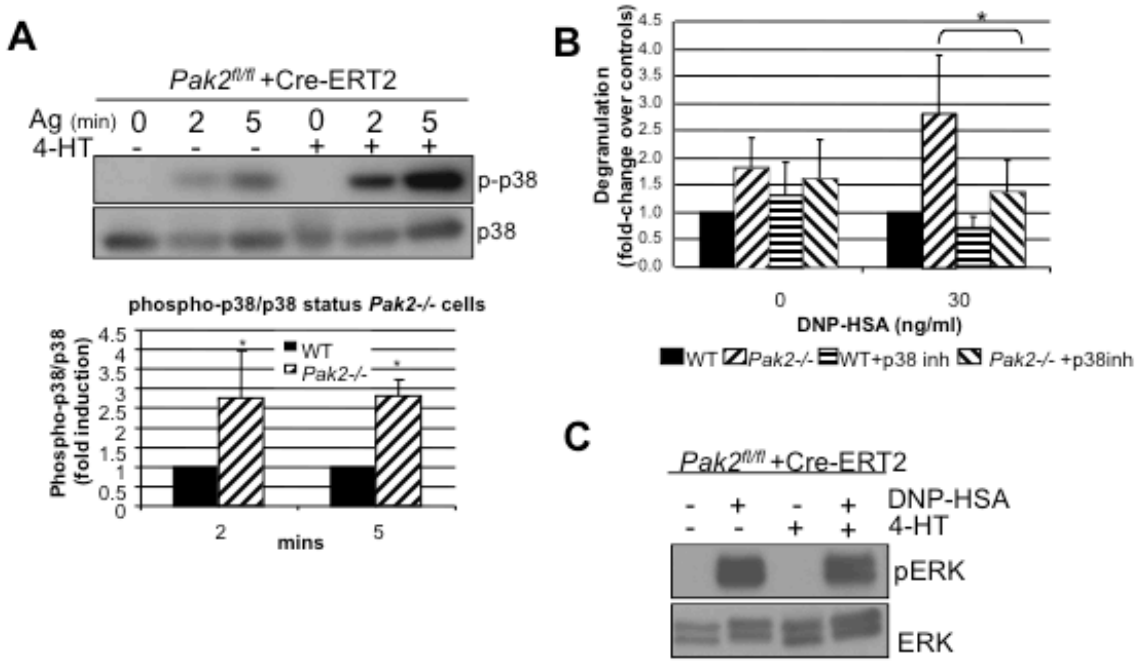

FIGURE 2.5. Effect of Pak2 on MAPK and p38 activity. *A,* IgE-primed *Pak2*$^{fl/fl}$ and *Pak2*$^{-/-}$ BMMCs were stimulated with 100 ng/mL DNP-HSA for 2 and 5 minutes, immunoblotted for anti-phospho-p38 (Thr180/182), anti-total p38, and anti-tubulin. Representative blot shown, along with the average of 3 independent experiments and standard deviations for relative fold induction over wildtype cells for phospho-p38/total p38. (p-value <0.05, Student *t* test) *B,* Degranulation of IgE-primed *Pak2*$^{fl/fl}$ and *Pak2*$^{-/-}$ BMMCs stimulated with 30 ng/ml DNP-HSA antigen and incubated with p38 inhibitor SB203580 (10 μM) 30 minutes prior to assay. Average fold change of 3 independent experiments and standard deviations (p-value<0.05, Student *t* test). *C,* ERK1/2 was probed on immunoblots from *Pak2*$^{fl/fl}$ and *Pak2*$^{-/-}$ stimulated for 10 minutes with 100 ng/mL DNP-HSA antigen. Representative blot shown, run for 3 independent experiments.

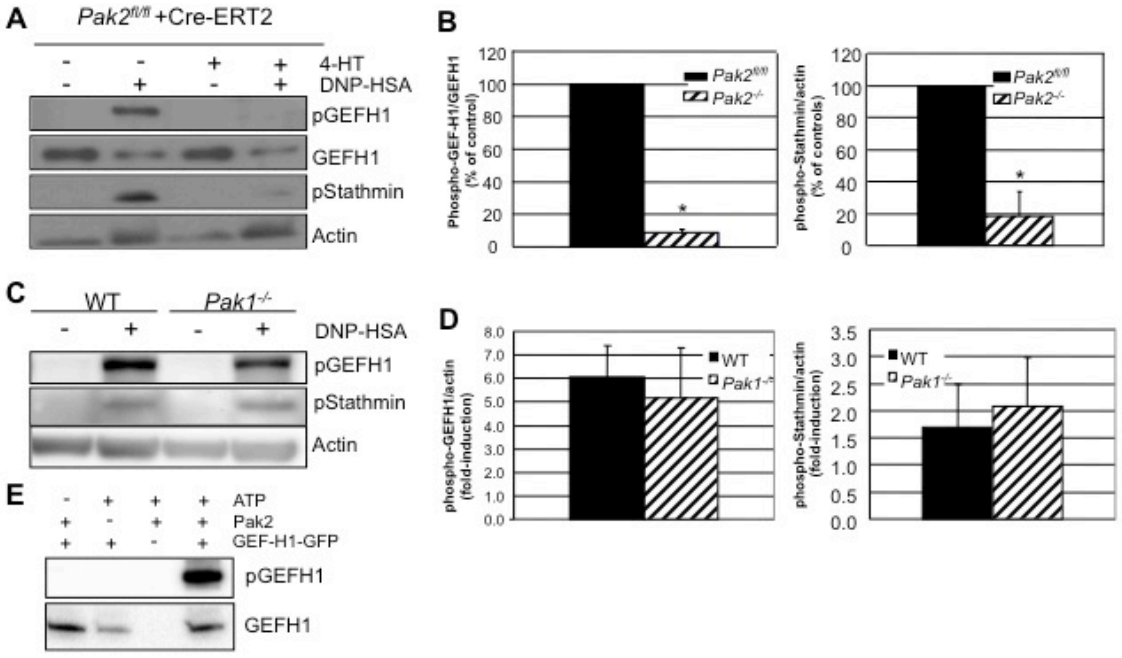

FIGURE 2.6. Pak2 phosphorylates GEF-H1 and Stathmin in antigen-stimulated mast cells. *A,* IgE-primed *Pak2*$^{fl/fl}$ and *Pak2*$^{-/-}$ BMMCs were stimulated with 100 ng/mL DNP-HSA for 10 minutes and immunoblotted for phospho-GEF-H1 (Ser885), total GEF-H1, phospho-stathmin (Ser16), and actin (loading control). Representative blots shown. *B, (left panel)* Graph depicts the average of 3 independent experiments and standard deviations for percent change in phospho-GEF-H1 (S885), normalized to total GEF-H1, relative to WT (p <0.05). *(right panel)* Graph depicts the average of 3 independent experiments and standard deviations for percent change in phospho-stathmin (S16) normalized to actin, relative to WT cells (p <0.05). *C,* Representative blot of IgE-primed *Pak1*$^{-/-}$ BMMCs stimulated with 100ng/mL DNP-HSA for 10 minutes and immunoblotted for phospho-GEFH1 (S885), phospho-Stathmin (S16) and actin (loading control). *D,* Graph depicts fold induction of phospho-GEFH1 and phospho-Stathmin normalized to actin, average of 3 independent experiments and standard deviations. *E,* In vitro kinase assay with recombinant Pak2 and GEF-H1-GFP, lysates run on parallel membranes were probed for phospho-GEF-H1 (S885) and total GEF-H1.

2.5C). These data differ with that reported for Pak1, where in BMMCs, Pak1 functions to activate p38 and Erk1/2.[11]

Pak2 regulates RhoA activation via phosphorylation and inactivation GEF-H1

How does Pak2 suppress RhoA activation? To answer this question, we looked towards phosphorylation status of a known regulator of RhoA GTPase activity, GEF-H1. GEFs (guanine nucleotide exchange factors) convert RhoA-GDP to RhoA-GTP. The RhoA-specific GEF-H1 can only activate RhoA in the non-phosphorylated state. When phosphorylated at serine 885, GEF-H1 localizes to microtubules and is inactivated, leading to inactive RhoA.[27,28] Pak1 was previously found to regulate GEF-H1 through phosphorylation at Ser-885, which induces 14-3-3 binding to GEF-H1 and relocation to the microtubules.[29] As shown in Figure 2.6A, when *Pak2* is deleted in BMMCs, phosphorylation of GEF-H1 is nearly abolished (p-value<0.05, Student *t* test). *Pak1* deletion, in contrast, did not affect GEF-H1 phosphorylation (Figure 2.6C). As impaired phosphorylation at Ser-885 is associated with constitutively active GEF-H1 and subsequent enhanced RhoA activity[30], it is likely that the observed impairment of GEF-H1 phosphorylation in *Pak2*-deleted cells is driving enhanced secretion. Interestingly, another Pak substrate associated with microtubules, stathmin/Op18, showed decreased phosphorylation at Ser-16 in *Pak2*$^{-/-}$ cells (Figure 2.6B, p-value<0.05 Student *t* test). Stathmin/Op18 remains in the active state upon dephosphorylation, suggesting that Pak2 deletion drives microtubule depolymerization via activated stathmin. *Pak1*-null cells fail to show a defect in either GEF-H1 or stathmin phosphorylation, indicating in mast cells that these are specific downstream targets of Pak2. Finally, a kinase assay to assess the ability of Pak2 to directly phosphorylate GEF-H1 at serine 885 demonstrated that GEF-

H1 can be phosphorylated by Pak2 *in vitro* (Figure 2.6E). Together these data demonstrate that Pak2 regulates RhoA-GTPase via regulation of microtubule-associated proteins and this regulation mediates mast cell FcεRI responses independent of Pak1's effects.

Passive systemic anaphylaxis

To determine if the effects observed in *Pak2*-deleted mast cells *in vitro* are consistent with effects *in vivo*, we performed a passive systemic anaphylaxis experiment to measure the capacity of mast cells to degranulate. In order to delete Pak2, we used an Mx1-cre recombinase expressing mouse model. Mx1-cre is activated by administration of double-stranded RNA (polyI:C). This allows for both temporal and spatial control of Pak2 deletion. Mx1-cre predominantly is expressed in hematopoietic tissue and spleen. After bone marrow recombination (day 10 post-injection of polyI:C), we administered IgE specific antibodies through retroorbital injection. After 16 hours, we injected antigen to induced systemic anaphylaxis. After 90 seconds, we extracted blood and isolated serum to measure for the level of histamine release. Our results indicate that *Pak2*-null mice have 50% decreased mast cell degranulation compared to wild-type polyI:C treated mice (Figure 2.7). These data are in contrast to the *in vitro* degranulation studies, which found *Pak2*-deficient mast cells in culture maintained a higher level of antigen-induced degranulation. There are a variety of theories and future experiments to explain these differences between *in vitro* and *in vivo* degranulation, which will be discussed below.

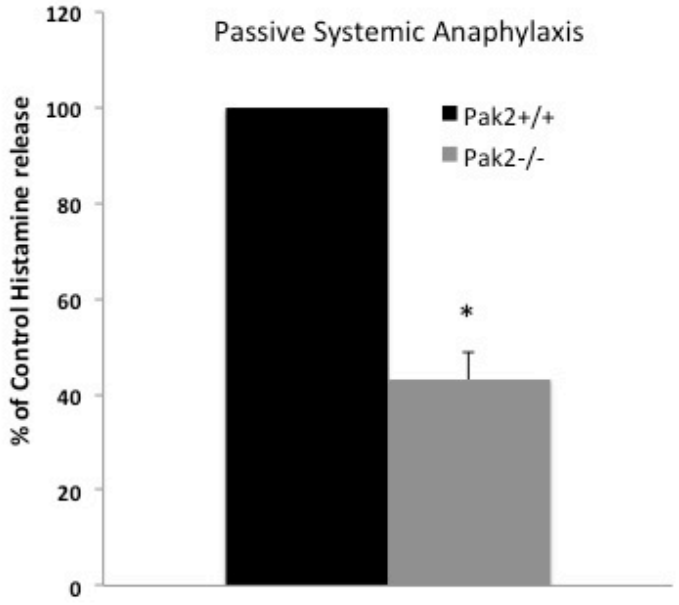

Figure 2.7 Passive systemic anaphylaxis in Mx1-cre+Pak2f/f mice demonstrate decreased antigen-induced degranulation. 24 hrs post-administration of IgE-DNP, mice were dosed with antigen (DNP-HSA, 10ug). 90 seconds after administration of antigen, blood was extracted, serum isolated and measured for histamine by ELISA. Amount of histamine calculated by a standard curve, and data displayed as percent of control. n=7 mice/genotype.

DISCUSSION

In this report, we describe the distinct functional roles of Pak1 and Pak2 in allergen-induced bone marrow-derived mast cell degranulation. Previous work found that Pak1 in mast cells modulates allergen- and stem cell factor- induced F-actin rearrangement, extracellular calcium flux and degranulation.[10,11] Here we show that disruption of the *Pak2* gene in BMMCs increases the allergen-induced degranulation response without effecting calcium flux, directly opposing Pak1 function. Pak1 functions to regulate mast cell secretion by promoting assembly of a PP2A phosphatase complex.[14] This phosphatase complex dephosphorylates Ezrin/Radixin/Moesin (ERM), resulting in uncoupling of the actin cytoskeleton from the plasma membrane and subsequent degranulation. Granule translocation to the plasma membrane is independent of calcium, however, fusion to the plasma membrane and release of granule content depends on the presence of calcium.[31] This partially explains why *Pak1*-null mast cells had deficient secretion, since they demonstrated a 40% reduction in antigen-induced calcium flux (Figure 2.2), however Pak2 has no effect on calcium flux. Therefore, our data seek to identify the alternative mechanism by which Pak2 functions to negatively regulate mast cell secretion.

Pak1 and Pak2 share over 93% sequence homology within their catalytic domains.[4] Pak2 however is the predominant isoform in BMMCs (Figure 2.1A), suggesting that small molecule Pak inhibitors would most likely demonstrate a Pak2 phenotype in mast cells, possibly resulting in severe anaphylaxis.[32-34] These findings also point to our limited understanding of the differences between Pak1 and Pak2 in terms of regulation and substrate specificity.

A strong phenotypic difference between *Pak1*- and *Pak2*-null mast cells is the difference in extracellular calcium flux. Pak1, but not Pak2, regulates calcium flux to influence the amount of degranulation a mast cell can undergo. Recent work on platelets and thrombin-induced calcium flux revealed that Pak inhibition with IPA-3 (small molecule inhibitor of Group I Pak2), impaired calcium flux.[35] These data suggest that inhibition of multiple Pak kinases may result in impaired calcium flux and downstream signaling effects.

Pak2 deletion in primary mast cells resulted in elevated RhoA-GTPase activity (Figure 2.4A). RhoA activity drives mast cell degranulation, as documented by various researchers nearly 20 years ago.[21-23,31] Constitutively-active (CA) forms of RhoA promoted degranulation in mast cell lines, and dominant-negative (DN) RhoA mutants impair secretion. The mechanism by which RhoA functions to regulate secretion is multifaceted. For one, RhoA controls microtubule formation independent of calcium signaling, allowing vesicle translocation and membrane fusion. DN-RhoA inhibited the FcεRI-induced microtubule formation resulting in reduced degranulation without affecting F-actin polymerization.

RhoA activation enhances MLC2 phosphorylation, known to regulate mast cell secretion by regulating the acto-myosin cortex (Figure 2.4B).[36,37] Additionally, *Pak2*-null mast cells displayed elevated adhesion to a fibronectin substrate in response to IgE-mediated stimulation (Figure 2.3). Upon antigen stimulation, mast cells decrease in height by 50%, and spread out on the substrate, increasing their surface area. Without proper adhesion, mast cells fail to fully degranulate. Interestingly, we found that *Pak1*-null mast cells failed to adhere well upon antigen stimulation, suggesting another reason

for isoform differences in degranulation. Previously, *Pak1* and *Pak2* inhibition with siRNA demonstrated that they play distinct roles in focal adhesions, mediated in part through their different regulation of MLC2 phosphorylation.[13] *Pak1* loss resulted in decreased MLC2 phosphorylation and failure to form focal adhesions, whereas *Pak2* loss resulted in elevated MLC activity leading to significantly larger focal adhesions.[13] These data support our findings, that reduced adhesion in *Pak1*-null cells leads to impaired exocytosis, where as increased adhesion in *Pak2*-null cells increases degranulation. These data suggest strongly that Pak1 and Pak2 function through different signaling pathways to regulate mast cell degranulation in response to antigen stimulation.

Since, MLC2 is activated upon calcium-ionophore induced mast cell secretion, we sought to determine if *Pak2*-null mast cells had changes in MLC2 phosphorylation status upon antigen stimulation.[38] As shown in Figure 2.4B, *Pak2*-null BMMCs had elevated MLC2 phosphorylation, possibly secondary to activation of the RhoA signaling pathway. MLC positively regulates mast cell degranulation via its control of the acto-myosin network and contractile forces. MLC inhibition with MLCK inhibitors KT5926 and ML-7 impaired mast cell degranulation.[36,37]

To test if Pak2 regulates MLC2 phosphorylation through RhoA signaling to control degranulation, we used the RhoA inhibitor, C3-exoenzyme on antigen-stimulated mast cells. We discovered that RhoA inhibition impaired the elevated degranulation observed in *Pak2*-null mast cells (Figure 2.4C). These data clearly demonstrate that Pak2 signals through RhoA to regulate degranulation.

MLC2 regulation by Pak1 and Pak2 remains controversial. MLC2 regulates the interaction between myosin and actin to drive ATP hydrolysis when phosphorylated at threonine 18 and serine 19, and this provides the driving force for cytoskeletal organization and contractility, cell motility, and migration. The phosphorylation state of MLC2 is regulated directly and indirectly by various factors, including MLC kinase (MLCK), MYPT1 phosphatase, RHO kinase (ROCK), and various Pak isoforms.[39-43] Recently, Chu et al. discovered that regulation of MLC2 phosphorylation by Pak1 (and not Pak2) depends on the stressed conditions to the cell, which lead to intestine smooth muscle contractility. In non-stressed conditions, Pak1 is a positive regulator of MLC2 by phosphorylating (inactivating) the phosphatase MYPT1, leading to elevated MLC2 phosphorylation and normal cellular contractility. In the disease/stressed state of intestinal smooth muscle, the relationship switches and Pak1 becomes a negative regulator of MLC2 by activating MYPT1 activity, resulting in decreased MLC2 phosphorylation, and decreased contractility. The complex interaction of Pak1 and Pak2 on MLC2 phosphorylation is diagrammed in Figure 1.3.

In addition to the Pak2-RhoA-MLC2 signaling axis, we also discovered that $p38^{MAPK}$ was elevated in antigen-stimulated Pak2-null mast cells (Figure 2.5A). These data are in direct contrast to *Pak1*-null mast cells, which were impaired for p38 phosphorylation upon stimulation with stem cell factor (SCF) to activate the c-kit receptor.[11] However, studies using dominant-negative mutant forms of Pak2 found an inverse relationship with p38 phosphorylation, suggesting Pak2 is a negative regulator of p38, and Pak1 is a positive regulator.[44] Mast cell activation of p38 in response to

antigen-stimulation is required for degranulation, as well as chemotaxis.[26,45] When p38 is phosphorylated for extended periods of time, there is an induction of degranulation.[26] Based on previous results of Pak1 and p38, as well as the known role of p38 in mast cells, we evaluated p38 phosphorylation status in antigen-stimulated mast cells.[11,46] Our results revealed elevated p38 activation in *Pak2*-null mast cells, opposing known roles of Pak1 and p38. Elevated p38 activation is one mechanism by which *Pak2*-null mast cells display increased degranulation, independent of RhoA activation (Figure 2.5A). We found that drug inhibition of p38 in *Pak2*-null mast cells rescued the enhanced degranulation phenotype to wildtype levels (Figure 2.5B). Together these data demonstrate that Pak2 is a negative regulator of degranulation by controlling the RhoA and p38 signaling pathways in antigen-stimulated mast cells.

Regulation of RhoA activity by Pak2 could be mediated by GAPs, GEFs, and/or GDIs. Since the RhoA-specific GEF, GEF-H1[27], was previously identified as a substrate of Pak1 and Pak4, we evaluated the role of Pak2 on GEF-H1 phosphorylation.[29,33,47] Phosphorylation of GEF-H1 was greatly inhibited in *Pak2*-deficient BMMCs (Figure 2.6A-B). Phosphorylation of GEF-H1 at Ser-885 is known to promote its localization to microtubules by binding 14-3-3, leading to inhibition of its exchange activity.[27-29] These results suggest that, in the absence of Pak2, GEF-H1 remains underphosphorylated and in an active state, thereby activating RhoA. Activated RhoA promotes enhanced degranulation in *Pak2*-deficient cells, as shown by the finding that enhanced degranulation was abolished by addition of the RhoA inhibitor, C3 exoenzyme (Figure 2.4C). An *in vitro* kinase assay demonstrated that Pak2 directly phosphorylates GEF-H1

at serine 885 (Figure 2.6E). Together these data suggest that Pak2 regulates GEF-H1, through phosphorylation, to negatively regulate mast cell degranulation.

A previously identified target of Pak1, Op18/Stathmin, destabilizes microtubules when in the unphosphorylated state by sequestering alpha and beta tubulin dimers.[48] Stathmin/Op18 also functions as a relay in various signal transduction pathways for extracellular signals and to regulate the microtubule (MT) network.[49] Stathmin/Op18 requires proximity to assembling MTs to become locally phosphorylated.[50] Many kinases and phosphatases, including ERK, are associated with microtubules, and therefore function only with intact MTs. Some kinases are activated in response to a change in the cellular MT network. In this context, it is interesting to note that Pak1 is a known negative regulator of stathmin via phosphorylation at Serine 16, a site critical for stathmin's microtubule-depolymerizing activity.[51] We found that this site was underphosphorylated in *Pak2*-deficient cells, but not in *Pak1*-deficient BMMCs, suggesting that stathmin is constitutively active in *Pak2*-deficient mast cells, destabilizing MTs and driving degranulation.

These results suggest two possible hypotheses to explain Pak2 function as a negative regulator of mast cell secretion. First, Pak2 could regulate RhoA activity in mast cells directly by phosphorylation of GEF-H1 at Serine 885, resulting in inhibition of this GEF with subsequent reduction in RhoA activity (Figure 2.6E). In addition, Pak2 might affect GEF-H1 indirectly through phosphorylation of stathmin, resulting in inhibition of stathmin, stabilization of MTs and retention of inactive GEF-H1 at these stabilized MTs (Figure 2.8). In *Pak2*$^{-/-}$ cells, underphosphorylated stathmin drives MT disassembly, generating active GEF-H1, leading to elevated RhoA activity and driving

secretion. These two hypotheses are not mutually exclusive. Recent work by Pathak et al. established that GEF-H1 orchestrates the interplay between the cytoskeleton, vesicle trafficking and fusion to the plasma membrane critical for mast cell degranulation. They found that GEF-H1 binds to exocyst component Sec5 and this interaction activates RhoA, which promotes vesicle assembly, translocation, membrane fusion and secretion.[52,53]

Microtubules serve as tracks for vesicle delivery to the plasma membrane, for fusion and release of contents. Since GEF-H1 is activated upon MT depolymerization, and has now been identified to influence exocyst formation, RhoA activation and translocation, one could postulate that GEF-H1 is in a position to promote vesicle-plasma membrane fusion.[52,53] These data provide yet another mechanism by which RhoA regulates mast cell degranulation, independent of F-actin polymerization.[22] Loss of GEF-H1 activity resulted in impaired endocytic recycling and exocytosis, resulting in the accumulation of vesicles in the cytoplasm. Constitutively active GEF-H1 resulted in increased binding of exocyst proteins, important for translocation of vesicles at the plasma membrane.[52,53] These data demonstrate that GEF-H1-RhoA-exocyst signaling axis could regulate secretion, by regulating exocyst assembly and maintenance through cytoskeletal modulation and fusion to the plasma membrane. Presumably, Pak2 regulation of GEF-H1 mediates these interactions during mast cell granule secretion.

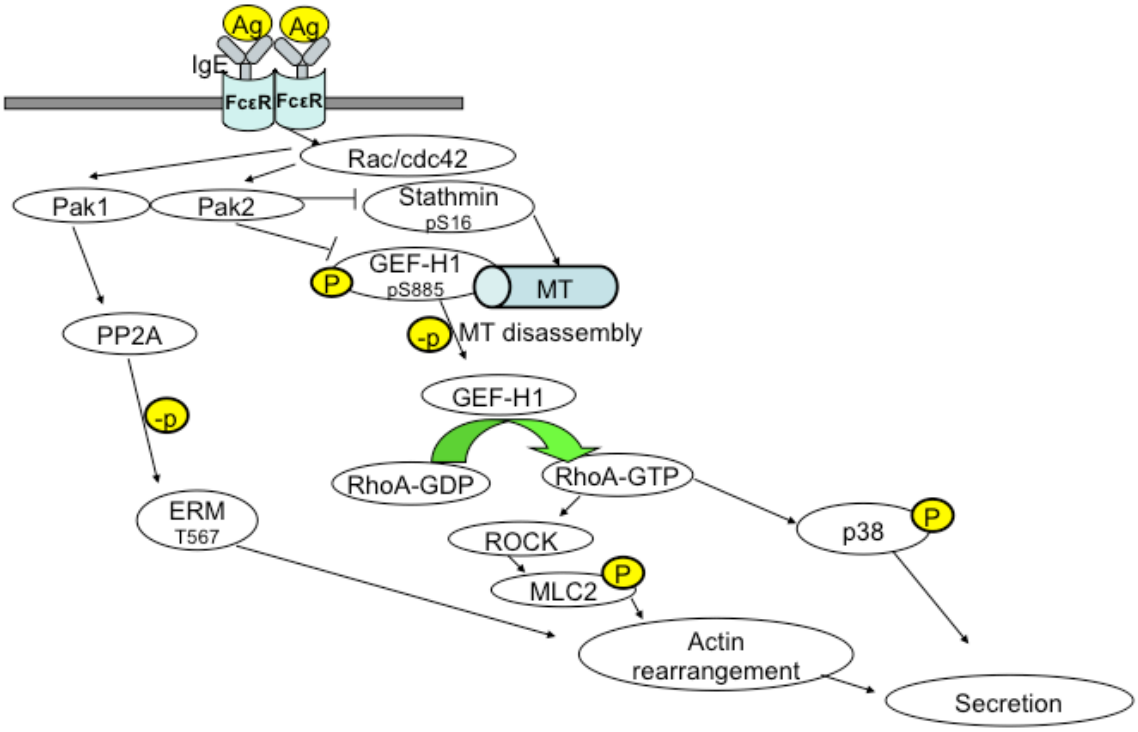

FIGURE 2.8. Model of divergent Pak1 and Pak2 signaling in mast cells. Model demonstrating how Pak1 and Pak2 regulate IgE-mediated secretion in mast cells. In this scenario, Pak1 and Pak2 play opposing roles. Pak1, which is present at lower levels than Pak2 in mast cells, act through association with PP2A to promote ERM phosphorylation and augment secretion. Pak2 acts primarily through phosphorylation of GEF-H1 and stathmin to limit RhoA activity, leading to diminished downstream activation of p38 and MLC2. Limiting the activity of these effectors results in diminished secretion. Differential spatial or temporal regulation of Pak1 *vs.* Pak2 might determine the output (*i.e.,* secretion or no secretion) under particular conditions.

What accounts for the opposing signaling effects of Pak1 and Pak2 in mast cells? These two kinases have very similar N-terminal p21-binding domains and C-terminal protein kinase domains, and have many binding partners and substrates in common. However, the primary intracellular localization of these two enzymes may differ.[54,55] For example, Pak1, unlike Pak2, is localized to cytosolic vesicular structures in unstimulated cells, and translocates to the nucleus following growth factor stimulation.[56,57] This unique feature may impart unique functions to Pak1. With respect to GEF-H1 phosphorylation, Pak1 (and Pak4) has been reported to catalyze Ser-885 phosphorylation, but, perhaps due to its low abundance compared to Pak2 in mast cells, plays little role in regulating GEF-H1 in this cell type (Figure 2.6C,D). In contrast, despite its relative low abundance in mast cells, Pak1, but not Pak2, is required for normal Erk1/2 activation (Fig. 2.5C and [11]).

Additionally, recent work demonstrated that the specific genetic background of the mouse model can alter the intensity of the degranulation response.[58] C57BL/6 (background of the Pak1KO mice) has decreased responsiveness to antigen-stimulated degranulation compared to Sv129 (Pak2f/f were in a mixed background C57BL/6 and Sv129, no backcrossing done prior to experimentation). Sv129 background mice have increased FceRI expression and increased serum IgE levels, partially explaining the increased degranulation observed in vitro. However, this phenotypic difference was only observed in degranulation studies and not observed in cytokine secretion analyses. Therefore, the differences observed in cytokine secretion between Pak1 and Pak2 knockout mast cells show an unambiguous role of Pak1 as a positive regulator and Pak2 as a negative regulator of mast cell antigen-induced secretion (Figure 2.1E). These data

inform future mast cell studies to take extra caution in matching controls based on genetic background of the mouse model in question.

In vitro studies presented here demonstrate that Pak2 is a negative regulator of mast-cell degranulation. To study degranulation in tissue resident mast cells, in vivo passive systemic anaphylaxis was performed. The results indicate a discrepancy between *in vitro* and *in vivo* antigen-mediated degranulation in *Pak2*-null mast cells. In vitro studies with bone marrow derived mast cells grown in culture for 5 weeks demonstrated increased degranulation (Figure 2.1D), where as *in vivo* studies with Mx1-cre induced *Pak2*-deletion demonstrated decreased antigen-induced anaphylaxis (Figure 2.9). There are myriad of explanations to describe these differences. One primary reason for the difference is that Mx1-cre promoter does not efficiently activate in mast cells upon polyI:C administration. Considering mast cells terminally differentiate in tissues and survive from months to years, activation of Cre may have been suboptimal in targeting tissue resident mast cells. There are several citations indicating that polyI:C (dsRNA) can activate toll-like receptor 3 (TLR3) on mast cells, and elicit activation of IRF3 (interferon regulatory factor 3), driving IFN-β activation of macrophages, CXCL8 secretion to recruit NK cells and co-stimulatory CD28/CD80 to recruit CD8+ T cells.[59,60] These activated mast cells can further recruit CD8+ T cells to lymph nodes and co-localize at sites of inflamed tissue. This activation elicited release of inflammatory cytokines and chemokines, but did not induce degranulation. 24 hours post-polyI:C exposure, mast cells had elevated surface expression of MHC class II, CD80/CD28 costimulatory molecules to shape adaptive immunity, complement receptor and FcγII/III.[59-61] Administation of polyI:C two weeks prior to antigen exposure resulted in increased IL-13

release and increased airway hyperresponsiveness.[61] Human mast cells treated with polyI:C demonstrated 50% reduction in adhesion to fibronectin and vitronectin, as well as reduced adhesion-dependent degranulation.[62] Clearly, activation of Mx1cre by administration polyI:C has many off-target effects on mast cells, rendering this model insufficient to study IgE-mediated systemic anaphylaxis. Future work using adoptive transfer of *in vitro* matured mast cells, or mast cell specific-Cre recombinase would be a viable option.

In addition to the off-target effects of polyI:C, there are other reasons for the differences between *in vitro* and *in vivo* mast cell degranulation. Our *in vivo* model collected serum 90 seconds post-antigen stimulation. It is possible that longer exposure to antigen, (i.e. 30 minutes) or increased antigen concentration may have resulted in elevated degranulation in $Pak2^{-/-}$ mice. However, there are reasons to believe that $Mx1cre^{+}Pak2^{f/f}$ mice are deficient in degranulation for reasons beyond the effect on mast cells. $Mx1cre^{+}Pak2^{f/f}$ mice suffer from various systemic problems, which result in death between day 14 and 20 post-polyI:C administration, described in detail in Chapter 4. Some changes that would affect the ability to respond to antigen is blood flow, reduced blood flow rate would reduce the rate at which antigen is presented to tissue resident mast cells. Also, vascular integrity is required for proper antigen presentation, mast cells must be properly positioned at lymphatic blood vessels and endothelium for antigen presentation.[63] $Pak2^{-/-}$ mice demonstrate vascular defects associated with lymphedema and pleural effusions, both indicators of improper vasculature. The *Pak2*-null mice have

decreased blood flow rate, determined by a tail bleed assay where *Pak2*-null mice demonstrated significantly slower blood flow from the tail cut.

An alternative hypothesis to explain differences observed between *in vitro* and *in vivo* degranulation include altered affinity of IgE to FcεRI in the different settings. If *in vivo*, the *Pak2*$^{-/-}$ mice fail to either distribute IgE to peripheral tissue resident mast cells, or the mast cells themselves are deficient in FcεRI, these could hinder anaphylaxis. There is also the possibility that *in vivo Pak2*$^{-/-}$ mast cells are not fully developed, have reduced numbers in circulation and tissues or have a weaker affinity for IgE. Additionally, *Pak2*$^{-/-}$ mast cells *in vivo* may not extravasate from endothelial cells into surrounding tissues as well as wildtype mice, and therefore do not mature fully. These are just a few examples to describe the discrepancy between *in vivo* and *in vitro* degranulation results.

However, to overcome the effects of systemic changes in *Pak2-/-* mice on mast cell degranulation and obtain data from *in vivo* degranulation, an alternative approach to study *Pak2*-null mast cells *in vivo*, would be to adoptively transfer *Pak2*-null mast cells in W^{sh}/W^{sh} mice, which lack functioning mast cells (*ckit*-null mice). This adoptive transplantation model would also clarify if the tissue resident mast cells were still wildtype, considering their longevity in tissues. However, the development of mouse strains wild-type for Kit, but selectively deficient for mast cells, and on defined genetic backgrounds would be advantageous to truly understanding the effects of gene deletion on mast cells. By constitutively expressing Cre recombinase under the control of mast cell specific genes, such as *A-Mcpt-5*-cre, mast cell-specific conditional gene modification can be been achieved in mature tissue resident mast cells, to varying degrees of efficiency and specificity, and avoids pathogenic effects of deregulated Pak2 signaling

in the hematopoietic progenitor cells.[64-67] Recent developments in this field have designed a tamoxifen-cre inducible mouse model that drives deletion of C-kit, a receptor on mast cells.[68] Recombination with tamoxifen administration took 2 weeks, and was sustained for at least 8 weeks in peritoneal and dermal mast cells. Hematopoietic c-kit+ cells were not affected by this deletion. There was low turnover and regeneration of mast cells from unaffected precursors. This genetic tool provides researchers the opportunity to study gene function in mature tissue resident mast cells without effecting other immune cells and bone marrow.

This study provides novel findings into signaling differences between Pak1 and Pak2 in mast cell FceRI signaling. Future *in vivo* experiments using mast cell-specific models to delete Pak2 solely in mast cells and *in vitro* experiments using chimeric versions of Pak1/2 hybrids may prove useful in mapping structural features within these kinases that impart signaling specificity.

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FOOTNOTES

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Abbreviations: BMMC, Bone-marrow derived mast cells; CRAC, calcium release-activated calcium; DNP-HSA, dinitrophenyl-human serum albumin; ELISA, Enzyme linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FcεRI, receptor for the Fc fragment of IgE; GEF, guanine-nucleotide exchange factor; GST, glutathione-*S*-transferase; 4-HT, 4-hydroxytamoxifen; IL, interleukin; myosin light chain, MLC; Pak, p21-activated kinase; PLCγ1, phospholipase Cγ1; Wild-type, WT.

Chapter 3: Pak2 restrains endomitosis during megakaryopoiesis

INTRODUCTION

Megakaryocytes are both the largest (50-100μm) and most rare cell type (~0.03-0.06%) in the bone marrow.[1,2] To produce sufficient numbers of platelets, these cells become polyploid and undergo massive nuclear proliferation, together with an enlargement of the megakaryocyte cytoplasm, which becomes filled with platelet-specific granules. Megakaryocytes undergo a complex maturation process by which their cytoplasmic contents are packaged into multiple elongated proplatelet processes.[3] As proplatelets extend through the sinusoid vessels, physiological shear force aids in dissociating proplatelets into circulating platelets.[4,5] MK maturation is triggered primarily through cellular signaling events initiated by the cytokine thrombopoietin (TPO).[6] TPO binds to the c-Mpl receptor on megakaryocytes to activate the Janus kinase (JAK)-2 signaling pathway and stimulation of PI3K/Akt and MAPK pathways.[7-9]

The p21-activated kinases (Paks) are serine/threonine kinases involved in a variety of key signaling pathways that effect cell shape, contractility, motility and survival.[10] Paks consist of six isoforms distributed into two groups, Group I (*Pak 1-3*) and Group 2 (*Pak 4-6*). Activation of Pak is primarily achieved through binding of the Rho GTPases, Rac1 and Cdc42, which relieve Pak autoinhibition and induce conformation changes that activate the catalytic domain. Once activated, Paks phosphorylate dozens of signaling proteins, including those that regulate the ERK pathway (Mek-1 and c-Raf), assembly of the mitotic spindle (Aurora A), and microfilament assembly (LIMK, GEF-H1).[11-14]

Paks regulate several hematopoietic biological processes, including hematopoietic stem cell engraftment and homing to the bone marrow, assembly of the actin cytoskeleton, and chemotaxis.[15] Paks promote hematopoiesis by regulating Raf-1 and Mek-1 activation to drive Erk1/2 activation, resulting in proper hematopoietic function.[16] Paks similarly have roles in platelet activation through the orchestration of platelet signaling and cytoskeletal dynamics.[17-22] Paks link Rac1, downstream of receptor GPVI activation, to activation of the MAP kinase pathway, granule secretion, and platelet aggregation.[17,18,23,24] Recently, mice with megakaryocyte-specific double knockout deletions of the Pak activating proteins, *Rac1/Cdc42*, were shown to develop macrothrombocytopenia, with abnormal megakaryocyte morphologies, a failure to form proplatelets and shortened platelet half-life.[25,26] Although Pak activation is compromised in megakaryocyte and platelet systems lacking Cdc42 and Rac1, a specific role for Paks in megakaryocyte development and function has yet to be defined.[23-25]

In this work, we sought to determine the contribution of group I Paks in the process of megakaryocyte maturation and polyploidization. We found that bone marrow specific deletion of *Pak2* is associated with macrothrombocytopenia, decreased platelet half-life, increased megakaryocyte ploidy, and altered microfilament and microtubule structures. These effects are accompanied by defective activation of the Pak substrates, LIMK and Aurora. Together, these findings suggest a unique function for *Pak2* in megakaryocyte development, a function that may need to be considered as small molecule inhibitors of Paks are developed as clinical agents.[27-31]

Material and Methods

Generation of mice with *Pak2*$^{-/-}$ bone marrow

Pak2-deficient mice were generated by crossing mice containing the *Pak2* gene flanked by loxP sites (*Pak2*$^{fl/fl}$), with mice carrying the Mx1cre transgene.[15,32,33] (Figure 4.1). In 8- to 12-week old *Mx1-cre*$^{+}$*Pak2*$^{fl/fl}$ mice (mixed background sv129/C57Bl/6), gene deletion was induced by 3 intraperitoneal injections of 400ug polyinosinic-polycytidylic acid (pIpC) in a 2-day interval. Littermates received the same treatment. Fourteen days after the first injection, mice were used in experiments. Additionally, a colony of CAGG-Cre-ERT2^{+}*Pak2*$^{fl/fl}$ mice were generated in order to delete *Pak2* in *in vitro* culture of bone marrow-derived megakaryocytes. To induce deletion, 500 nM 4-hydroxytamoxifen was administered to bone marrow cultures for 5 days. For Pak inhibitor treatments, administration of Frax1036 (Genentech) was done by daily oral gavage (30 mg/kg) diluted in 20% (2-Hydroxypropyl)-β-cyclodextrin (Sigma) for three weeks. Age-matched control animals were treated with vehicle alone. All animal studies were performed according to protocols approved by Fox Chase Cancer Center institutional animal care and use committee.

Antibodies and Reagents

All reagents were purchased from Sigma-Aldrich unless otherwise stated. Polyclonal antibodies against phospho-LIMK (T508), phospho-Cofilin (S3), and phospho-Aurora A/B/C (T288/T232/T198) and total proteins (LIMK, Cofilin) were purchased from Cell Signaling Technology. A mouse monocloncal antibody against Aurora A was purchased from BD Biosciences. Recombinant mouse thrombopoietin (rmTPO) was purchased from Shenandoah Biotechnology (Warwick, PA). IL-3 was purchased from PeproTech (Rocky Hill, NJ). Antibodies for flow cytometry were from

Ebioscience (antibody name (clone)) murine hematopoietic lineage eFluor 450 cocktail, c-Kit-APC (2B8), sca-1-PE-Cy7 (D7), CD150-FITC (BioLegend-TC15-12F12.2), CD41-APC-Cy7 and CD41-eFluor 450 (MWReg30), CD105-PE (MJ7/18), FcγII/III-PerCP-eFluor 710(clone 93). Antibodies for immunofluorescence (CD41 and β1-tubulin) were purchased from Abcam.

Analysis of platelet clearance and production

To determine platelet clearance in both *Pak2*$^{-/-}$ and wild-type mice, an *in vivo* biotinylation approach was used.[34] Briefly, 8- to 12-week-old *Pak2*$^{-/-}$ and wild-type mice, 10 days after pIpC injection were injected via tail vein with 35 μg/g body weight sulfo-NHS-biotin (Pierce Chemical). Retro-orbital bleeds were used to collect blood daily into 3.8% sodium citrate. After collection, the blood was diluted 20× in PBS and incubated with streptavidin-PE (BD Biosciences) to label biotinylated platelets for 30 minutes at 4°C. Thiazole orange (10 μg/mL) was then added to measure reticulated platelets, and the samples were incubated for 15 minutes at room temperature (RT). After fixation in 1% formalin, the samples were analyzed via flow cytometry with appropriate color compensation.

Bone marrow collection and MegaCult-C assays

Bone marrow cell suspension was isolated from the tibias and femurs of pIpC treated mice. Bones were flushed with DMEM/2% FBS followed by filtration through a 100-μm nylon strainer. RBC lysis was done prior to antibody staining, bone marrow culture and colony formation assays. For MegaCult-C assays, a total of 10^5 unsorted bone marrow cells were used, according to the manufacturer's protocols (Stem-Cell Technologies); 50 ng/ml murine thrombopoietin (TPO) (Shenandoah Biotechnology,

Warwick, PA), 10 ng/ml murine IL-3 (Peprotech, Rocky Hill, NJ) were used in these assays. The cultures were incubated for 6 to 8 days. Colonies were fixed and stained for acetylcholinesterase according to the manufacturer's protocol (StemCell Technologies). Duplicate assays were performed for each mouse.

Measurement of hematological parameters

Mice were euthanized with CO_2 and blood was extracted via the hepato-portal vein with a syringe containing ACD (acid-citrate dextrose) (10% final). Whole blood was added into tubes containing EDTA. The complete blood count was performed using a VetScan HM5 (Abaxis) within 1-2 hours of collection at room temperature.

***In vitro* culture of bone marrow-derived megakaryocytes**

Primary megakaryocytes derived from bone marrow of *Mx1-cre*$^{+}$*Pak2*$^{fl/fl}$ mice 10 days after the last pIpC injection were cultured in DMEM, 10% FBS, penicillin/streptomycin, and 100 ng/ml TPO for 5 days. Megakaryocytes cultured from Cag-Cre-ERT2;Pak2f/f bone marrow were treated with 500nM 4-hydroxytamoxifen during the 5 day culture with TPO to delete Pak2. Cultures were then fractionated on a 1.5% / 3% discontinuous bovine serum albumin (BSA) gradient, and cultured for an additional day.

Proplatelet formation

Proplatelet-displaying megakaryocytes were defined as cells exhibiting one or more cytoplasmic processes with areas of constriction. After 5 days in culture, fetal liver derived megakaryocytes were separated on a BSA gradient, followed by 24 hours of growth. Proplatelet extensions were quantified by calculating the percentage of megakaryocytes with such processes on inverted microscope at a magnification of 20X.[35]

Megakaryocyte ploidy analysis

For ploidy measurements, cells were analyzed as described previously.[36] DNA content in $CD41^+$ megakaryocytes was determined by labeling RBC-lysed bone marrow with anti-CD41 4°C for 30 minutes, followed by fixation with 0.5% formalin for 15 minutes at room temperature. Cells were then permeabilized with 70% ice-cold methanol for 1 hour on ice. After washing cells, they were then incubated with 10ug RNase A at 37°C for 30 minutes, followed by incubation with 1 μg/μl propidium iodide for 15 minutes at room temperature. Cells were analyzed for ploidy on an LSR-II with proper fluorochrome compensation.

Analysis of megakaryocyte progenitor cells

To analyze megakaryocyte stem cells, freshly isolated bone marrow was stained with mouse hematopoietic eFluor-450 lineage cocktail, anti-c-Kit-APC, anti-sca-1-PE-Cy7, anti-CD150-FITC, anti-CD41-APC-Cy7, anti-CD105-PE, anti-FcγII/III-PerCP-eFluor 710. PreMegE cells were gated as $lin^-sca1^-ckit^+CD41^-FcγII/III^-CD105^-CD150^+$ and megakaryocyte progenitors were gated as $lin^-sca1^-ckit^+CD150^+CD41^+$.[37] Mature megakaryocytes were analyzed by anti-CD41-APC-Cy7 and calculated as percent of total bone marrow. Flow cytometric data collection was performed on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

Western Blotting

In vitro cultured megakaryocytes on day 5 post-BSA gradient were lysed, clarified by centrifugation and denatured. Samples were separated by SDS–polyacrylamide gel electrophoresis (PAGE) on a 4% to 20% gradient gel (BioRad, Carlsbad, CA) and transferred to PVDF membrane. Membranes were blocked and

probed with primary antibodies overnight. Secondary HRP-conjugated antibodies were incubated for one hour. ECL (Millipore) was used to resolve bands and imaged on ProteinSimple imager. Phosphorylated protein band density was quantified with Fiji (NIH Image software; National Institutes of Health, Bethesda, MD) and calculated relative to total protein.

Confocal microscopy

In vitro cultured bone marrow-derived megakaryocytes were adhered to fibrinogen coated slides (200 ng/ml) for 5 hours with TPO. Cells were fixed with 2% PFA and centrifuged onto coated slides for 5 minutes at 1000 x *g*. Cells were permeabilized with 0.5% triton X-100 for 5 minutes prior to blocking for 1 hour and antibody incubation overnight at 4°C. Slides were counterstained with DAPI and phalloidin, and mounted in ProLong gold prior to visualization. Confocal microscopy was done on an inverted Leica SP8 3 channel confocal system at 100X magnification. Image analysis was performed with Fiji (ImageJ) software (NIH).

Histology

Five μm sections of paraffin-embedded sternum and spleen were stained with Hematoxylin and Eosin and analyzed for megakaryocytes using an Olympus BX53 microscope with the 40X objective.

Statistical analysis

The data presented in this report are the results of at least 3 independent experiments with separate mice used per replicate. Statistical analysis was performed using a 2-tailed Student *t* test, and a P value <0.05 was considered statistically significant.

RESULTS

Inducible deletion of *Pak2* from bone marrow results in macrothrombocytopenia

To study the function of the *Pak2* gene in various hematopoietic-derived cells, mice carrying conditional $Pak2^{fl/fl}$ alleles were crossed with mice harboring the interferon-inducible Mx1-Cre transgene to produce Mx^{Tg};$Pak2^{f/f}$ ($Pak2^{-/-}$) and Mx^{Tg};$Pak2^{+/+}$ controls (wild-type).[32, 38](M. Radu and J. Chernoff, manuscript in preparation). Deletion of the floxed exon 2, which encodes the start site of *Pak2*, was achieved by IP administration of plpC. Pak2 protein levels in the bone marrow, platelets and megakaryocytes were below the level of detection of Western blotting with anti-Pak2 antibodies at 14 days post-plpC (Figure 3.1A). Deletion in megakaryocytes of $Pak2^{-/-}$ mice was confirmed by culturing bone marrow extracted at 14 days post-pIpC.

Complete blood count analysis of $Pak2^{-/-}$ and wild-type mice displayed moderate thrombocytopenia (wild-type, 997 $\times 10^3/\mu l$ [$\pm 98 \times 10^3$] *n=29*; $Pak2^{-/-}$, 498 $\times 10^3/\mu l$ [$\pm 25 \times 10^3$] *n=52*; Table 3.1). There was also a statistically significant increase in platelet size as determined by mean platelet volume (MPV; wild-type, 6.56±0.05 fL vs. $Pak2^{-/-}$ 7.1±0.07 fL, $p<0.001$). Additional changes in blood counts included increased neutrophils and monocytes and decreased lymphocytes (Table 3.1). Deletion of the related gene, *Pak1,* did not affect peripheral blood indices, including no changes in platelet counts or megakaryopoeisis.[39] (J. Kostyak unpublished data)

Pak2 plays a role in megakaryocyte maturation

Mice with megakaryocyte-specific deletions of the Pak activating proteins, Rac1/Cdc42, are macrothrombocytopenic, with megakaryocytes that fail to form proplatelets, but mature properly with normal polyploidization.[25,26] As Paks are

downstream effectors of these GTPases, we sought to determine if Pak activation is required for megakaryocyte development and function, as well as to maintain platelet counts.[23-25] As *Pak1*$^{-/-}$ mice do not have a notable platelet or megakaryocyte phenotype[39], we examined the effects of genetic deletion of *Pak2 in vivo* on megakaryocyte differentiation. We evaluated the bone marrow for changes in megakaryocyte number and morphology. Bone marrow from *Pak2*$^{-/-}$ mice had increased number of mature megakaryocytes (CD41^{+}) with increased size (Figure 3.1B-D). Flow cytometry analysis demonstrated an increased proportion of cells expressing the megakaryocyte-specific antigen, CD41^{+} (Figure 3.1D).

We next sought to determine if Pak kinase activity changed during stem cell maturation into fully differentiated megakaryocytes. We isolated CD34^{+} stem cells by flow cytometry from the bone marrow and cultured the sorted cells with TPO for 5 days. Sample lysates were collected and analyzed for activated phosphorylated Pak1/2/3, total Pak2 and CD41 protein expression (Figure 3.1E). Our results demonstrated Pak1/2/3 activation increased during the maturation process, until eventually decreasing at the fully mature state (D5). Pak2 was substantially autophosphorylated in CD34^{+} stem cells (D0), but this level, like that of Pak1/3, also increased during maturation (Figure 3.1E, lower phospho-Pak band).

To determine the localization of Pak2 in mature megakaryocytes, proplatelets and platelets, we performed confocal microscopy. Our results indicated that Pak2 is expressed throughout these three stages of megakaryocyte maturation (Figure 3.1F). Together, these data demonstrate that Pak2 has a role in megakaryocyte development,

from the stem cell state to the mature megakaryocyte, and functions as a negative regulator of megakaryopoiesis.

Table 3.1. Complete blood count profile for Pak2-deficient mice

Parameters	$Mx1Cre^{Tg}Pak2^{+/+}$	$Mx1cre^{Tg}Pak2^{f/f}$
WBC, $x10^9$/L	8.97 ±0.44	7.6 ±0.4
Neutrophils, %	12.66±0.93	29.23±1.63§
Lymphocytes, %	79.22±1.72	62±2.5§
Monocytes, %	2.28±0.23	3.18±.28
RBC, $x10^{12}$/L	9.87±0.27	9.67 ±0.25
Hb, g/L	14.43 ±0.42	14.57 ±0.29
HCT, %	44.9 ±1.3	45.73 ±0.85
MCV (fL)	44.37 ±1.12	46.09 ±0.39
Platelets, $x10^9$/L	997±98	498 ±25 §
MPV (fL)	6.56±0.05	7.1±0.07 §

§ $P < 0.05$

Complete blood counts from primary $Mx1\text{-}cre^{Tg}pak2^{f/f}$ mice and corresponding controls were performed 13 to 16 DPI with polyI:C as described in methods. Hematologic measurements were performed on a Hemavet 850 Hematology Analyzer. The data are means ±SEM.

WBC indicates white blood cells; RBC, red blood cells; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpusal volume; MPV, mean platelet volume.

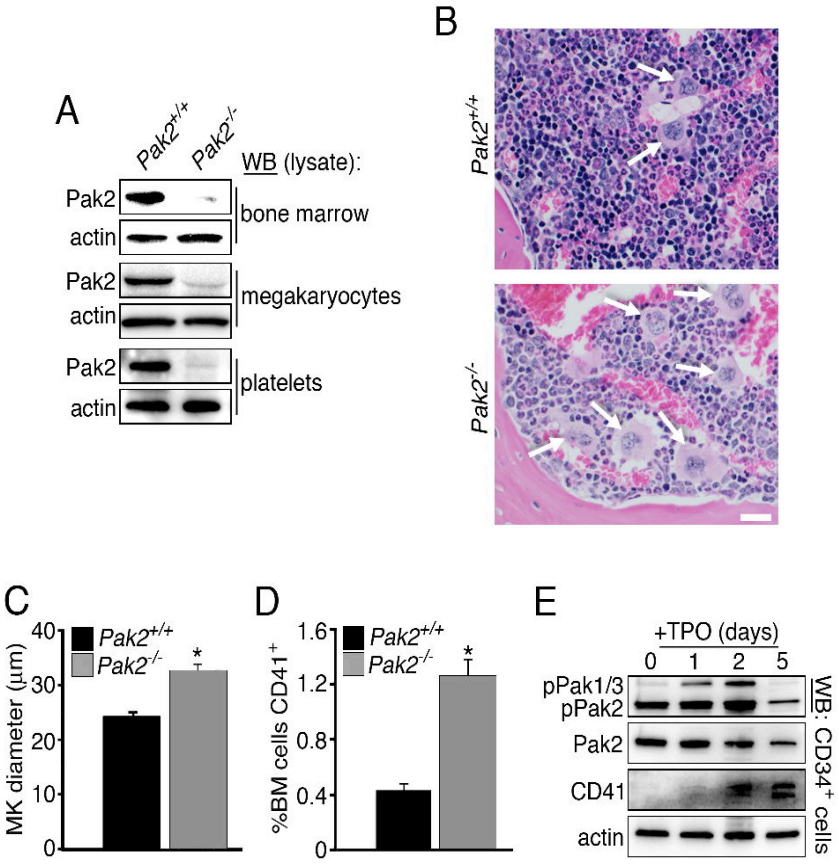

Figure 3.1. *Pak2* deletion stimulates megakaryopoiesis *in vivo*

A, Western blot to detect Pak2 levels in bone marrow (top), megakaryocytes (middle) and platelet (bottom) lysates at 14 days post-pIpC (DPI). Actin serves as a loading control for relative protein levels. *B*, Representative bone marrow histology from wild-type and *Mx1-cre*$^{+}$*;Pak2*$^{fl/fl}$ mice (n=5 mice/genotype), both treated with plpC and analyzed 14 DPI. White arrows indicate megakaryocytes. Scale bar = 20 µm (40x original magnification). *C*, Measurement of megakaryocyte (MK) diameter in the bone marrow was performed by H&E sections.(n=5 mice/genotype). *D*, Percent of bone marrow (BM) expressing CD41 measured by flow cytometry 14 DPI 14. (E) Replicate samples of CD34+ bone marrow stem cells sorted by flow cytometry and cultured with 100 ng/ml TPO for 5 days. Representative WB of phospho-Pak1/2/3 (Serine141) and Pak2 expression over a 5 day time course. Actin loading control. CD41 expression marks mature megakaryocytes.

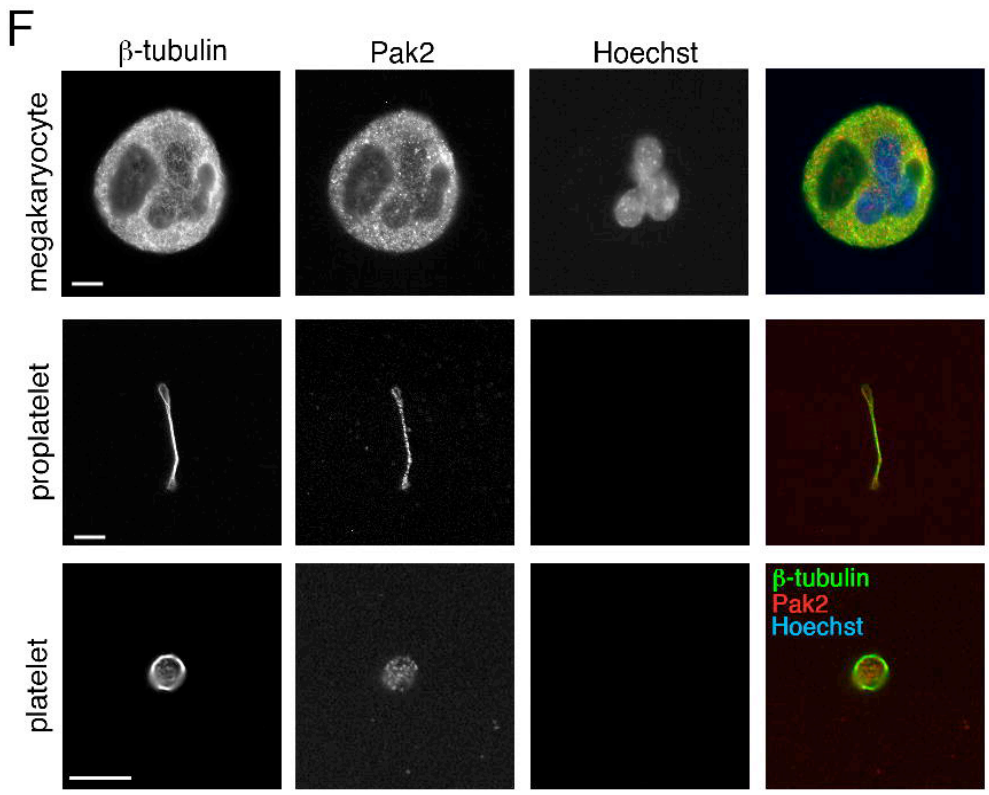

Figure 3.1. (Continued). *F*, Confocal microscopy of a megakaryocyte (top), proplatelet (middle) and platelet (bottom) stained for β1-tubulin, Pak2 and nuclei (Hoechst). Scale bar = 10μm. Image contributed by Joseph Aslan, Oregen Health and Sciences University. All values are mean ± SEM for at least 5 mice/genotype. * *p-value <0.05*

***Pak2*-deficiency results in increased platelet clearance rate and thrombopoiesis**

The combination of increased bone marrow megakaryocyte size, number, along with a decrease in peripheral platelet count, suggested that *Pak2*$^{-/-}$ mice produce abnormal platelets with decreased survival in the circulation. To test whether platelet clearance was altered, we measured platelet life span, as well as the production of reticulated platelets.[34] The amount of platelets remaining in circulation was calculated as a percentage of labeled platelets from 24 hours post-NHS biotin injection. *Pak2*$^{-/-}$ platelet life span was significantly reduced relative to wild-type mice (Figure 3.2A). These data are comparable to the shortened platelet life span in the *Rac1/Cdc42*$^{-/-}$ mice.[25] To assess if platelet production was altered by hematopoietic deletion of *Pak2*, we labeled blood samples *ex vivo* with thiazole orange.[40] As shown in Figure 3.2B, there was nearly double the amount of reticulated platelets in *Pak2*$^{-/-}$ mice compared to controls (wild-type, 5.8 ± 0.4% vs. *Pak2*$^{-/-}$, 11.5 ± 0.6%; mean ± SEM) at 10 days post-pIpC injection. Young reticulated platelets are larger than older non-reticulated platelets[41,42]; therefore we evaluated mean platelet volume (MPV) and found it to be significantly increased in *Pak2*$^{-/-}$ mice (Figure 3.2C). The mouse spleen acts as a site for platelet clearance and production following bone marrow damage. Therefore, we evaluated the spleen for extramedullary megakaryopoiesis and discovered that *Pak2*$^{-/-}$ null mice had significantly increased production of mature megakaryocytes throughout the spleen, along with significantly decreased spleen size (Figure 3.2D-F). Together, these data demonstrate that *Pak2*$^{-/-}$ mice are macrothrombocytopenic due to decreased platelet life span.

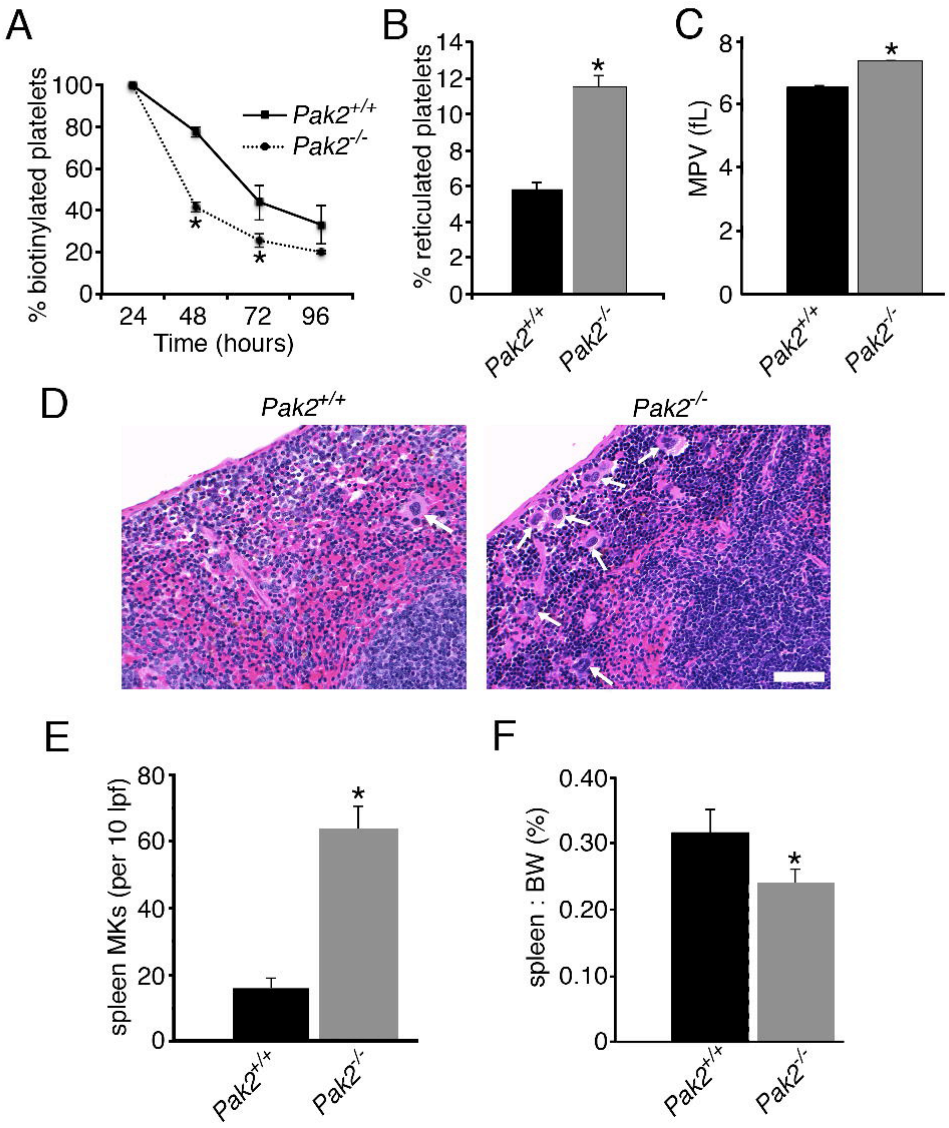

Figure 3.2. Clearance rate and production of platelets is increased in *Pak2-null* mice

(A) Quantification of *in vivo* biotinylated platelets 24, 48, 72, and 96 hours after NHS-biotin injection. Data expressed as percentage of baseline (24 hours post-injection). (n=4-8 mice/genotype per time point; mean±SEM). (B) Quantification of the percentage of new, reticulated platelets, as a percentage of total platelets in wild-type and *Pak2*$^{-/-}$ mice (n>5; mean±SEM). (C) Mean platelet volume (MPV) of wild-type and *Pak2*$^{-/-}$ platelets. (D) Representative images of hematoxylin and eosin stained spleen sections from wild-type and *Pak2*$^{-/-}$ mice D14 post-pIpC injection. Arrows indicate splenic megakaryocytes, scale bar = 50 μm (40x original magnification). (E) Quantification of megakaryocytes in spleen sections counted in 10 low power fields (lpf). (F) Ratio of spleen weight to body weight (BW) on day 14 post-plpC injections. All data from at least 3 mice/genotype; mean ± SEM. * *p-value <0.05.*

Megakaryocyte progenitor populations increased in *Pak2*-deficient mice

Extensive flow cytometry studies have identified surface markers that constitute bone marrow megakaryocyte progenitors. Since we observed significantly more mature megakaryocytes in *Pak2*$^{-/-}$ bone marrow compared with their wild-type littermate controls (Figure 3.1D), we investigated whether this increase was associated with an increase in megakaryocyte progenitors. The bipotential megakaryocyte/erythroid progenitor, PreMegE, is the main progenitor cell type that produces mature megakaryocytes and has the highest capacity to form megakaryocyte colonies *in vitro*.[37] This population is defined as Lin$^-$c-Kit$^+$Sca1$^-$CD41$^-$FcγRII/III$^-$CD150$^+$CD105$^-$. We found that *Pak2*$^{-/-}$ bone marrow had significantly more bipotential PreMegE cells than wild-type bone marrow (Figure 3.3A). We next examined committed megakaryocyte progenitors (MkP) by surface expression of CD41 after gating for Lin$^-$c-Kit$^+$Sca1$^-$CD150$^+$. These progenitor cells represent an intermediate stage between the bipotential precursor and the fully differentiated megakaryocytes. This committed population was significantly increased in the *Pak2*$^{-/-}$ bone marrow (Figure 3.3B). Both the PreMegE and MkP populations were elevated in *Pak2*$^{-/-}$ bone marrow, suggesting that there is enhanced production of bone marrow megakaryocyte progenitors in the *Pak2*$^{-/-}$ mice.

Given the increase in megakaryocyte stem cell progenitors, we next tested whether these progenitors were capable of producing mature megakaryocyte colonies. To address this question, we cultured unsorted bone marrow from wild-type and *Mx1cre*$^+$*Pak2*$^{fl/fl}$ animals, 14 days post-pIpC injection, and seeded an equal number of cells in a megakaryocyte specific colony-formation assay (CFU-MK). Analysis of the

colonies with acetylcholinesterase staining after 8 days of growth with TPO and IL-3 demonstrated an increased number of colonies in *Pak2*$^{-/-}$ bone marrow, relative to wild-type (Figure 3.3C). These results suggest that the number and proliferative potential of MK progenitors are increased in *Pak2*-deficient mice.

Since *Pak2*$^{-/-}$ mice demonstrated altered megakaryopoiesis, we next asked if loss of the *Pak2* gene was associated with abnormal hematopoiesis. Total BM hematopoietic stem cells [LSK (Lin^-/C-kit^+/$Sca1^+$) (p-value<0.01) and LK (Lin^-/C-kit^+/$Sca1^-$) (p-value<0.001)] were evaluated and found to be significantly elevated in *Pak2*$^{-/-}$ bone marrow compared with wild-type (Figure 3.3D). Additional evaluation of LSK stem cells, divided into HSC ($CD150^+CD105^+$) and multipluripotent stem cells (MPP) ($CD150^-CD105^-$) also demonstrated significantly increased populations in the bone marrow of *Pak2*-null mice (Figure 3.3D, p-value<0.005). Representative flow cytometry gating schemes are shown in Figure 3.3E. Collectively, these findings identify *Pak2* as a negative regulator of megakaryocyte maturation via regulation of the bipotential precursors (PreMegE) and megakaryocyte committed progenitor (MkP) cells in the bone marrow. Additionally, Pak2-deletion results in expansion of the hematopoietic stem cell compartment.

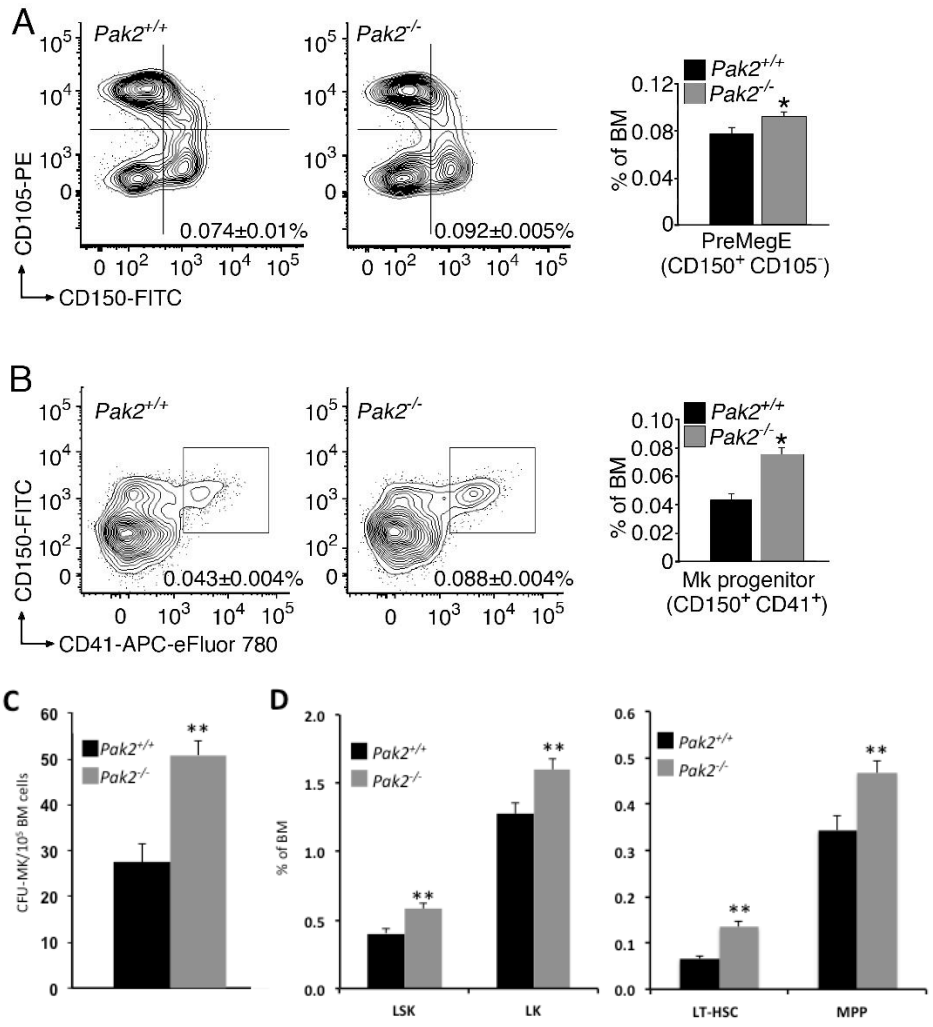

Figure 3.3. *Pak2*-deficiency increases megakaryocyte precursors and hematopoietic stem cells (A) Percent of bone marrow cells from wild-type or *Pak2*$^{-/-}$ mice that are Pre-megakaryocyte/erythroid cells (PreMegE) identified by CD150^{+} and CD105^{-} after gating for Lin^{-}C-kit^{+}Sca1^{-}. (* p<0.05) (B) Percent of bone marrow from wild-type or *Pak2*$^{-/-}$ mice that are from the committed megakaryocyte progenitor (MkP) population, defined by CD41^{+} and CD150^{+}, after gating for Lin^{-}C-kit^{+}Sca1^{-} (* p<0.0001) (C) Methylcellulose colony formation assays of bone marrow cells from wild-type and *Pak2*$^{-/-}$ mice. Numbers of megakaryocyte colony forming units (CFU) were counted after staining with acetylcholinesterase. (*p<0.0002). (D) Hematopoietic stem cells in bone marrow of *Pak2*$^{+/+}$ and *Pak2*$^{-/-}$ mice. LSK (Lin^{-}/C-kit^{+}/Sca1^{+}); LK (Lin^{-}/C-kit^{+}/Sca1^{-}). LSK cells were then analyzed for CD150 and CD105 to distinguish long-term HSCs (LT-HSC, LSK;CD150^{+}CD105^{+}) and multipluripotent cells (MPP, LSK;CD150^{-}CD105^{-}).

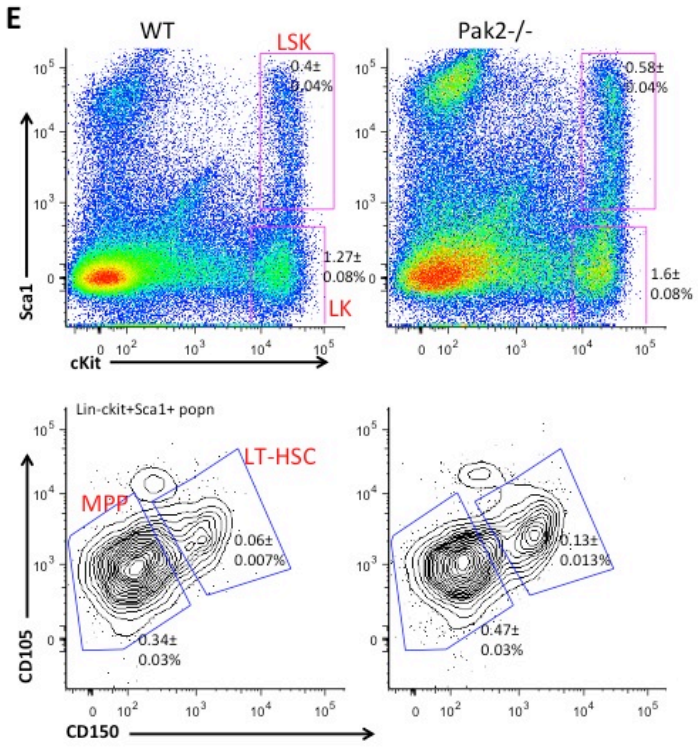

Figure 3.3 Continued (E)Representative flow cytometry plot (mean±SEM, n>13) for bone marrow hematopoietic stem cells, LSK, LK, LT-HSC and MPP. All bone marrow measured at day 14 post-pIpC. Experiments included a minimum of n=10 mice/genotype, mean ± SEM, * $p<0.05$; ** $p<0.01$.

Megakaryocyte endomitosis is negatively regulated by *Pak2*

Normal megakaryocytes progress through development to an endomitotic phase, where cells are programmed to fail cytokinesis and accumulate DNA in a single polylobulated nucleus prior to proceeding to a final maturation state, consisting of proplatelet formation and platelet release.[43,44] Accordingly, we examined the process of endomitosis (polyploidization) in megakaryocytes from *Pak2*$^{-/-}$ mice. We found that *Pak2* deletion in hematopoietic cells *in vivo* resulted in markedly increased polyploidization of megakaryocytes (% of CD41^{+} cells containing >8N DNA content) (Figure 3.4A). To determine if increased polyploidization *in vivo* was due to cell-intrinsic effects, we measured polyploidy in megakaryocytes deleted *in vitro* with tamoxifen-regulated CAG-Cre recombinase and found *Pak2* regulated polyploidization in a cell-intrinsic manner (Figure 3.4B). *Pak2*$^{-/-}$ megakaryocytes demonstrated significantly increased 8N, 16N and 32N populations (*p-value* <0.03). These *in vitro* data suggest that increased polyploidization observed *in vivo* is from cell-intrinsic signaling, independent of changes in peripheral platelet counts or in the bone marrow stroma.

Small molecule inhibitors to specifically inhibit group I Paks (isoform 1-3), are currently being developed for treatment of tumors overexpressing Pak proteins or with gene amplification of Pak.[10,29-31,45,46] These include, but are not restricted to, breast, pancreatic, Braf-wildtype melanoma, neurofibromatosis type 1 and 2, and colon cancers.[10,47] We examined if mice treated with Frax1036, the most specific *Pak1-3* kinase inhibitor known to date, also displayed altered megakaryocyte polyploidization (H.Y. Chow and J. Chernoff, manuscript submitted August 2014). Treatment of animals

with Frax1036 for 3 weeks increased polyploidization in megakaryocytes, as well as increased $CD41^+$ megakaryocyte population (Figure 3.4C-D). Frax1036 treatment effectively ablated *Pak1-3* phosphorylation in the bone marrow (Figure 3.4C, inset). Bone marrow-derived megakaryocytes treated with Frax1036 in culture for 5 days also demonstrated increased polyploidization at 8N and 16N ploidy stages ($p<0.008$ and $p<0.003$, respectively) (Figure 3.4E). Collectively, these findings indicate that genetic deletion of *Pak2*, as well as pharmacologic kinase inhibition, causes increased polyploidization in $CD41^+$ bone marrow megakaryocytes *in vivo* and *in vitro*. Since Pak kinase inhibition resulted in increased polyploidy, this indicates that Pak is required in a kinase-dependent function, rather than an alternative function, such as a scaffold protein.

Altered microtubule structure in *Pak2*$^{-/-}$ megakaryocytes

Pak kinases are principally known for their regulation of actin and tubulin cytoskeleton networks.[48] During megakaryocyte maturation into circulating platelets, these cells undergo massive cytoskeletal alterations critical for formation of proplatelet extensions, which transverse the sinusoid vessels to release platelets into circulation.[44,49,50] As *Pak2*$^{-/-}$ megakaryocytes demonstrated increased ploidy, we analyzed if *Pak2* had an effect on the megakaryocyte cytoskeleton. Previous work demonstrates a role for cytoskeletal regulation in megakaryocyte polyploidization.[51-56] Mature megakaryocytes were analyzed for β1-tubulin expression, the tubulin isoform primarily expressed in megakaryocytes. As shown in Figure 3.5A, β1-tubulin expression was altered in *Pak2*$^{-/-}$ megakaryocytes compared to wild-type megakaryocytes.

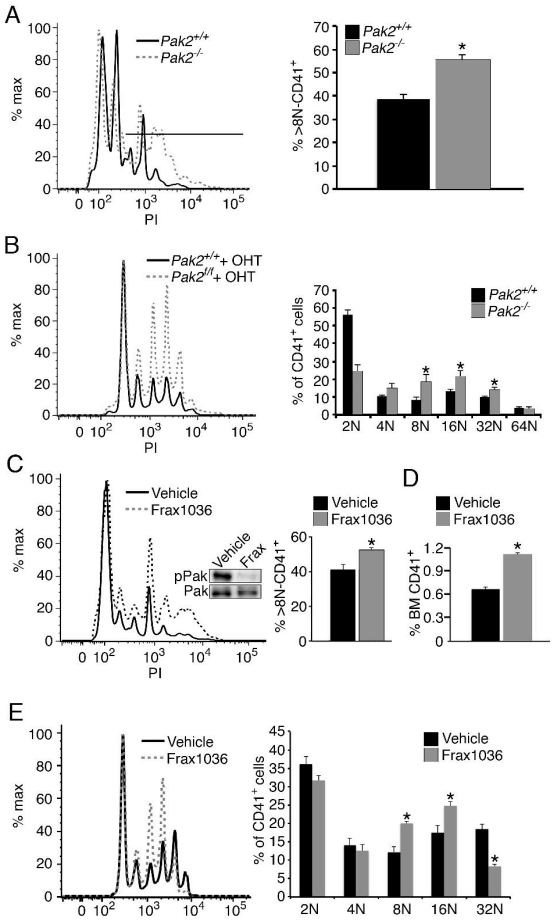

Figure 3.4. *Pak2* is a negative regulator of megakaryocyte endomitosis

(A) Megakaryocyte DNA content in wild-type and *Pak2*$^{-/-}$ mouse bone marrow, 14 days post-pIpC, was measured in CD41-positive bone marrow cells by flow cytometry. Line indicates 8N+ cells (n >10; mean ± SEM, **p*<0.05). (B) Megakaryocyte DNA content in wild-type and *Pak2*$^{-/-}$ *in vitro*-derived MKs. Bone marrow cultured for 5 days with TPO and 500nM 4-hydroxytamoxifen (OHT) to activate *Cag-Cre-ERT2;Pak2*$^{fl/fl}$ transgene (n>4, mean ± SEM, **p*<0.05). (C) Megakaryocyte DNA content in vehicle and Pak1/2/3 inhibitor Frax1036-treated mouse bone marrow. Mice were dosed with Frax1036 via oral gavage daily for 21 days. Frax1036 (Frax) ablates Pak1/2/3 Serine 141 phosphorylation (pPak) in the bone marrow relative to total Pak1/2/3 (Pak) (WB inset). % >8N DNA content, mean ± SEM, *p<0.05.* (D) Percentage of bone marrow expressing CD41 after Frax1036 treatment. 4 mice/genotype, mean ± SEM, *p<0.001* (E) Polyploidization in bone marrow derived megakaryocytes cultured with 1uM Frax1036 for 5 days, representative FACS plot of CD41^{+} cells stained with propidium iodide and ploidy data for n>5 mice/genotype. 8N and 16N populations significantly increased in Frax treated bone marrow (p <0.008 and p< 0.003, respectively).

Proplatelet extensions were also evaluated for β1-tubulin expression, and found to have decreased expression and altered structures in *Pak2*$^{-/-}$ megakaryocytes adherent to fibrinogen (Figure 3.5B). Staining for actin filaments indicated decreased actin polymerization in these cells (Figure 3.5C). To determine if group I Pak inhibitors would recapitulate the phenotype seen in the genetic model, we cultured fetal liver cells with TPO and Frax1036 for 5 days, using a drug dose that effectively suppressed Pak activity (Figure 3.5E). Similar to *Pak2*$^{-/-}$ bone marrow-derived megakaryocytes, we observed altered β1-tubulin staining in Frax1036 treated fetal-liver derived megakaryocytes (Figure 3.5D).

Confocal microscopy demonstrated significant alterations in β1-tubulin and actin expression and organization (Figure 3.5). Proplatelet formation is the final step in megakaryocyte maturation to platelets. This is a cytoskeleton intense process, as cells undergo profound reorganization of the cytoskeleton to form long protrusions to extend into the sinusoid vessels. Microtubules provide the sliding power for the megakaryocyte membranes to extend into long protrusions and actin functions in elongation and shaft bifurcation of proplatelet extensions.[49,57,58] Therefore, we evaluated the amount of proplatelet extensions with both genetic deletion of *Pak2 in vitro* and therapeutic inhibition of Group I Paks with Frax1036. After five days in culture with TPO, megakaryocytes were grown on fibrinogen-coated slides for five hours, and the number of megakaryocytes with proplatelets were counted as a ratio of total megakaryocytes present. We found the extent of proplatelet formation in both conditions was reduced significantly compared to wild-type controls (Figure 3.6A-C). Together these data

demonstrate that Pak2 functions to regulate polyploidization and proplatelet formation through regulation of the cytoskeleton.

Altered signal transduction in *Pak2*$^{-/-}$ megakaryocytes

To examine the potential mechanism underlying the effects of *Pak2* deletion on polyploidization and proplatelet formation, megakaryocyte turnover in the bone marrow and spleen, and the effects of Pak2 deletion on the cytoskeleton, we next investigated the phosphorylation status of various downstream effectors of Pak2 in wild-type and *Pak2*-deficient megakaryocytes (deleted *in vitro* using a tamoxifen inducible form of CAG-cre-recombinase[59]). Accordingly, we evaluated signaling networks that regulate actin and microtubule dynamics. We found that LIMK phosphorylation was significantly reduced (>60% reduction) in *Pak2*$^{-/-}$ megakaryocytes, and associated with reduced phosphorylation of cofilin (>40% reduction) (Figure 3.7A-B). Cofilin is active in the non-phosphorylated state to associate with F-actin and promote actin severing and turnover. These results suggest that *Pak2*$^{-/-}$ megakaryocytes undergo more rapid actin severing and depolymerization compared with wildtype megakaryocytes. These properties have previously been associated with enhanced polyploidization in megakaryocytes and decreased proplatelet formation.[52-54,58,60]

In addition to elevated actin depolymerization in *Pak2*-null megakaryocytes promoting polyploidization and impaired proplatelet formation, enhanced microtubule depolymerization with colchicine, nocodazole and vincristine have all been shown to enhance megakaryocyte ploidy and impair proplatelet foramtion.[51,54,56,58] Microtubule dynamics are regulated by a variety of proteins, including the Pak substrate Aurora A kinase.[14,61] We evaluated *Pak2*$^{-/-}$ megakaryocytes for Aurora A, B, C phosphorylation

and found markedly decreased levels in *Pak2*$^{-/-}$ cells compared to wild-type levels (>48% reduction) (Figure 3.7A-B). Consistent with our results, pharmacologic and genetic inhibition of Aurora A and Aurora B were recently identified as negative regulators of polyploidization.[62,63]

These data strongly suggest that Pak2 regulates multiple functions in the developing megakaryocyte by regulating both the actin and microtubule networks through its catalytic domain (Figure 3.8).

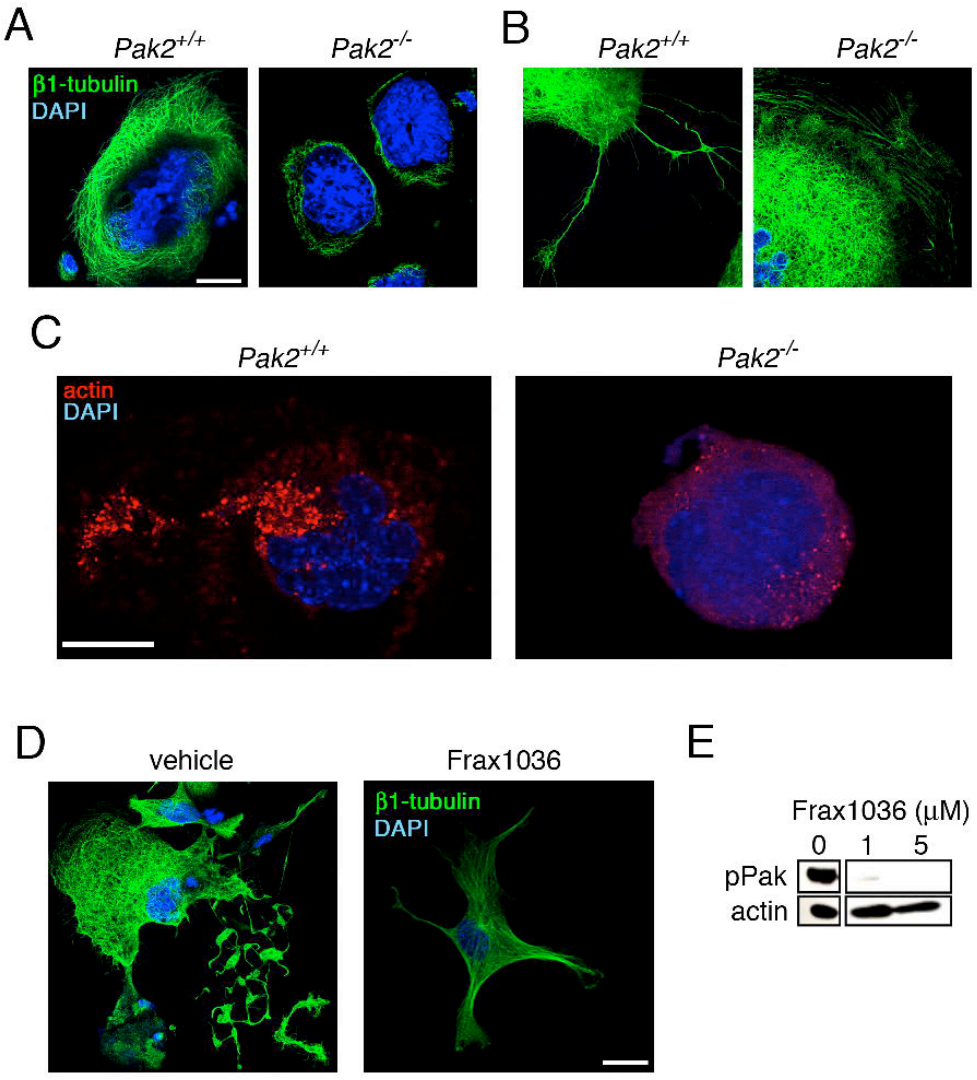

Figure 3.5. Altered cytoskeleton in *Pak2*-null megakaryocytes.

(A) Analysis of β1-tubulin structure and (B) proplatelet structure by confocal microscopy of wild-type and *Pak2*$^{-/-}$ megakaryocytes. Bone marrow treated with 500nM 4-hydroxytamoxifen to induce Cag-Cre-ERT2 expression and delete *Pak2*$^{fl/fl}$. Representative β1-tubulin (green) and DAPI nuclear (blue) staining from 3 different mouse samples per genotype. Scale bar = 20 μm. (C) Representative TRITC-phalloidin staining for actin (red) and DAPI (blue) analyzed by fluorescence confocal microscopy of wild-type and *Pak2*$^{-/-}$ megakaryocytes. (D) Fetal liver derived megakaryocytes treated with Frax1036 for duration of culture (4 days) and stained for β1-tubulin (green) and DAPI (blue). (E) Western blot detection of Serine 141 phosphorylated Pak1/2/3 (pPak) of FL-MKs treated with Frax1036. Actin serves as a control for protein loading. Scale bar = 20μm for all images.

DISCUSSION

Here we identify a role for the p21-activated kinase-2, Pak2, in megakaryocyte biogenesis, cytoskeletal remodeling, proplatelet formation and endomitosis. Using a conditional knockout mouse model, as well as small molecule inhibitors, we established *Pak2* as a negative regulator of megakaryocyte development. We demonstrate that *Pak2*$^{-/-}$ mice develop macrothrombocytopenia, which manifests *in vivo* with decreased platelet lifespan, along with increased megakaryocyte stem cells and mature cells in bone marrow and spleen, and increased megakaryocyte ploidy. Using *in vivo* and *in vitro* genetic deletion models, we found that Pak2 negatively regulates megakaryocyte polyploidizaton and proplatelet formation in a cell-intrinsic manner and gives rise to platelets with decreased survival in the circulation, (Figures 3.2 and 3.4). Evidence from signaling studies suggest that at least two signaling pathways that regulate cytoskeletal function in megakaryocytes are regulated by Pak2: Aurora A and B, which regulates microtubule dynamics, and LIMK/cofilin, which regulate actin dynamics (Figure 3.8). Similar to Pak2, these proteins play a restraining role in megakaryocyte polyploidization.[62,64-67] Therefore, our model proposes that deletion of *Pak2* results in inhibition of Aurora activation, leading to increased microtubule depolymerization and increased polyploidization, and activation of cofilin drives actin severing and impairs proplatelet formation (Figure 3.8).

Multiple signaling cascades are involved in the intricate process of polyploidization. One such kinase cascade is regulated by Aurora A and B, found to negatively regulate megakaryocyte polyploidization, in part due to its regulation of

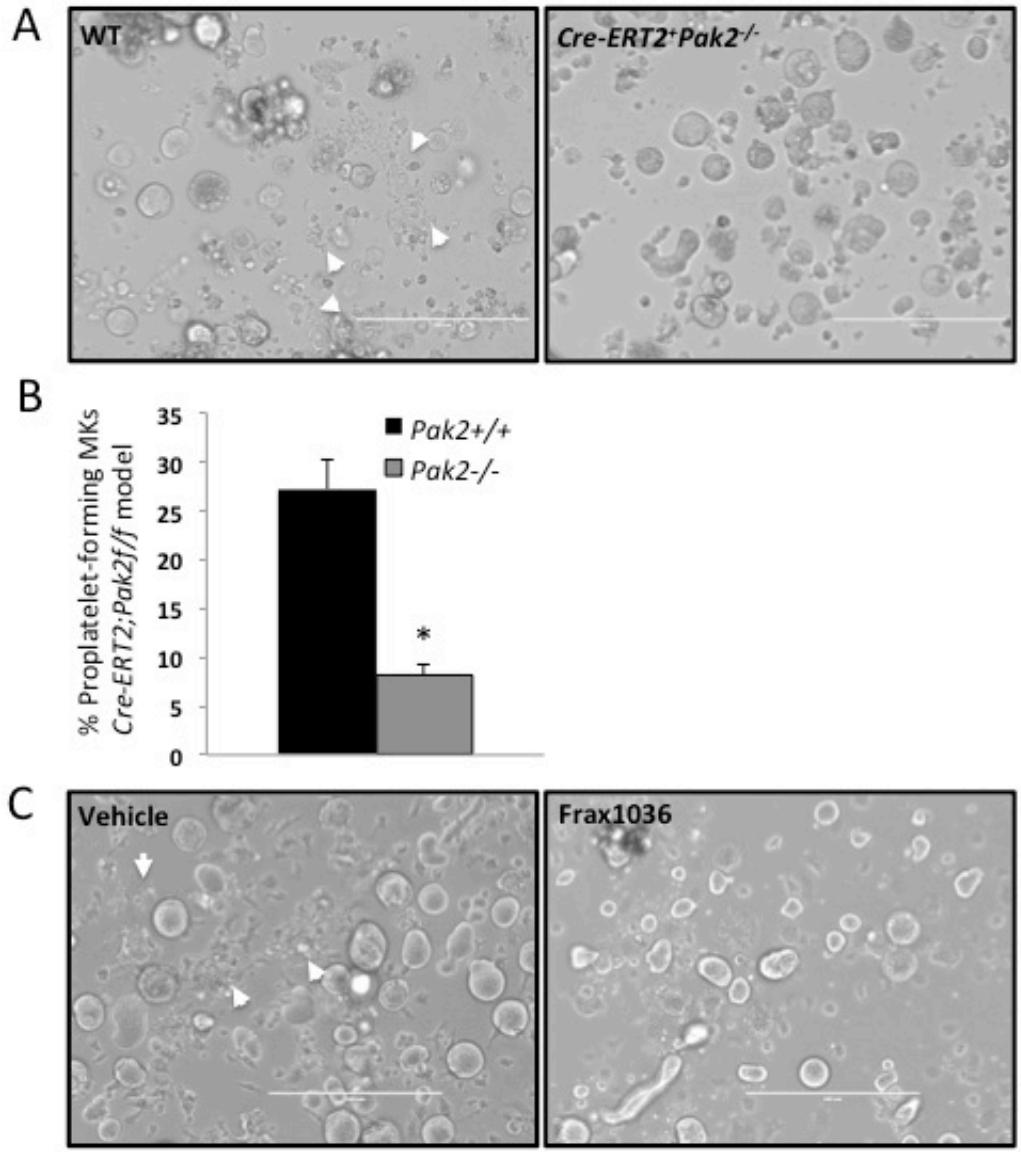

Figure 3.6. Proplatelet formation is impaired in Pak2-null megakaryocytes and with Frax1036 inhibitor. (A) Bone marrow-derived megakaryocytes deleted for Pak2 *in vitro* with tamoxifen, demonstrated decreased proplatelet formation (Day 6). Arrows indicate proplatelets. 20X magnification (B) Percentage of megakaryocytes with cytoplasmic proplatelet extensions, mean±SEM of n=5/per genotype. (C) Representative image of proplatelet-containing megakaryocytes in vehicle-treated fetal liver derived megakaryocytes (Day 5). Arrows indicate proplatelets, 20X.

microtubule dynamics.[62,66] Pak kinases directly phosphorylate and activate Aurora A and regulate mitotic entry.[14] Studies using genetic knockdown techniques, along with selective inhibitors of Aurora A and B, demonstrated that these kinases negatively regulate megakaryocyte polyploidization.[62] Therefore, we evaluated the effect of *Pak2* deletion on Aurora phosphorylation. We found that *Pak2*$^{-/-}$ megakaryocytes had decreased Aurora phosphorylation, explaining one possible mechanism driving increased polyploidization. One mechanism by which Aurora could negatively regulate polyploidization is by inhibition of microtubule destabilizing proteins (i.e. stathmin and MCAK), thereby preventing microtubule destruction.[64-67] During mitosis, Aurora A is important for bipolar spindle formation, histone H3 phosphorylation and regulation of the microtubule- organizing center (MTOC).[68]

Aurora A and Aurora B have distinct functions in mitosis, and have therefore been investigated for possible therapeutic targets to drive differentiation and polyploidization in acute megakaryoblastic leukemia (AMKL).[62,63] Unlike many blood cancers, AMKL patients rarely have known mutations, making it challenging to identify targeted therapeutic intervention. Pharmacologic inhibition of Aurora A kinase with MLN8237 and Aurora B kinase with AZD1152-HQPA in acute megakaryoblastic leukemia (AMKL) cells both impaired proliferation, induced polyploidization and increased megakaryocyte differentiation. Additionally, inhibition of both Aurora A and B in human primary CD34^{+} stem cells resulted in increased megakaryocyte polyploidization.[62]

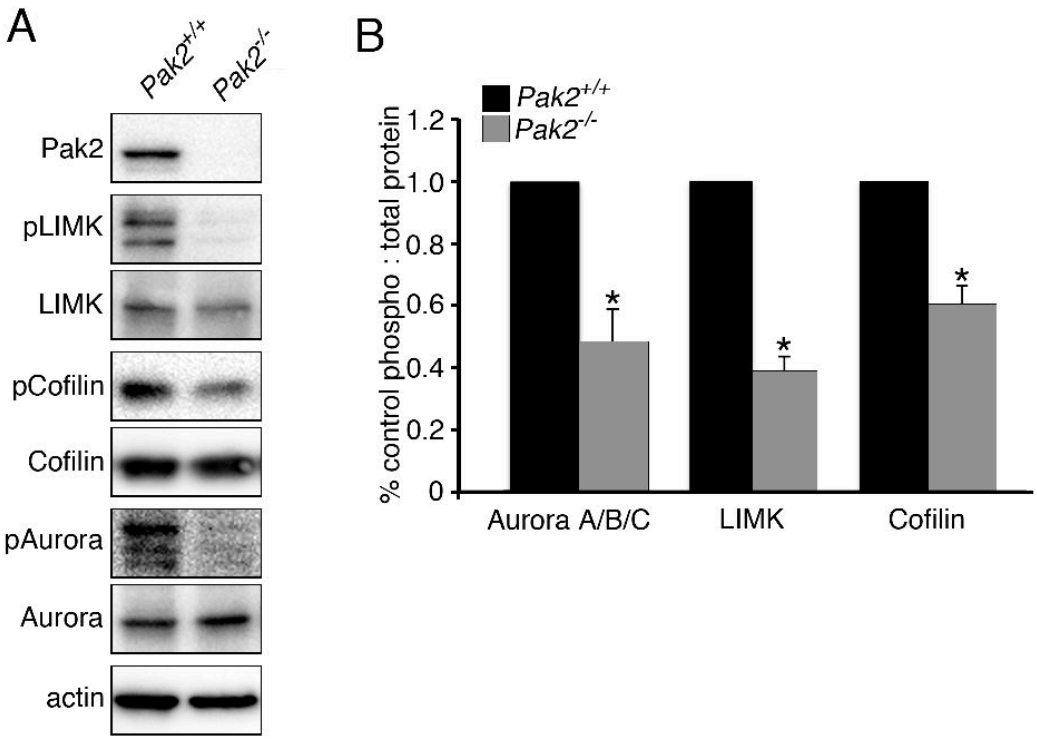

Figure 3.7. Altered phosphorylation of cytoskeleton regulatory proteins in *Pak2*-null megakaryocytes. (A) Western blot analysis of phosphorylation levels of cytoskeletal regulatory proteins LIMK, Cofilin and Aurora A/B/C in *in vitro*-deleted bone marrow derived megakaryocytes (*CAGG-Cre-ERT2*$^{+}$*;Pak2*$^{f/f}$). Equal quantities of total cellular protein were loaded and phospho-protein content was detected with phospho-specific antibodies (pLIMK, pCofilin and pAurora) and total protein antibodies. Actin served as a control for equal loading. Blots are representative of at least four independent experiments. (B) Band densities quantified as a ratio of phospho:total protein and calculated as percent of control. Densitometry was quantified with Fiji-Image J Software (National Institute of Health, USA). *n=4* per genotype * $p<0.01$

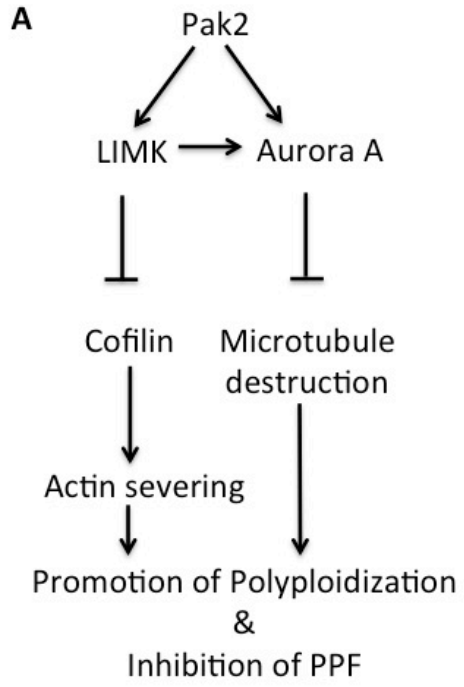

Figure 3.8. Model depicting Pak2 regulation of megakaryocyte polyploidization and proplatelet formation (PPF) through control of actin and microtubule network.

Our data demonstrate decreased Aurora phosphorylation at threonine-232 in *Pak2*-null megakaryocytes (Figure 3.7). This site was previously identified to be activated at late stages of mitosis and endomitosis at the metaphase/anaphase transition, and to phosphorylate histone H3 to ensure proper chromosomal segregation. Phosphorylation mutants of Aurora B, which fail to activate threonine-232, result in multi-nucleated cells.[69] These data demonstrate that phosphorylation of Aurora B is essential for mitosis and cytokinesis due to its involvement in chromosome alignment during metaphase and separation during anaphase. Aurora B inhibition in cancer cell lines results in chromosomal mis-segregation, abnormal cytokinesis followed by endoreplication. Aurora B also controls RhoA at the cleavage furrow through phosphorylation of its substrate, MgcRacGAP, to activate RhoA. Another mechanism by

which Aurora B inhibition results in increased mean megakaryocyte ploidy is possibly through hypophosphorylated Rb allowing abnormal mitosis and endomitosis to occur.[66] Together these data demonstrate the critical role Aurora A and B play in negatively regulating megakaryocyte maturation. Our data demonstrate that Aurora A and B in primary megakaryocytes is regulated by Pak2, implicating the utilization of Pak inhibitors to regulate Aurora.

Another pathway by which Pak2 regulates megakaryocyte development is through LIMK and its substrate cofilin. Previous studies found that megakaryocyte specific *cofilin* gene knockout impaired proper proplatelet production.[60] LIMK negatively regulates cofilin by phosphorylation at serine 3, thereby directly inhibiting actin severing, and leading to normal levels of actin polymerization. In *Pak2*-null megakaryocytes, LIMK activity was decreased resulting in increased activation of cofilin mediated actin-severing activity. Enhanced actin depolymerization in megakaryocytes with various therapeutic compounds results in elevated polyploidization. Incubation of cell lines and primary megakaryocytes with an inhibitor of actin polymerization (Cytochalasin B) resulted in elevated polyploidization without stimulation of expression of CD41 and CD61 mature megakaryocyte markers.[52-55] At the end of mitosis during normal cell division, cytoplasmic separation requires the integrity of cytoskeleton-associated proteins for the formation of the tubulin spindle and actin contractile ring. Inhibition of actin with cytochalasin B therefore inhibits normal separation of cytoplasm and induced polyploidization.

In addition to signaling directly to cofilin, it is important to note that LIMK can also directly phosphorylate Aurora A to stabilize the microtubule network.[70-72] LIMK

inhibition was shown to cause microtubule hyper-stability through Aurora A inhibition, leading to chromosomal fragmentation and apoptosis in cancer cells. Thus, loss of LIMK activation in *Pak2*$^{-/-}$ megakaryocytes likely contributes to altered polyploidization, via its role as a signaling node between actin and microtubules, by affecting both Aurora and cofilin (Figure 3.8). The impact of Pak2 inhibition on LIMK, and Aurora A/B phosphorylation demonstrates that Pak2 influences multiple signaling networks to control the actin and microtubule cytoskeleton.

The cytoskeleton intense process of proplatelet formation requires Pak2 signaling, as demonstrated in Figures 3.5 and 3.6. Both microtubules and actin polymerization are required for extensions to form and bifurcate to produce functioning platelets. Treatment of patients with Bortezomib resulted in thrombocytopenia, induced by a failure of megakaryocytes to produce proplatelet extensions.[73] This failure was due to an accumulation of active RhoA in the cells, as incubation with a RhoA kinase (ROCK) inhibitor was able to rescue proplatelet formation. Additional studies evaluating the role of RhoA found a similar phenotype.[57,74-76] Active RhoA, through its effectors ROCK and mDia regulate stress fiber formation and actomyosin contractility, as well as microtubule dynamics and focal adhesions.

Given that group I Paks phosphorylate LIMK and are activated by Rac1 and Cdc42, it is curious that, contrary to expectation, *Rac1/Cdc42*$^{-/-}$ megakaryocytes display increased, rather than decreased, LIMK phosphorylation, as well as increased phosphorylation of the LIMK target cofilin.[25] Additionally, the *Rac1/Cdc42*$^{-/-}$ megakaryocytes did not demonstrate changes to polyploidization like *Pak2*$^{-/-}$ megakaryocytes. In contrast to these reports, we found that *Pak2*$^{-/-}$ megakaryocytes had

markedly decreased phosphorylation of LIMK and cofilin, consistent with the well-established role for group I Paks in phosphorylating and activating LIMK.[12,70,77] At present, we do not fully understand the reasons for these seemingly discrepant signaling results, but will note that the double knockout of *Rac1* and *Cdc42* would be expected to have more complex and far-reaching phenotypic consequences than deletion of their effector *Pak2* alone. Additionally, Pak kinases are activated by a variety of kinases and other proteins, in addition to canonical Rac1 and Cdc42 GTPases.

These data provide a mechanism to describe how *Pak2* regulates megakaryocyte endomitosis and maturation by means of regulating signal transduction cascades involved in cytoskeletal network regulation. Additionally, these data suggest that Pak2 inhibition represents a plausible target for treatment of AMKL (acute megakaryoblastic leukemia) by increasing polyploidization and increasing megakaryocyte maturation in the bone marrow by regulating a variety of signal transduction pathways, including those that affect actin filaments (LIMK/cofilin) and microtubules (Aurora activation).[62,72]

Expansion of the hematopoietic stem cell compartment in *Pak2*-null mice is an intriguing finding (Figure 3.3D-E). Deletion using the inducible Mx1-cre resulted in increased HSC populations. These populations are defined as lineage$^-$ckit$^+$Scal$^-$ (LK) or lineage$^-$ckit$^+$Scal$^+$ (LSK). Further analysis of the LSK population, defined by cell surface expression of CD150 (SLAM) and CD105 (Endoglin) found increased LT-HSC (long-term-HSC) (CD150$^+$CD105$^+$) and MPP(CD150$^-$CD105$^-$) populations in *Pak2*-null mice. These 2 proteins (CD150 and CD105) are highly expressed in the primitive HSC compartment (LT-HSC), as opposed to the slightly more differentiated, multipluripotent stem cell (MPP), and are therefore valid markers for distinguishing these populations.[37]

Research into HSC homing, engraftment, and proliferation/quiescence have identified that megakaryocytes are both positive and negative regulators of this process. After transplantation into irradiated mice, HSCs localize preferentially in the bone marrow near megakaryocytes[78] and inhibition of megakaryocytes impairs HSC engraftment.[79] Megakaryocytes expand the osteoblastic niche after irradiation, suggesting that megakaryocytes indirectly regulated HSC proliferation. However, more research into this field recently discovered that megakaryocytes also play a direct role on HSC expansion through secretion of growth factors.[80,81] Recent findings by two independent groups found that mature megakaryocytes maintain HSC quiescence by secretion of CXCL4 and TGF-β and induce HSC proliferation and recovery with FGF-1 secretion.[80,81] These novel findings demonstrate that terminally differentiated megakaryocytes directly regulate HSC activity, as well as provide another location for quiescent HSCs in the sinusoidal-megakaryocyte niche besides the typical arteriolar niche.

Considering Pak2 deletion resulted in increased megakaryocytes in the bone marrow and spleen, feedback to promote expansion of HSCs is one possibility to explain our findings of increased HSC populations. A validation experiment inhibiting TPO production to reduce megakaryocyte load would confirm if HSC increases in Pak2-null mice are due to megakaryocytes positive feedback to HSCs or from a different mechanism.

Discoveries into pathways that regulate HSC activity are necessary for the development of therapeutics to enhance HSC recovery after myeloablative therapy or stem cell transplantation. Since Pak2 deletion in an *in vivo* mouse model resulted in enhanced megakaryocyte progenitors and HSCs, there is the potential that small molecule

inhibitors developed for Group I Pak kinase inhibition could possible be used in the setting of HSC recovery.

Increased megakaryocyte mass in the bone marrow is indicative of some disorders, including pernicious anemia, immune thrombocytopenia, reactive thrombocytosis, essential thrombocythemia, and primary myelofibrosis. Interestingly, several of these disorders, primary myelofibrosis and essential thrombocythemia carry a risk of progression to acute myeloid leukemia. Myelodysplastic syndrome is also associated with increased megakaryopoiesis and HSC cycling. A possible explanation is that the increased megakaryocyte load is regulated HSC cycling and contributing to these hematological malignancies. A common side effect of chemotherapy is the loss of megakaryocytes, possibly leading to decreased HSC recovery. Megakaryocyte-ablated mice showed decreased HSC recovery and regeneration after chemotherapy.[80] These data suggest, that therapeutics to boost megakaryocyte recovery after myeloablative therapy may also promote HSC regeneration.

Under conditions of stress rapid expansion of HSCs is required for survival. Zhao et al. recently discovered that the dominant secreted factor to drive HSC expansion under stress is FGF1.[80] Interestingly, FGF1 is predominantly secreted from megakaryocytes in order to promote HSC expansion and survival. In our mouse model of $Mx1cre^{+}Pak2^{f/f}$ there is evidence of systemic stress, displayed by rapid platelet clearance and thrombocytopenia, resulting in increased megakaryocyte maturation in the bone marrow. Along with increased megakaryocytes, we also observed increased HSCs. Future work to evaluate if expansion of *Pak2*-null megakaryocytes promotes HSC expansion in our mouse model will be of interest. Additional evaluation of TGF-β1 and FGF-1 secretion

from *Pak2*-null megakaryocytes under non-stress and stress conditions would also be interesting to study in order to identify if Pak2 functions in a megakaryocyte-specific manner to regulate HSC expansion via growth factor secretion. Previous reports demonstrated that megakaryocytes drive HSC proliferation in an *in vitro* culture system.[78] To this end, co-culture of *Pak2*-null megakaryocytes with HSCs could be done to understand if there is a direct role of Pak2 in regulating secretion from megakaryocytes leading to HSC expansion. These are interesting data in light of the development of Pak inhibitors, implicating the use of these inhibitors in hematopoietic malignancies requiring expansion of HSCs.

In summary, our results demonstrate that *Pak2* regulates megakaryocyte polyploidization through control of signaling networks that regulate actin and microtubule cytoskeleton. This multi-network influence of Pak2, through phosphorylation of LIMK and Aurora, demonstrates how Pak2 controls the dynamic cytoskeleton network in developing megakaryocytes and plays a potentially novel role in megakaryocyte biology. In addition to enhanced ploidy, Pak2 negatively regulates megakaryocyte expansion in the bone marrow. Increased megakaryocyte mass in Pak2-null mice may lead to observed HSC expansion, providing a possible therapeutic link between Pak2 inhibition and HSC expansion.

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Chapter 4: Phenotype of Pak2 deletion in an inducible mouse model

Introduction

Pak2 is ubiquitously expressed in the adult animal, and plays a critical role in embryogenesis. *Pak2*$^{-/-}$ embryos do not progress past E8.5-9.5, whereas deletion of the highly homologous isoform, *Pak1*, does not cause lethality in the embryo nor in the adult.[1] *Pak2*$^{-/-}$ embryo lethality is due to failure to properly establish blood vessel vasculature due to endothelial apoptosis. (unpublished, M. Radu and J. Chernoff) This chapter will investigate the phenotype observed in the Mx1-cre;Pak2-floxed mouse model. A previously demonstrated in Chapter 3, *Pak2*$^{-/-}$ adult mice rapidly clear platelets from circulation resulting in thrombocytopenia.

Recent research into lymphatic blood vessel integrity elegantly describes a vital role for platelets in regulating lymphatic and endothelial blood vessel separation during both neonatal development and adult blood vessel homeostasis.[2-4] We identified that *Pak2*-null adult mice suffer from thrombocytopenia and lymphedema. Ongoing research in the Chernoff laboratory has discovered that *Pak2*-null neonates die from failure of the vasculature to form by day E9.5 (M. Radu and J. Chernoff, manuscript in preparation). Recent work by several groups found that deletion of Clec-2, Slp-76, and Syk[5-7], specifically in platelets, led to a failure of lymphatic and blood vessel separation. We hypothesize that Clec-2 receptor signaling activates Pak through Vav-GEF and Rac1/Cdc42 GTPases, known activators of Pak kinases, to regulate the vasculature in embryos and adults, platelet actin cytoskeleton and lymphatic integrity.[8] Our study utilized two mouse models to study these questions, the inducible Mx1-cre model to temporally control induction of cre-recombinase in an adult, and PF4-cre, a platelet and

megakaryocyte specific cre recombinase, which constitutively deletes at the embryonic stage of development and in the adult.

Methods

Generation of mice with *Pak2*$^{-/-}$ bone marrow

Pak2-deficient mice were generated by crossing mice containing the *Pak2* gene flanked by loxP sites (*Pak2*$^{fl/fl}$), with mice carrying the Mx1-cre transgene.[9-11](M. Radu and J.Chernoff, manuscript in preparation) (Figure 4.1). In 8- to 12-week old *Mx1-cre*$^{+}$*Pak2*$^{fl/fl}$ mice (mixed background sv129/C57Bl/6), gene deletion was induced by 3 intraperitoneal injections of 400ug polyinosinic-polycytidylic acid (pIpC) in a 2-day interval. Littermates received the same treatment. Fourteen days after the first injection, mice were sacrificied and tissues were fixed in 10% neutral-buffered formalin. In the case of survival curve calculations, mice were monitored daily until they were non-responsive. Additionally, to evaluate the effects of Pak2 deletion specifically in platelets and megakaryocytes, we crossed the *Pak2*$^{f/f}$ animals with PF4-cre (platelet-factor 4) animals.

All animal studies were performed according to protocols approved by Fox Chase Cancer Center institutional animal care and use committee. To determine if the observed thrombocytopenia was due to cell-intrinsic defects in *Pak2*-null megakaryocytes and platelets we crossed *Pak2*-floxed animals with a platelet and megakaryocyte-specific cre (PF4-cre). This specific cross will reveal if the increased platelet clearance rate observed in *Mx1cre*$^{+}$*Pak2*$^{fl/fl}$ animals was intrinsic to megakaryocytes and platelets, or caused by a non-cell autonomous defect in *Pak2*-null mice.

Measurement of hematological parameters

Mice were euthanized with CO_2 and blood was extracted via the hepato-portal vein with a syringe containing ACD (acid-citrate dextrose) (10% final). Whole blood was added into tubes containing EDTA. The complete blood count was performed using a VetScan HM5 (Abaxis) within 1-2 hours of collection at room temperature.

Granulocyte-Myeloid progenitors (GMP) measured by flow cytometry

Red blood cell lysed bone marrow from *Mx1-cre*$^{+}$*;Pak2*$^{fl/fl}$ animals was analyzed for granulocyte/myeloid progenitors with the following antibodies purchased from Ebioscience (antibody name (clone)) murine hematopoietic lineage eFluor 450 cocktail, c-Kit-APC (2B8), sca-1-PE-Cy7 (D7), FcγII/III-PerCP-eFluor 710(clone 93), CD34-FITC.

Immunohistochemistry

Formalin fixed, paraffin-embedded tissue was sectioned at 5 microns and adhered to glass slides. Tissues were stained for anti-podoplanin (Abcam) according to standard IHC techniques.

Megakaryocyte ploidy measurements

For ploidy measurements, cells were analyzed as described previously.[12] DNA content in CD41^{+} megakaryocytes was determined by labeling RBC-lysed bone marrow with anti-CD41 on ice for 30 minutes, followed by fixation with 0.5% formalin for 15 minutes at room temperature. Cells were then permeabilized with 70% ice-cold methanol for 1 hour on ice. After washing, cells were then incubated with 10ug RNase A at 37°C for 30 minutes, followed by incubation with 1µg/µl propidium iodide for 15 minutes at

room temperature. Cells were analyzed for ploidy on an LSR-II with proper fluorochrome compensation.

Proplatelet formation

Proplatelet-displaying megakaryocytes were defined as cells exhibiting one or more cytoplasmic processes with areas of constriction. After 5 days in culture, fetal liver derived megakaryocytes were separated on a BSA gradient, followed by 24 hours of growth. Proplatelet extensions were quantified by calculating the percentage of megakaryocytes with such processes on inverted microscope at a magnification of 20X.[13]

Results/Discussion

Phenotype of Mx1-cre induced deletion of Pak2

The model used to develop a *Pak2-floxed* animal under the control of Mx1-cre is described in Figure 4.1A. Induction of Mx1-cre recombinase was done with pI:pC injection. All of the $Pak2^{-/-}$ mice died within 20 days of the initial injection, whereas no wild-type mice injected with pI:pC died (Figure 4.1B).

$Mx1\text{-}cre^{+};Pak2^{f/f}$ animals injected with pI:pC to activate Mx1-cre all demonstrated increased neutrophil counts in peripheral blood and subsequent increase in GMP (granulocyte-myeloid progenitors) in bone marrow, which are the precursors to neutrophils (Figure 4.2A-C). GMP progenitor cells are defined here as Lin-c-$kit^{+}Sca1^{-}$ FcγRII/$III^{hi}CD34^{hi}$.[14] Neutrophil percentages of total white blood count increased from 12.6±0.9% in wildtype mice to 29.2±1.6% in $Pak2^{-/-}$ mice (Figure 4.2B,C). Evaluation of GMP progenitor cells revealed about a 30% increase in $Pak2^{-/-}$ mice (Figure 4.2A). These data are consistent with bone marrow changes observed in a recent publication with our collaborators.[15] Upon deletion of *Pak2* in transplanted bone marrow, they observed

significantly increased peripheral blood granulocytes and increased bone marrow granulocyte progenitors (GMP).[15] Curiously, deletion only in bone marrow did not cause lethality in the adult animals.

Considering the bone marrow of *Pak2*$^{-/-}$ animals appeared hypocellular in histological sections, we evaluated the stem cell populations by flow cytometry and found increased early hematopoietic stem cell populations in the bone marrow (Figure 3.3D-E). The process of hematopoiesis involves many cell-intrinsic and cell-extrinsic factors, to control transcription factor networks involved in blood cell lineage fate determination. Identification of how *Pak2* controls hematopoiesis is beyond the scope of this dissertation, but it is helpful to inform future work to identify baseline changes in the bone marrow of *Pak2*$^{-/-}$ mice. Work with our collaborators discovered that *Pak2* functions to regulate HSC engraftment and homing to the stem cell niche in the bone marrow, but research into bone marrow homeostasis has not been done.[10]

Our data from day 14 post-pIpC injection demonstrated significantly increased levels of long-term hematopoietic stem cell population (LT-HSC), along with the multi-pluripotent progenitor population (MPP) (*p*-value 0.002 and 0.005, respectively) (Figure 3.3D-E). LT-HSC were defined as Lin$^-$C-kit$^+$Scal$^+$CD150$^+$CD105$^+$ bone marrow cells, and MPP were defined as Lin$^-$C-kit$^+$Sca1$^+$CD150$^-$CD105$^-$ bone marrow cells (identified in Pronk et al.[16]). Together these data provide evidence that deletion of *Pak2* in the bone marrow increases the stem cell population in various lineages though a yet undefined mechanism. Regulation of *Pak2* in the hematopoietic stem cell compartment has never been demonstrated prior to our work presented here.

The effect of *Pak2* on hematopoiesis is similar to changes observed in *Cdc42*-null mice and *Rac1*-null mice, both activators of Group I Paks. *Rac1*$^{-/-}$ mice are embryonic lethal at day 8.5, and inducible deletion in the adult results in rapid lethality.[17] Another GTPase activator of Pak, Cdc42, also has a critical role in hematopoiesis. Cdc42 deletion results in rapid lethality between day 12 and 26 in an Mx1-cre inducible Cdc42 floxed model.[18] Prior to death, *Cdc42*$^{-/-}$ mice demonstrate increased hematopoietic stem cells (LK and LSK populations) and GMP (Granulocyte/myeloid progenitors), leading to altered myeloid and erythroid homeostasis. Similarly, *Pak2*-null mice have increased stem cell progenitors, GMP and megakaryocyte precursors (figures 3.3 and 4.2). However, Cdc42 bone marrow chimeras of *Mx1cre*$^{+}$*Cdc42*$^{f/f}$ transplanted bone marrow into WT recipients also died around 22 days post-polyI:C. Recently published work with our collaborators (Yi Zeng, University of Arizona) found that *Pak2*-bone marrow chimeras (wildtype animals transplanted with *Mx1-cre*$^{+}$*;Pak2*$^{f/f}$ bone marrow) do not die after Cre-induction with polyI:C. This indicates that lethality in *Mx1-cre*$^{+}$*;Pak2*$^{f/f}$ animals is due to factors independent of changes to the bone marrow. Additionally, polyI:C is not causing lethality in the chimeric model, providing further evidence that the phenotype is not due to polyI:C injections.

Regardless of the chimeras, these data indicated that Pak2, similarly to Rac1 and Cdc42, regulates the bone marrow hematopoiesis. Therefore, I evaluated bone marrow hematopoietic stem cells in *Pak2*-deleted mice, and found increased levels of all stem cell populations. Future work is needed to identify if *Pak2*-null mice have myeloproliferative disorder (MPD), identified by massive infiltration of myeloid cells of varying degree of maturation into liver, lung, spleen, bone marrow and peripheral blood.

In addition to increased neutrophil progenitors and mature cells, peripheral blood demonstrated decreased platelet counts (Table 3.1). The cause of thrombocytopenia was described in Chapter 3, Figure 3.2 A-C. These data demonstrate that *Pak2*$^{-/-}$ mice have reduced platelet lifespan, and to compensate for the loss of platelets, they have increased production of newly synthesized, reticulated platelets. Additionally, spleen and bone marrow megakaryocytes are increased, possibly in response to increased TPO levels caused by the decrease in circulating platelets. (Figure 3.1B,D and Figure 3.2D) Future analysis of serum TPO levels will indicate if the increase in megakaryocytes is in response to elevated TPO.

Along with decreased platelet counts, the *Mx1-cre*$^{+}$*;Pak2*$^{-/-}$ mouse large intestines were edematous and hemorrhagic. (Figure 4.3A). In addition, the pulmonary cavity was filled with large ("lethal") quantity of pleural effusion and congestion, suggesting that *Pak2*-null mice suffered from generalized vascular leakage. It is of interest to note that our previous studies demonstrating hyperactivated *Pak2*-null mast cells in Chapter 2, suggest that the presence of intestinal mucosal mast cells could have a pathogenic role by either increased presence of mast cell infiltrate or exaggerated release of inflammatory cytokines by hyperactive mast cells. Immune cell infiltrate was observed in histology H&E sections, (Figure 4.3A), but specific identification of mast cells was not pursued. Future genetic crosses with mast cell specific Cre-recombinase, such as *A-Mcpt-5*-cre, could elucidate the function of *Pak2*-deletion in mast cells on intestinal inflammation.

The pleural effusion observed around day 14 post-pI:pC injection in *Mx1cre*$^{+}$*Pak2*$^{f/f}$ was of a transudate quality, defined by 60% large mononuclear cells/macrophages, 30% small mononuclear cells/small lymphocytes with low nucleated

cellularity and low protein, which places this fluid in the transudate category. Transudative effusions typically occur as a result of increased vascular hydrostatic pressure and/or low plasma oncotic pressure (severe hypoalbuminemia), compression of the vasculature, cardiovascular disease, or pulmonary inflammation. The mechanism by which *Pak2* regulates these processes has yet to be discovered. Given the role of Pak2 in vasculogenesis, it is possible that these events are related to the direct effect of Pak2 on vascular integrity, both by regulating endothelial junctions and apoptosis. (Radu, M and Chernoff, J, *manuscript in preparation*).

Disseminated intravascular coagulation in *Pak2*$^{-/-}$ mice

Pak2-null mice are able to synthesize new platelets, however, overall platelet counts were decreased by 50% and spleen weight was minimally decreased (Figure 3.2F and Table 3.1). We hypothesize that platelet loss is not due to destruction in the spleen, which would normally be accompanied by splenomegaly, but instead is due to consumption into microclots, referred to medically as DIC, disseminated intravascular coagulation.[19] DIC is a syndrome, not a disease, and is caused by sepsis, or trauma and results in morbidity and mortality in 25-50% of patients. DIC initiates when a stimulus disrupts vascular integrity, or cytokines and chemokines activate systemic coagulation and inflammation. Initial release of IL-6, followed by TNF-α and IL-1 are the prevailing inducers of DIC in sepsis. Preliminary studies evaluating IL-6 and TNF- α levels in the serum of wild-type and *Pak2*$^{-/-}$ showed no elevation (data not shown). These data suggest, that vascular damage, independent of cytokine storm, could be driving DIC in *Pak2*$^{-/-}$ mice.

DIC manifests by rapid acceleration of the coagulation cascade, massive consumption of platelets and coagulation factors exceeding the body's ability to replenish from the bone marrow and liver.[19] Systemically extensive micro-vascular blood clots emerge due to dysregulation of the coagulation cascade. Ultimately, the result of this widespread thrombosis is multiple organ failure. Risk of hemorrhage is elevated due to consumption of platelets and coagulation factors. Currently, the only approved therapy is activated protein C. Considering what is known about DIC, we evaluated the possibility of this disease state in *Pak2*$^{-/-}$ mice. We found that these mice had severe lymphedema throughout the body, with enlarged lymphatic vessels in the intestine, along with inflammatory stroma (Figure 4.3A). To evaluate for lymphatic endothelial damage, we stained for podoplanin (PDPN), and found increased surface expression (Figure 4.3B). Podoplanin expression on lymphatics activates platelets and recruits them to the site of damage through the podoplanin-CLEC-2 signaling cascade.[2,3,20]

To quantitatively test for DIC, measurements of decreased fibrinogen and/or increased D-Dimer are typically done. As the coagulation cascade proceeds, thrombin cleaves fibrinogen into fibrin, and cross-linked fibrin is referred to as D-Dimer. Fibrin degradation products and D-dimer are two markers of fibrinolysis that indicate microthrombus formation in DIC. Further evaluation of other DIC markers are needed to confirm if DIC is occurring in the *Pak2*$^{-/-}$ mice.

Vascular damage on endothelial cells can also activate the coagulation cascade by expressing tissue factor-FVIIa on the surface. Typically, under physiologic conditions, tissue factor (TF) is tightly regulated and only expressed on the subendothelial surfaces. In DIC, TF exposed on the endothelial cell surface changes vascular permeability and

additionally can be expressed on macrophages and monocytes. Upon damage, the protein is exposed to circulation, and drives the coagulation cascade. TF is also released in response to cytokines (IL-1, TNF-α, and endotoxin).[21] Administration of anti-TF antibody functions to fully abrogate activation of the coagulation cascade in vivo, as well as attenuate inflammation and endothelial cell injury by reducing serum plasma levels of IL-6 and VCAM-1, in mice with sickle cell disease.[21] Future work with administration of anti-TF antibody into *Mx1-cre*$^{+}$*;Pak2*$^{f/f}$ would be informative towards the understanding of the source of vascular damage.

Neutrophils can also be a triggering agent to activate vascular damage-induced coagulation. These innate immune cells adhere to damaged blood vessels, and initiate a coagulation cascade. Since *Pak2*$^{-/-}$ mice had elevated circulating neutrophils, there is a possibility that this is an early event that contributes to the cause of death. In order to study the effect of neutrophils in vascular integrity in our mouse model, we could inject a neutrophil inhibitory antibody to disrupt the neutrophil-vasculature binding cycle and inhibit DIC. Such neutrophil blocking antibodies are anti-Ly6(G/C) and ICAM-1. There is also the possibility that the observed reduced fibrinogen levels in serum resulted in hyperactivated neutrophils, implicating fibrinogen as a negative regulator of neutrophil function and the inflammatory response.[22]

Platelets function to protect blood vessels from injury. This was demonstrated by the transfusion of normal resting platelets or degranulated platelets into a mouse model with highly vascularized tumor. The authors discovered that in the absence of platelets, tumor vasculature was damaged, but this phenotype was rescued by the transfusion of resting platelets, but not with the transfer of already degranulated platelets.[23] Platelet

granule secretion protects blood vessels by secreting soluble factors that inhibit excessive endothelial permeability and/or prevent vascular injury induced by inflammation. Platelets secrete VEGF, angiopoietin-1, TGF-β, platelet basic protein, serpins, serotonin and sphingosin-1-P to support vascular barrier function and/or have immunomodulatory properties. This research indicates that platelets are required to maintain blood vessel integrity through secretion of growth factors.

Based on these studies, future experiments with *Mx1cre*$^{+}$*;Pak2*$^{fl/fl}$ mice can evaluate if polyI:C is promoting inflammation due to IFNalpha activation. This exacerbated immune response, along with thrombocytopenia, could possibly result in vascular damage observed in *Pak2*$^{-/-}$ mice. We hypothesized that transfusion of wildtype platelets every other day into *Mx1cre*$^{+}$*;Pak2*$^{fl/fl}$ mice could rescue the vascular defects observed, such as pleural effusion and lymphedema.

Pak2 does not regulate vascular integrity via alterations in platelet function in vivo

Using a Cre recombinase system driven by platelet factor 4 (PF4), expressed solely in platelets and megakaryocytes, we were able to evaluate two questions: (1) Is embryonic lethality due to failure of *Pak2*-null platelets to regulate lymph-blood vessel separation downstream of CLEC-2 receptor and (2) Is the observed reduction in peripheral platelet count in *Pak2*-null adult mice due to a platelet specific effect or a systemic effect?

Our results demonstrated that *PF4cre*$^{+}$*;Pak2*$^{fl/fl}$ mice are viable adults with normal litter size. Under non-stressed conditions, these mice do not demonstrate a decrease in peripheral platelet count. However, upon evaluation of their CD41+ megakaryocytes we

did observed slightly increased ploidy (Figure 4.4). Evaluation of proplatelet formation (PPF) in fetal liver-derived megakaryocytes demonstrated decreased formation of these cytoskeleton-rich extensions (Figure 4.5A). These data demonstrate that Pak2 functions in megakaryocytes in a cell-instrinsic manner to regulate ploidy and proplatelet formation, but does not effect the production or clearance of platelets.

Platelet clearance, Reticulated platelets and Mean platelet volume

The observation that *Mx1-cre*$^{+}$*;Pak2*$^{-/-}$ animals experience increased platelet clearance rate and increased reticulated (young) platelets could be induced by multiple mechanisms (Figure 3.2). The observed dramatic increase in platelet clearance rate could be due to clearance-induced antiplatelet antibodies (CIAA).[24] Platelets can be taken up and destroyed by bone marrow-derived macrophages. CIAA could explain why *Pak2*-null mice demonstrate increased clearance along with increased production. As new platelets are being produced to handle the loss of platelets, they are being destroyed by CIAA, and therefore not measured in the peripheral blood counts. The *Pak2*-null phenotype is typical of CIAA, as platelets are consumed new platelets are produced, however quickly cleared via CIAA.

We also observed a significant increased in mean platelet volume (Figure 3.2). Increased mean platelet volume is typically indicative of young, reticulated platelets.[25,26] Increased platelet size (~1 fL difference) is correlated with increased risk for various acute vascular complications, including cardiovascular disease and peripheral arterial disease.[27,28] Increased platelet volume is associated with increased platelet aggregation and increased expression of adhesion molecules. A study using rat models revealed that lung damage can also reduce circulating platelets, suggesting that the lungs play an active

role in the regulation of platelet counts.[29] The reduced platelet numbers in *Pak2*$^{-/-}$ mice could be a result of lung damage, observed by extensive amounts of pleural effusion and lung congestion. Increased mean platelet volume in *Pak2*$^{-/-}$ mice could correlate with the vascular disruption in these mice, observed by lymphedema, pleural effusion, and hemorrhagic intestines.

Future mouse rescue experiments to define cause of mortality

To further investigate the cause of mortality in *Mx1-cre*$^{+}$*;Pak2*$^{f/f}$ mice, we propose several rescue experiments. These animals displayed signs of lymphedema and vascular damage (with lung pleural effusion), a proposed rescue experiment would be to infuse wildtype platelets, and evaluate if lymphatic damage is rescued. Additionally, to evaluate if circulating anti-platelet antibodies are destroying platelets (CIAA), we proposed to transfuse serum from *Mx1-cre*$^{+}$*;Pak2*$^{f/f}$ into wildtype recipients and measure platelet count after 16-24 hours.

If DIC and vascular damage is caused by an activated immune system, the rescue experiment would involve transplantation of wild-type bone marrow into the *Mx1-cre*$^{+}$*Pak2*$^{f/f}$ animals. In the presence of a wild-type immune system (derived from the new wildtype bone marrow), we propose that survival time would increase indefinitely if the source of DIC is an activated immune system secreting inflammatory cytokines, such as IL-6 and TNFα.

In summary, identification of the initiating events leading to lethality in *Pak2*$^{-/-}$ mice is critical to understanding potential side effects from inhibiting Group I kinase function in various cancer types. Specifically, vascular damage and changes to the bone

marrow could be exacerbated by inhibition of Group I Paks. Identification of the impact of Pak2 deletion on signaling networks using phosphoproteomic analyses would be the next step in identifying which signaling networks are most effected by deletion of Pak2. Use of additional hematopoietic cre-recombinase systems, such as *Vav*-cre, would also demonstrate if lethality were from the hematopoietic system or from another compartment.

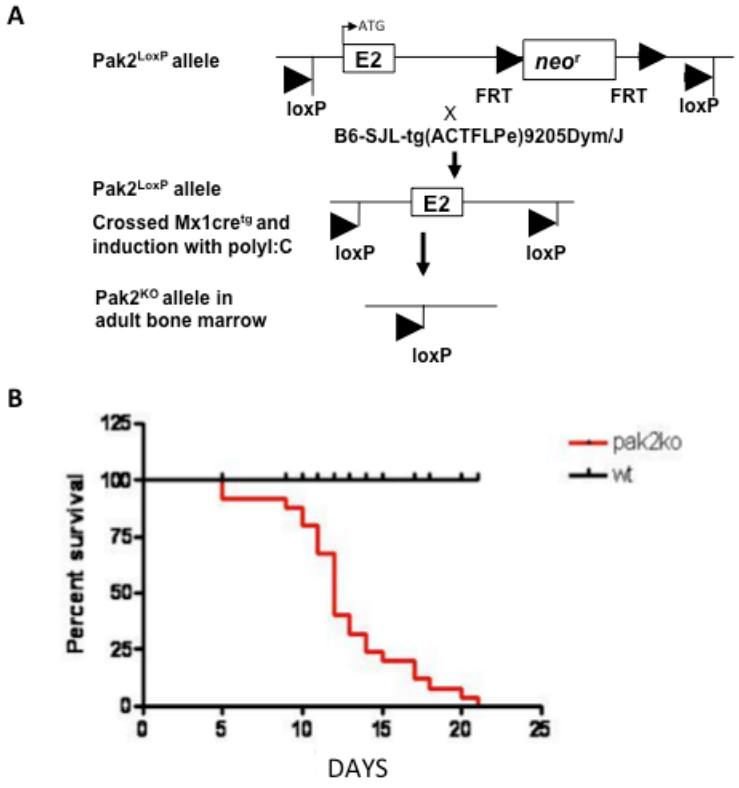

Figure 4.1. A. Model depicting the genetic structure for the inducible Pak2 floxed alleles. Upon Cre-induction with administration of polyI:C, exon 2 of Pak2 gene is excised, resulting in complete ablation of Pak2. **B.** Survival curve in adult Pak2fl/fl mice after induction of cre-recombinase with polyI:C. Wildtype (wt) and Pak2fl/fl both treated with equal concentration of polyI:C. n>30.

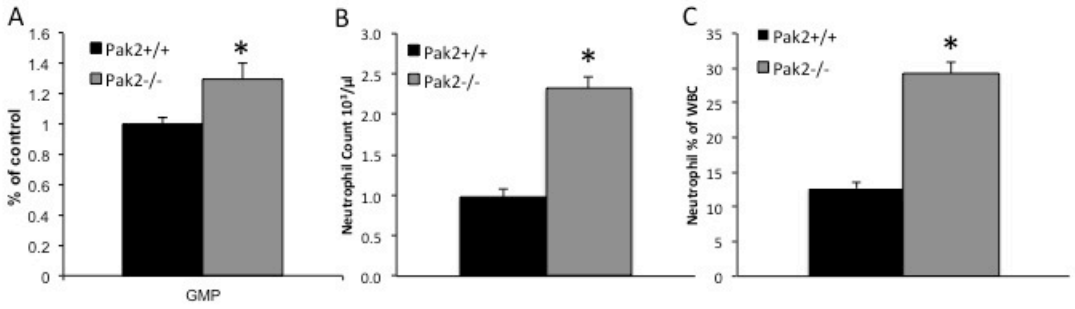

Figure 4.2 Increased stem and progenitor cell numbers in bone marrow of *Pak2*$^{-/-}$ mice. (A) Increased granulocyte/myeloid progenitor cells in bone marrow from polyI:C-treated Pak2$^{-/-}$ (gray bar) mice compared to WT(black bar) mice 14 DPI. (B) Increased neutrophil count in peripheral blood in Pak2$^{-/-}$ animals 14 DPI. (C) Increased neutrophils as a percentage of total white blood count (WBC) in Pak2$^{-/-}$ mice compared to WT mice, 14 DPI. DPI: Days post-injection of polyI:C. *p*-value <0.01

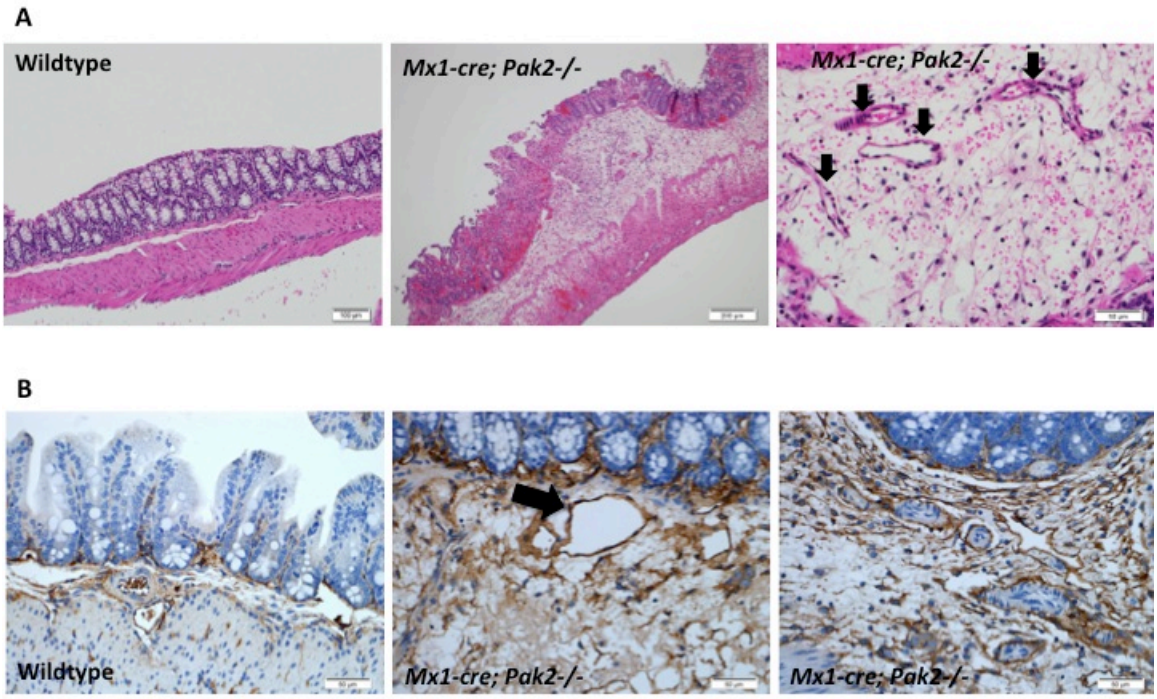

Figure 4.3 Pak2-null mice develop lymphedema in intestinal tissue. (A) H&E of mouse large intestine from wildtype (pIC injected) and Mx1cre+Pak2f/f (pIC injected). Intestines fixed at day 14 post-pIC. Arrows indicate enlarge lymphatic vessels in Pak2-knockout animals. (B) Podoplanin IHC, a glycoprotein expressed on the surface of damaged lymphatic vessels. Arrow indicates enlarge lymphatic vessel in Pak2-null animals. Overall increased staining in Pak2-null tissues, along with fibroblastic reticular cells, and inflammatory cells.

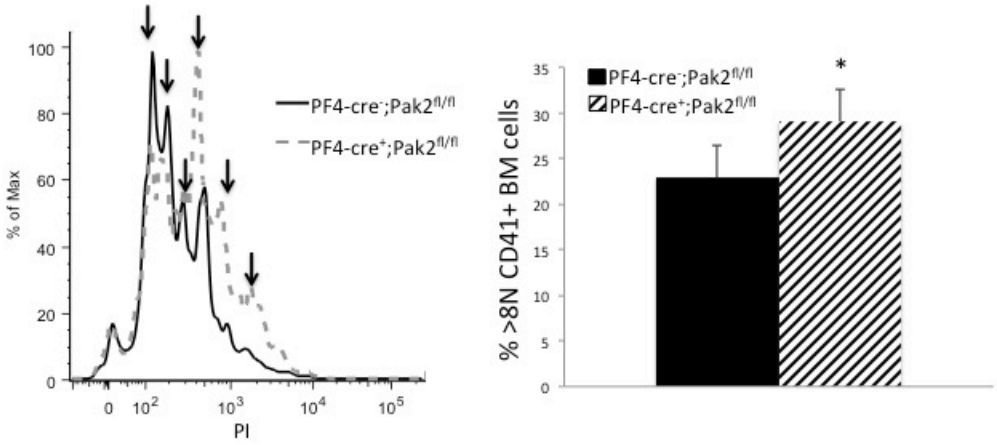

Figure 4.4 Increased ploidy observed with Pak2-deletion using a megakaryocyte and platelet specific cre-recombinase (PF4-cre). CD41+ cells selected and measured for ploidy with propidium iodide. Average of 8N+ population of CD41+ bone marrow cells.

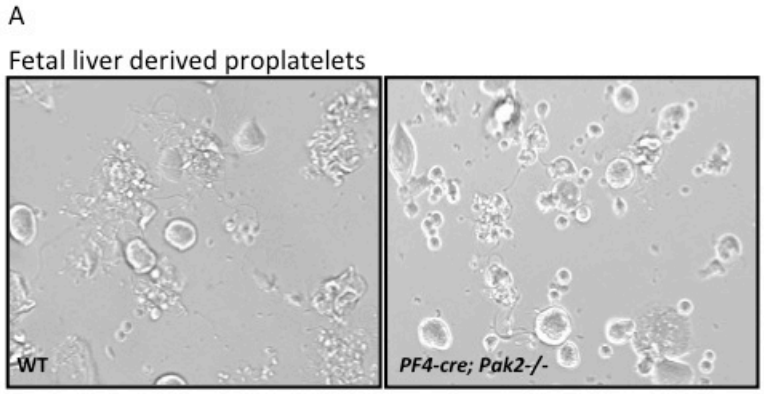

Figure 4.5 Impaired proplatelet formation in PF4-cre;Pak2-/- megakaryocytes in vitro. (A) Representative image of wild-type (WT) and *PF4-cre*$^{+}$*;Pak2*$^{-/-}$ in vitro cultured megakaryocytes. WT megakaryocytes display multiple long extensions, which are mostly absent from *PF4-cre+;Pak2*$^{-/-}$ megakaryocytes in culture.

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Chapter 5: Conclusions and Future Direction

Immune cells elicit a response to various external stimuli through many signaling cascades, however these networks, no matter what the cell type, require activation and remodeling of the cytoskeleton. Defects in the actin and microtubule cytoskeleton result in severe immunodeficiency due to the pivotal role for actin in immune cell recruitment, migration, intracellular signaling and activation.[1] The actin cytoskeleton forms the basis of the cell cortex, microtubules and intermediate filaments connect with actin to form a network, which organizes the internal cell structure. A major requirement to respond to immunogenic stimuli is a rapid reorganization of cell shape, over the time scale of seconds. Actin turnover occurs in a half-life of 15 seconds, myosin II turnover is less than 10 seconds, and proteins that crosslink actin filaments can turn over as fast as every second. Maintaining this multifaceted functionality requires the regulated interaction of over 100 proteins.[1]

The work presented here investigated the role of p21-activated kinases in two immune cells from distinct hematopoietic lineages, the Granulocyte/Myeloid-derived mast cell and the Meg/Erythroid progenitor-derived megakaryocyte. In Chapter 2, we showed that Pak2 functions to negatively regulate mast cell FcεRI mediated secretion by stimulating RhoA-GTPase. In Chapter 3, we discovered that Pak2 is involved in the maturation process of bone marrow megakaryocytes, proplatelet formation, and regulation of polyploidization, likely through effects on LIMK and Aurora phosphorylation. Finally, in chapter 4, we described the lethality phenotype observed in an inducible model of *Pak2* deletion in an adult.

Our research investigating isoform differences in mast cell function showed that Pak2 is the predominant Pak isoform expressed in mast cells, and functions to impair secretion via negative regulation of the RhoA-MLC2 signaling cascade and $p38^{MAPK}$, without effecting calcium flux (chapter 2). These findings are in contrast to our previous findings on Pak1, and show that Pak1 and Pak2 function in different manners during antigen-induced mast cell secretion. Pak1 deletion impaired mast cell secretion through regulation of F-actin and calcium flux, whereas Pak2-deletion enhanced secretion through negative regulation of RhoA activity. Since Pak1 and Pak2 share high sequence identity, in both their regulatory and catalytic domains (Appendix Figure 1), as well as the same substrate phosphorylation recognition sequences, it is of great interest to identify functional differences in primary cells. With such similarities, it is of interest to understand the source of biological differences. Observed differences may stem from subcellular localization (i.e. nuclear localization vs. plasma membrane bound), binding to different multiple protein complexes to elicit differential effects, and/or gene and protein expression differences. Identification of the exact source of these functional differences is an important goal of future studies. Future work with chimeric cells (knockout cells reconstituted with vectors expressing chimeras of Pak1 and Pak2) and bone marrow chimera studies, will elucidate the function of each isoform in cellular functions *in vitro*, as well as maintaining hematopoiesis *in vivo*.

Our studies presented in Chapter 2 clearly demonstrate that Pak2, but not Pak1, regulates RhoA activity to promote mast cell IgE-mediated secretion. This regulation might be regulated through two plausible pathways. One pathway is via direct

phosphorylation and inhibition of GEF-H1, the primary GTP exchange factor (GEF) and activator of RhoA. Additionally, Pak2 may regulate GEF-H1 indirectly by regulating microtubule stability though stathmin. Stathmin, a previously identified substrate for Pak1[2], is a microtubule destabilizing protein which, in the unphosphorylated state, sequesters soluble tubulin into an assembly-incompetent complex. Inhibition of stathmin results in polymerized microtubules during thrombin stimulation. These intact microtubules retain GEF-H1 in the inactive state, thereby inhibiting RhoA activation.[3] Recent findings by Meiri et al.[4] indicate that GEF-H1 activation and displacement from intact microtubules may not actually require microtubule depolymerization as previously thought. With a short stimulus of LPA or thrombin, GEF-H1 was activated and release in the presence of intact microtubules.

These findings are of interest to our observations in *Pak2*-null mast cells. *Pak2* deletion in primary mast cells resulted in impaired phosphorylation of stathmin at S^{16}, thereby increasing its microtubule destabilizing activity. Without fully polymerized microtubules, *Pak2*-null mast cells expressed activated GEF-H1, which is unable to bind and be inhibited by polymerized microtubules. Elevated GEF-H1 activity is a critical factor driving constitutively-active RhoA GTPase activity.[5,6] Studies evaluating the effect of MT polymerization and RhoA activity found that drug-induced MT depolymerization resulted in robust RhoA activation due to activated GEF-H1.[7] GEF-H1 overexpressed cells had increased stress fiber formation, focal adhesions and actomyosin contraction.[7] Given the time course of mast cell stimulation of 10 minutes, it is unclear if Pak2 regulation of GEF-H1 was through direct phosphorylation and release from microtubules or indirectly through stathmin-induced microtubule depolymerization. Our findings

demonstrate that Pak2 negatively regulates microtubule associated proteins GEF-H1 and stathmin, thereby driving antigen-induced mast cell secretion through RhoA activation. Activated RhoA by Pak2 inhibition could have important therapeutic implications for solid tumors and hematological malignancies.

Using primary bone marrow derived mast cells as a model, we provide the first demonstration that Pak2 is a negative regulator of RhoA through phosphorylation and inhibition of both GEF-H1 and stathmin. This control leads to negative regulation of mast cell secretion. Beyond mast cell biology, the Stathmin-GEFH1-RhoA signaling axis is a critical regulator of thrombin induced-vascular permeability and endothelial cell junctions.[3,5] It will be interesting for future studies to determine if this signaling network is also influenced by Pak2 in the vasculature.

Biochemically, future work to expand this field will need to identify if Pak2 directly signals through GEF-H1 and stathmin to regulate secretion. In order to do this, we can use shRNA to silence hyperactivated GEF-H1 and stathmin in *Pak2*-null mast cells to rescue IgE-mediated secretion to wild-type levels. Our data demonstrate that both proteins are involved in FcεRI crosslinking signal transduction, as both proteins are phosphorylated (inhibited) upon IgE-stimulation. This inhibition results in stabilization of microtubules and inhibition of RhoA hyperactivation. Recent work with a proteomics screen found activation of Stathmin gene expression upon FcεRI crosslinking.[8] Additionally, GEF-H1 was found to regulate endocytic and exocytic vesicle trafficking.[9,10] Future work using Pak1 and Pak2 chimeric vectors will help elucidate the origins of protein isoform differences, in particular why they have opposite functions, in mast cell secretion.

In Chapter 3 we sought to evaluate the function of Pak2 in another bone marrow derived immune cell, the megakaryocyte. The main function of a megakaryocyte is to synthesize platelets through a maturation process that involves endomitosis, or duplication of DNA without cytokinesis, before terminal differentiation into platelets. Using an inducible model of *Pak2*-deletion *in vivo*, we discovered that Pak2, but not Pak1, is the predominant isoform in megakaryocytes important for polyploidization and proplatelet formation. We discovered that Pak2 likely functions through LIMK to regulate actin and Aurora to regulate the microtubule network, both leading to negative regulation of polyploidization. By promoting polyploidization, with the inhibition of Pak2 kinase, this provides a novel mechanism for treatment of acute megakaryoblastic leukemia, described by an accumulation of immature, non-polyploid megakaryocytes. Aurora kinase overexpression was found in many hematological malignancies, including acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, acute megakaryocytic leukemia, multiple myeloma, aggressive non-Hodgkin lymphoma and Hodgkin's lymphoma.[11] Therefore, identification of upstream activators of Aurora, such as Paks and LIMK, provides additional therapeutic targets for hematological malignancies with elevated Aurora activity. Future work to understand the role of Pak2 in regulating polyploidization and megakaryocyte maturation, as it pertains to AMKL, is still needed. However, preliminary work suggests Pak could be a valuable target for therapeutic intervention in some Aurora-driven hematological malignancies, since Pak2 inhibition resulted in inhibited Aurora phosphorylation.

Recent work evaluating neoplasms driven by gain-of-function mutations in KIT receptor tyrosine kinase found constitutively-active Pak downstream of KIT activation.

For the first time in hematological malignancies, Pak was found to drive tumor growth in a mouse model of KITD814V-mutation, and human leukemic cells with KITD816V.[12] Pak inhibition was investigated as a possible therapeutic target for these very aggressive tumors, which are resistant to imatinib (Gleevac) and show little therapeutic efficacy in response to dasatinib (Sprycel). They discovered that Pak inhibition, with allosteric inhibitiors, was successful in inhibiting systemic mastocytosis, myeloproliferative neoplasms (MPN) and acute myeloid leukemia (AML) in mice.[12] Mice bearing myeloid cells expressing both KITD814V developed severe MPNs, whereas this phenotype was rescued by expression of Pak kinase mutant, PakK299R. Inhibition of Pak kinase function resulted in increased survival and a significant delay in disease onset. Overall, the MPN disease phenotype was reversed with the inhibition of Pak1 kinase function with both genetic and pharmacologic inhibition.[12]

In addition to the use of Pak inhibitors to reverse MPN progression, it is possible that Pak inhibition could be used to promote hematopoietic stem cell (HSC) expansion after chemotherapy or myeloablation therapy. These therapies often result in decreased HSC proliferation and recovery. Recent discoveries into the drivers of HSC expansion found that megakaryocytes secrete growth factors (FGF-1, TGF-β1 and CXCL4) to maintain or expand the HSC niche during stress.[13-16] Curiously, HSCs ($Lin^-CD48^-CD41^-CD150^+$) are generally located close to megakaryocytes, with a considerable fraction (20%) directly adjacent to a megakaryocyte.[15] This percentage is greater than any random distribution of HSCs and MKs in the bone marrow. HSC dependency on MKs is also evident during bone marrow engraftment studies, where it was found that HSCs

preferentially home to the MK-rich endosteal areas, and host MKs facilitate donor HSC engraftment after lethal irradiation.[14,16]

Considering the close association between MKs and HSCs, this provides evidence that therapeutic intervention to expand megakaryocytes may function well to expand the HSC niche as well. Our data demonstrate that *Mx1cre*$^{+}$*;Pak2*$^{f/f}$ mice have expansion of their megakaryocyte progenitors and mature cells, as well as HSCs. Further evaluation will look into the direct role of Pak2 on megakaryocyte growth factor secretion and its potential role on expansion of the HSC niche. Since Pak2 is a negative regulator of mast cell cytokine secretion, we hypothesize that future analysis of growth factor secretion from megakaryocytes may also demonstrate enhanced secretion with impaired Pak2 function.

Future research to elucidate the function of Pak2 in microtubule integrity and barrier dysfunction

Upon induction of *Pak2*-deletion using Mx1-cre recombinase, we observed that these animals have a disruption to their endothelial barrier function, identified by lymphedema, pleural effusion in the lungs, and intestinal bleeding, accompanied by rapid platelet clearance (described in Chapter 4). Considering the disruption of normal regulation of GEF-H1 and Stathmin in mast cells identified in Chapter 2, it is interest to look at the function of these substrates on endothelial barrier function. Upon deletion of *Pak2* in the adult animal, we observed lethality in 100% of the mice between 14 and 20 days post-polyI:C administration (similar lethality is observed using tamoxifen-regulated CAG-Cre). Based on the phenotype observed, our future hypotheses are to look at the

role of GEF-H1 and Stathmin suppression in a *Pak2*-null adult animal. We hypothesize that elevated GEF-H1 and Stathmin activity in *Pak2*-null animals is driving endothelial barrier dysfunction, resulting in acute lethality.

The RhoA-specific GEF, GEF-H1, localizes to microtubules (MT) when inactivated (phosphorylated) and remains in the suppressed state, inhibiting RhoA activation.[17] GEF-H1 becomes activated and dephosphorylated when released from MTs as they undergo disassembly (Figure 5.1). Activated GEF-H1 is a mechanotransducer, which connects depolymerized microtubules with RhoA activation and actin filament depolymerization, driving endothelial barrier dysfunction.[18] *In vivo* experiments to elucidate the role of GEF-H1 in barrier function are not possible since *GEF-H1*-null animals are embryonic lethal. Instead, researchers using intravenous injection of siRNA against GEF-H1 were able to preserve lung endothelial barrier function and prevent vascular leakage upon damage with LPS stimulation.[19] LPS induces MT disassembly and the release of active GEF-H1 from microtubules, leading to barrier dysfunction. Protection against MT disassembly by MT stabilizing compounds was also able to protect against compromised endothelial barriers in drug and disease induced states.[20] These data suggest that future experiments with injection of MT stabilizing compounds in mouse models of *Pak2*-deletion could partially rescue the lethality in *Pak2*-null mice, if death is primarily due to vascular damage or elevated microtubule depolymerization.

Another regulator of microtubule stability is Stathmin, the microtubule depolymerizing protein which functions by binding microtubule heterodimers preventing polymerization.[21] Stathmin can also drive microtubule polymerization by increasing the switching frequency (catastrophe frequency) from growth to shortening at plus and minus

ends by binding directly to the microtubules. Phosphorylation of stathmin on one or more of its four serine residues (S^{16}, S^{25}, S^{38}, and S^{63}) reduces its microtubule-destabilizing activity, suggesting a cooperative nature of stathmin phosphorylation at different sites to control its effects on MT depolymerization.[22] We demonstrated in Chapter 2 that stathmin phosphorylation was completely ablated in *Pak2*-null antigen-stimulated mast cells. Prior to my work, Pak1 was identified as an effector of stathmin phosphorylation.[2] Recently, depletion of endogenous Pak1 abolished both HGF-induced stathmin phosphorylation and the increase in the pool of acetylated tubulin, demonstrating enhanced stathmin depolymerizing activity in the absence of Pak1.[5] Murine knockdown of stathmin *in vivo* with siRNA demonstrated that lung endothelial barrier dysfunction induced with thrombin was protected against when stathmin was depleted which preserved lung endothelial barrier function, similar to the GEF-H1-silenced mice.[3] Thrombin treatment of human pulmonary ECs induces rapid stathmin dephosphorylation and activation. Constitutively-active stathmin (a phosphorylation-deficient mutant) expressed in endothelial cells resulted in exacerbated thrombin-induced barrier dysfunction. Inhibition of stathmin activity caused MT stabilization and down-regulation of exacerbated RhoA signaling. Together, these data suggest that stathmin hyperactivity in *Pak2*-null cells may drive endothelial barrier dysfunction, along with GEF-H1 hyperactivity resulting in lethality.

Stathmin and GEF-H1 are mechanistically interconnected; stathmin-knockdown provides a protective effect to thrombin-induced endothelial cell permeability.[3] This protective effect is abrogated by expression of constitutively-active GEF-H1. Additionally, activated mutants of stathmin exacerbated the effect of thrombin, and this

elevated effect was abolished by depletion of GEF-H1. These data strongly suggest that GEF-H1 is a major regulator of endothelial cell permeability downstream of stathmin. Thrombin stimulation activates stathmin to depolymerize microtubules, and results in subsequent decrease in microtubule-bound GEF-H1, yielding active GEF-H1 to promote RhoA signaling and phosphorylation of myosin light chain, ultimately leading to endothelial barrier dysfunction. This thrombin-induced decrease in MT-bound GEF-H1 was abolished by depletion of stathmin.[3]

Future directions to fully understand the observed lethality in *Pak2*-deficient mice might include various informative genetic crosses. For example, one of our hypotheses implicates stathmin activation as a potential cause of the observed phenotype. We propose to cross *Stathmin*-deleted mice with the $Mx1cre^{+}Pak2^{fl/fl}$ to identify if endothelial barrier dysfunction observed in the *Pak2*-null mice is mediated through stathmin over-activation, leading to enhanced microtubule depolymerization and endothelial barrier dysfunction. Interestingly, analysis of *Stathmin*-deficient mice showed a reduction in thymocyte cellularity and peripheral T cell numbers.[23,24] Similarly, T-cell specific deletion of Pak2 (*Lck-cre* and *CD4-cre*) also resulted in lymphocytopenia due to a failure to undergo proper maturation.[25] These similarities provide evidence to support stathmin as both an *in vivo* and *in vitro* Pak2 substrate, as well as a possible driver of adult lethality. However, further work is needed to identify the initiating factor activating the pathway to lethality.

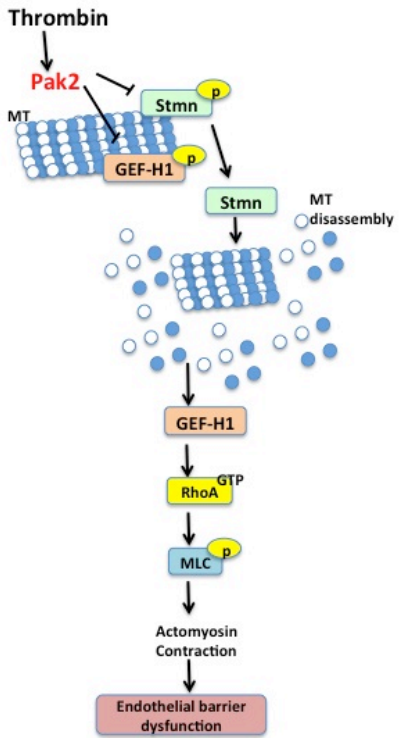

Figure 5.1 A model depicting the mechanism by which Pak2 regulates GEF-H1 and Stathmin to negatively regulate RhoA signaling and MT stability. This is a potential mechanism to explain the observed endothelial barrier dysfunction and lethality in Mx1cre^{+}Pak2$^{f/f}$ mouse model. This same mechanism also describes the elevated secretion observed in IgE-stimulated mast cells.

Future Directions: Bone marrow Chimeras

We observed numerous changes to the bone marrow compartment in Pak2-deleted mice. To further understand these changes, future work using bone marrow transplantation studies with chimeric forms of Pak1 and Pak2 mutant bone marrow can address many questions. Similar research with Cdc42, an activator of Group I Paks, was found to cause rapid lethality in the adult due to suppressed erythroid differentiation using an *Mx1cre*$^{+}$*Cdc42*$^{fl/fl}$ mouse model. The authors utilized a bone marrow chimera model (WT animal transplanted with *Mx1cre*$^{+}$*Cdc42*$^{f/f}$ bone marrow) and discovered that lethality in the *Cdc42*-floxed animal was due to changes in the bone marrow, specifically failure to differentiate erythroid progenitors and expansion of myeloid progenitors. To address these questions in *Pak2*-floxed animals, bone marrow transplantation models

could be used, where *Mx1cre*$^{+}$*;Pak2*$^{f/f}$ bone marrow will be transplanted into an irradiated wild-type host, and vice versa, wild-type bone marrow into a *Mx1cre*$^{+}$*;Pak2*$^{f/f}$ host. After engraftment (6 weeks), deletion would be induced with polyI:C, and the peripheral blood changes will be monitored weekly, along with survival. This type of experiment would help to identify the location of the cell type initiating lethality in the *Mx1-cre*$^{+}$*Pak*$^{f/f}$ mice.

Another key aspect for future research is our data suggesting that differences exist between two models of inducible Cre-recombinase, Mx1-cre (polyI:C inducible) and Cag-Cre-ERT2 (tamoxifen inducible). The two models have similar rates of lethality, (100% lethality observed, between day 14 and 25). However, their phenotypes are strikingly distinct. These differences include lymphedema, pleural effusion and decreased platelet counts in *Mx1cre*$^{+}$*Pak2*$^{f/f}$ mice, which is not observed in tamoxifen-induced *Cre-ERT2*$^{+}$*Pak2*$^{f/f}$ animals. Both models do show signs of vascular damage. One key difference on activation of Cre-recombinase is that Mx1-cre is induced with polyI:C, which elicits an interferon-alpha (IFN-α) response and tamoxifen administration does not elicit immune activation. Perhaps the simultaneous activation of IFN-α along with *Pak2* deletion results in an enhanced TLR3 response resulting in vascular injury. Experimentally one can address this by inducing Pak2 deletion with tamoxifen in the *Cre-ERT2*$^{+}$*;Pak2*$^{f/f}$ animals along with simultaneous injections of polyI:C. After 14-16 days post-tamoxifen administration (DPI), measurements of peripheral blood changes, platelet counts, weight gain, lymphedema, and pleural effusion can be assessed. Since it was shown that *Cre-ERT2*$^{+}$*;Pak2*$^{f/f}$ do not have decreased platelet counts at 14 DPI (HY Chow, unpublished), it is of interest to observe if the addition of polyI:C to this model of *Pak2* deletion exacerbates the phenotype to yield decreased platelet counts and

lymphedema. A caveat to work with the Cag-Cre-ERT2 model to induce Pak2 deletion, is that bone marrow and megakaryocytes were not evaluated for the level of Pak2 recombination and deletion. It is possible that this dose and time course of tamoxifen dosage was not sufficient to delete in this compartment.

It is of interest to understand how the combination of immune activation with polyI:C, along with simultaneous deletion of *Pak2* in the bone marrow, results in a gross pathology quite distinct from the tamoxifen-induced *Pak2* mouse model. Since Pak inhibitors will be administered to patients with varying degrees of immune reactivity, caution should be taken. These studies provide insight into a possible therapeutic side effect of Group I Pak inhibition with simultaneous immune activation.

Conclusion

Individual isoform differences among immune cells, epithelial cells, and many other cell types are just being discovered with the aide of isoform-specific knockout mouse models. Years of prior work helped identify the multitude of Pak substrates phosphorylated in cell culture or in *in vitro* kinase assays, however, this work was often never verified in an *in vivo* setting. Data presented here represents advances in the understanding of isoform differences, and identification of novel signaling pathways for Pak2 regulation of mast cell secretion (Chapter 2) and megakaryocytes maturation/polyploidization (Chapter 3). Distinct differences exist between Pak1 and Pak2 isoforms identified with the use of mouse models, as opposed to RNAi and dominant-negative mutants. This dissertation describes how Pak2 is the predominant isoform signaling in mast cells and megakaryocytes. Our findings, combined with recent

collaborations utilizing the *Pak2*-knockout mouse model in various tissue types, have led to the discovery that Pak2 is critical for survival during embryogenesis and in adult homeostasis, for the development and survival of T-cells and HSC bone marrow engraftment.[25,26] As the *Pak2*-floxed animal continues to be crossed with tissue specific Cre-recombinases, we will continue to make novel discoveries regarding the importance of this isoform in proper development and maturation, and potentially elucidate possible therapeutic implications for inhibition of Pak2 kinase function.

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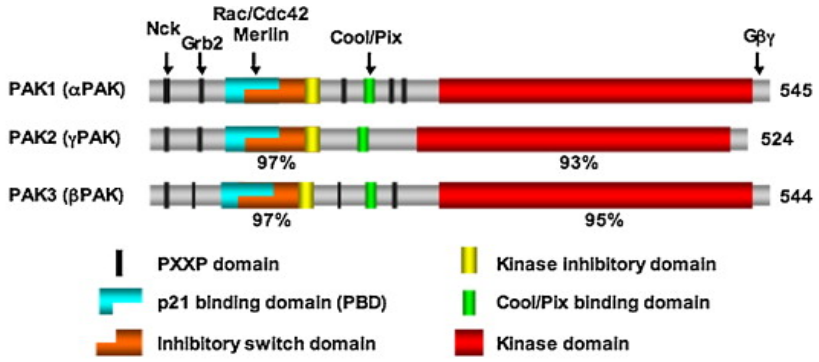
A



B

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Pak1 MSNNGVDIQDKPPAPPMRNTSTMIGAGSKDTGTLNHGSKPLPPNPEEKKKKDRFYRSILP  60
Pak2 MSDNG-ELEDKPPAPPVRMSSTIFSTGGKDPLSANHSLKPLPSVPEEKKPRNKII-SIFS  58

Pak1 G-DKTNKKREKERPEISLPSDFEHTIHVGFDAVTGEFTGMPEQWARLLQTSNITKSEQKK  119
Pak2 GTEKGSKKKEKERPEISPPSDFEHTIHVGFDAVTGEFTGMPEQWARLLQTSNITKLEQKK  118

Pak1 NPQAVLDVLEFYNSKKTSNSKKYMSFTDKSAEDYNSSN-TLNVKTVSETPAVPPVSEDDE  178
Pak2 NPQAVLDVLKFYDSNTVK--QKYLSFTPPEKDGFPSGTPALNTKG-SETSAV--VTEEDD  173

Pak1 DDDDDATPPPVIAPRPEHTKSVYTRSVIEPLPVTPTRDVATSPISPTENNTTPPDALTRN  238
Pak2 DDEDAA--PPVIAPRPDHTKSIYTRSVIDPIP---------APVG--DSNV---DSGAKS  217

Pak1 TEKQKKKPKMSDEEILEKLRSIVSVGDPKKKYTPFEKIGQGASGTVYTAMDVATGQEVAI  298
Pak2 SDKQKKKAKMTDEEIMEKLRTIVSIGDPKKKYTRYEKIGQGASGTVFTATDVALGQEVAI  277

Pak1 KQMNLQQQPKKELIINEILVMRENKNPNIVNYLDSYLVGDELWVVMEYLAGGSLTDVVTE  358
Pak2 KQINLQKQPKKELIINEILVMKELKNPNIVNFLDSYLVGDELFVVMEYLAGGSLTDVVTE  337

Pak1 TCMDEGQIAAVCRECLQALEFLHSNQVIHRDIKSDNILLGMDGSVKLTDFGFCAQITPEQ  418
Pak2 TCMDEAQIAAVCRECLQALEFLHANQVIHRDIKSDNVLLGMEGSVKLTDFGFCAQITPEQ  397

Pak1 SKRSTMVGTPYWMAPEVVTRKAYGPKVDIWSLGIMAIEMIEGEPPYLNENPLRALYLIAT  478
Pak2 SKRSTMVGTPYWMAPEVVTRKAYGPKVDIWSLGIMAIEMVEGEPPYLNENPLRALYLIAT  457

Pak1 NGTPELQNPEKLSAIFRDFLQCCLEMDVEKRGSAKELLQHQFLKIAKPLSSLTPLMHAAK  538
Pak2 NGTPELQNPEKLSPIFRDFLNRCLEMDVEKRGSAKELLQHPFLKLAKPLSSLTPLILAAK  517

Pak1 EATKNNH  545
Pak2 EAMKSNR  524
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CRIB Rac1/Cdc42 binding domain
Auto-inhibitory domain
Catalytic domain

Appendix Figure 1: Sequence homology of murine Pak1 and Pak2. (A) Schematic of protein structure, including regulatory domain and kinase domain. Percent sequence identity depicted below kinase domains. Adapted from Ci, Y et al. 2010. (B) Protein sequence for Pak1 and Pak2 (murine), yellow depicts sequence homology. Regulatory domain shown with blue bar, auto-inhibitory domain shown with red bar, and catalytic domain shown with green bar.

Appendix 2: Table 1
Mouse Models of Paks

Group	Pak Isoform	Knockout phenotype	Ref.
I	***Pak1*$^{-/-}$**	Reduce mast cell mediated anaphylaxis; macrophage defects, glucose homeostasis	1, 2,3
	αMYH6-cre; Pak1$^{fl/fl}$	Cardiac hypertrophy	4
	MMTV-ErbB2; Pak1$^{-/-}$	breast cancer tumor regression and prolonged survival	5
	K5-tet-on;tet-K-ras^{G12D}*;Pak1*$^{-/-}$	Decreased tumor initiation and progression of SCC	6
	NF1$^{+/-}$*;Pak1*$^{-/-}$	Reduction of mast cell dermal accumulation	7
	***Pak2*$^{-/-}$**	Lethal E8.5	8
	MSCV-Cre-ERT2; Pak2$^{fl/fl}$	mast cells hyperresponsive to IgE stimulation (ex vivo)	9
	MSCV-Cre; Pak2$^{fl/fl}$	Failure of bone marrow engraftment	10
	Lck-cre;Pak2$^{fl/fl}$	severe T-cell lymphopenia, loss of regulatory T-cells	11
	CD4-cre;Pak2$^{fl/fl}$	severe T-cell lymphopenia	11
	Mx1-cre;Pak2$^{fl/fl}$	Lethal 3 weeks after deletion, thrombocytopenia	Kosoff dissertation
	Mx1-cre;Pak2$^{fl/fl}$	Chimeric bone marrow (wildtype recipient with Pak2-/- bone marrow); neutrophil and GMP expansion, survival.	12
	***Pak3*$^{-/-}$**	Learning and memory defects, abnormalities in synaptic plasticity	13
	Pak1$^{-/-}$*;Pak3*$^{-/-}$	Learning and memory defects, hyperactivity	14
	Camk2a-PID; FMR1$^{-/-}$	Rescues fragile X syndrome phenotype	15
II	***Pak4*$^{-/-}$**	Lethal E10.5, heart and neural tube defects	16
	***Pak5*$^{-/-}$**	Viable healthy	17
	***Pak6*$^{-/-}$**	Viable healthy	18
	***Pak5*$^{-/-}$*;Pak6*$^{-/-}$**	Impaired learning and locomotion	18

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