Identification of Novel Regulators in Hematopoiesis: Roles for Gfer in Hematopoietic Stem Cell Proliferation and CaMKK2 in the Restriction of Granulopoiesis

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
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ABSTRACT

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Abstract

Hematopoiesis is the process in which billions of blood cells are produced on a daily basis, and is vital for sustaining life. This process is tightly regulated by a dynamic balance between hematopoietic stem cell (HSC) self-renewal and differentiation, and maintenance of this balance is of critical importance as dysregulation of HSCs can lead to hematopoietic deficiencies or malignancies such as leukemogenesis. While the signaling mechanisms that regulate HSC homeostasis and function are not well understood, our previous studies have identified a calcium/calmodulin (CaM)-dependent protein kinase, CaMKIV, that is intrinsically required for regulating normal proliferation and survival in HSCs. These findings suggest not only the importance of calcium-initiated pathways including CaMKIV-dependent signaling in hematopoietic cells, but also the potential for other calcium/CaM-dependent effector proteins such as other CaM-kinases to be involved in regulating HSCs and hematopoiesis.

The first major section of this dissertation work presented herein was based on the usage of RNA interference (RNAi) technology to specifically deplete HSCs of growth factor erv1-like (Gfer), a gene whose expression appeared to be absent in CaMKIV null HSCs based on comparative microarray analysis with wild-type HSCs, and seemed a potential target of CaMKIV. We showed that depletion of Gfer in HSCs compromised their *in vivo* engraftment potential and triggered a hyper-proliferative response that led

to their exhaustion. We further assessed Gfer-depleted HSCs by using microscopy techniques and found that these cells possessed significantly reduced levels of the cyclin-dependent kinase inhibitor (CDKI) $p27^{kip1}$. In contrast, ectopic over-expression of Gfer in HSCs resulted in significantly elevated total and nuclear $p27^{kip1}$. We next performed immunoprecipitation-immunoblot analyses to determine whether alteration of Gfer levels would affect $p27^{kip1}$'s binding with its inhibitor, the COP9 signalosome subunit jun activation-domain binding protein 1 (Jab1), that would subsequently lead to its ubiquitination, and determined that depletion of Gfer resulted in enhanced binding of $p27^{kip1}$ to Jab1. Conversely, over-expression of Gfer resulted in its enhanced binding to Jab1 and inhibition of the Jab1-$p27^{kip1}$ interaction. Furthermore, normalization of $p27^{kip1}$ in Gfer-KD HSCs rescued their *in vitro* proliferation deficits. These results provide evidence for a novel Gfer-Jab1-$p27^{kip1}$ pathway present in HSCs that functions to restrict abnormal proliferation.

The second major section of this dissertation work describes our studies of a CaMKIV kinase, CaMKK2, and its role in HSCs and hematopoietic development. These studies were largely based on the usage of mice genetically ablated for the *Camkk2* gene in the germline. Herein, we identified a role for CaMKK2 in the restriction of granulocytic fate commitment and differentiation of myeloid progenitor cells. We performed bone marrow transplantation studies and discovered that engraftment by *Camkk2*$^{-/-}$ donor cells resulted in the increased production of mature granulocytes in the

bone marrow and peripheral blood. Similarly, we used fluorescence activated cell sorting (FACS) to determine that *Camkk2*$^{-/-}$ mice possessed elevated numbers of common myeloid progenitor cells, and exhibited an accelerated granulopoietic phenotype in the bone marrow. Expression of ectopic CaMKK2 in *Camkk2*$^{-/-}$ common myeloid progenitors was sufficient to rescue aberrant granulocyte differentiation, and when over-expressed in 32Dcl3 cells was also sufficient to impede granulocyte differentiation in a kinase activity-dependent manner. Collectively, our results reveal a novel role for CaMKK2 as an inhibitor of granulocytic fate commitment and differentiation in early myeloid progenitors.

While our original intent was to identify and link a downstream target and upstream kinase to CaMKIV in HSCs, our results ultimately did not suggest that Gfer or CaMKK2 function in the same pathway in HSCs as discussed in the following chapters. Nonetheless, our findings represent a considerable advance in identifying and characterizing the functions of two novel regulators, Gfer and CaMKK2, that are important for HSC proliferation and the commitment and early differentiation steps of granulopoiesis, respectively.

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List of Acronyms and Abbreviations

32Dcl3	32D Clone 3
AMPK	AMP-activated protein kinase
APC	allophycocyanin
Bcl-2	B cell lymphoma 2
BM	bone marrow
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
°C	degrees Celsius
Ca^{2+}	calcium
CaM	calmodulin
CaMK	Ca^{2+}/CaM dependent protein kinase
CaMKK	Ca^{2+}/CaM dependent protein kinase kinase
cAMP	cyclic AMP
CBP	CREB binding protein
CKLiK	CaMKI-like kinase
CFU-G	colony-forming unit-granulocyte
CFU-M	colony-forming unit-macrophage
CLP	common lymphoid progenitor

CMP	common myeloid progenitor
CREB	cyclic AMP response element-binding protein
C-terminal	carboxy-terminal
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
FACS	fluorescent activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
Gfer	growth factor *erv1*-like
GMP	granulocyte-monocyte progenitor
GFP	green fluorescent protein
HBSS	Hank's Buffered Salt Solution
HSC	hematopoietic stem cell
IL	interleukin
Jab1	jun activation binding protein 1
KD	knock-down
KLS	c-Kit^{+}Lin$^{-/lo}$Sca1^{+}
Lin	lineage
MAPK	mitogen-activated protein kinase
MEP	megakaryocyte-erythroid progenitor

MPP	multipotent progenitor
mRNA	messenger RNA
MSCV	murine stem cell virus
N-terminal	amino-terminal
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PE	phycoerythrin
PI	propidium iodide
rm	recombinant mouse
RNA	ribonucleic acid
RT	reverse transcription
SCF	stem cell factor
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Ser	serine
shRNA	short hairpin RNA
Thr	Threonine

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1. Introduction

1.1 The Calcium/Calmodulin-Dependent Protein Kinase Cascade

Calcium (Ca^{2+}) is a ubiquitous and essential second messenger that is utilized in all cells to initiate regulatory pathways that alter important processes including cell proliferation, development, motility, secretion, and apoptosis. Stimuli such as hormones, growth factors, cytokines, and neurotransmitters all elicit increases in intracellular Ca^{2+} levels that can vary widely based on the amplitude, temporal, and spatial nature of the Ca^{2+} release (Thomas, Bird et al. 1996; Macrez and Mironneau 2004). Elevations in Ca^{2+} trigger signaling cascades to occur wherein Ca^{2+} can either act directly on target proteins (Ca^{2+} effectors), or its effects can be mediated by a number of intracellular Ca^{2+}-binding proteins that function as Ca^{2+} buffers or transducers.

One of the major Ca^{2+}-binding proteins that transduces Ca^{2+}-signaling and acts as a primary Ca^{2+} receptor and in the cell is calmodulin (CaM), a highly conserved protein consisting of four EF-hand, high affinity Ca^{2+}-binding motifs (Chin and Means 2000). The binding of Ca^{2+} results in a conformational change in CaM that modulates CaM's effects on its targets (bound previously in its Ca^{2+}-free state), and also allows it to interact with many Ca^{2+}-directed targets including phosphodiesterases, adenylyl cyclases, ion channels, protein kinases, and protein phosphatases. An important group of cellular targets positively regulated by Ca^{2+}-bound CaM (Ca^{2+}/CaM) is the family of Ca^{2+}/CaM-dependent protein kinases (CaMK).

CaMKs are Ser/Thr protein kinases, and can be grouped into two main classes based on whether they phosphorylate either a very restricted ('dedicated') or wide ('multifunctional') range of substrates. The dedicated CaMKs include phosphorylase kinase, myosin light chain kinase (MLCK), and eukaryotic elongation factor-2 kinase (also known as Ca^{2+}/CaM-dependent kinase III (CaMKIII)), whereas the multifunctional CaMKs include broad-substrate enzymes: CaMKI (subfamily including α, β, γ, and δ genes), CaMKII (subfamily including α, β, γ, and δ genes), CaMKIV, and CaMK-kinases (CaMKK) (subfamily including α and β genes; proteins also known as CaMKK1 and CaMKK2, respectively) (Means 2000). The multifunctional CaMKs share considerable sequence homology (20-40% global identity) as well as very similar domain structures, in that each kinase contains a conserved N-terminal kinase domain and a central regulatory domain comprised of overlapping autoinhibitory and Ca^{2+}/CaM-binding regions (Hook and Means 2001; Chow and Means 2007). In the absence of bound Ca^{2+}/CaM, each CaMK is inhibited by intramolecular interactions. However when Ca^{2+}/CaM binds, it releases the CaMK from intrasteric autoinhibition and activates the enzyme by inducing a conformational change, removing the autoinhibitory domain from the catalytic pocket to allow substrate access.

Despite their structural similarities and common regulation by Ca^{2+}/CaM, CaMKs are highly functionally distinct based on their varied tissue distributions, subcellular localizations, substrate specificites, and the different mechanisms by which they are

regulated (Hook and Means 2001). For instance, unlike CaMKI and CaMKIV, CaMKII is a 10-12-subunit multimeric enzyme that upon binding Ca^{2+}/CaM becomes fully activated from holoenzymatic, intersubunit autophosphorylation (Braun and Schulman 1995; Hudmon and Schulman 2002). In contrast, maximal kinase activation of CaMKI and CaMKIV requires phosphorylation on a specific threonine reside, present on the activation loop of each, by another upstream Ca^{2+}/CaM-dependent kinase, CaMKK. In such a way, the sequential involvement of two CaM kinases in the same signaling pathway constitutes what has become known as a "CaM-dependent kinase cascade" (Soderling 1999; Means 2000), based on its similarity to the mitogen-activated protein kinase (MAPK) cascade wherein three enzymes are sequentially activated and phosphorylate each other on their respective activation loops. Thus, the components of CaMK cascades include – both CaMKKs (CaMKK1 and CaMKK2) and their substrates, CaMKI, CaMKIV, and the more recently reported AMP-dependent kinase (AMPK; described below), but not CaMKII. As the goal of this dissertation research was to advance our understanding of the CaMK cascade's putative biological roles, the focus of the discussion herein will pertain to what is currently known about the CaMK cascade and its members.

1.1.1 CaMKKs

As previously mentioned, the upstream activating kinases of CaMKI and CaMKIV are two known CaMKK isoforms, CaMKK1 and CaMKK2, that are encoded by

distinct genes (*Camkk1* and *Camkk2*, respectively). The *Camkk2* gene produces several splicing isoforms depending on cell type, which increases the number of isoforms of CaMKKs (Hsu, Chen et al. 2001; Ishikawa, Tokumitsu et al. 2003). CaMKKI and CaMKK2 are approximately 50% identical at the amino acid level, and generally have similar broad tissue distribution with highest expression levels in the brain, followed by moderate levels in the testis, thymus, and spleen, and low levels in many other cells and tissues (Tokumitsu, Enslen et al. 1995; Edelman, Mitchelhill et al. 1996; Anderson, Means et al. 1998). However, CaMKK1 and CaMKK2 are differentially expressed in tissue subregions. For example, in the brain, CaMKK1 is most highly expressed in the hippocampus and outer cortex, while expressed at low levels in the cerebellum. In contrast, CaMKK2 is most highly expressed in the cerebellum and outer cortex, while expressed at a low extent in the hippocampus (Anderson, Means et al. 1998). Both isoforms are predominantly cytoplasmic, although CaMKK2 on occasion has been reported to be present in the nucleus (Sakagami, Umemiya et al. 2000; Nakamura, Okuno et al. 2001).

In addition to phosphorylating CaMKI and CaMKIV, CaMKK has been identified in as an upstream activator for both protein kinase B (PKB; or Akt), a protein involved in cell proliferation, apoptosis, angiogenesis, and glucose homeostasis, as well as AMP-dependent kinase (AMPK), a central regulator in sensing and maintaining intracellular energy balance (Yano, Tokumitsu et al. 1998; Hawley, Pan et al. 2005; Hong,

Momcilovic et al. 2005; Witters, Kemp et al. 2006). In particular, AMPK has been shown in multiple cell types to be a direct, physiological substrate of CaMKK2 (Means 2008). Given the roles of CaMKK in regulating these additional pathways, and that CaMKK appears to have a greater affinity for Ca^{2+}/CaM than either CaMKI or CaMKIV (Matsushita and Nairn 1998), this suggests the notion that CaMKK may be activated under circumstances where neither CaMKI nor CaMKIV can be activated but where CaMKK is important as an upstream kinase for PKB, AMPK, or other potential substrates.

1.1.2 AMPK

Under metabolically stressful conditions such as exercise or nutrient shortage, the cellular AMP:ATP ratio increases and leads to activation of an AMPK-kinase (AMPKK) that phosphorylates AMPK. AMPK is a heterotrimeric enzyme with a catalytic α-subunit, a β-subunit that acts as a scaffold, and a γ-subunit that binds AMP/ATP. When AMPK is activated, it in turn stimulates ATP-producing catabolic pathways, such as fatty acid β-oxidation, glycolysis, glucose transport, and food intake, while inhibiting ATP-consuming processes like protein synthesis, fatty-acid synthesis, and glucose-regulated transcription, altogether functioning to replenish ATP levels in the cell (Hardie, Scott et al. 2003).

Numerous studies have shown that LKB1 is the prototypic AMPKK when AMPK is responding to acute changes in energy balance that occur at the cellular level (Shaw,

Kosmatka et al. 2004; Sakamoto, McCarthy et al. 2005; Shaw, Lamia et al. 2005). This is the case in liver and skeletal muscle, where in response to increased AMP:ATP, LKB1 phosphorylates AMPK on Thr172. However, multiple studies have also demonstrated that CaMKK can act as an AMPK-kinase in cells, and that this function is likely unique to CaMKK2 (Hawley, Pan et al. 2005; Hurley, Anderson et al. 2005; Woods, Dickerson et al. 2005). When CaMKK2-mediated activation of AMPK occurs, this process is initiated by an increase in intracellular Ca^{2+} and is independent of changes in AMP:ATP ratio, in contrast to the LKB1/AMPK complex that is sensitive to AMP and not Ca^{2+}. Moreover, CaMKK2 regulation of AMPK seems to utilize Ca^{2+}/CaM as the allosteric activator of AMPK rather than AMP, given the lack of AMPK's γ-subunit in the CaMKK2/AMPK complex (Anderson, Ribar et al. 2008). Altogether, these observations reveal interestingly distinct mechanisms for AMPK phosphorylation by either LKB1 in an AMP-dependent manner, or Ca^{2+}/CaM-directed activation by CaMKK2.

For instance in MCF-7 human breast cancer cells, addition of vitamin D_3 increases intracellular Ca^{2+} and leads to macroautophagy in a CaMKK2/AMPK-mediated process (Hoyer-Hansen, Bastholm et al. 2007). Increased Ca^{2+} in the cell leads to increased Ca^{2+}/CaM complex formation, which activates CaMKK2. CaMKK2 then phosphorylates and activates AMPK, which in turn inhibits the activity of the mammalian target of rapamycin (mTOR), a negative regulator of macroautophagy, through tuberous sclerosis protein 2 (TSC2) and Rheb (Hoyer-Hansen, Bastholm et al. 2007). These studies also

suggested that the consequent accumulation of autophagosomes in the cell appeared to require CaMKK2/AMPK, as siRNA depletion of CaMKK2 or inhibition of CaMKK2 or AMPK using pharmacological inhibitors (STO-609 or Compound C, respectively) resulted in attenuated autophagy. In other findings of CaMKK2/AMPK effects in cells, it has also been demonstrated that this cascade participates in the regulation of energy balance by functioning downstream of ghrelin stimulation in hypothalamic neurons of the arcuate nucleus (Anderson, Ribar et al. 2008). Binding of ghrelin to its receptor on neuropeptide Y (NPY) neurons leads to increased intracellular Ca^{2+} levels through phospholipase C (PLC)/inositol triphosphate (IP_3) and subsequent activation of CaMKK2, which in turn phosphorylates AMPK and leads to transcription of genes encoding NPY and Agouti-related peptide (AgRP), two orexigenic peptide hormones that affect energy balance (Kahn, Alquier et al. 2005). Thus, these findings and others illustrate the important, diverse roles that CaMKK2-AMPK plays for various Ca^{2+}-dependent cellular functions.

1.1.3 CaMKI

Four CaMKI isoforms, encoded by distinct genes, have been identified so far in mammals, and are known as CaMKIα, CaMKIβ (PNCK), CaMKIγ (CLICK III), and CaMKIδ (CKLiK) (Picciotto, Czernik et al. 1993; Haribabu, Hook et al. 1995; Verploegen, Lammers et al. 2000; Takemoto-Kimura, Terai et al. 2003). Each CaMKI gene also produces one or more splice variants. CaMKIα, the first isoform identified and the most

well studied (usually referred to simply as CaMKI), is broadly expressed in all tissues and is subcellularly localized in the cytoplasm. CaMKI is completely dependent on Ca^{2+}/CaM for its activity, and binding of Ca^{2+}/CaM has two primary effects – it relieves the steric autoinhibition of CaMKI thereby generating an active enzyme, as well as exposes the activation loop threonine (Thr177) of CaMKI which can then be phosphorylated by CaMKK (Goldberg, Nairn et al. 1996; Matsushita and Nairn 1998).

The biological roles of CaMKI have not been thoroughly explored, but in recent years there have been roles uncovered for a CaMKK-CaMKI cascade, particularly in the brain. For example, CaMKK and CaMKI appear to be important for neuronal motility, as inhibition of either kinase, by means of pharmacological inhibition or dominant-negative mutant proteins, significantly decreased neuronal outgrowth and branching in hippocampal and cerebellar neurons (Wayman, Kaech et al. 2004). In these same studies, the effects of CaMKK inhibition on neuronal morphology were rescued by the overexpression of a full-length constitutively active CaMKI mutant, suggesting that CaMKK functions upstream of CaMKI in neuronal branching. Moreover, CaMKK2 and CaMKI have also been demonstrated to be involved in spine formation and synaptogenesis in hippocampal neurons (Saneyoshi, Wayman et al. 2008), such that downstream of the *N*-methyl-D-aspartic acid (NMDA) receptor, CaMKK2 and CaMKI form a multiprotein signaling complex with the guanine nucleotide exchange factor (GEF) βPIX and GIT1, a scaffold protein, that is localized in spines. CaMKI-mediated

phosphorylation of Ser516 in βPIX enhances its GEF activity, thereby promoting GTP-loading and activation of Rac1, a small GTPase and established enhancer of spinogenesis, leading to subsequent actin/cytoskeleton remodeling.

In addition to its roles in neuronal processes, CaMKI has been implicated as a regulator of cell-cycle progression. In WI-38 human fibroblasts, both the selective CaMK inhibitor, KN-93, and dominant-negative CaMKI mutant led to the inhibition of cyclin D/CDK4 activation and arrest of the cell cycle in G1 phase (Kahl and Means 2004). Moreover, a dominant-negative mutant of CaMKII did not elicit this inhibitory effect, and WI-38 cells do not express CaMKIV (which can also be inhibited by KN-93), suggesting that CaMKI plays a role in CDK4 activation during G1 to S phase transition.

The other mammalian CaMKI isoforms, CaMKIβ, CaMKIγ, and CaMKIδ, are less well characterized, though it is likely the biological roles they mediate are quite different from CaMKIα since each isoform displays different tissue expression patterns and subcellular localizations. Similar to CaMKIα, CaMKIβ is broadly distributed through all tissues, and localized in both the nucleus and cytoplasm (Naito, Watanabe et al. 1997; Ueda, Sakagami et al. 1999). On the other hand, CaMKIγ and CaMKIδ are more restricted in their tissue expression, such that CaMKIγ is most highly expressed in certain subregions of the brain and to a lesser degree in skeletal muscle, kidney, spleen, and liver (Takemoto-Kimura, Terai et al. 2003), while CaMKIδ has only been shown to well expressed in the brain and granulocytes (Sakagami, Umemiya et al. 2000;

Verploegen, Lammers et al. 2000). CaMKIγ has been observed localized in the cytoplasm, and is enriched at membrane compartments such as the Golgi and plasma membrane (Nishimura, Sakagami et al. 2003; Takemoto-Kimura, Terai et al. 2003). For instance in cortical neurons, membrane-bound CaMKIγ has been shown to be important for dendrite formation by promoting Rac activity and actin/cytoskeletal remodeling (Takemoto-Kimura, Ageta-Ishihara et al. 2007). In the case of CaMKIδ, it is largely cytoplasmic but has been observed, following CaMKK-dependent activation, to translocate to the nucleus during KCl-induced depolarization of hippocampal neurons (Sakagami, Kamata et al. 2005). Moreover, CaMKIδ has also been implicated to play a role in neutrophil maturation and function (Verploegen, Ulfman et al. 2005; Gaines, Lamoureux et al. 2008). Collectively, these results demonstrate the versatile roles CaMKK-CaMKI cascades play in different cellular processes.

1.1.4 CaMKIV

CaMKIV's most widely recognized function is its role as a transcriptional regulator. It is encoded by a single gene that contains alternate transcriptional initiation sites, giving rise to three isoforms of CaMKIV - CaMKIVα, CaMKIVβ, and calspermin, a testis-specific protein of unknown function (Sun, Means et al. 1995). It is unknown whether the regulation or function of CaMKIVα and CaMKIVβ differ significantly, although one study found that the two isoforms had different patterns of expression in the brain (Sakagami, Umemiya et al. 1999). CaMKIV, unlike CaMKI, is expressed only

in specific tissues. It is most highly expressed in distinct regions of the brain, but is also found in the thymus, testis, spleen, ovary, and certain bone marrow and immune cells (Means, Ribar et al. 1997; Wang, Ribar et al. 2001). CaMKIV displays predominantly nuclear sublocalization, though it has also been detected in the cytoplasm in some instances (Matthews, Guthrie et al. 1994; Wu, Gonzalez-Robayna et al. 2000).

As mentioned before, binding of Ca^{2+}/CaM to CaMKIV relieves the enzyme from autoinhibitory interactions, and this leads to basal CaMKIV activity. The conformational change that occurs from Ca^{2+}/CaM-binding also exposes the CaMKIV activation loop threonine (Thr196 in mouse; Thr200 in human) that allows for phosphorylation by CaMKK, resulting in maximal CaMKIV activation.

While both CaMKK1 and CaMKK2 are able to phosphorylate CaMKIV, it has been thought that CaMKK2 may be the physiologically relevant kinase for CaMKIV since CaMKK2 expression in subregions of the brain tracks most closely with CaMKIV, such that both are highly expressed in the cerebellar granule cell layer where little CaMKK1 is detected (Anderson, Means et al. 1998; Sakagami, Saito et al. 1998). Conversely though, CaMKK1 expression in brain regions more closely mirrors CaMKIα expression patterns notably where CaMKK2 is less abundant, such as in the hippocampus, brain stem, and olfactory bulb (Picciotto, Zoli et al. 1995; Sawamura, Sakagami et al. 1996; Anderson, Means et al. 1998). In addition, relatively more

CaMKK2 than CaMKK1 has been found expressed in CaMKIV-expressing tissues such as thymus, testis, and spleen (Anderson, Means et al. 1998).

Indeed, there have been cases suggesting the existence of a CaMKK2/CaMKIV cascade functioning in cells. For instance, mouse genetic studies have shown that this cascade plays an important role in hippocampal memory formation, such that CaMKIV is required for hippocampus-dependent spatial memory formation and contextual fear memory formation, while CaMKK2 is necessary for spatial memory but not contextual fear memory in male mice (Kang, Sun et al. 2001; Wei, Qiu et al. 2002; Peters, Mizuno et al. 2003). In another set of studies, it has also been shown that in cerebellar granule cell development, CaMKK2/CaMKIV-dependent phosphorylation of cAMP response element-binding protein (CREB) correlates with the transcription of brain-derived neurotrophic factor (BDNF), which is required for normal development of cerebellar granule cell neurons (Kokubo, Nishio et al. 2009). In mice null for either CaMKK2 or CaMKIV, developing cerebellar granule cells in the external granule cell layer are unable to cease proliferation and begin migration to the internal granule cell layer. These results show specifically that Ca^{2+}-dependent regulation of BDNF through CaMKK2/CaMKIV is required for cerebellar granule cell development. Hence, in light of these findings and possibilities for distinct CaMKK/CaMK pairings, further research is needed to elucidate what the cellular contexts and differences are between CaMKK1 vs. CaMKK2 regulation of their shared CaMK substrates *in vivo*.

Nonetheless, while it is known that CaMKK phosphorlyation of CaMKIV's activation loop Thr results in maximized CaMKIV activity, studies have shown that the phosphorylation event also generates Ca^{2+}/CaM-independence or "autonomy" (Chow, Anderson et al. 2005), and it is only the autonomously active form of CaMKIV that is translocated to the nucleus and participates in the regulation of transcription by activating several transcription factors such as CREB, AP-1, myocyte enhancer factor 2A (MEF2), and retinoid-related orphan receptor (ROR) (Kane and Means 2000; Passier, Zeng et al. 2000; Corcoran and Means 2001; Lonze and Ginty 2002; Chow, Anderson et al. 2005). Among identified CaMKIV functions, the role of CaMKIV in regulating CREB-dependent transcription has been the best characterized.

For instance in hippocampal neurons, CaMKIV as well as MAPK are activated by depolarizing stimuli and are the dominant pathways that act in tandem to contribute to the CREB phosphorylation required for subsequent CREB-dependent transcription and long-term potentiation (LTP) (Kasahara, Fukunaga et al. 2001; Wu, Deisseroth et al. 2001). Moreover, CaMKIV has also been implicated in CREB-dependent events essential for neuronal survival, dendritic growth, T-cell function, and long-term depression (LTD) in Purkinje cells (Ahn, Ginty et al. 1999; See, Boutillier et al. 2001; Yu, Shih et al. 2001; Tremper-Wells, Mathur et al. 2002). The importance of CaMKIV in CREB-dependent processes is supported by phenotypes of CaMKIV-deficient mice, wherein CaMKIV-null mice display decreased basal levels of phospho-CREB, and many of the physiological

defects displayed by these mice (such as impaired learning and memory and defective T-cell responses) appear related to a failure to adequately induce CREB phosphorylation in response to stimuli (Ho, Liauw et al. 2000; Ribar, Rodriguiz et al. 2000; Kang, Sun et al. 2001; Anderson and Means 2002).

Similarly, our group has also found that CaMKIV knock-out mice exhibit significant survival and proliferation defects in hematopoietic stem cells (HSCs; discussed in Chapter 1.2) that are associated with decreased levels of phospho-CREB as well as its co-activator CREB-binding protein (CBP) and B-cell lymphoma protein 2 (Bcl-2) (Kitsos, Sankar et al. 2005). These studies demonstrated that the loss of CaMKIV leads to a decrease in HSC number, and that HSCs deficient in CaMKIV are functionally impaired due to abnormal hyper-proliferation and exhaustion, resulting in their inability to regenerate blood cells. However, re-expression of CaMKIV in these cells was able to rescue the proliferation defects *in vitro* as well as restore the levels of CBP and Bcl-2. Thus, these studies revealed a critical role for CaMKIV as a regulator of HSC homeostasis. Based on these intriguing findings and the important cellular functions of CaMK cascades as previously described, we were excited about further investigating the potential involvement of a CaMKIV-mediated CaMK cascade in HSCs and hematopoiesis, a vital process in animals that will be discussed in the following section.

1.2 Hematopoietic Stem Cells and Hematopoiesis

Hematopoiesis is the physiological process that produces all mature blood cell types from HSCs, a rare population functionally defined by their lifelong ability to self-renew as well as differentiate to produce all blood cell lineages (multipotency). HSC homeostasis is tightly regulated by growth factors, signaling molecules, and transcription factors that must synergistically act to maintain and propagate the HSC pool in a dynamic balance of self-renewal and differentiation to produce enough mature effector cells. However when signaling pathways are dysregulated in HSCs, it can lead to the pathogenesis of several hematological disorders or cancer (Haurie, Dale et al. 1998; Reya, Morrison et al. 2001). These processes are not fully understood, but recent studies have underscored the importance of cellular functions such as cell cycle, apoptosis, and oxidative stress response in HSC homeostasis.

The findings that have been made in understanding the biology of HSCs are in large part due to their successful isolation and the delineation of the stages through which they progress during development. The staining of bone marrow with a combination of cell surface antigen-specific antibodies led to the prospective isolation of HSCs as c-Kit^{+}Sca-1^{+}Lin$^{-/lo}$ (KLS) cells (Spangrude, Heimfeld et al. 1988; Morrison and Weissman 1994; Morrison, Hemmati et al. 1995). KLS cells can be further subdivided into long-term (LT) and short-term (ST) repopulating HSCs and multi-potent progenitors (MPPs) by defining them with the addition of other markers (Osawa,

Hanada et al. 1996; Kiel, Yilmaz et al. 2005; Yang, Bryder et al. 2005). Similar approaches led to the identification of lineage-restricted progenitors with more restricted differentiation potential, and ultimately a paradigm for hematopoiesis was proposed by I. L. Weissman and collaborators, wherein the development and differentiation of all blood cells occurs in a hierarchical and lineage-based fashion characterized by a cascade of binary decisions (Figure 1; (Spangrude, Heimfeld et al. 1988; Morrison and Weissman 1994; Kondo, Weissman et al. 1997; Akashi, Traver et al. 2000; Weissman and Shizuru 2008)).

Studies in the bone marrow that led to this model first uncovered the identification of a common progenitor for all lymphoid lineages (CLP) (Kondo, Weissman et al. 1997), as well as a common myeloid progenitor (CMP) that generates granulocytic-macrophage (GM) and megakaryocytic-erythroid (MegE) lineages (Akashi, Traver et al. 2000). CLPs give rise to pro-B and pro-T cells, uncommitted lymphoid progenitors that will differentiate further into mature B and T cells, and also produce NK lineage cells (Kondo, Weissman et al. 1997). CMPs in turn generate two more restricted progenitors: granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs), generating GM and MegE cells, respectively (Akashi, Traver et al. 2000). The offspring of GMPs also includes granulocytes such as neutrophils, eosinophils, and basophils/mast cells, as well as monocytes, macrophages, and dendritic cell subsets (Denburg, Telizyn et al. 1985;

Iwasaki, Mizuno et al. 2005). The observation that CMPs and CLPs derived from adult bone marrow generate mutually exclusive progeny (Kondo, Weissman et al. 1997; Akashi, Traver et al. 2000) suggests that their diversification represents the earliest branching point during hematopoietic differentiation.

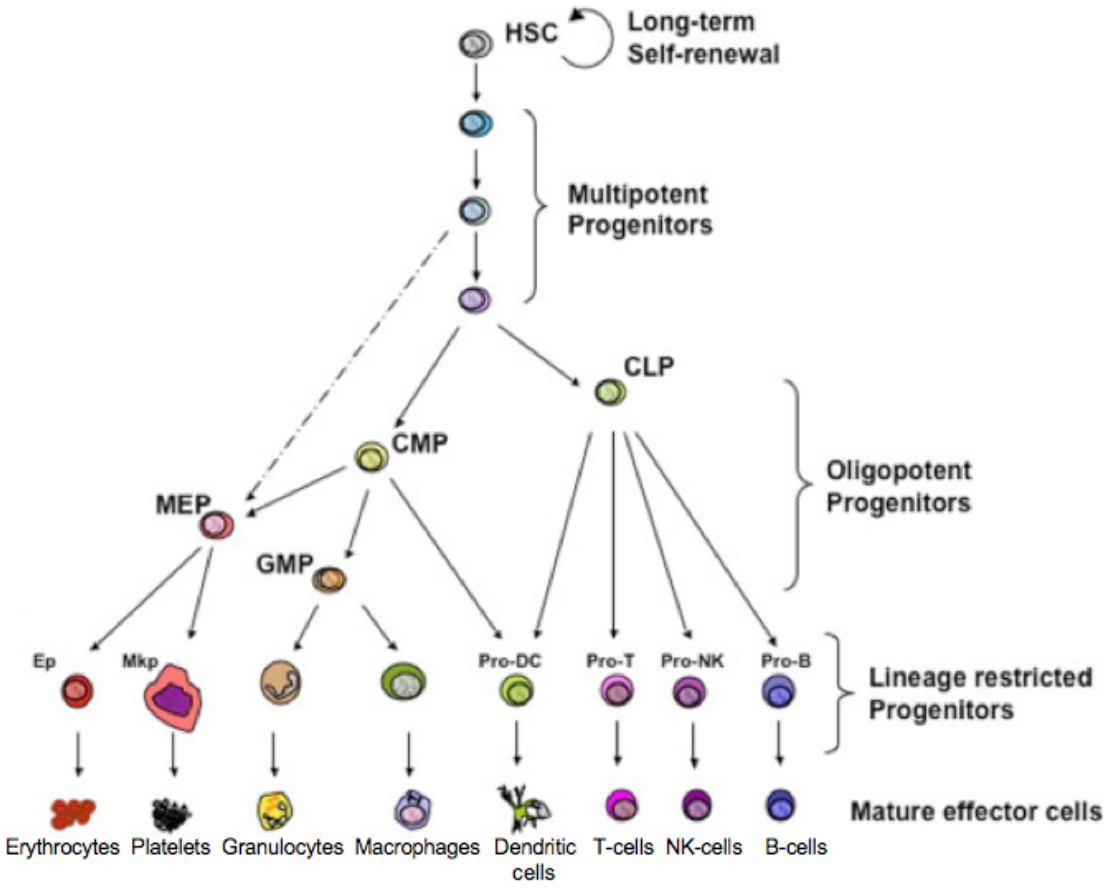

Figure 1: Model for hematopoietic development including intermediates in the hierarchy of hematopoietic differentiation

HSC represents long-term reconstituting, self-renewing; MPP, multipotent progenitors with limited self-renewal leading to transient but multilineage reconstitution; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; BLP, B lymphocyte

protenitor; ProT, T-cell progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocyte/erythroid progenitor; MkP, megakaryocyte progenitor; EP, erythroid progenitor.

While considerable progress has been made in delineating developmental pathways of several lymphoid and myeloid lineages, as well as identifying transcription factors and gene expression "programs" that establish and maintain their fate, the developmental branching points between these two large cell compartments remain controversial, and little is known about how their diversification is induced (Enver, Heyworth et al. 1998; Metcalf 1998; Miyamoto, Iwasaki et al. 2002; Laiosa, Stadtfeld et al. 2006). Lineage commitment could either be induced by extracellular factors, including cytokines, direct cell-cell interactions, or other environmental cues. Alternatively, it could be induced by intrinsic mechanisms, such as the stochastic upregulation of transcription factors or other regulatory molecules. This leads to another currently unresolved debate in the field, as to whether extrinsic and intrinsic factors play an instructive role and actively induce commitment and differentiation, or are merely permissive for the outgrowth of pre-committed progenitors by promoting cell survival and/or expansion (Enver, Heyworth et al. 1998). Nonetheless, whether lineage decisions are induced by extracellular cues, intrinsic events, or a combination of both, further studies are needed to uncover the pathways directly involved in regulating

hematopoietic development - a process that beings with HSC homeostasis and includes lineage commitment and differentiation.

1.3 CaMKIV and Implications for the CaMK Cascade in Hematopoietic Cells

As discussed previously in Chapter 1.1.4, our studies have identified a novel role for CaMKIV as an important regulator of HSC homeostasis, as its intrinsic absence results in marked repopulating failure of CaMKIV null HSCs after transplantation. CaMKIV deficiency in HSCs led to abnormal hyperproliferation and exhaustion effects that were rescued when CaMKIV was re-introduced into CaMKIV null HSCs. Associated with these defects, levels of phosphorylated CREB and Bcl-2 levels were decreased in CaMKIV null HSCs. These findings point to the existence of a Ca^{2+}-dependent signaling cascade in HSCs that minimally includes CaMKIV-CREB-Bcl-2 and is required for normal HSC homeostasis.

Interestingly, a separate set of studies has also identified the importance of this pathway in another hematopoietic cell type, in this case dendritic cells (Illario, Giardino-Torchia et al. 2008). These studies showed that CaMKIV null mice have a decreased number of dendritic cells (DCs) in lymphoid tissues, similar to the observed decrease in HSCs, and these mice fail to accumulate mature DCs in spleen on *in vivo* exposure to lipopolysaccharide (LPS), an agonist of Toll-like receptor 4 (TLR4) that regulates the lifespan of DCs. Isolated *Camk4*$^{-/-}$ DCs also failed to accumulate phospho-CREB, Bcl-2, and Bcl-xL, and displayed marked survival defects. Thus, these results collectively

suggest the important roles of Ca^{2+}-dependent pathways such as CaMKIV-mediated signaling in hematopoietic cells, as well as implicating the potential for other Ca^{2+}/CaM-dependent enzymes to be involved in regulating hematopoietic development.

Hence, the goal of this dissertation research was to better elucidate the pathway in which CaMKIV functions in HSCs, as well as determine the biological role of the CaMK cascade, mediated at least in part by CaMKIV, in hematopoietic stem and progenitor cells. To this end, we first set out to identify potential downstream targets of CaMKIV in HSCs by conducting a comparative microarray analysis of freshly isolated wild-type (WT) and *Camk4*$^{-/-}$ HSCs. This led to our preliminary findings of a gene, known as augmenter of liver regeneration/growth factor erv1-like (ALR/Gfer) and previously reported as a "stemness" gene due to its enriched expression in neuronal, embryonic, and hematopoietic stem cells (Ramalho-Santos, Yoon et al. 2002), that seemed absent in *Camk4*$^{-/-}$ HSCs. Our studies later showed that re-expression of CaMKIV in *Camk4*$^{-/-}$ HSCs was unable to rescue levels of Gfer, suggesting that the two proteins are not present in the same pathway. However, our studies also revealed a novel and critical role for Gfer, similar to CaMKIV, in the regulation of HSC proliferation, though through a Jab1/p27-dependent process as discussed in Chapter 3. Afterwards in Chapter 4, our studies of an upstream kinase to CaMKIV, CaMKK2, in hematopoietic development will be discussed. Finally, in Chapter 5 the significance of

these findings, as well as where these results should take us in the future will be described.

2. Materials and Methods

2.1 Mice

C57BL/6 (CD45.2) and B6.SJL-Ptprc^{a} Pep3^{b}/BoyJ (CD45.1) strains of mice were purchased from Jackson Laboratories or Taconic Farms. Wild-type (WT) and *Camkk2*$^{-/-}$ mice were generated as previously described (Anderson, Ribar et al. 2008). The animals were housed under a 12-h light, 12-h dark cycle and provided food and water *ad libitum*. All animal care and experimental procedures were performed in compliance with National Institutes of Health and Duke University Institutional Animal Care and Use Committee guidelines. Unless otherwise noted, experiments were performed using 8- to 12-week-old, gender-matched mice.

2.2 Flow Cytometry and Primary Cell Isolation

Murine BM hematopoietic cell subsets were analyzed and/or isolated by fluorescence-activated cell sorting (FACS) based on the expression pattern of cell-surface markers as previously described (Osawa, Hanada et al. 1996; Kondo, Weissman et al. 1997; Akashi, Traver et al. 2000; Christensen and Weissman 2001). Nucleated BM cell suspensions were prepared from hindlimb bones in Hank's Buffered Salt Solution (HBSS) (Mediatech) plus 5% fetal bovine serum (FBS) (Gemini Bio-products).

Prior to staining and sorting stem-progenitor cells by FACS, c-Kit^{+} (CD117) cells were enriched from BM cell suspensions using an autoMACS cell sorter to separate cells

bound to anti-CD117 conjugated microbeads (both from Miltenyi Biotec). c-Kit$^+$ cells were then stained with fluorescent antibodies, which herein came from eBioscience unless otherwise noted. For KLS cell isolation and/or analysis by FACS, c-Kit$^+$ cells were stained with allophycocyanin (APC)-conjugated c-Kit (2B8), phycoerythrin (PE)-Cy5-conjugated Sca-1 (D7), and Lineage (Lin) cocktail in PE [Lin: CD3 (145-2C11), CD4 (GK1.5), CD5 (53-7.3), CD8 (53-6.7), B220 (RA3-6B2), Mac1 (M1/70), Gr1 (RB6-8C5), and Ter119 (TER-119)]. HSCs and MPPs were stained with c-Kit-APC, Sca-1-Pacific Blue (BioLegend), CD48-FITC (HM48-1), and CD150-PE-Cy7 (TC15-12F12.2). CLPs were stained with c-Kit-FITC, Sca-1-PE-Cy5, B220-PE-Cy7, and IL-7Rα-biotin (A7R34), followed by streptavidin APC-Cy7. Myeloid progenitors were stained with c-Kit-APC, Sca-1-PE-Cy5, CD16/32-eFluor450 (93), and CD34-FITC (RAM34). Primary granulocytes and differentiating 32Dcl3 cells were stained with Gr1-FITC and Mac1-PE or -Pacific Blue. Stem and progenitor cell populations were immunophenotypically defined as follows - HSC: CD150$^+$c-kit$^+$Sca-1$^+$CD48$^-$Lin$^-$; MPP: CD150$^-$c-kit$^+$Sca-1$^+$CD48$^-$Lin$^-$; KLS: c-Kit$^+$Sca-1$^+$Lin$^{-/lo}$; CLP: Lin$^-$IL-7R$^+$B220$^-$Sca-1^{lo}c-Kit^{lo}; CMP: Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$CD16/32^{lo}; GMP: Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$CD16/32$^+$; MEP: Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^-$CD16/32$^-$. Analyses were performed using a FACScan or FACSCanto II cytometer from BD, and cell sorting performed with a FACSVantage cell sorter from BD. Data analyses were performed using CellQuest Software (BD), and FlowJo Software (Tree Star).

For isolation of granulocytes used for immunoblotting experiments, BM cells were collected from WT and *Camkk2*$^{-/-}$ mice as described above. Granulocytes were isolated by performing two sequential density-gradient centrifugations. Firstly, whole BM cells were stratified on Lympholyte (Cedarlane). The high density fraction containing granulocytes and erythrocytes were recovered and further fractionated by using a 50% Percoll (GE Healthcare) gradient. Granulocytes were obtained from the less dense fraction, and purity was confirmed shortly thereafter by FACS analysis.

2.3 Cells, Cell Lines, and Culture Conditions

HSCs, KLS, myeloid progenitor subsets (CMP, GMP), and granulocytes were isolated as previously mentioned, and were maintained accordingly as described in "Section 2.5 Cellular-Based Assays". The EML cell line used is a stem cell factor (SCF)-dependent lympho-hematopoietic progenitor cell line, and contains progenitors capable of differentiation along B-lymphocyte, erythrocyte, neutrophil, macrophage, mast cell, and megakaryocyte lineages, thus resembling the multi-potentiality of HSCs. They are the only known SCF-dependent cell line with both lympho and myelo-erythroid potential. EML cells were maintained in Iscove's modified Dulbecco's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate containing 200 ng/ml mouse stem cell factor and 20% heat-inactivated fetal bovine serum. The murine myeloid cell line 32D Clone 3 (32Dcl3; herein referred to as 32D) is IL-3 dependent, and is one of the few cell lines that can terminally differentiate into neutrophils. 32D cells

were propagated in RPMI-1640 containing 10% FBS, 1% penicillin-streptomycin, and 5 ng/mL rmIL-3. Both 32D and EML cell lines were obtained from American Type Culture Collection (ATCC).

2.4 Virus Production and Infection of Cells

For knock-down studies of Gfer in KLS cells, FG12-GFP^{+}-Gfer1/2 shRNA and -LacZ shRNA lentiviruses were produced as previously described using 293T cells (Qin, An et al. 2003; Todd, Damin et al. 2010a). For over-expression studies of Gfer in KLS cells, murine *Gfer* and p27^{kip1} cDNA constructs were obtained from Open Biosystems, PCR-amplified, and cloned into MSCV-internal ribsome entry site (IRES)-GFP or MSCV-IRES-YFP vectors. Retrovirus production was performed as previously described (Kitsos, Sankar et al. 2005). Approximately 30,000 freshly isolated KLS cells were sorted out into a 96-well U-bottom plate (BD Biosciences) and cultured at 37°C in the presence of X-Vivo-15 (Cambrex) media supplemented with 2% FBS, 30 ng/ml stem cell factor (SCF), 30 ng/ml Flt-3 ligand, and 50 μM 2-mercaptoethanol. They were then infected with the appropriate lenti- or retro-viruses at an MOI of 5:1 along with 4 μg/ml polybrene (Sigma-Aldrich). Viable (PI^{neg}) GFP and/or YFP positive cells that were also positive for c-Kit and Sca-1, and negative for Lineage markers (GFP/YFP^{+}KLS) were re-sorted for appropriate experiments after 72 h of infection. Uninfected control cells were also cultured for 72 h and sorted for positive expression of c-Kit and Sca-1 and negative for Lineage markers.

For studies of CaMKK2 in 32D myeloblasts, cells were lentivirally-transduced with either CaMKK2-shRNA or the corresponding empty vector, pLKO.1, from Open Biosystems, then selected with 2 μg/mL puromycin (Sigma-Aldrich) for 5-7 days. For over-expression experiments, cells were transduced with lentiviruses containing either CaMKK2 (WT) or CaMKK2^{D311A} (inactive) that were constructed as previously described (Kokubo, Nishio et al. 2009), and after 3 days were selected by GFP marker expression using FACS.

2.5 Cellular-Based Assays

In vivo Bone Marrow Transplantation Assays

CD45.1^{+} recipient mice were lethally irradiated with two doses of 5 Gy whole-body irradiation (10 Gy total) from a ^{137}Cs source administered approximately 24 h apart, and 8-12 h after the second dose, the recipients were transplanted with freshly isolated donor murine BM cells. For transplantation experiments of Gfer-knockdown (KD)-HSCs, we used 6-8 recipient mice per sample genotype. Uninfected or lentivirus-infected FG12-GFP^{+}-Gfer or -LacZ shRNA KLS cells were re-sorted by FACS, as mentioned before, after 72 h in culture. Approximately 10,000 donor cells (CD45.2^{+}), along with 300,000 rescuing CD45.1^{+}Sca1-depleted whole BM cells obtained from non-irradiated recipient mice (Duncan, Rattis et al. 2005), were retro-orbitally injected into groups of 8 recipient mice. We used a high number of donor cells (10,000) to initiate the transplant in order to ensure successful engraftment by GFP-lentivirus-infected KLS

cells. Transplant recipients were monitored daily and were maintained on antibiotic water. All recipients were bled at 3, 9, 12, 15 and 19 weeks post-transplant to measure the percentage of donor/host allelic chimerism (based on expression of CD45.2/CD45.1, respectively) and blood reconstitution by FACS. At 15 weeks post-transplant, whole BM cell suspensions were prepared and pooled from three transplant recipients per cohort, and Lineage$^-$CD45.2$^+$ cells were isolated from the BM by FACS to analyze Gfer mRNA levels.

For transplantation experiments of *Camkk2*$^{-/-}$ HSCs, CD34$^-$KLS donor cells were isolated from three WT or *Camkk2*$^{-/-}$ mice (CD45.2$^+$), then pooled, respectively. Subsequently, 1,000 CD45.2$^+$ donor cells along with 300,000 CD45.1$^+$ Sca-1-depleted BM cells obtained from non-irradiated recipient mice were injected retro-orbitally into 5-10 irradiated recipients per cohort. To measure blood cell repopulation based on donor engraftment and lineage reconstitution, peripheral blood was obtained from recipient tail veins every 3 weeks for at least 15 weeks, and at each time-point nucleated blood cells were stained and analyzed by FACS. After 15 weeks, recipient BM cells were harvested and stained for FACS analysis of CD45.2$^+$ lineage populations.

Proliferation Assays

To measure the proliferation of KLS cells *in vitro*, either GFP$^+$-shRNA lentivirus-infected, GFP$^+$/YFP$^+$ MSCV retrovirus-infected, and/or GFP$^-$ uninfected KLS cells were FACS sorted at 10 cells per well into Terasaki plates (Nalgene Nunc). The cells were

grown in serum free media (SFEM; Stem Cell Technologies) supplemented with 90 ng/ml SCF, 30 ng/ml Flt-3 ligand (both from R&D Systems), 50 U/ml penicillin/streptomycin (Invitrogen), and 50 μM 2-mercaptoethanol (Millipore) for 8 days. The proliferation rate of the plated cells was estimated by counting the number of cells in each well at the 2, 4, 6, and 8 days of culture. On the 6th day of proliferation, cells from all wells of an extra Terasaki plate were pooled, stained with antibodies for KLS as described above, fixed in 1% paraformaldehyde (PFA; Polysciences), and analyzed for percentage of KLS vs. differentiated (Lineage^{+}) cells by flow cytometry.

For experiments measuring proliferation of KLS cells *in vivo*, mice were administered a single intraperitoneal injection of bromodeoxyuridine (BrdU; Sigma-Aldrich) at 1 mg per 6 g of mouse weight (Jankovic, Ciarrocchi et al. 2007). Three hours later, the mice were sacrificed and KLS cells were isolated and fixed in 70% ethanol at -20°C. Analysis of *in vivo* BrdU incorporation was performed using a FITC BrdU Flow Kit (BD) according to manufacturer's instructions, followed by FACS.

Apoptosis Assays

To determine the viability of KLS cells in culture, cells from all wells of an extra Terasaki plate were pooled on days 0, 6, and 8. Staining for Annexin V/7-AAD on KLS cells was performed using the Annexin V-APC Apoptosis Detection Kit I (BD Biosciences) following the manufacturer's suggested protocol. Harvested cells were washed twice in cold PBS and suspended in 1X Annexin V Binding Buffer (provided in

the kit) at a concentration of 1 x 10^6 cells/ml. Samples were then analyzed within one hour by flow cytometry. KLS cells that were freshly isolated from mice were immediately stained with anti-Annexin V-FITC (BD) and propidium iodide (Sigma-Aldrich) for 15 min. according to the manufacturer's instructions (BD). Stained cells were analyzed by flow cytometry within 30 min.

Reactive Oxygen Species (ROS) Detection Assay

On days 0, 6 and 8 of *in vitro* culture, KLS cells originally plated at a density of 5,000 cells per well in a 96-well plate were harvested and loaded with 5 μM dihydrorhodamine 6G (Molecular Probes, Invitrogen) in HBSS for 45 min. under normal growth conditions. Cells were then washed, re-suspended in fresh HBSS, and analyzed by flow cytometry. Intracellular oxidation of this non-fluorescent dye yields the fluorescent, oxidized rhodamine 6G (R6G) which localizes to the mitochondria.

Cytological Analyses

To examine the morphology of developing granulocytes, nucleated BM cells were obtained from individual WT and *Camkk2*$^{-/-}$ mice and early-stage granulocytes were sorted based on B220$^-$CD11c$^-$Gr1$^+$Mac1$^{-/lo}$ cell surface expression. Isolated granulocytes were cytospun, air-dried, fixed in methanol, and subjected to Giemsa staining (Sigma-Aldrich) for analysis of nuclear morphology.

Myeloid Progenitor Differentiation and Colony-Forming Assays

For experiments to determine the differentiation potential of common myeloid progenitors (CMPs), FACS sorted WT and *Camkk2*$^{-/-}$ CMPs were cultured as previously described (Zhou, Li et al. 2008) on murine OP9 BM stromal cells plated the day before at a density of 20,000 cells/well in a 24-well plate. OP9 cells were propagated in Alpha-Minimum Essential Medium (α-MEM) (Invitrogen) containing 20% FBS, and 1% penicillin-streptomycin (Mediatech). Five hundred CMPs were co-cultured with OP9 cells for 8-12 days in RPMI-1640 medium (Invitrogen) containing 10% FBS, 1% penicillin-streptomycin, and cytokines that support cell growth and myeloid differentiation including recombinant (rm) SCF (50 ng/mL), rmFlt3 ligand (30 ng/mL), rmIL-3 (10 ng/mL), rmIL-7 (10 ng/mL), and rmGM-CSF (10 ng/mL) (R&D Systems). All cells were harvested from each well, enumerated using a Z1 Coulter Counter (Beckman Coulter), stained with Gr1-FITC, Mac1-Pacific Blue, and CD45-PE (to exclude OP9 CD45^{-} cells), then analyzed by FACS.

To examine the proliferation and differentiation potential of granulocyte-monocyte progenitors (GMPs), we tested their *in vitro* colony-forming ability. 1,500 sorted GMP cells were plated in triplicate into gridded 35 mm cell culture dishes (Nalgene Nunc) and grown in methylcellulose media (Complete Methocult® Media M3434; StemCell Technologies) containing SCF, IL-3, IL-6, and erythropoietin (Epo). Seven to 10 days later, colonies were classified and enumerated based on morphology.

To assess granulocytic differentiation potential, 32Dcl3 cells growing in normal culture media were washed with HBSS to remove IL-3, and 200,000 cells were plated in RPMI-1640 containing 10% FBS, 1% penicillin-streptomycin, and 40 ng/mL rmG-CSF. After 6 days in culture, cells were harvested, cytospun, then Wright's-stained (EMD Chemicals) for analysis of nuclear morphology to determine the extent of granulocytic differentiation.

2.6 Microscopy Applications

Electron Microscopy

Approximately 20,000 freshly re-sorted KLS cells were washed with phosphate buffered saline (PBS) and pelleted. This cell pellet was then fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate overnight at 4°C and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 h (Sigma-Aldrich). Cells were briefly rinsed with 0.1 M sodium cacodylate and then dehydrated using a series of graded alcohols, embedded in LX-112 epoxy resin (Ladd Research Industries), and approximately 8 mμ sections were cut on an LKB microtome. Sections were then stained using uradyl acetate and lead citrate before viewing on a Phillips CM12 electron microscope equipped with a digital camera.

Immunocytochemistry

Immunocytochemistry of cytospun and PFA-fixed GFP^+ or YFP+ virus infected KLS cells was done as previously described (Kitsos, Sankar et al. 2005) using anti-$p27^{kip1}$,

anti-$p21^{cip1}$, anti-Jab1 (all diluted 1:100; Rabbit polyclonal; Santa Cruz Biotechnology) and anti-Gfer (diluted 1:100; Rabbit polyclonal; PTG Labs) primary antibodies. Cy3-conjugated secondary antibody (Jackson Immunoresearch) was used and samples were mounted using Vectashield Mounting Media with DAPI (Vector Laboratories). Cells were examined using a fluorescence Axio Observer Z1 microscope with ApoTome assembly (Carl Zeiss), and digital optical slice images were captured at 630X magnification. The signal intensities were calculated using the AxioVision 2.0 software (Carl Zeiss) and analyzed using Microsoft Excel spreadsheet. For relevant experiments, KLS cells were treated for 1 h with 10 μM MG132 (Calbiochem, EMD Chemicals), cytospun immediately thereafter, and fixed in 1% PFA for immunocytochemistry.

2.7 Biochemical and Molecular Assays

Immunoprecipitation and Immunoblot Assays

For experiments testing Gfer, Jab1, and p27 interactions, approximately 2 million EML cells were infected with MSCV-Control, MSCV-Gfer, FG12-LacZ and/or FG12-Gfer viruses and sorted for GFP expression 72 h later. Equal numbers of cells were lysed with Tween 20 lysis buffer, sonicated, and pre-cleared with protein A sepharose (GE Healthcare). About 500 μg of whole cell lysates were incubated with anti-$p27^{kip1}$ or anti-Jab1 antibodies (both mouse monoclonal, Santa Cruz) for 2 h at 4°C and the IP complexes were captured using protein A Sepharose beads. The IP complexes were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

and transferred to Immobilon polyvinylidene fluoride membrane (Millipore). Membranes were incubated in Blocking solution containing 5% milk in tris-buffered saline plus 0.2% Tween 20 (TBS-T) (Sigma-Aldrich) for 1 h at room temperature, and subsequently probed with rabbit polyclonal antibodies, diluted at 1:500 in Blocking solution, against $p27^{kip1}$, Jab1, Ubiquitin (all Santa Cruz), or Gfer (PTG Labs) overnight at 4°C. Next, the membranes were washed 4x with TBS-T for 5 min. per wash. Primary antibodies were detected with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted in Blocking solution using enhanced chemiluminescence (ECL) (Amersham Biosciences). Signal intensities of bands were quantified using the ImageQuant Capture Software (GE Healthcare) and analyzed using Microsoft Excel spread sheets.

For immunoblotting experiments from 32D cells, cells were lysed in 0.5% NP-40 lysis buffer, homogenized with syringe and needle, and whole cell extracts prepared following centrifugation. Equal amounts of protein lysates per lane (unless otherwise noted) were denatured and resolved by SDS-PAGE. Proteins were transferred onto Immobilon-FL membranes (Millipore), and membranes were incubated in Licor-Blocking solution containing 0.1% casein (MP Biomedicals) and 0.2% fish gelatin (Sigma-Aldrich) dissolved in 0.2X phosphate-buffered saline (PBS) for 1 h at room temperature. All primary antibodies (see below) were diluted at 1:1000 except anti-β-actin, which was diluted at 1:50000, in Licor-Blocking solution plus 0.2% Tween 20, and added to

membranes for overnight incubation at 4°C. Next, the membranes were washed 4x with TBS-T for 5 min. per wash. Primary antibodies were detected with the appropriate infrared dye-conjugated secondary antibody diluted in primary antibody buffer containing 0.02% SDS. Immunoblots were washed, scanned, and quantified using the Odyssey Infrared Western Blotting detection system (LI-COR Biosciences). All primary antibodies used were monoclonal, and are listed as follows: anti-CaMKK (BD), anti-β-actin (Sigma-Aldrich), anti-phospho-AMPKα (Thr172) (Cell Signaling, Danvers, MA, USA), anti-AMPKα (Cell Signaling), anti-CaMKIV (BD), and anti-eukaryotic translation initiation factor 4E (eIF-4E) (BD). Polyclonal anti-CaMKI was generated by Christina Kahl, laboratory of A.R. Means. Secondary antibodies used were anti-mouse IgG Alexa Fluor 680 (Invitrogen) and anti-rabbit IgG IRDye800 conjugated (Rockland Immunochemicals).

Real-time Quantitative RT-PCR Assays

Total RNA was isolated from HSCs and myeloid progenitor using the RNAqueous-Micro kit (Ambion), and from 32D cells using the RNeasy Mini kit (Qiagen). The first strand cDNA was prepared using SuperScript III Reverse Transcriptase (Invitrogen) or High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time PCR-based gene expression analysis was performed using IQ SYBR Green Supermix (BioRad

Laboratories) with the respective primers, and the reactions were performed using an IQ5 I-Cycler System (BioRad Laboratories).

2.8 Statistical Analyses

Student's *t* test was used for all statistical analyses, and level of significance was set at $P < 0.05$. Bar graphs represent mean ± SEM, with the number of experiments indicated in figure legends.

Table 1: Primers

Name	Sequence (5′→3′)	Description
Gfer-1 (top strand)	ACCGGCCAGAACAACAACAGGATATTTCAAGAG AATATCCTGTTGTTGTTCTGGCTTTTTC	shRNA construction
Gfer-1 (bottom strand)	TCGAGAAAAAGCCAGAACAACAACAGGATATTC TCTTGAAATATCCTGTTGTTGTTCTGGC	shRNA construction
Gfer-2 (top strand)	ACCGCCAGGTGCCTCGTACCCTTCATTCAAGAGA TGAAGGGTACGAGGCACCTGGTTTTTC	shRNA construction
Gfer-2 (bottom strand)	TCGAGAAAAACCAGGTGCCTCGTACCCTTCATCT CTTGAATGAAGGGTACGAGGCACCTGG	shRNA construction
LacZ (top strand)	ACCGGTGACCAGCGAATACCTGTTTCAAGAGAA CAGGTATTCGCTGGTCACTTTTTC	shRNA construction
LacZ (bottom strand)	TCGAGAAAAAGTGACCAGCGAATACCTGTTCTCT TGAAACAGGTATTCGCTGGTCAC	shRNA construction
Gfer-Fwd	TCTAGCCTGGTTCTATGGGCAACA	Real-time PCR
Gfer-Rev	TCAGATGACAGCGCCTCTGAAACT	Real-time PCR
β-actin-Fwd	GGGAAATCGTGCGTGACATC	Real-time PCR
β-actin-Rev	CCAAGAAGGAAGGCTGGAAAAG	Real-time PCR
Jab1-Fwd	GAATACCCTGAGTTCCTCTAGC	Real-time PCR
Jab1-Rev	CTAAGCCCAACATGAAACTGC	Real-time PCR

Table 1: Primers, cont'd

Name	Sequence (5′→3′)	Description
$p27^{kip1}$-Fwd	TGGACCAAATGCCTGACTC	Real-time PCR
$p27^{kip1}$-Rev	GGGAACCGTCTGAAACATTTTC	Real-time PCR
β2MG-Fwd	ACCGGCCTGTATGCTATCCAGAAA	Real-time PCR
β2MG-Rev	GGTGAATTCAGTGTGAGCCAGGAT	Real-time PCR
Camkk2-Fwd	CATGAATGGACGCTGC	Real-time PCR
Camkk2-Rev	TGACAACGCCATAGGAGCC	Real-time PCR
C/EBPα-Fwd	TATAGACATCAGCGCCTACATCGA	Real-time PCR
C/EBPα-Rev	GTCGGCTGTGCTGGAAGAG	Real-time PCR
PU.1-Fwd	GCATCTGGTGGGTGGACAA	Real-time PCR
PU.1-Rev	TCTTGCCGTAGTTGCGCAG	Real-time PCR
Pax5-Fwd	CCGCCAAAGGATAGTGGAACTTG	Real-time PCR
Pax5-Rev	CACAGTGTCATTGTCACAGACTCGC	Real-time PCR

Table 1: Primers, cont'd

Name	Sequence (5′→3′)	Description
E2A-Fwd	CACAGAGACCTCCCGACTCC	Real-time PCR
E2A-Rev	CGGCTACTGATGCGATTTCC	Real-time PCR

3. Gfer Inhibits Jab1-Mediated Degradation of $p27^{kip1}$ to Restrict Proliferation of Hematopoietic Stem Cells[1]

3.1 Preface

Gfer, the mammalian homologue of the yeast Erv1 protein, is a highly conserved flavin adenine dinucleotide (FAD)-dependent sulfhydryl oxidase (Lisowsky, Lee et al. 2001). In yeast, *erv1* has roles in cytoplasmic Fe-S cluster assembly, mitochondrial biogenesis and, along with its partner Mia-40, forms a critical disulfide redox system instrumental in the import of small proteins such as cytochrome C into the mitochondrial intermembrane space (IMS) (Becher, Kricke et al. 1999; Lange, Lisowsky et al. 2001; Lisowsky, Lee et al. 2001; Mesecke, Terziyska et al. 2005). In higher eukaryotes, Gfer and its homologues play roles in tissue regeneration (Klissenbauer, Winters et al. 2002; Klebes, Sustar et al. 2005; Gatzidou, Kouraklis et al. 2006).

Gfer maps within the stem cell-rich t-haplotype region of mouse chromosome 17 and is highly enriched in embryonic and adult stem cells (Polimeno, Lisowsky et al. 1999; Ivanova, Dimos et al. 2002; Ramalho-Santos, Yoon et al. 2002). In pluripotent mouse embryonic stem cells (ESCs), Gfer plays a specific, pro-survival role by regulating mitochondrial fission-fusion balance through its modulation of the key fission GTPase,

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[1] Teng, E. C., L. R. Todd, et al. (2011). "Gfer inhibits Jab1-mediated degradation of p27kip1 to restrict proliferation of hematopoietic stem cells." Mol Biol Cell **22**(8): 1312-1320. The results presented in this chapter were previously published in the above manuscript. Permission to reproduce the material here was kindly granted by The American Society of Cell Biology.

Drp1 (Todd, Damin et al. 2010a; Todd, Gomathinayagam et al. 2010b). In multipotent HSCs, quantitative trait loci (QTL) mapping studies aimed at uncovering regulatory pathways that affect cell function predicted Gfer to be important in proliferation and cell turnover (Bystrykh, Weersing et al. 2005). However, its involvement in HSC proliferation and/or function has yet to be verified.

To evaluate the precise role of Gfer in murine HSCs, we utilized gene knockdown and over-expression approaches. We report herein that short hairpin (sh) RNA-mediated knockdown (KD) of Gfer in $cKit^{+}Lineage^{-}Sca1^{+}Flk2^{-}CD34^{-}$ (KLS-$Flk2^{-}$-$CD34^{-}$) cells (a population enriched for long-term HSCs) compromises their ability to successfully engraft the bone marrow of lethally irradiated recipient mice. While Gfer KD in KLS-$Flk2^{-}$-$CD34^{-}$ cells did not affect cell survival, it did result in an unexpected, enhanced proliferation response *in vitro* that is followed by exhaustion. Whereas KD of Gfer in KLS-$Flk2^{-}$-$CD34^{-}$ cells significantly reduced $p27^{kip1}$, over-expression of Gfer enhanced the total and nuclear levels of this cell cycle inhibitor. Further investigations in hematopoietic progenitor EML cells revealed that Gfer interferes with the binding of $p27^{kip1}$ with its inhibitor Jab1, thus enhancing nuclear retention and/or stability of this CDKI. Furthermore, restoration of normal $p27^{kip1}$ levels in Gfer-KD KLS-$Flk2^{-}$-$CD34^{-}$ cells resulted in restoration of normal proliferation *in vitro*. Thus, our cumulative results support a role for Gfer in the restriction of unwarranted HSC proliferation through its inhibition of Jab1-mediated turnover of $p27^{kip1}$.

3.2 Experimental Results

Knockdown of Gfer expression in KLS cells significantly impairs in vivo function

To evaluate the biological function of Gfer in HSCs, we knocked down Gfer using a previously reported FG12-Lentivirus-green fluorescent protein (GFP)-shRNA system (Qin, An et al. 2003; Todd, Damin et al. 2010a). KLS cells freshly isolated from wild-type (WT) mice were infected with lenti-shRNA-GFP viruses containing Gfer1 (Gfer1-KD; targeting exon 2); Gfer2 (Gfer2-KD; targeting the 3' untranslated region) and/or non-specific LacZ (LacZ-KD) shRNAs. Uninfected WT KLS cells (WT-U) cultured similarly to the virus-infected cells served as controls in these experiments. Approximately 72 h later, viable GFP^- (WT-U) or GFP^+ virus-infected (LacZ-KD and Gfer-KD) KLS cells were re-isolated and evaluated for Gfer expression. We achieved approximately 72% and 57% KD of Gfer mRNA and protein using Gfer1 and 2 shRNAs, respectively (Figure 2A, B).

To investigate whether down-regulation of Gfer in KLS cells affected their *in vivo* function, we performed competitive bone marrow transplantation (BMT) by transplanting approximately 10,000 CD45.2 WT-LacZ-KD, WT-Gfer-KD, and WT-U KLS cells along with 300,000 competing CD45.1 total bone marrow cells into cohorts of 8 lethally irradiated CD45.1 recipient mice. We analyzed bone marrow cells from

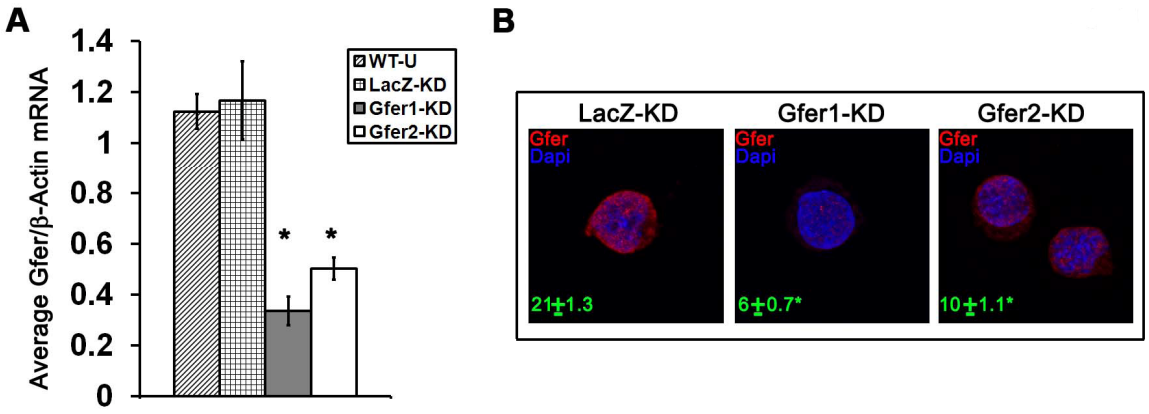

Figure 2: Depletion of Gfer by RNAi in KLS cells

(A) Average Gfer mRNA, normalized to those of β-actin, from uninfected (U) WT (WT-U), LacZ-KD and Gfer-1/2KD KLS cells, measured by qRT-PCR at 72 h post viral infection. Error bars represent standard deviation (SD) of the average values (n=4); *p<0.05. (B) Digital optically sectioned immunocytochemistry images (630X) depicting Gfer protein levels in red and Dapi in blue from LacZ-KD and Gfer-1/2KD KLS cells at 72 h after viral infection. Average signal intensities ± SD from approximately 150 cells (n=3) are shown in green.

recipient mice 48 h after the transplant and confirmed that CD45.2-donor-derived cells from all genetic backgrounds "homed" to the bone marrow of host mice (Figure 3A). Flow cytometric analysis of peripheral blood from transplant recipients revealed that while WT-U and WT-LacZ KLS cells reconstituted at similar rates, achieving an average of 45% donor chimerism by 3 weeks, and 90% by 15 weeks after BMT. KD of Gfer resulted in a significant reduction in reconstitution, with an average of 8-13% CD45.2 donor reconstitution during the 19-week period (Figure 3B, C). Analysis of recipient

bone marrow at the end of the 19-week BMT period revealed that an average of 2.3% LacZ-KD CD45.2^{+} KLS cells and 0.2% Gfer-KD CD45.2^{+} KLS cells (Figure 3D) remained in the recipient bone marrow, indicating that the fundamental defect of engraftment following KD of Gfer occurred in the KLS cells. To verify whether the remaining CD45.2^{+} Gfer-KD cells in transplant recipients represented "escapers" from knockdown, we isolated CD45.2 Lineage^{Negative} cells from transplant recipients at week 15 and measured the levels of Gfer mRNA. As shown in Figure 3E, Gfer mRNA was still expressed 2-fold lower in CD45.2^{+} Lineage^{Negative} Gfer-KD than in LacZ-KD cells, indicating that the shRNA was still active in these cells. Collectively, these data support a role for Gfer in the maintenance of HSC function *in vivo*.

Diminished Gfer in KLS cells triggers enhanced proliferation followed by exhaustion

Down-regulation of Gfer in the ESCs triggers apoptosis accompanied by a loss of mitochondrial function (Todd, Damin et al. 2010a). To understand whether KD of Gfer triggers apoptosis in KLS cells, we analyzed LacZ- and Gfer-KD KLS cells (freshly re-sorted 72 h post virus infection) for Annexin V/ 7-amino actinomycin D (7AAD) reactivity by flow cytometry. As indicated in Figure 4A, down-regulation of Gfer did not result in a significant loss of cell viability through apoptosis. We further examined the mitochondrial morphology in LacZ- and Gfer-KD KLS cells by transmission electron microscopy (TEM). LacZ-KD KLS cells possessed an average of 6-10, round to oval, mitochondria with well-defined cristae. In contrast, while the majority of Gfer-KD

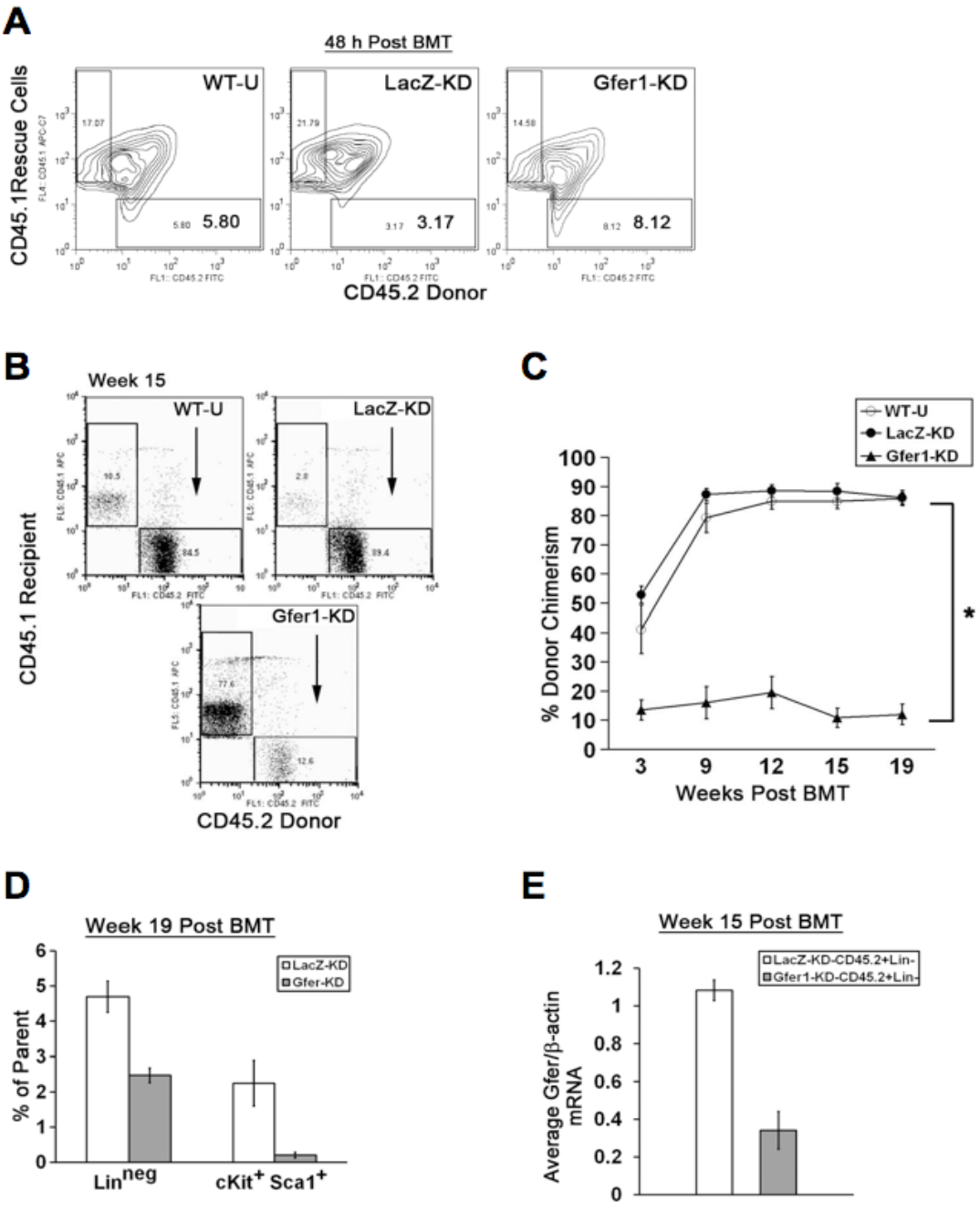

Figure 3: Gfer is a novel regulator of HSC function

(A) Flow cytometry histograms showing homing by CD45.2 donor cells of indicated genotypes assessed 48 h post BMT; representative histograms from two experiments are

shown. (B) Representative histograms depicting total CD 45.2 donor engraftment (X-axis) at 15 weeks post-BMT by donor cells from indicated genotypes. (C) Line graph depicting average CD45.2 donor reconstitution by the indicated donor genotype at 3, 9, 12, 15 and 19 weeks post bone marrow transplant. Donor reconstitution by uninfected WT donor cells is also shown. Error bars represent standard error of the mean of reconstitution by individual recipients (n=8) within each group; *p<0.05. (D) Lineage negative and c-Kit/Sca1 double positive cells isolated from pooled bone marrow of LacZ and Gfer KD KLS transplant recipients, euthanized at week 19 of the BMT assay. The SSC-FSC gated BM cells were first selected for CD45.2 expression before analyzing for KLS status. (E) Average Gfer mRNA levels, normalized to β-actin, in CD45.2 Lineage$^-$ cells of the indicated genotype when isolated at 12-weeks post BMT.

mitochondria appeared swollen with intact overall morphology, the remaining mitochondria in these cells appeared to be in various stages of degeneration (Figure 4B). However, down-regulation of Gfer did not result in a significant loss of mitochondrial function (data not shown). Overall, these results indicate that KD of Gfer resulted in alteration of mitochondrial morphology without compromising overall cell survival.

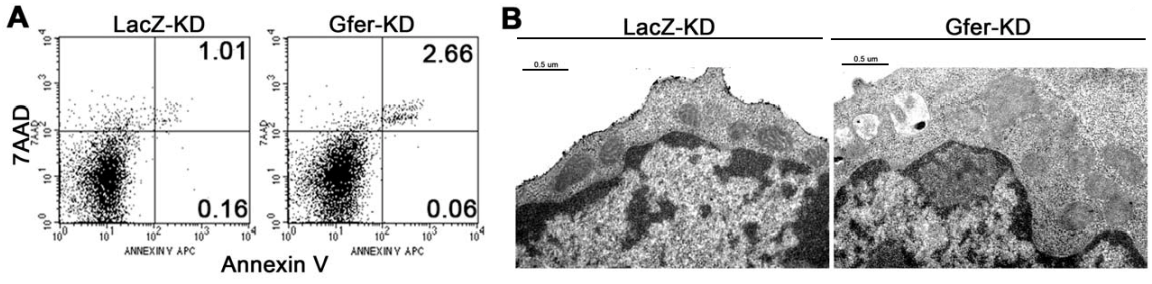

Figure 4: Knockdown of Gfer does not affect cell viability, but leads to altered mitochondrial morphology in KLS cells

(A) Apoptosis assessed in re-sorted GFP$^+$ LacZ- and Gfer-KD KLS cells by measuring Annexin V and 7AAD reactivity. Representative histograms from n=3 experiments

shown. (B) TEM images, 25000X magnifications, depicting the ultrastructural details in LacZ- and Gfer-KD KLS cells. Scale bars represent 0.5 μm.

Next, we evaluated whether down-regulation of Gfer in KLS cells affected their ability to proliferate. To this end, equal numbers of LacZ-, Gfer1-, and Gfer2-KD KLS cells were grown *in vitro* in serum-free media containing stem cell factor (SCF) and Flt3 ligand for 8 days. Comparison of the KLS profiles of these cells at days 0 and 6 of the *in vitro* proliferation assay revealed that differentiation occurred at a similar rate in LacZ- and Gfer-KD cells (Figure 5A). Surprisingly, down-regulation of Gfer expression resulted in significantly enhanced proliferation *in vitro*, such that by day 4, there were twice as many Gfer-KD as there were LacZ-KD cells (Figure 5B). Furthermore, by day 6, there were 2-2.5 fold higher numbers of Gfer-KD than LacZ-KD cells in culture, respectively (Figure 5B). In contrast, by day 8, the majority of the Gfer1/2-KD cells underwent exhaustion (Figure 5B, C). We additionally verified that down-regulation of Gfer was similar in Gfer-KD cells at days 0 and 6 in culture (data not shown). Increased proliferation followed by loss of function has been observed in *ataxia-telangiectasia mutated* (*Atm*$^{-/-}$) and *Foxo3a*$^{-/-}$ HSCs, where diminished quiescence is accompanied by elevated reactive oxygen species (ROS) (Ito, Hirao et al. 2006; Miyamoto, Araki et al. 2007). To evaluate whether an increase in oxidative stress in Gfer-KD cells triggers a hyper-proliferative response, we measured ROS levels in LacZ- and Gfer-KD cells on days 6

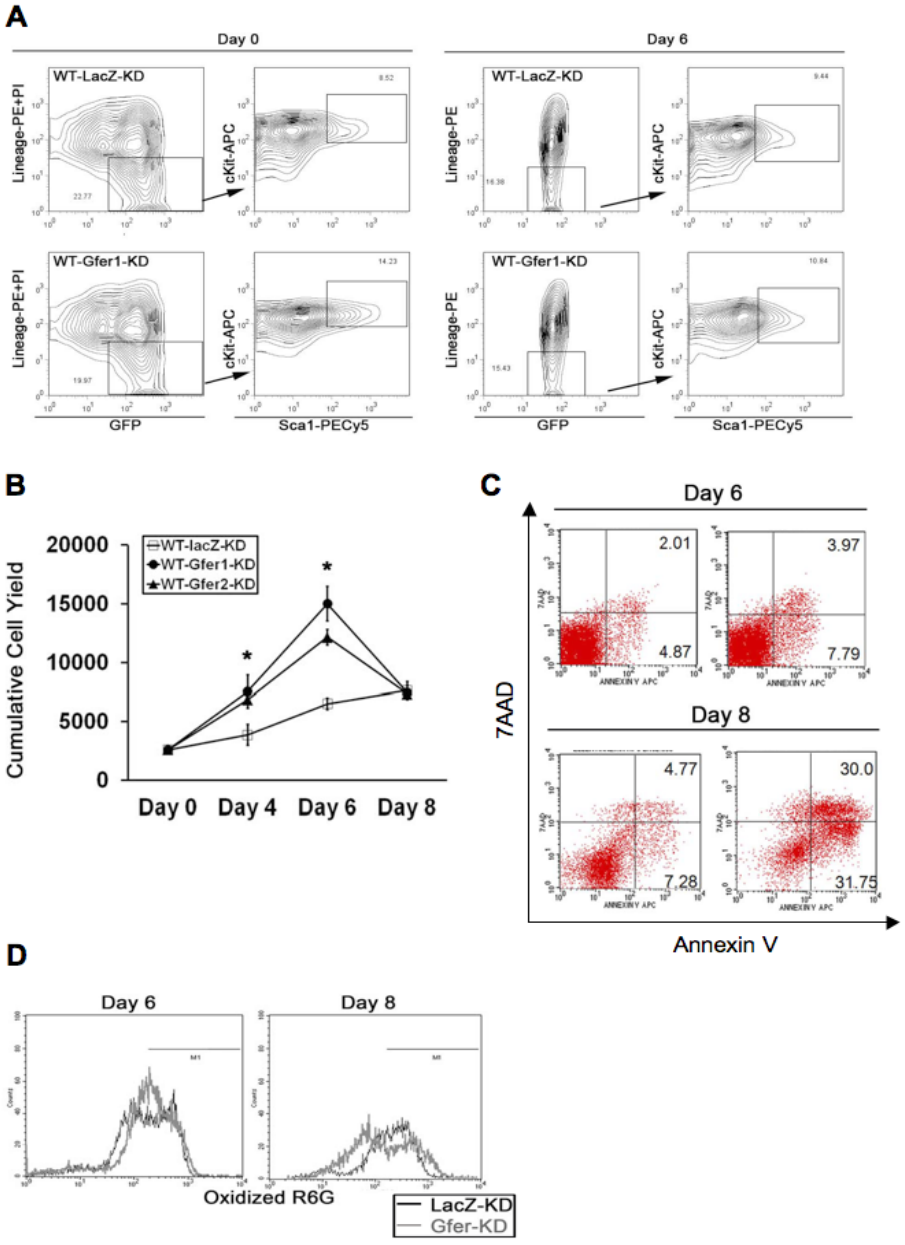

Figure 5: Gfer KD does not affect KLS cell differentiation, but results in enhanced proliferation followed by increased apoptosis

(A) Representative histograms depicting lineage analysis of WT-LacZ-KD or WT-Gfer1-KD cells during a typical proliferation assay. On the left is representation of GFP^+, propidium iodide $(PI)^-$, $Lineage^-$, $cKit^+$, $Sca1^+$ KLS cells, sorted 72 h post Lenti-GFP-shRNA virus transduction, to setup the proliferation assay (Day 0). On day 6 of the

proliferation assay (right), cells from all wells from an entire Terasaki plate were pooled, stained for KLS markers, and analyzed by flow cytometry. (B) Average (n=3) cumulative cell yield from indicated genotypes during *in vitro* proliferation assays performed in Terasaki plates for 8 days in serum free media; *$p<0.05$. (C) Histograms representing apoptosis assays performed based on Annexin V-7AAD reactivity on LacZ- and Gfer-KD cells, pooled from entire Terasaki plates, on Days 6 and 8 of culture. (D) Representative histograms (n=2) indicating intracellular ROS levels in LacZ and Gfer KD KLS cells on days 6 and 8 of the proliferation assay. Measurements were based on the production of oxidized rhodamine 6G by the intracellular oxidation of the non-fluorescent dihydrorhodamine 6G.

and 8 of growth in culture using the redox-active fluorescent probe dihydrorhodamine 6G by flow cytometry. We did not observe significant differences in R6G fluorescence intensities between LacZ- and Gfer-KD cells on days 6 and 8 of proliferation *in vitro* (Figure 5D), indicating that the enhanced proliferation in Gfer-KD cells is not due to oxidative stress.

Gfer modulates total and nuclear levels of $p27^{kip1}$ in KLS cells

Our data suggest that KD of Gfer from KLS cells triggers enhanced cell proliferation that results in exhaustion and loss of *in vivo* function. But what might be the mechanism that elicits a hyper-proliferative response in Gfer-KD KLS cells? Gfer directly interacts and co-localizes in the nucleus with Jab1, the 5th subunit of the highly conserved COP9 signalosome (CSN) protein complex (Lu, Li et al. 2002; Wang, Lu et al. 2004). The CSN complex consists of a multi-subunit protease that functions in the ubiquitin–proteasome pathway by directly regulating the activities of the cullin–RING ligase (CRL) family of ubiquitin E3 complexes (Wei, Serino et al. 2008; Kato and Yoneda-

Kato 2009; Schwechheimer and Isono 2010). Jab1, which is expressed in HSCs (Mori, Yoneda-Kato et al. 2008), binds to the cell cycle inhibitor $p27^{kip1}$ through its C-terminal domain, coordinating nuclear export and subsequent proteasome-dependent degradation of this CDKI (Tomoda, Kubota et al. 1999; Tomoda, Kubota et al. 2002).

We hypothesized that in normal KLS cells, Gfer binds to and inactivates Jab1 resulting in stabilization of $p27^{kip}$ and cell quiescence. To test this, we first assessed $p27^{kip1}$ and Jab1 protein levels in LacZ and Gfer-KD KLS cells by fluorescent immunocytochemistry. Down-regulation of Gfer (Figure 6vii-viii) resulted in a significant 2-fold reduction in overall (total and nuclear) $p27^{kip1}$ levels (Figure 6i-ii); without significant alterations in Jab1 levels (Figure 6iv-v; Figure 7A). Further, treatment of Gfer-KD KLS cells with the proteasome inhibitor MG132 resulted in significantly increased nuclear and total $p27^{kip1}$ levels, consistent with its increased turnover in the absence of Gfer (Figure 7Bi-iv). We then asked whether $p27^{kip1}$ and Jab1 levels were altered in KLS cells over-expressing Gfer. Murine stem cell virus (MSCV)-mediated over-expression of Gfer (Figure 6ix) resulted in a significant 1.6-fold increase in $p27^{kip1}$, 85% of which was retained in the nucleus (Figure 6iii), while total Jab1 protein levels did not change (Figure 6vi). Conversely, levels of $p21^{cip1}$, a member of the cip/kip-cell cycle inhibitor family with demonstrated roles in HSC quiescence (Cheng, Rodrigues et al. 2000), did not change when Gfer was down-regulated or over-expressed

(Figure 8). Altogether, these results indicate that Gfer expression significantly modulates total and nuclear $p27^{kip1}$ in KLS cells.

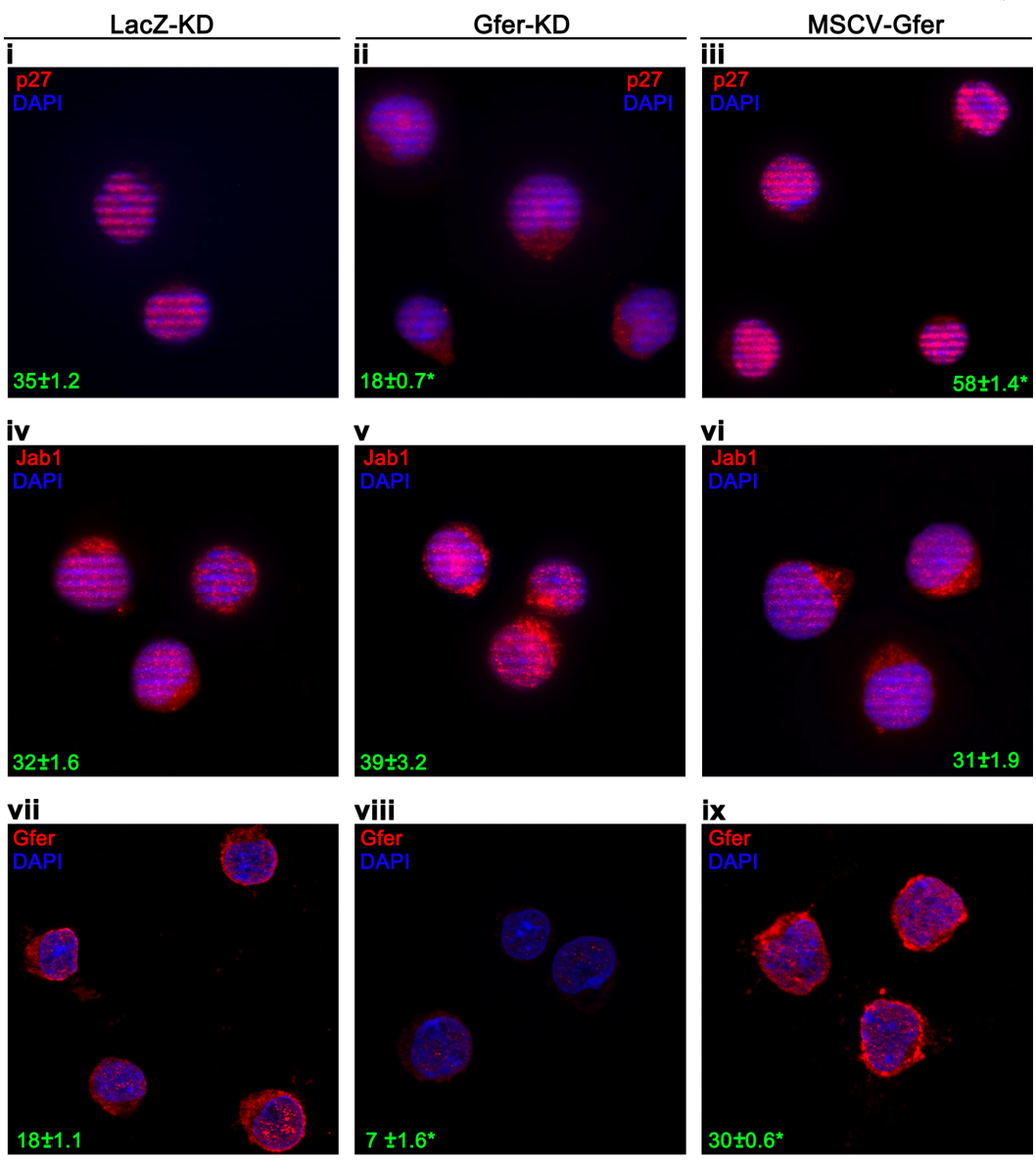

Figure 6: Gfer expression modulates total and nuclear $p27^{kip1}$ in KLS cells

Digital optical section (Apotome; 630X) immunocytochemistry images depicting $p27^{kip1}$ (i-iii); Jab1 (iv-vi); and Gfer (vii-ix) levels in LacZ and Gfer KD and MSCV-Gfer KLS cells. Protein levels were detected by probing with secondary antibodies conjugated to Cy3 (Red) fluorophore. Dapi is shown in Blue and average signal intensities ± SD calculated from at least 250 cells from three independent experiments are indicated in Green. *p<0.05.

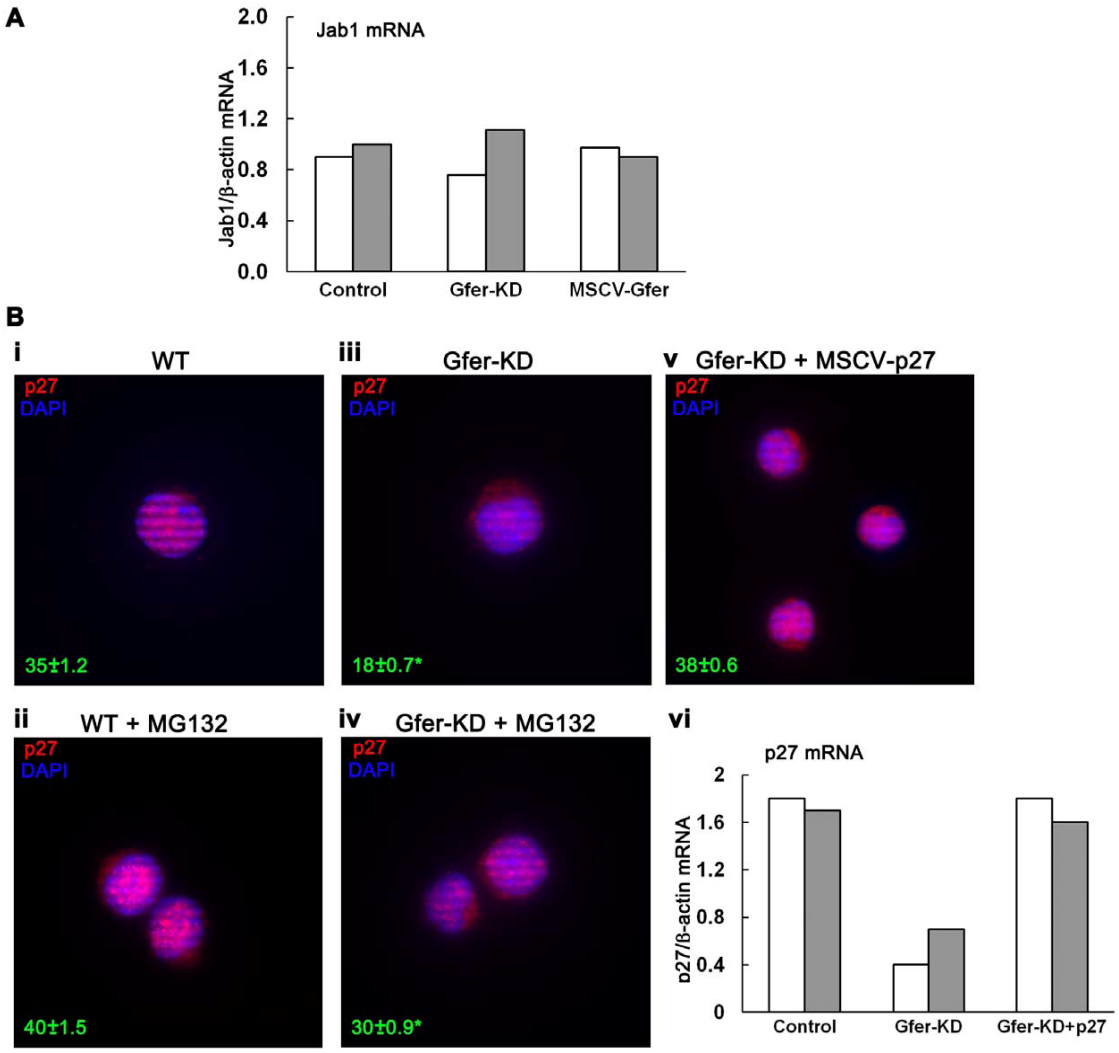

Figure 7: Depletion of Gfer results in increased turnover of $p27^{kip1}$, without altering total Jab1 mRNA

(A) Jab1 mRNA levels were measured using real-time qRT-PCR from WT, Gfer-KD and MSCV-Gfer KLS cells, immediately after re-sorting fro KLS status and virus infection and normalized to the β-actin mRNA levels. Values from two independent experiments are shown. (B) Digital Apotome immunocytochemistry sections (630X) depicting $p27^{kip1}$ (Red) in WT KLS cells (i), WT KLS cells treated with 10 μM MG132 treated (ii), and Gfer-KD KLS cells, in the absence (iii) or presence of 10 μM MG132 (iv); and Gfer-KD KLS cells infected with the MSCV-p27-YFP retrovirus (v). Dapi is shown in Blue and signal intensities ± SD calculated, from over 150 cells from three independent experiments, are shown in Green. (Bvi) $p27^{kip1}$ mRNA levels, normalized to respective β-actin mRNA, in control KLS cells, Gfer-KD KLS cells and Gfer-KD KLS cells infected with MSCV-p27-YFP were measured by real-time qRT-PCR and results from two independent experiments are shown.

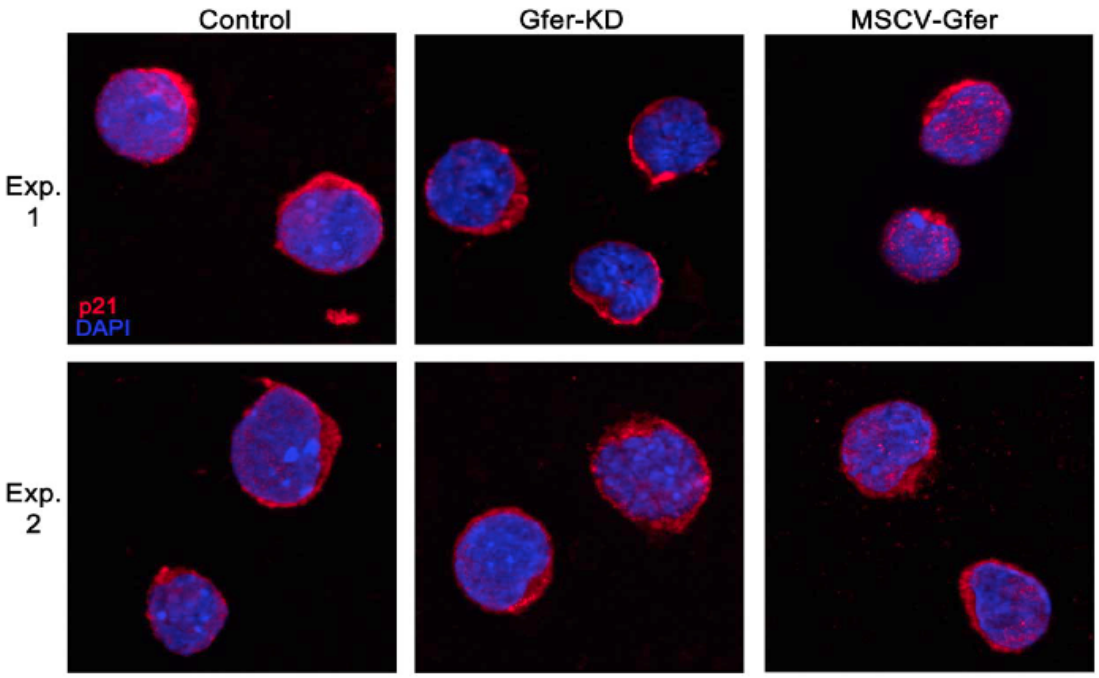

Figure 8: Gfer expression does not alter $p21^{cip1}$ levels

Digital immunocytochemistry images (630X) depicting $p21^{cip1}$ (red) and nuclei (Dapi-blue) in control, Gfer-KD, and MSCV-Gfer KLS cells from 2 independent experiments are shown.

Gfer binds to Jab1 and inhibits its destabilization of p27^{kip1}

Testing the hypothesis that Gfer regulates p27^{kip1} levels via its interaction with Jab1 would require traditional biochemical techniques such as immunoprecipitation (IP) followed by SDS-PAGE and immunoblot analyses of the precipitates. However, such tasks are impractical to accomplish in HSCs because of their rarity. HSCs constitute only 0.01% of the total bone marrow amounting to an approximate yield of 10,000 cells per mouse. Moreover, being very small (average diameter of 4 μM), and largely quiescent, HSCs possess very limited amount of protein (Viatour, Somervaille et al. 2008; Wilson, Laurenti et al. 2008). Hence we turned to the hematopoietic progenitor EML1 cell line (Tsai, Bartelmez et al. 1994) to study the mechanism of regulation of p27^{kip1} stability by Gfer. First, we performed KD and over-expression of Gfer in EML cells using the previously mentioned FG12-Lentivirus and MSCV systems (Figure 9Ai-Aii). Over-expression of Gfer increased p27^{kip1} and its KD decreased the levels of the CDKI, without a significant alteration of Jab1 levels (Figure 9Ai-Aii). We then analyzed Jab1-IP complexes from these EML cells for the presence of Gfer and p27^{kip1}. As shown in Figure 9Bi-Bii, endogenous Gfer bound to Jab1 in MSCV control (MSCV-C) and FG12-LacZ infected EML cells; this interaction was enhanced upon Gfer over-expression and diminished upon Gfer KD. In contrast, the amount of p27^{kip1} present in Jab1-IP complexes decreased in MSCV-Gfer infected EML cells and increased in Gfer-KD cells

(Figure 9Bi-Bii). Similarly, there was a marked reduction of Jab1 in p27^{kip1}-IP complexes from MSCV-Gfer compared to MSCV-C cells and an increase in Jab1 present in these complexes in Gfer-KD compared to the non-specific LacZ-KD EML cells (Figure 9Ci-Cii). Further, ubiquitination in p27^{kip1}-IP complexes, a mechanism previously evidenced for p27 turnover (Zhou, Yang et al. 2009), was significantly higher in Gfer-KD EML cells, while it was almost abolished in p27^{kip1} immunocomplexes from cells over-expressing Gfer (Figure 9Ci-Cii). Thus, increasing the concentration of Gfer resulted in sequestration of Jab1, leaving less of this COP9 signalosome subunit available for association with p27^{kip1} and orchestration of its nuclear export and degradation.

Normalization of p27^{kip1} levels rescues the enhanced proliferation deficit in Gfer-KD KLS cells

We next determined whether expression of p27^{kip1} in Gfer-KD KLS cells will rescue their *in vitro* proliferation defects by using a modified MSCV-IRES-yellow fluorescent protein (YFP) to achieve restoration, but not over-expression, of p27^{kip1} in these cells (Figure 7Bi, iii, iv, vi). We also confirmed that infection of Gfer-KD KLS cells with MSCV-p27^{kip1}-YFP did not cause enhanced differentiation and/or apoptosis (data not shown). Normalization of p27^{kip1} levels in Gfer-KD KLS cells to those found in control cells resulted in a rescue of the enhanced proliferation deficit in the mutant cells (Figure 10A). Moreover, Gfer-KD KLS cells expressing MSCV-p27^{kip1}-YFP did not undergo exhaustion by day 8 of the proliferation assay, suggesting that enhanced

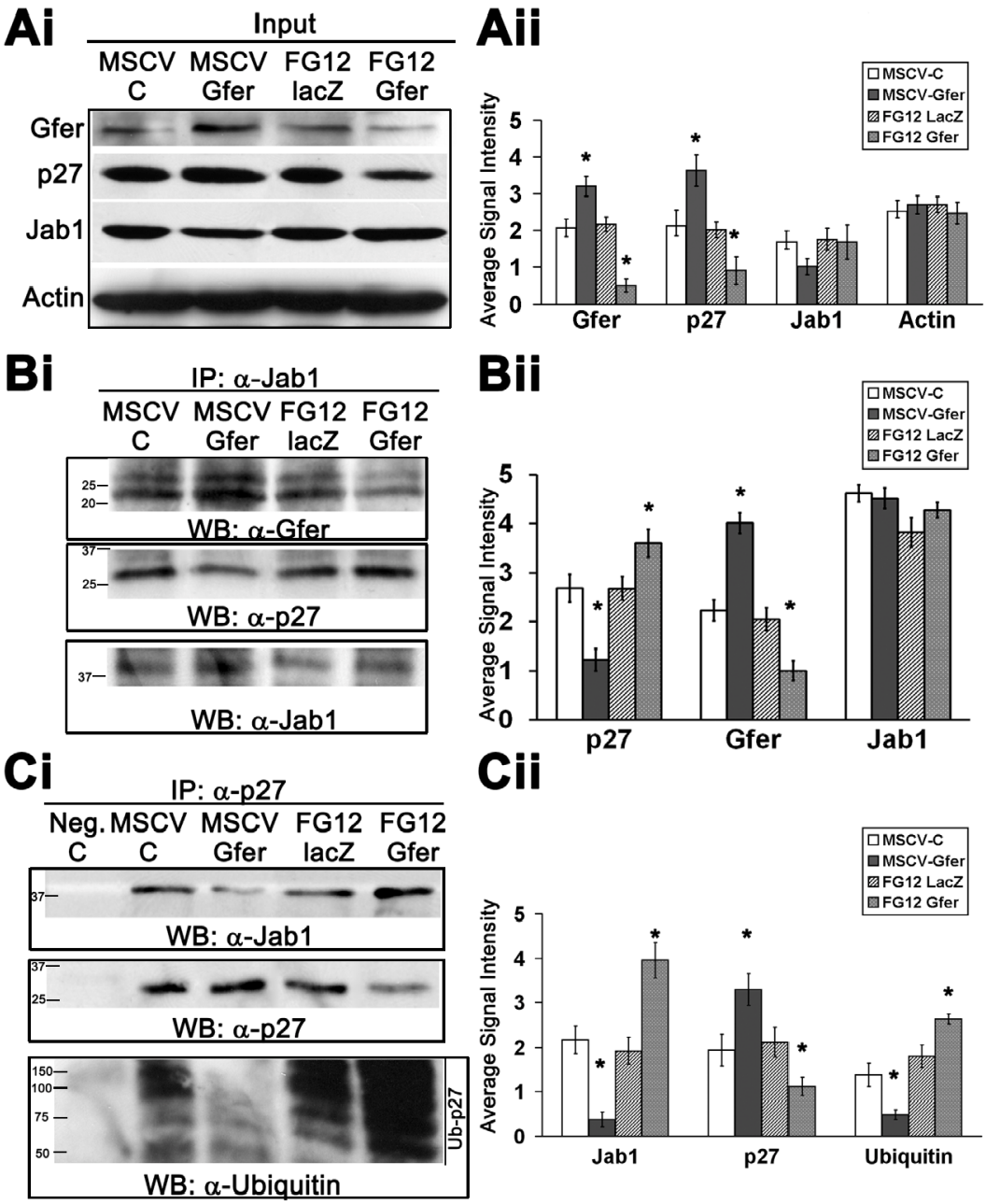

Figure 9: Gfer regulates the stability of p27^{kip1} through its interaction with Jab1

(Ai) Immunoblots showing Gfer, p27^{kip1}, Jab1 and actin (loading control) levels in whole cell lysates from MSCV-C, MSCV-Gfer, FG12-LacZ, and FG12-Gfer infected EML

hematopoietic progenitor cells; (Aii) Average signal intensities of bands from corresponding immunoblots of the indicated genotypes from n=3 experiments; *p<0.05. (Bi) Immunoblots depicting the levels of Gfer, $p27^{kip1}$, and Jab1 present in anti-Jab1 immunoprecipitates from EML cells of the indicated genotypes. (Bii) Average signal intensities of bands showing Gfer, $p27^{kip1}$, and Jab1 levels in Jab1-IP complexes from 3 experiments; *p<0.05. (Ci) Western blot images indicating the levels of Jab1, $p27^{kip1}$, and ubiquitin associated with anti-$p27^{kip1}$ immunoprecipitates in control, Gfer-KD, or Gfer over-expressing EML cells. Negative control indicates a beads-only control with no primary antibody performed with MSCV-C EML cells. (Cii) Graphs showing average signal intensities of bands showing Jab1, $p27^{kip1}$, and ubiquitin levels in p27-IP complexes from 3 experiments; *p<0.05.

proliferation by the mutant cells may have caused their exhaustion and, perhaps, loss of *in vivo* function. Furthermore, MSCV-Gfer KLS cells possessed enlarged, mostly round or oval mitochondria (Figure 10B), indicating that the altered mitochondrial morphology may partially contribute to the enhanced differentiation of these cells (Mantel, Messina-Graham et al. 2010). Nonetheless, our cumulative data suggest that in normal HSCs, Gfer acts to counter Jab1-mediated nuclear export and de-stabilization of the CDKI $p27^{kip1}$, by directly binding to and sequestering the COP9 signalosome subunit (Figure 11). Through this mechanism, Gfer promotes quiescence in HSCs, thus playing a critical role in maintaining the functional integrity of these important cells.

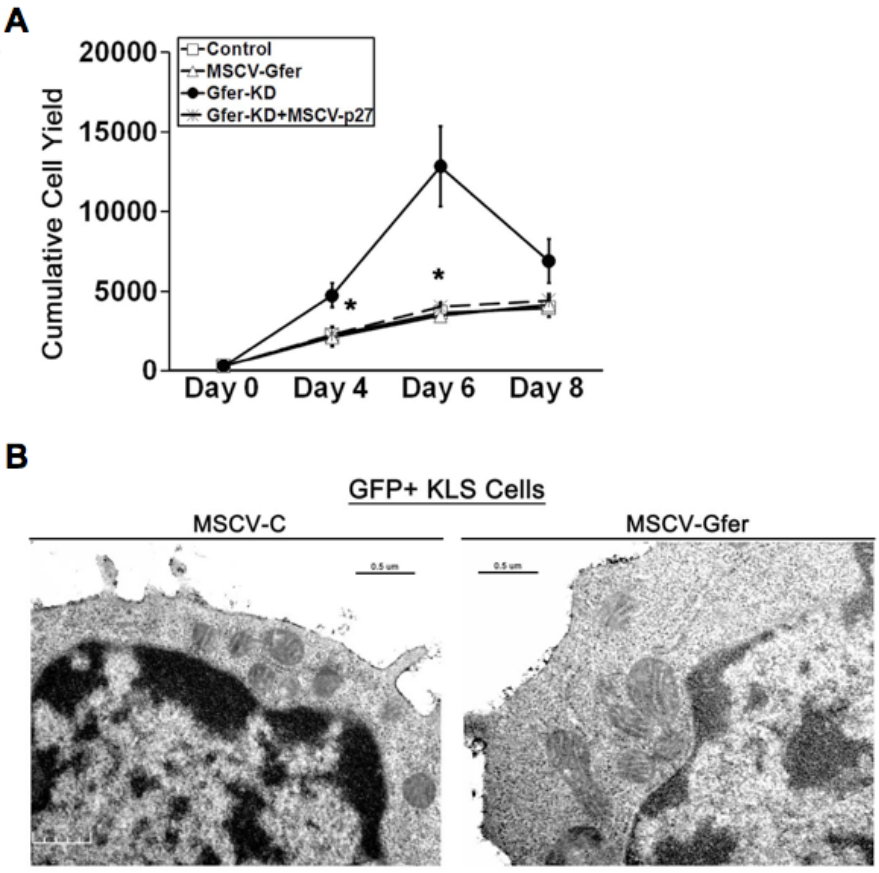

Figure 10: Normalization of p27^{kip1} levels in Gfer KD KLS cells rescues their proliferation/exhaustion deficit, while Gfer over-expression results in enlarged mitochondria

(A) Average cumulative cell yield (n=3) from control, MSCV-Gfer, Gfer-KD KLS cells and in Gfer-KD KLS cells expressing MSCV-p27^{kip1}-YFP virus. Cumulative cell yield was calculated by counting total number of cells per well of Terasaki plates initially plated with KLS cells of the indicated genotypes cultured on serum free media containing SCF and Flt3 ligand; *p<0.05. (B) High magnification (25000X) TEM images showing mitochondrial morphology in MSCV-C and MSCV-Gfer infected KLS cells fixed after resort at 72 h post infection. Enlargement of mitochondria following Gfer over expression can be seen.

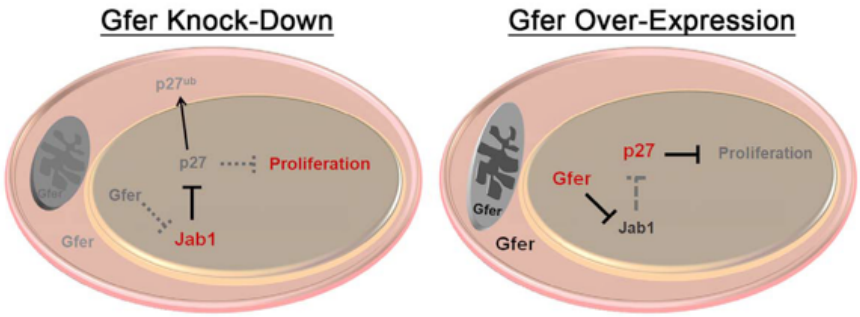

Figure 11: Model proposing a novel role for Gfer in the restriction of unwarranted HSC proliferation through its inhibition of Jab1-mediated ubiquitination and turnover of $p27^{kip1}$

Down-regulation of Gfer in KLS cells results in its inability to efficiently inhibit Jab1 from binding to and causing the nuclear export and subsequent degradation of $p27^{kip1}$. This results in increased proliferation of HSCs during homeostasis, resulting in their eventual exhaustion and loss of *in vivo* function. On the other hand, increased concentration of Gfer in KLS cells results in its increased binding and sequestering of Jab1, leading to increased nuclear retention and stability of $p27^{kip1}$, which then blocks the ability of G1 cyclin-Cdk complexes to organize the progression of the cell cycle.

3.3 Conclusions

HSC quiescence, tightly regulated by various intrinsic and extrinsic mechanisms, ensures the maintenance of long-term genetic, epigenetic and mitochondrial stability (Wilson and Trumpp 2006). Consequently, loss of quiescence in the long-term repopulating HSCs results in their exhaustion and/or loss of function. Genes that execute the fundamental task of restricting unwarranted HSC proliferation thus play important roles in the maintenance of their function during homeostasis and periods of hematological stress.

In this study, we show that the highly conserved sulfhydryl oxidase Gfer performs a novel, fundamental role in the restriction of HSC proliferation through its modulation of Jab1-mediated turnover of the key cell cycle inhibitor, $p27^{kip1}$. KD of Gfer from KLS cells results in a loss of cell quiescence and *in vivo* function. Gfer-KD cells display reduced $p27^{kip1}$ levels while its over-expression significantly elevates $p27^{kip1}$ levels and enhances its nuclear retention. In hematopoietic EML cells, Gfer binds to the COP9 signalosome subunit Jab1, preventing its association with $p27^{kip1}$, thereby inhibiting Jab1-mediated nuclear export and increased turnover of this CDKI.

Accordingly, restoration of $p27^{kip1}$ in KLS cells down-regulated for Gfer expression normalizes their *in vitro* proliferation kinetics, indicating a pro-quiescence role for the novel Gfer-Jab1-$p27^{kip1}$ pathway in HSCs. Indeed, decreased $p27^{kip1}$ correlates with the loss of HSC quiescence in *Foxo3a*$^{-/-}$ mice, underscoring a role for this CDKI in regulating HSC proliferation (Miyamoto, Araki et al. 2007). However, unlike Gfer-KD KLS cells, HSCs from $p27^{kip1}$-null mice did not undergo abnormal proliferation (Cheng, Rodrigues et al. 2000). Enhanced $p21^{cip1}$ levels restricted unwanted proliferation in $p27^{kip1}$-null HSCs, whereas the levels of $p21^{cip1}$ did not increase in Gfer-KD KLS cells. On the other hand, this discrepancy could be due to genetic differences, as our HSCs were derived from C57BL/6 mice while the $p27^{kip1}$-null mice were generated in the SV129 background.

Besides its predominant localization in the IMS of mitochondria, Gfer is also present in the cytoplasm and nucleus, and a non-mitochondrial activity is associated with its role in spermatogenesis (Klissenbauer, Winters et al. 2002). The mitochondrial morphology in KLS cells down-regulated for or over-expressing Gfer was not nearly as dramatic as those observed in ESCs. We also did not observe loss of mitochondrial membrane potential, elevation of intracellular ROS or decrease in cell viability in Gfer-KD KLS cells. Our data strongly suggest that the nuclear function of Gfer, inhibiting Jab1 and stabilizing $p27^{kip1}$ to restrict proliferation, is the most important one in HSCs. In fact, Gfer binds Jab1 through its divergent N-terminal domain, as opposed to the highly conserved FAD domain (Wang, Lu et al. 2004). On the other hand, in ESCs, the mitochondrial function of Gfer is the most relevant as its down-regulation resulted in extensive mitochondrial fragmentation and apoptosis (Todd, Damin et al. 2010a), indicating that the role of Gfer in maintaining mitochondrial integrity may be associated with the more primitive status of the ESCs. Further, Gfer is largely dispensable for maintenance of mitochondrial function and cell survival in more differentiated cells (Todd, Damin et al. 2010a).

While our studies reveal Gfer to be a positive regulator of $p27^{kip1}$ in HSCs, it has also been reported that Bcl-2 up-regulates $p27^{kip1}$ (Greider, Chattopadhyay et al. 2002). As discussed in Chapter 1, a reduction in the levels of Bcl-2 is associated with hyper-proliferation in CaMKIV-null HSCs, a phenotype also displayed by Gfer-KD HSCs.

Thus, one of CaMKIV's downstream effectors may be $p27^{kip1}$ through the regulation of Bcl-2 levels. In Chapter 4, we further investigate the role of calcium/CaM signaling in hematopoiesis by evaluating the function of CaMKIV's upstream activator, CaMKK2.

4. A Cell-Intrinsic Role for CaMKK2 in Granulocyte Lineage-Commitment and Differentiation[1]

4.1 Preface

As described previously, hematopoiesis is the lifelong process in which hematopoietic stem cells (HSCs) self-renew or differentiate into all mature blood cell lineages through the production of intermediate progenitor blood cells (Till and McCulloch 1961; Metcalf 2007). This process is tightly regulated in the BM by several factors, including microenvironment, paracrine interactions, cytokines, transcriptional programs, and signal transduction pathways. While the signaling cascades that regulate hematopoietic cell maintenance and lineage specification are largely unclear, there have been implications for Ca^{2+} and its intracellular receptor, calmodulin (CaM), to be important mediators of hematopoietic stem-progenitor cell processes such as growth and migration (Katayama, Nishikawa et al. 1990; Ueda, Mizuki et al. 2002; Adams, Chabner et al. 2005), suggesting that Ca^{2+}/CaM-dependent pathways may play significant and diverse roles in hematopoiesis.

Indeed, we previously identified a CaM kinase, CaMKIV, as a critical cell-intrinsic signaling component that regulates HSC survival and maintenance (Kitsos,

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[1] Teng, E. C., L. Racioppi, et al. (2011). " A cell-intrinsic role for CaMKK2 in granulocyte lineage-commitment and differentiation." J Leukoc Biol The results presented in this chapter were previously published and in part modified from the above manuscript. Permission to reproduce the material here was kindly granted by the Society for Leukocyte Biology.

Sankar et al. 2005). *Camk4*$^{-/-}$ mice possessed a significantly decreased number of c-Kit^{+}Sca1^{+}Lin$^{-/lo}$ (KLS) cells, which include HSCs and multipotent progenitors, and *Camk4*$^{-/-}$ HSCs failed to reconstitute hematopoiesis when transplanted into lethally irradiated mice. These HSC defects were due to deregulated proliferation and increased apoptosis as a result of deficiencies in phospho-cyclic AMP response element-binding protein (CREB), CREB-binding protein, and Bcl-2. Furthermore, our previous studies demonstrated that CaMKIV signaling through CREB regulates the lifespan and function of several mature hematopoietic cell types, including dendritic cells, T cells, and thymocytes, indicating a broader role for this CaMK in hematopoiesis (Anderson, Ribar et al. 1997; Anderson and Means 2002; Illario, Giardino-Torchia et al. 2008).

CaMKIV belongs to a family of Ca^{2+}/CaM-dependent, multifunctional serine/threonine kinases that also include CaMKI and CaMKII (Chow and Means 2007; Colomer-Font and Means 2007). However, only CaMKI and CaMKIV require phosphorylation on a single activation-loop threonine to achieve maximal activation. This phosphorylation event occurs via an upstream CaMK kinase, CaMKK1 and/or CaMKK2, which is also a Ca^{2+}/CaM-binding protein (Anderson, Means et al. 1998). This method of regulation has been termed a "CaM kinase cascade" in cells, analogous to the MAPK cascade (Means 2008). Indeed, we recently showed that CaMKK2-CaMKIV signaling to CREB is required for normal cerebellar granule cell development (Kokubo,

Nishio et al. 2009). However, the importance of CaMKK2-mediated signaling in hematopoiesis has not been evaluated.

To elucidate the physiological relevance of CaMKK2 and the CaMK cascade in early hematopoietic stem-progenitor cells, we investigated the effects of CaMKK2 deletion on the mouse hematopoietic system. Similar to CaMKIV, mice null for CaMKK2 possessed reduced numbers of total KLS and bone marrow (BM) cells. However, depletion of CaMKK2 did not elicit abnormal cell proliferation, or impaired survival or function of HSCs. Interestingly, unlike *Camk4*$^{-/-}$ mice, *Camkk2*$^{-/-}$ mice displayed marked defects in early myeloid progenitor (CMP and GMP) populations in the BM. Engraftment of *Camkk2*$^{-/-}$ HSCs in BM transplantation (BMT) led to increased repopulation of myeloid lineages, and particularly in the granulocyte compartment. Moreover, *Camkk2*$^{-/-}$ myeloid progenitors differentiated into greater numbers of CFU-G and Gr1^{+}Mac1^{+} granulocytes *in vitro*. Restoration of CaMKK2 in *Camkk2*$^{-/-}$ CMP cells was sufficient to rescue the enhanced granulopoietic phenotype of *Camkk2*$^{-/-}$ progenitors. Similarly, over-expression of wild-type, but not kinase-deficient, CaMKK2 in 32Dcl3 myeloid progenitor cell line resulted in a pronounced inhibition of granulocyte differentiation. Thus, our findings demonstrate a novel, cell-intrinsic role for CaMKK2 in myeloid progenitors as a negative regulator of granulopoiesis, and provide key insights for potential therapies of granulopoietic and/or myelogenous disorders.

4.2 Experimental Results

CaMKK2 is not required for homeostatic KLS cell proliferation or survival

We confirmed that CaMKK2 mRNA is expressed in KLS cells, a population enriched for HSCs (Figure 12A), which was compared to β2MG expression as a loading control (data not shown). We next evaluated whether the hematopoietic compartment was altered in the absence of CaMKK2 by analyzing the KLS and BM cellularity of WT and *Camkk2*$^{-/-}$ mice. While our results showed a similar frequency of KLS cells in WT and *Camkk2*$^{-/-}$ BM (Figure 12B, C), the total number of KLS and BM cells was significantly reduced in *Camkk2*$^{-/-}$ mice (Figure 12D, E), similar to what was observed in *Camk4*$^{-/-}$ mice (Kitsos, Sankar et al. 2005). This decrease in *Camkk2*$^{-/-}$ BM cellularity did not result from a runted body size, as these mice were comparable in weight to WT on a standard chow diet as previously described (Anderson, Ribar et al. 2008). Because the relative decrease in total BM cellularity was proportional to the reduction in KLS cells, the frequency of KLS cells was not significantly different in WT versus *Camkk2*$^{-/-}$ mice.

However, given that the total number of KLS cells was similarly reduced in both *Camkk2*$^{-/-}$ and *Camk4*$^{-/-}$ mice, we tested whether CaMKK2-deficient KLS cells exhibited defective proliferation and/or survival under homeostatic conditions like *Camk4*$^{-/-}$ KLS cells. BrdU-incorporation experiments revealed no significant differences in the percentage of proliferating KLS cells between WT and *Camkk2*$^{-/-}$ mice (Figure 13A, B). To test whether CaMKK2 was necessary for KLS cell survival, freshly isolated WT and

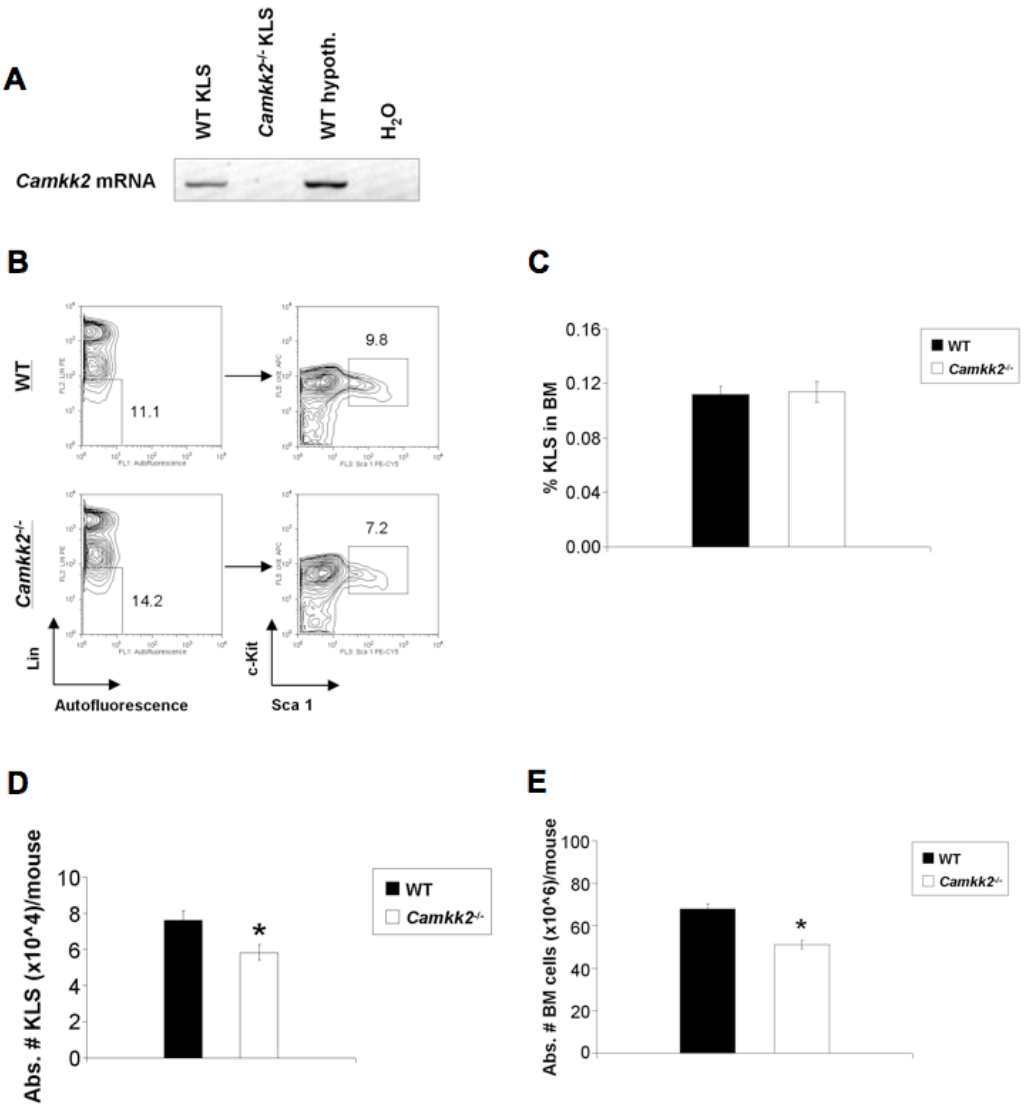

Figure 12: *Camkk2*$^{-/-}$ mice exhibit reduced KLS and BM cellularity, but normal frequency of KLS cells

(A) RT-PCR analysis of *Camkk2* mRNA expression in WT and *Camkk2*$^{-/-}$ KLS cells. WT hypothalamus (hypoth.) cDNA was included as a positive control, and H_2O as a negative control. β2MG expression was also measured and used as a loading control (data not shown). (B) Representative FACS analysis of KLS cells in c-Kit$^+$-enriched BM from WT and *Camkk2*$^{-/-}$ mice. (C) Percentages of KLS cells in BM of WT and *Camkk2*–/– mice ($n \geq 8$). (D) Total numbers of KLS cells in WT and *Camkk2*$^{-/-}$ mice ($n \geq 8$). (E) Absolute numbers of nucleated BM cells per mouse in WT and *Camkk2*–/– mice ($n \geq 8$; *$p<0.001$). (A-E) Data are representative of at least three independent experiments.

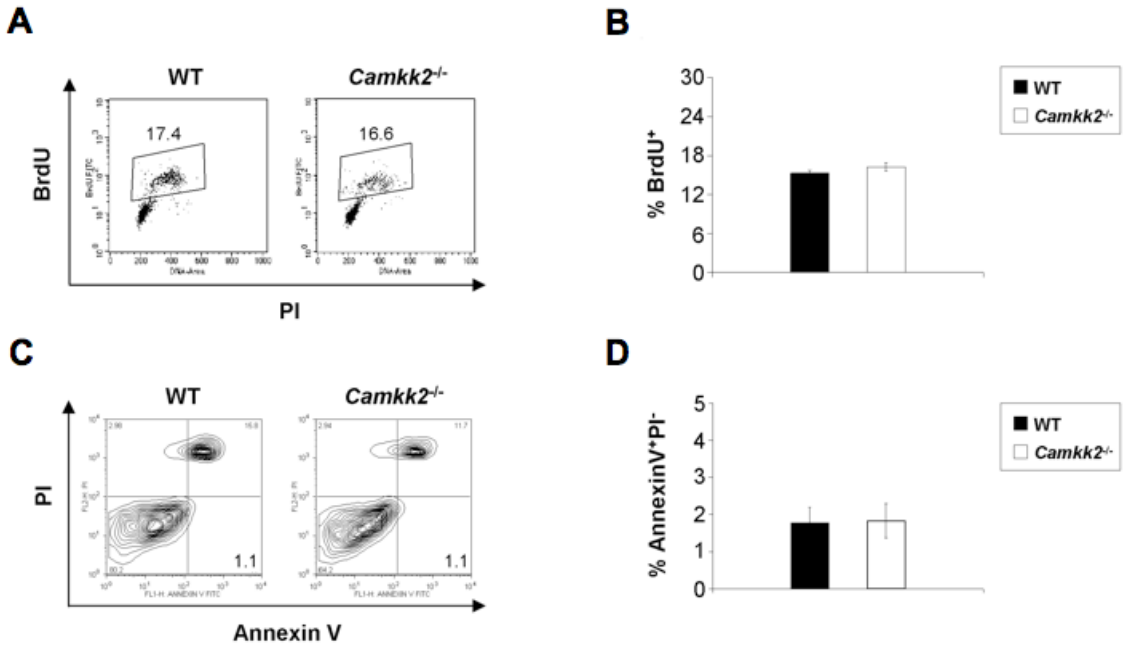

Figure 13: Loss of CaMKK2 does not affect cell proliferation or survival of KLS cells

(A) To assess proliferation, KLS cells in WT and *Camkk2*$^{-/-}$ mice were pulse labeled with BrdU *in vivo*, then isolated, stained with anti-BrdU-FITC and PI, and analyzed by FACS. (B) Quantification of (A) showing percentages of BrdU-positive KLS cells ($n \geq 9$). (C) To evaluate apoptosis, WT and *Camkk2*$^{-/-}$ KLS cells were freshly isolated and stained with anti-Annexin V-FITC and PI, then analyzed by FACS. (D) Quantification of (C) showing percentages of (Annexin V^{+}PI^{-}) KLS cells undergoing apoptosis ($n \geq 9$). (A-D) Data are representative of at least three independent experiments.

Camkk2$^{-/-}$ KLS cells were analyzed for Annexin V and PI reactivity to evaluate the percentage of apoptotic cells. We observed no significant differences in the percentage of early apoptotic (Annexin V^{+}PI^{-}) cells in WT versus *Camkk2*$^{-/-}$ mice (Figure 13C, D). Since KLS cell proliferation and survival did not appear altered, the decreased number

of KLS cells in the *Camkk2*$^{-/-}$ BM may reflect the overall decrease in total BM cellularity, and/or be suggestive of altered self-renewal or repopulation ability. Collectively, these data indicate that CaMKK2 is not necessary for regulating the proliferation or survival of KLS cells.

CaMKK2 deficiency does not impair HSC repopulating ability, but causes enhanced myelopoiesis in vivo during BMT

Although the loss of CaMKK2 did not affect KLS cell proliferation or viability, the reduced number of *Camkk2*$^{-/-}$ KLS cells suggested the possibility that CaMKK2-null HSCs were functionally compromised relative to WT. Moreover, the long term self-renewal potential and multi-lineage repopulating properties of *Camkk2*$^{-/-}$ HSCs remained unknown. Therefore, to determine whether loss of CaMKK2 affects the *in vivo* repopulation potential of HSCs, we performed a non-competitive transplantation assay in the same manner that allowed us to identify marked defects in the blood reconstitution ability of *Camk4*$^{-/-}$ HSCs (Kitsos, Sankar et al. 2005). To this end, we transplanted an equal number of CD34^{-}KLS cells (enriched for long-term HSCs (Osawa, Hanada et al. 1996)) from CD45.2^{+} WT and *Camkk2*$^{-/-}$ mice along with Sca-1-depleted BM cells from non-irradiated WT CD45.1^{+} mice into lethally irradiated CD45.1^{+} recipients. Donor:host blood cell chimerism (CD45.2^{+} versus CD45.1^{+}) and hematopoietic lineages in the peripheral blood were analyzed by FACS once every three weeks for 15 weeks. Based on the mean percentage of CD45.2^{+} cells in the blood, overall donor engraftment

was significantly higher in *Camkk2*$^{-/-}$-reconstituted mice compared to WT (Figure 14A, B), suggesting that repopulation and/or self-renewal abilities of *Camkk2*$^{-/-}$ HSCs were not impaired but perhaps enhanced.

However, further analysis showed that increased engraftment by *Camkk2*$^{-/-}$ donor cells in irradiated recipients was lineage-specific and largely due to increases in the total number of myeloid (Mac1^{+}) rather than lymphoid cells in the peripheral blood, although by week 15, recipient mice also exhibited a significant increase in circulating *Camkk2*$^{-/-}$ B cells (B220^{+}) (Figure 14C). While assessing the short-term (6 weeks post-BMT), rapid repopulation potential of donor cells (Yang, Bryder et al. 2005) (Figure 14C, *left*), we observed engraftment by *Camkk2*$^{-/-}$ HSCs that was 3-fold higher in the myeloid lineages, when compared with WT HSC transplantation. In addition, a transient decrease in *Camkk2*$^{-/-}$ engraftment of T cells (CD3^{+}) was observed at this time point, but later became comparable with WT CD3^{+} numbers. By 15 weeks after BMT, the long-term reconstitution ability as well as the self-renewal potential of HSCs could be evaluated (Osawa, Hanada et al. 1996), and these results showed a persistent increase in the number of CD45.2^{+} *Camkk2*$^{-/-}$ myeloid cells compared to CD45.2^{+} WT (Figure 14C, *right*). We additionally observed a significant enhancement in the long-term B cell repopulation potential of *Camkk2*$^{-/-}$ donor cells. Furthermore, analysis of recipient spleens revealed differences consistent with those found in the peripheral blood (Figure 14D). Interestingly, we also found that host T cells (CD3^{+}) were significantly increased

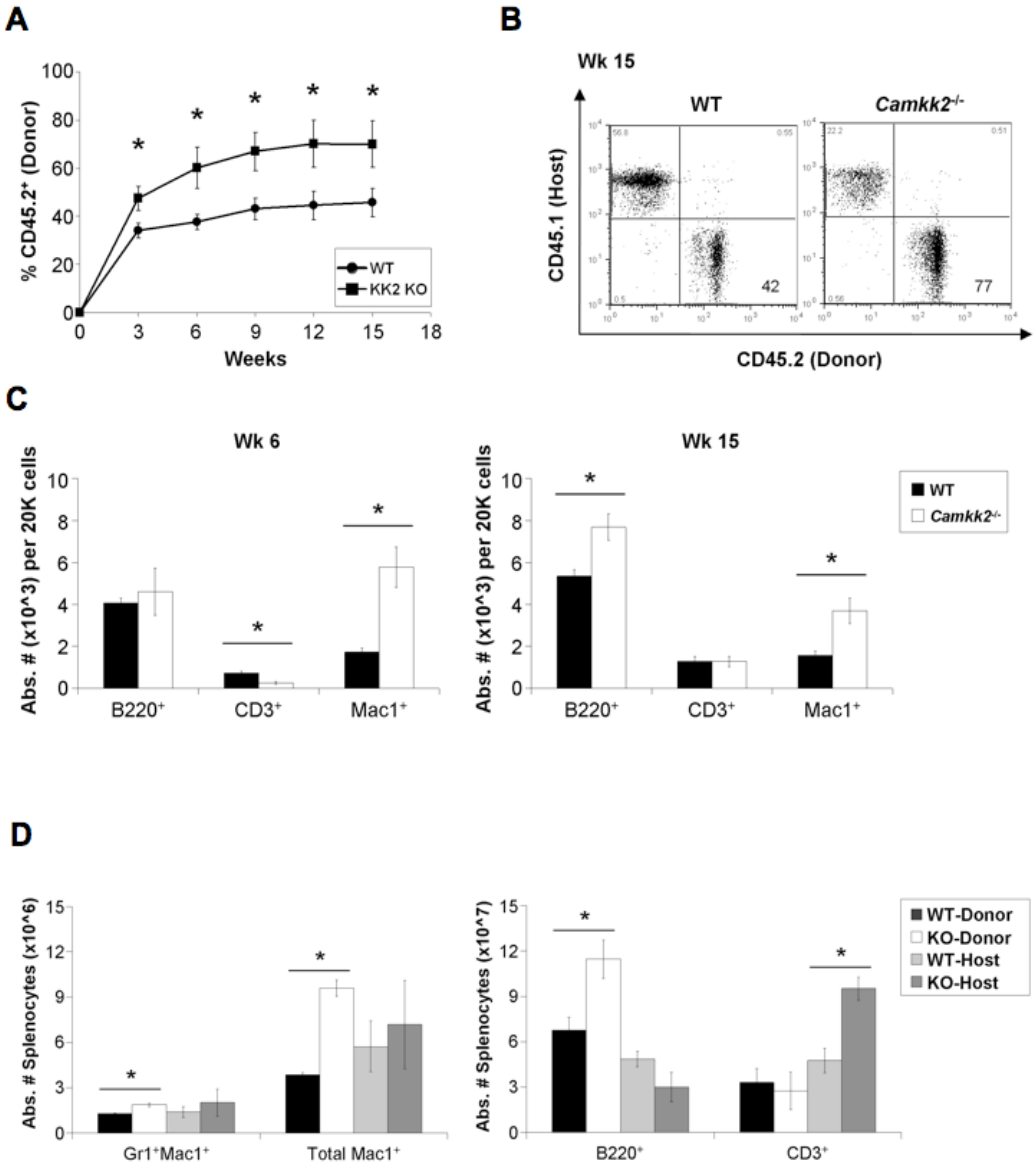

Figure 14: Loss of CaMKK2 alters hematopoietic engraftment and results in the expansion of myeloid and granulocyte lineages during *in vivo* BMT

Donor WT and *Camkk2*$^{-/-}$ (CD45.2$^+$) HSCs were transplanted into irradiated WT (CD45.1$^+$) recipient mice and monitored for ≥ 15 weeks post-BMT. (A) Donor (CD45.2$^+$) chimerism in recipients was measured by FACS analysis of peripheral blood cells stained with anti-CD45.2-APC and anti-CD45.1-FITC. KK2 KO: CaMKK2 knock-out. (B) Representative FACS plots of WT and *Camkk2*$^{-/-}$ donor engraftment in (A). (C) Numbers of CD45.2$^+$ B cells (B220$^+$), T cells (CD3$^+$), and myeloid cells (Mac1$^+$) in

recipients' peripheral blood 6 weeks (*left*) and 15 weeks (*right*) after transplantation (*$p<0.01$). (D) Absolute numbers of donor (CD45.2+) and respective host (CD45.1+) granulocytes (Gr1+Mac1+), total myeloid cells (Mac1+), B cells (B220+), and T cells (CD3+) in the spleens of transplant recipients after 15 weeks post-transplantation ($n > 3$; *$p<0.05$). (A-D) Data are representative of at least three independent experiments ($n \geq 15$; *$p<0.03$).

in the spleens of recipients with *Camkk2*$^{-/-}$ but not WT donor cells. This suggested an accumulation of T lymphocytes occurred, possibly resulting from the increased number of supporting myeloid cells (including macrophages and dendritic cells) from *Camkk2*$^{-/-}$ engraftment.

To understand whether the increases in *Camkk2*$^{-/-}$ mature myeloid and B cells in the recipients were derived from an elevation of stem or progenitor cells, we examined the composition of the donor-reconstituted recipient BM after 15 weeks post-BMT. While the total BM cellularity in WT and *Camkk2*$^{-/-}$ recipients was not different, we found an approximately 2-fold increase in total *Camkk2*$^{-/-}$ donor cells compared to WT (Figure 15A), which correlates with the higher engraftment by *Camkk2*$^{-/-}$ cells observed in the peripheral blood. In our analysis of donor stem/progenitor populations, we did not find statistically significant differences in the number of CD45.2^{+} *Camkk2*$^{-/-}$ HSC, MPP, or KLS cells in the recipient BM, compared to engraftment by WT (Figure 15B), which suggested that the increase in total *Camkk2*$^{-/-}$ engraftment was not due to any proliferation or survival advantages of *Camkk2*$^{-/-}$ HSCs. Furthermore, there were no significant differences in the number of CD45.2^{+} CLP or MEP cells in WT- and *Camkk2*$^{-/-}$-engrafted

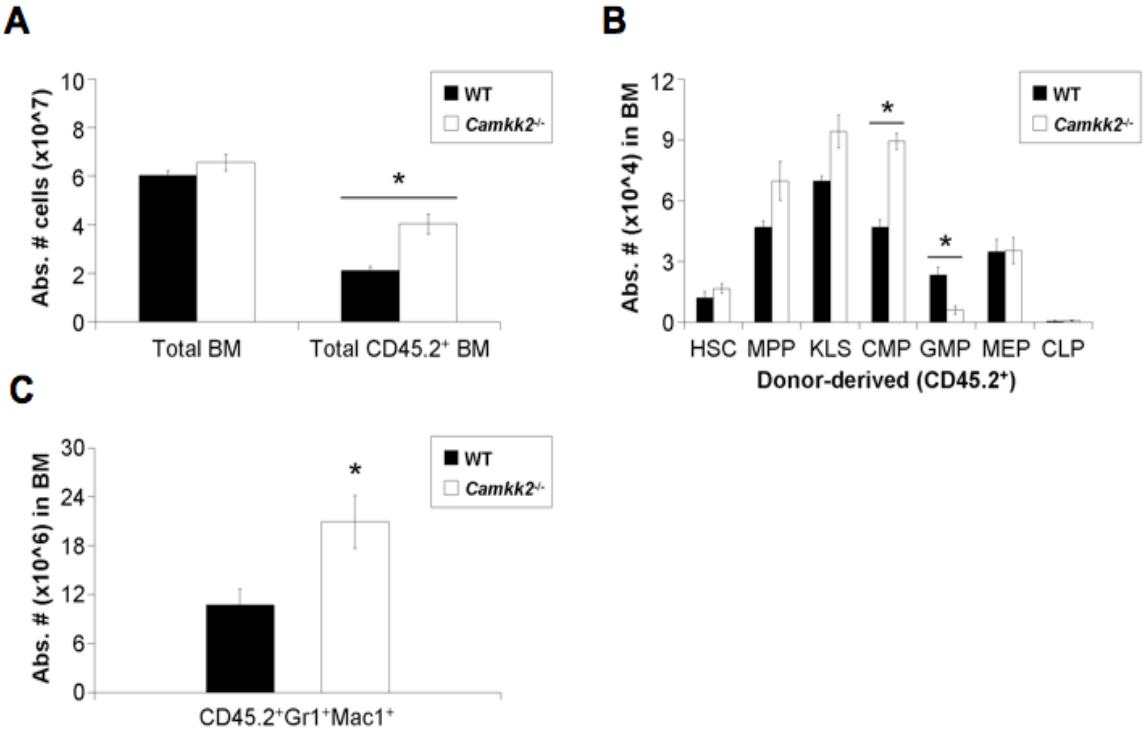

Figure 15: Transplantation of *Camkk2*$^{-/-}$ HSCs leads to enhanced repopulation of the BM, specifically in the myeloid lineage

(A-C) Analysis of recipient BM populations after 15 weeks post-transplantation. (A) Total number of whole BM cells and CD45.2$^+$ BM cells. (B) Total number of CD45.2$^+$ stem and progenitor cells of different subsets. (C) Total number of CD45.2$^+$ granulocytes. (A-C) Data are representative of at least three independent experiments ($n \geq 15$; *$p<0.03$).

recipients. Interestingly, our data did indicate a significant 2-fold increase in the number of CD45.2$^+$ *Camkk2*$^{-/-}$ CMPs and conversely a 4-fold reduction of GMPs (differentiated progeny of CMPs) in the recipient BM, compared to WT. Additionally, we observed a significant 2-fold increase in Gr1$^+$Mac1$^+$ donor granulocytes in *Camkk2*$^{-/-}$ recipient BM relative to WT (Figure 15C), presumably contributing to the increased

number of myeloid cells in the *Camkk2*$^{-/-}$-repopulated peripheral blood. Altogether, these data suggest that CaMKK2 plays a modulatory role in myelopoiesis, particularly in the suppression of granulocyte development. Thus, based on the intriguing differences in myeloid progenitor subsets and mature myeloid cells without a change in lymphoid progenitor cell number compared to WT, we began to evaluate a potential role for CaMKK2 in myelopoiesis and granulocyte development.

Loss of CaMKK2 promotes cell autonomous granulocyte commitment and differentiation from myeloid progenitors

Since the transplantation of *Camkk2*$^{-/-}$ HSCs resulted in increased engraftment in the CMP and granulocyte lineages in the recipient BM and peripheral blood, we surmised that the absence of CaMKK2 results in the accelerated differentiation of CMPs to mature granulocytes. Consistent with our findings in *Camkk2*$^{-/-}$-reconstituted recipient BM, *Camkk2*$^{-/-}$ mice possessed significantly increased frequency and number of CMPs, as well as decreases in GMPs compared to WT (Figure 16A-C). A possible explanation of this anomaly is that in the absence of CaMKK2, GMPs undergo more rapid differentiation into mature granulocytes, which would trigger an expansion of the parental CMPs in order to compensate for the decrease in GMPs. To test this, we first measured the percentage of proliferating CMPs in the BM of WT and *Camkk2*$^{-/-}$ mice and found a significantly higher percentage of BrdU-positive CMPs in *Camkk2*$^{-/-}$ mice, suggesting an increased expansion of *Camkk2*$^{-/-}$ CMPs does occur (Figure 16D).

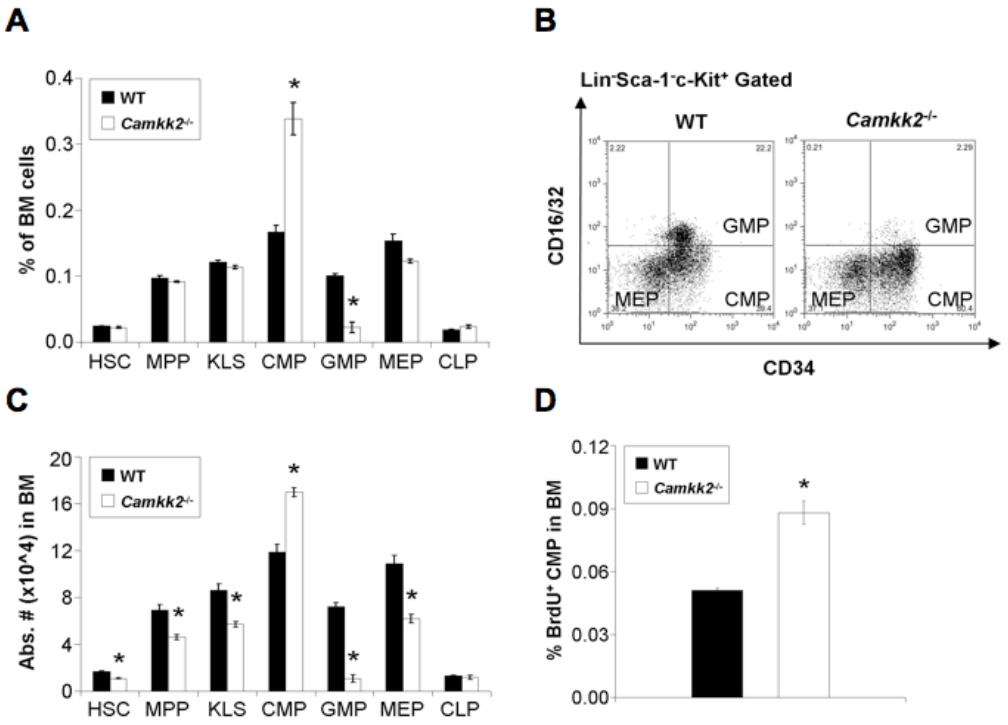

Figure 16: *Camkk2*$^{-/-}$ mice display defects in common myeloid and granulocyte/monocyte progenitors

(A) Percentages of HSC and progenitor populations in BM of WT and *Camkk2*$^{-/-}$ mice (n > 8; *$p<0.01$). (B) Representative FACS analysis of Lin$^-$Sca-1$^-$c-Kit$^+$-gated myeloid progenitor populations (CMP, GMP, and MEP) in BM of WT and *Camkk2*$^{-/-}$ mice. (C) Total number of stem and progenitor cells of different subsets in the BM (n > 8; *$p<0.01$). (D) Percentages of BrdU-positive CMPs in BM of WT and *Camkk2*–/– mice (n ≥ 3; *$p<0.01$).

To confirm whether loss of CaMKK2 resulted in accelerated granulocyte differentiation, we performed Giemsa staining of the immature, developing Gr1^{lo}Mac1$^{lo/+}$ granulocytes (Hestdal, Ruscetti et al. 1991; Kastner, Lawrence et al. 2001) isolated from the BM of WT and *Camkk2*$^{-/-}$ mice. As indicated in Figure 17A and 17B, the Gr1^{lo}Mac1$^{lo/+}$

population of early committed granulocytes from *Camkk2*$^{-/-}$ mice consisted of a 4-fold higher number of cells displaying condensed band or segmented nuclear morphology, a hallmark of terminally differentiated, mature granulocytes (Hestdal, Ruscetti et al. 1991). Moreover, *Camkk2*$^{-/-}$ mice had a significantly higher frequency and number of mature granulocytes (Gr1^{+}Mac1^{+}) in the BM than WT mice (Figure 17C-E), as seen before in the BM and peripheral blood of *Camkk2*$^{-/-}$-transplanted recipients (Figure 14C; Figure 15C). These developmental defects in the BM of *Camkk2*$^{-/-}$ mice appeared to be specific to the myeloid and granulocyte populations, as there were no significant differences in B lymphocyte and erythroid (Ter119^{+}) lineages (Figure 17C-E). We also measured circulating granulocytes in the peripheral blood to assess if the *Camkk2*$^{-/-}$ mice exhibited neutrophilia, but found no significant differences (Figure 17F, G). Nonetheless, our data corroborate that the *in vivo* deficiency of CaMKK2 leads to enhanced granulocytic differentiation in the BM.

The accelerated granulocyte differentiation observed in *Camkk2*$^{-/-}$ mice suggested that the intrinsic deficiency of CaMKK2 in myeloid progenitors increases their commitment and differentiation towards the granulocyte lineage. To directly test this hypothesis, WT and *Camkk2*$^{-/-}$ CMP cells were isolated and co-cultured with OP9 cells in media supplemented with myeloid growth factors to induce differentiation. The cells were then harvested and stained for FACS analysis of Gr1^{+}Mac1^{+} granulocytes versus Gr1^{-}Mac1^{+} monocytic populations. Similar to our earlier results of enhanced myeloid

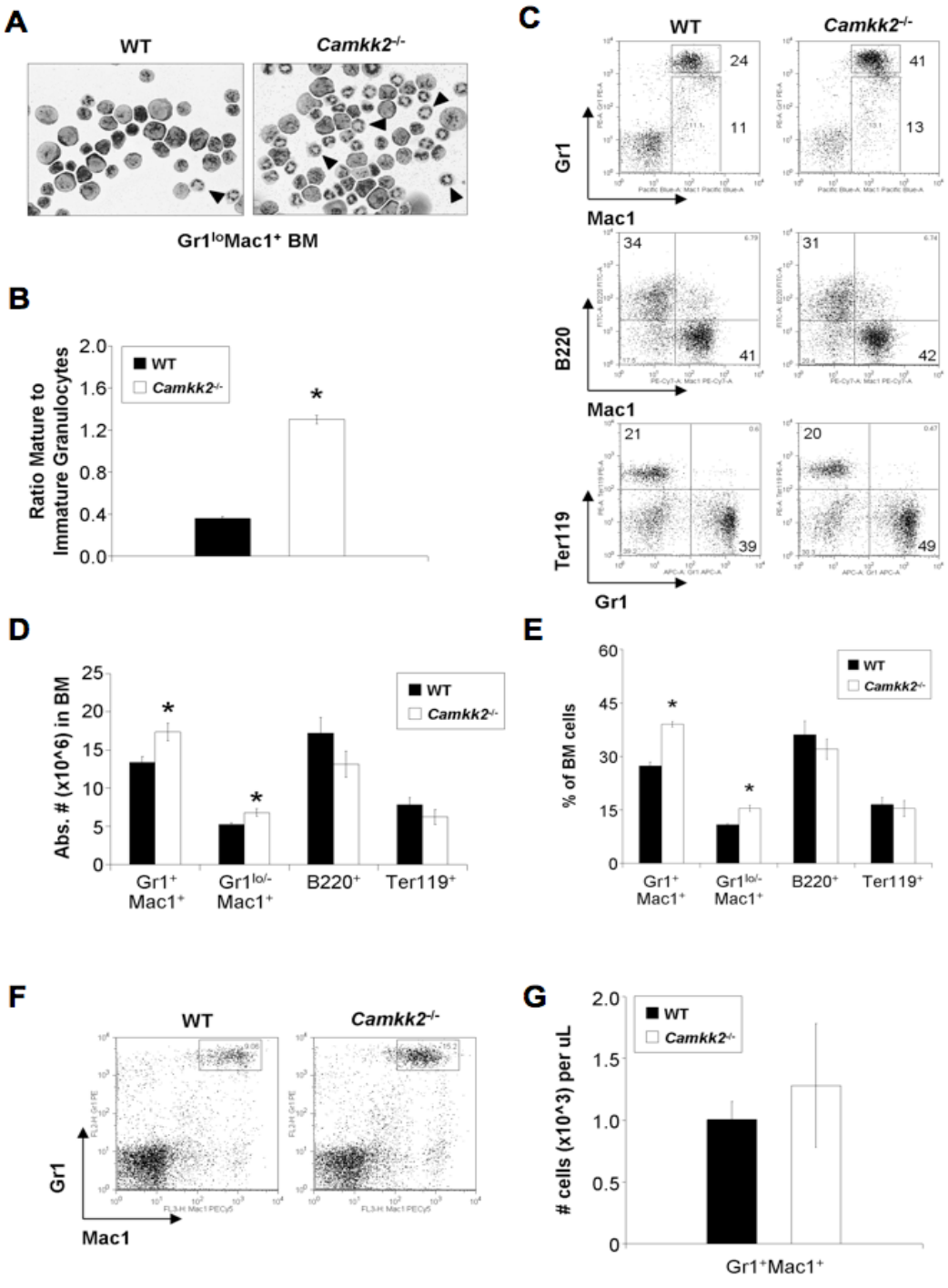

Figure 17: *Camkk2*$^{-/-}$ mice display enhanced granulopoiesis in the BM

(A) Cytological images demonstrating the morphology of immature, developing granulocytes ($Gr1^{lo}Mac1^{+}$) that were sorted from the BM of WT and *Camkk2*$^{-/-}$ (KO) mice, then subjected to Giemsa staining. Arrowheads indicate mature granulocytes, which exhibit band or segmented nuclear morphology. Images were acquired with an Axio Observer Z1 microscope (Carl Zeiss) at original magnification x 630. (B) Quantification of (A) showing ratios of mature granulocytes over immature granulocytes (i.e. promyelocytes/myelocytes/metamyelocytes) (n = 4; ≥ 300 cells scored per individual; *$p<0.001$). (C) Representative FACS analysis of mature myeloid, B, and erythroid lineages in BM. (D) Total number of mature granulocytes ($Gr1^{+}Mac1^{+}$), monocyte-lineage myeloid cells ($Gr1^{lo/-}Mac1^{+}$), B cells ($B220^{+}$), and erythroid cells ($Ter119^{+}$) in the BM of WT and *Camkk2*$^{-/-}$ mice, 3-4 weeks old (n = 5; *$p<0.05$). (E) Percentages of granulocytes (Gr1+Mac1+), monocyte-lineage myeloid cells (Gr1lo/-Mac1+), B cells (B220+), and erythroid (Ter119+) populations in WT and *Camkk2*$^{-/-}$ BM (n ≥ 8; *$p<0.01$). (F) Representative FACS plots of Gr1 x Mac1 myeloid cells in the peripheral blood of WT and *Camkk2*$^{-/-}$ mice. (G) Complete blood count (CBC) showing numbers of granulocytes in WT and *Camkk2*$^{-/-}$ peripheral blood.

repopulation in *Camkk2*$^{-/-}$-reconstituted recipients, these results indicated a significant increase in both granulocyte and monocyte populations (Figure 18A). Yet, while we observed a 19-fold increase in *Camkk2*$^{-/-}$ versus WT granulocytes, the increase in monocytic cells was smaller (i.e. 6-fold) in *Camkk2*$^{-/-}$ versus WT, suggesting a preferential lineage commitment of *Camkk2*$^{-/-}$ myeloid progenitors to differentiate into granulocytes rather than monocytes. To ensure that the granulocytic differentiation phenotype was specifically due to the absence of CaMKK2, we re-expressed CaMKK2 in *Camkk2*$^{-/-}$ CMPs and assessed their function *in vitro*. After confirming that CaMKK2 was restored (~2.5-fold relative to WT-Control) in *Camkk2*$^{-/-}$ CMPs (Figure 18B), we tested their differentiation potential and confirmed that the add-back of CaMKK2 to *Camkk2*$^{-/-}$ CMPs was able to significantly reverse enhanced granulopoiesis (Figure 18C, D).

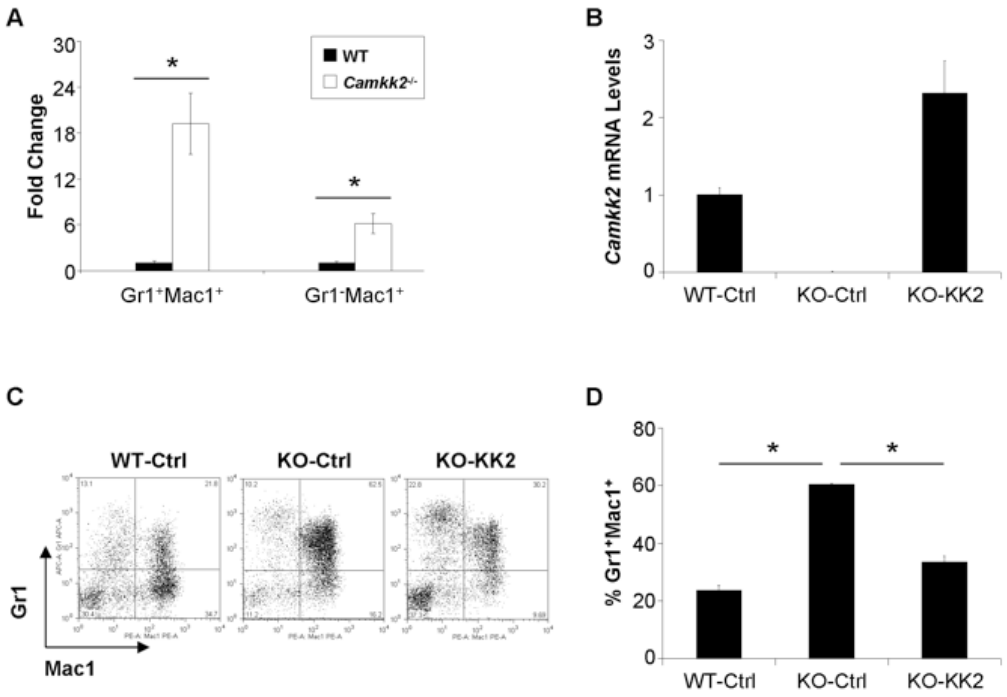

Figure 18: CaMKK2 deficiency in CMP cells results in increased granulocytic differentiation, and its re-expression in these cells is sufficient to rescue abnormal granulopoiesis *in vitro*

(A) Fold change relative to WT of $Gr1^+Mac1^+$ and $Gr1^-Mac1^+$ cells that arose from *Camkk2*$^{-/-}$ CMPs cultured and differentiated for 8-12 days *in vitro* (n = 3; *$p<0.001$). (B) Quantitative RT-PCR analysis of *Camkk2* mRNA in WT-Control (WT-Ctrl), CaMKK2 KO-Ctrl, and CaMKK2 KO-CaMKK2 (added back) CMP cells. (C) Representative FACS analysis of Gr1/Mac1 cells differentiated *in vitro* from WT-Ctrl, CaMKK2 KO-Control, and CaMKK2 KO-CaMKK2 (added back) CMP cells. (D) Quantified average percentage of $Gr1^+Mac1^+$ cells from (C).

In addition to evaluating the effects of the absence of CaMKK2 in CMPs, we examined the differentiation potential of WT and *Camkk2*$^{-/-}$ GMPs via *in vitro* methylcellulose-based CFU assays. In the presence of growth factors, GMPs

differentiate into CFU-G and CFU-M of the granulocyte and macrophage/monocyte lineages, respectively (Figure 19A). We found that *Camkk2*$^{-/-}$ GMP cells gave rise to a significant 3-fold higher number of CFU-G, and 1.5-fold higher number of total colonies than WT (Figure 19B). There was also a decrease, albeit not significant, in the number of CFU-M that formed from *Camkk2*$^{-/-}$ GMPs compared to WT. Collectively, our findings thus far indicate that the absence of CaMKK2 in myeloid progenitors leads to increased granulocytic commitment and differentiation in a cell autonomous manner.

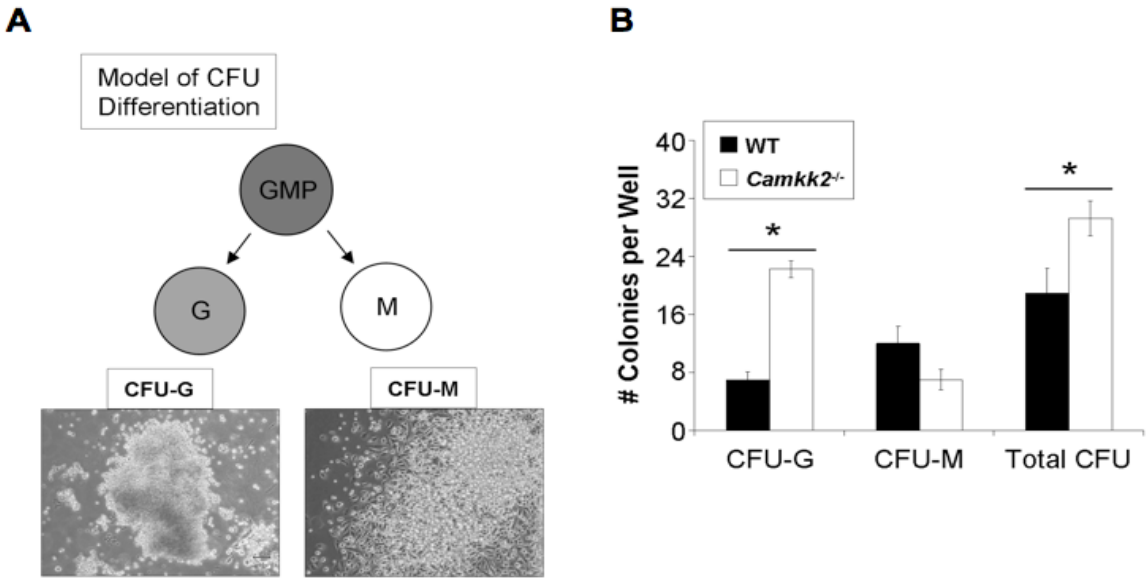

Figure 19: Loss of CaMKK2 in GMP cells leads to increased formation of granulocyte-lineage colonies *in vitro*

(A) Schematic model of *in vitro* colony-forming unit (CFU) differentiation. Representative images of CFU-G (granulocytic) and CFU-M (macrophage/monocytic) were acquired with a CK40 microscope (Olympus) at original magnification x 100. (B) Total numbers of lineage specific- and total CFUs that developed from WT and *Camkk2*$^{-/-}$ GMPs after 7 days of culture in Complete Methocult media (n = 4; *$p<0.05$).

CaMKK2 is expressed in myeloid progenitors, but is absent in terminally differentiated granulocytes

The enhanced granulopoiesis phenotype resulting from the cell-intrinsic loss of CaMKK2 in myeloid progenitors supports the idea that CaMKK2 functions as an inhibitor of granulocyte differentiation in myeloid progenitors. To test this idea during granulopoiesis, we first evaluated CaMKK2 gene expression in progenitor cells by quantitative RT-PCR and found that *Camkk2* mRNA was expressed in WT CMP, GMP, and MEP cells, but was vastly decreased (>30-fold) in mature granulocytes, suggesting that CaMKK2 expression becomes down-regulated when myeloid progenitors differentiate into granulocytes (Figure 20A). Next, we evaluated whether C/EBPα and PU.1, two master regulators of early myeloid lineage commitment that are essential for granulocyte development (Scott, Simon et al. 1994; Zhang, Zhang et al. 1997; DeKoter, Walsh et al. 1998; Wang, Scott et al. 1999), were altered between WT and *Camkk2*$^{-/-}$ CMP cells. Compared to WT, *Camkk2*$^{-/-}$ CMPs showed a significant 2-fold increase in C/EBPα and PU.1 mRNA, whereas the levels of Pax5 and E2A, which are involved in early lymphoid lineage differentiation and included in the experiment as negative controls (Urbanek, Wang et al. 1994; O'Riordan and Grosschedl 1999), were not different (Figure 20B). These data confirm our earlier contention that the absence of CaMKK2 primes myeloid progenitors for granulocyte fate commitment and accelerated granulopoiesis.

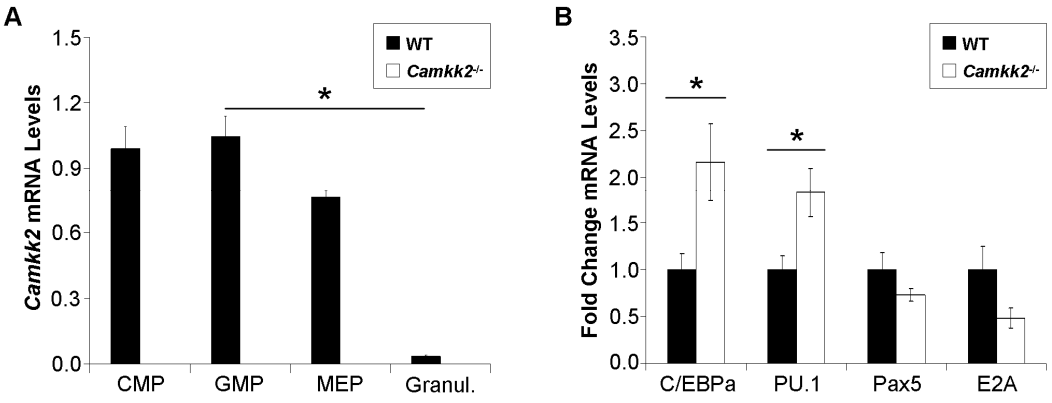

Figure 20: *Camkk2* mRNA is expressed in myeloid progenitors but is absent in fully differentiated granulocytes, while *Camkk2*$^{-/-}$ CMPs contain higher mRNA levels of transcription factors essential for granulocyte-lineage commitment

(A) Quantitative RT-PCR analysis of *Camkk2* mRNA levels in myeloid progenitor subsets and mature granulocytes from the BM, normalized by β2-microglobulin ($n \geq 3$). (B) CMPs were isolated and analyzed by quantitative RT-PCR for the expression of genes involved in hematopoietic cell differentiation and fate commitment, normalized by β2-microglobulin (n = 6 mice from 2 independent experiments; $*p<0.05$).

We next questioned the presence of CaMKK2 in mature Gr1$^+$Mac1$^+$ BM granulocytes from WT, *Camkk2*$^{-/-}$, and *Camkk1*$^{-/-}$ mice (where both knock-out genotypes were used as a control). After cell isolation, we determined the purity of the granulocyte preparation from BM to be > 90% Gr1$^+$Mac1$^+$ by FACS (Figure 21A). Granulocytes were then immunoblotted for CaMKK2 using a pan-CaMKK antibody, and as a control for CaMKK2 immunoblotting, we included lysates from 32Dcl3 myeloid progenitor cells that expressed either control- or CaMKK2-shRNA. These results revealed CaMKK2 to be undetectable at the protein level in terminally differentiated granulocytes (Figure

21B). Taken together, these data reinforce the hypothesis that loss of CaMKK2 in myeloid progenitor cells promotes differentiation as evidenced by increased levels of C/EBPα and PU.1, and reveal that CaMKK2 is decreased during granulocyte development *in vivo*.

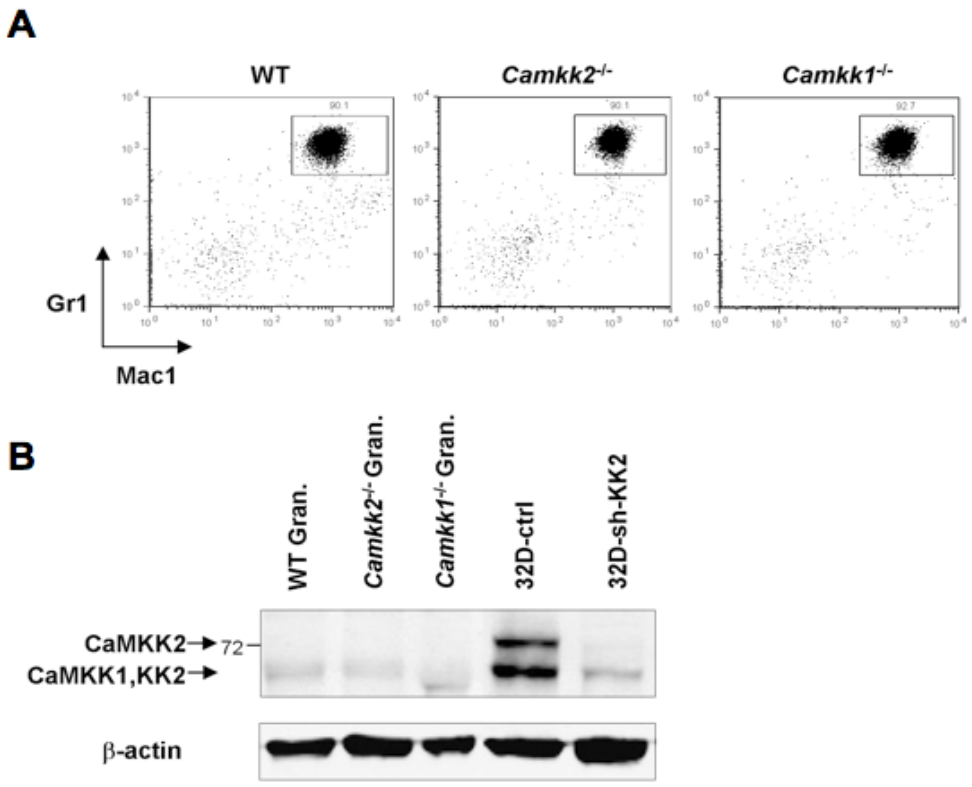

Figure 21: CaMKK2 is undetectable at the protein level in mature granulocytes

(A) Representative FACS analysis of granulocytes purified by density-gradient centrifugation from WT, *Camkk2*$^{-/-}$, and *Camkk1*$^{-/-}$ BM. (B) Representative immunoblot of 2 experiments to evaluate expression of CaMKK2 in Gr1^{+}Mac1^{+} terminally differentiated granulocytes purified from the BM of WT, *Camkk2*$^{-/-}$, and *Camkk1*$^{-/-}$ mice (the latter generated in our lab, and shown here as a control for CaMKK band discrimination). Lysates of 32Dcl3 myeloblast cells ± control-shRNA (ctrl) or CaMKK2-shRNA (sh-KK2) served as controls for CaMKK2 immunoblotting, and β-actin served as a loading control.

Granulocyte differentiation of 32Dcl3 myeloblast cells results in decreased CaMKK2

To better understand the role of CaMKK2 in myeloid progenitors at the transition point of early granulocyte differentiation, we utilized the well-established murine 32Dcl3 (herein referred to as 32D) myeloid progenitor cell line as a secondary model to study the role of CaMKK2 in granulocyte development (Valtieri, Tweardy et al. 1987; Nakajima and Ihle 2001). When 32D cells were treated with G-CSF, they terminally differentiated into granulocytes as indicated by the continuous increase in percentage of $Gr1^{+}Mac1^{+}$ cells over at least 9 days following G-CSF treatment (Figure 22A, B). To evaluate whether CaMKK2 decreased during granulocyte differentiation of 32D cells as was suggested by the preceding *in vivo* data, we examined CaMKK2 mRNA and protein in G-CSF treated 32D cells as a function of time. Our results revealed that *Camkk2* mRNA is significantly decreased (~ 2.3 fold) after 6 days of G-CSF treatment (Figure 22C). However, CaMKK2 protein was markedly down-regulated (~ 65%) within 3 days and became undetectable after 6 days of G-CSF treatment (Figure 22D, E). These data are consistent with our earlier observations that CaMKK2 cannot be detected in mature BM granulocytes (Figure 21B) and suggest that G-CSF stimulates the post-transcriptional down-regulation of CaMKK2 during granulocyte differentiation.

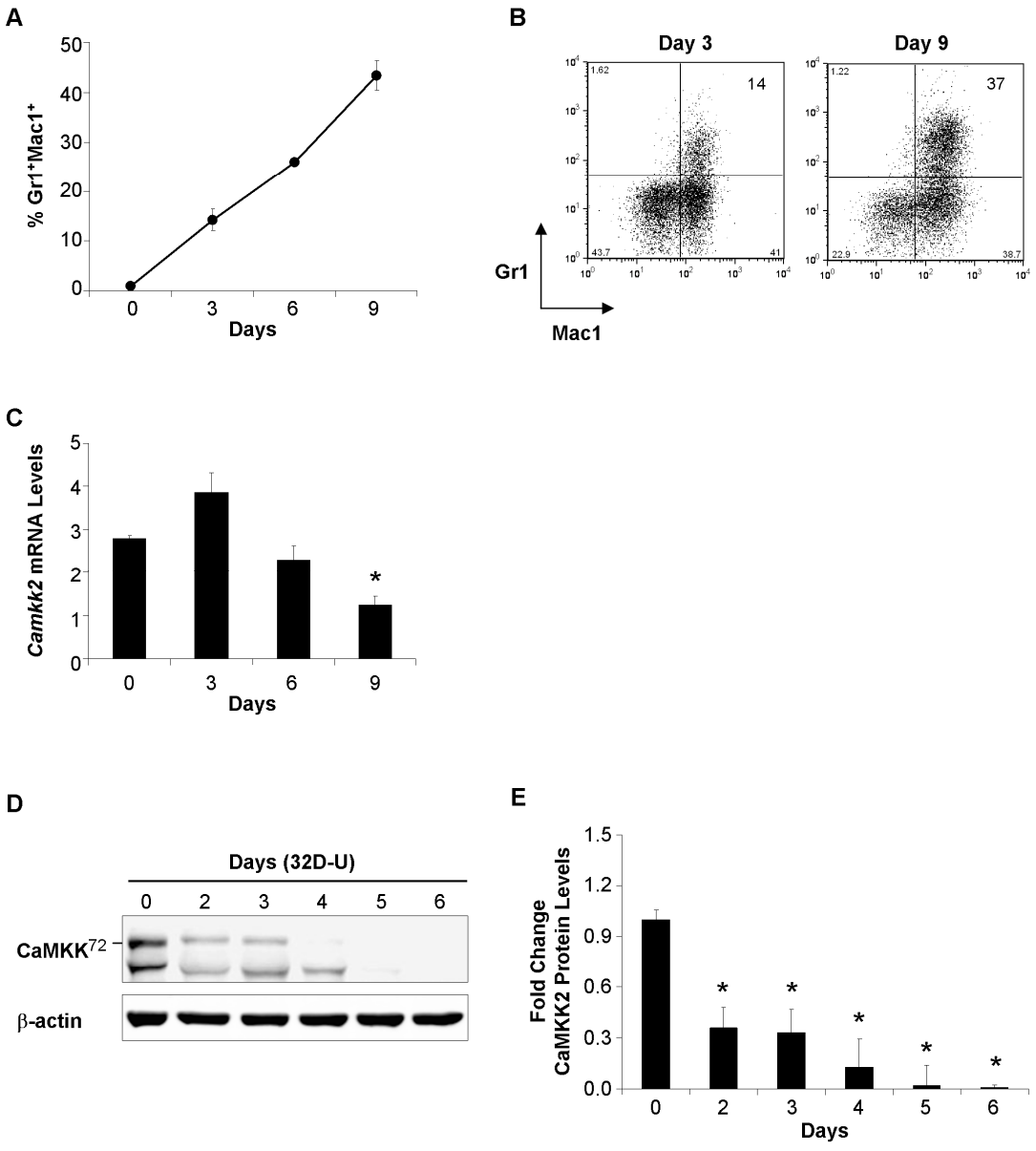

Figure 22: CaMKK2 is markedly down-regulated during early granulocyte differentiation of 32Dcl3 myeloblast cells

(A) Percentages of 32D cells that differentiated into $Gr1^+Mac1^+$ cells over a time-course of 9 days after G-CSF treatment (n = 3, plated in triplicate). (B) Representative FACS plots demonstrating 32D cell differentiation in (A). (C) Quantitative RT-PCR analysis of *Camkk2* mRNA levels during 32D cell differentiation over 9 days, normalized by β2-microglobulin (n = 2, plated in triplicate; *$p<0.01$). (D) CaMKK2 protein levels assessed

by immunoblot analysis as a function of time during G-CSF-induced 32D cell differentiation. (E) Quantification of (D) showing CaMKK2 protein levels relative to Day 0, normalized by β-actin (n = 3, *$p<0.02$).

CaMKK2 represses granulocytic differentiation, independently of AMPK, CaMKI, or CaMKIV

Since a reduction in CaMKK2 occurs as 32D cells undergo granulocytic differentiation, we investigated whether forced expression of CaMKK2 in these cells would inhibit granulocyte differentiation. To this end, 32D cells were transduced with lentiviruses encoding control green fluorescent protein (Ctrl), WT CaMKK2 (KK2), or catalytically inactive CaMKK2 (D311A (Anderson, Means et al. 1998)). Uninfected (U) and lentivirus-infected 32D cells were stimulated with G-CSF for 6 days, and cell samples in each category from Day 0 and Day 6 of treatment were Wright's stained, then analyzed to quantify immature and mature granulocytes based on nuclear morphology. Immature granulocytes such as myeloblasts, promyelocytes, and myelocytes contain rounded nuclei while mature granulocytes such as metamyelocytes and neutrophils display band or segmented-shaped nuclei (Bainton, Ullyot et al. 1971). While there were no significant differences in the percentage of mature granulocytes among 32D-U, -Ctrl, or -D311A, after 6 days of G-CSF treatment, we observed a significant, 3-fold decrease in the percentage of mature granulocytes among 32D-KK2 cells (Figure 23A, B), indicating repression of granulopoiesis by CaMKK2 in a kinase-dependent manner.

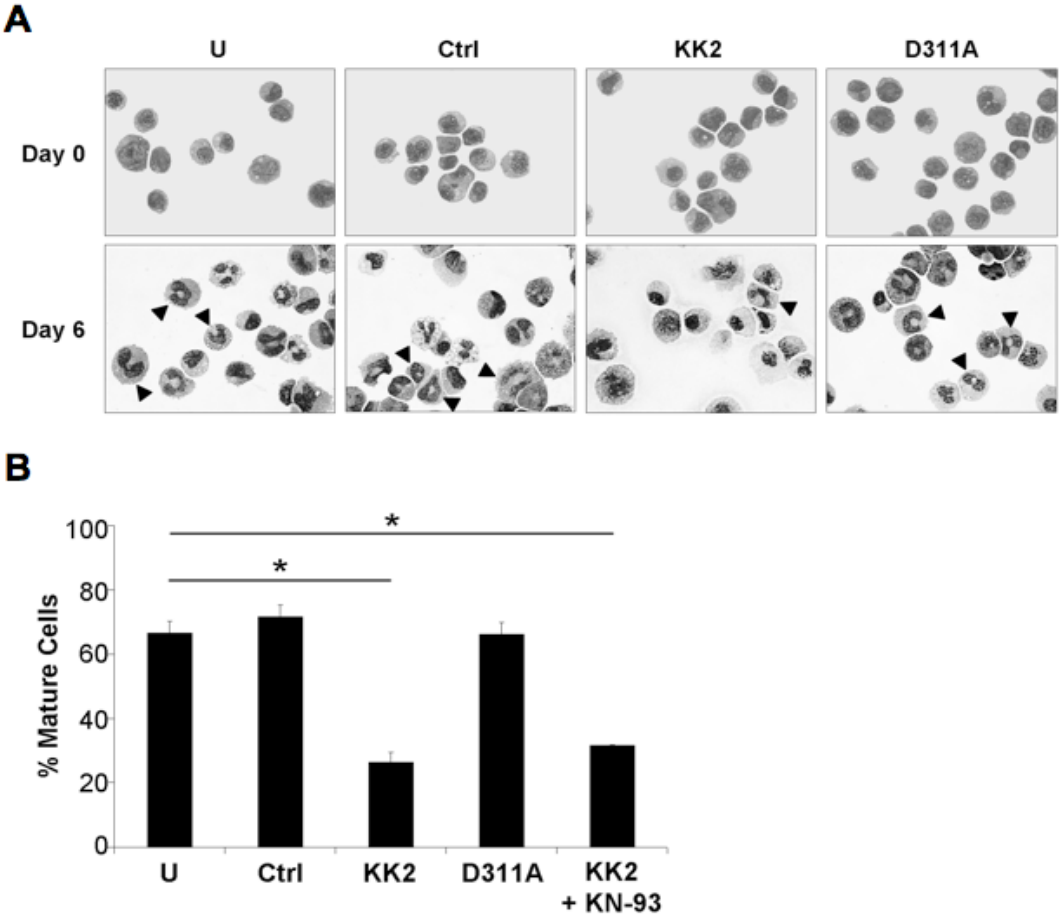

Figure 23: CaMKK2 over-expression in 32Dcl3 cells is sufficient to repress granulocyte differentiation in a kinase-dependent manner

A) Uninfected (U) or 32D cells transduced with lentiviral empty vector-green fluorescent protein control (Ctrl), CaMKK2 (KK2), or kinase-inactive CaMKK2^{D311A} (D311A) were induced to differentiate with G-CSF, then Wright's-stained. Arrowheads indicate cells with mature nuclear morphology. Images were taken at original magnification x 630. (B) Quantification of (A) and (Supplemental Fig. 4B) showing percentages of mature cells on Day 6 (n = 3, and ≥ 150 cells scored per sample; *$p<0.01$).

To determine whether this CaMKK2-mediated effect occurred through its downstream targets, CaMKI or CaMKIV, we first evaluated if these substrates are expressed in 32D cells by immunoblot (Figure 24A). These results indicate that CaMKI, which has been reported to be ubiquitously expressed (Hook and Means 2001; Chow and Means 2007), is present in 32D cells. However, CaMKIV was not detectable in these cells, which is consistent with previous findings that it is more tissue-restricted in its expression than is CaMKI (Means, Ribar et al. 1997; Means 2000). We next investigated whether inhibiting the function of CaMKI would be sufficient to relieve CaMKK2-mediated inhibition of granulocyte differentiation, and performed G-CSF-dependent granulocyte differentiation of 32D-KK2 cells in the presence of KN-93, a pharmacological inhibitor of CaMKs I, II, and IV (Anderson, Means et al. 1998; Colomer-Font and Means 2007). KN-93 did not abrogate CaMKK2-mediated repression of granulocyte differentiation suggesting that neither CaMKI (nor IV or II) was functioning downstream of CaMKK2 (Figure 24B; Figure 23B). As a positive control for CaM kinase inhibition of 32D cells, we confirmed that 2.5 μM KN-93 (which was the maximal dose these cells could tolerate without cytotoxicity) inhibited the Ca^{2+}-induced autophosphorylation of CaMKII, a CaMK not phosphorylated by CaMKK2 but inhibited by KN-93 with a similar inhibition constant (K_i) to CaMKI and CaMKIV (Figure 24C, D) (Means 2000). Altogether, these data indicate that CaMKK2 represses granulocyte differentiation of 32D cells in a CaMK-independent manner, although this

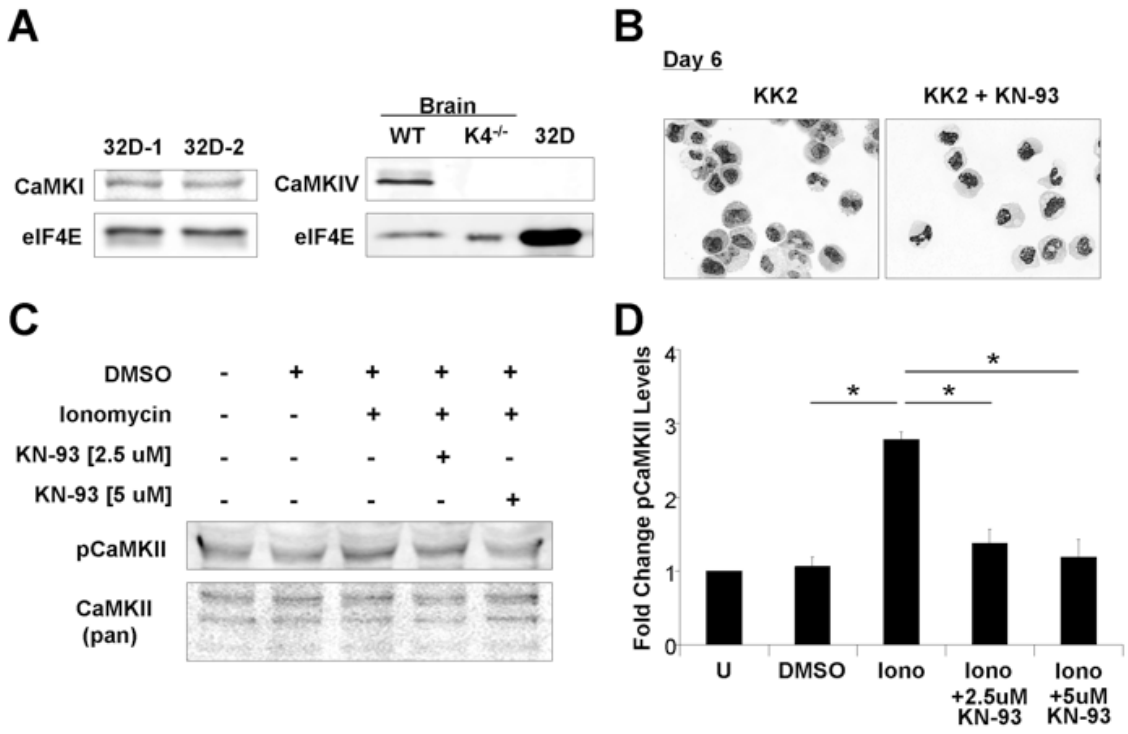

Figure 24: CaMKI is expressed in 32D cells, however KN-93 does not affect CaMKK2-mediated inhibition of 32D differentiation

(A) Immunoblot analysis of CaMKI (shown here in duplicate as 32D-1 and 32D-2) and CaMKIV expression in 32D cells. eIF4E served as a loading control. Brain lysates from WT and *Camk4*–/– mice, generated as previously described (Kitsos, Sankar et al. 2005), were included as controls for CaMKIV immunoblotting. (B) Representative Day 6 images of G-CSF-stimulated CaMKK2-transduced 32D cells (KK2) and KK2 cells that were treated with 2.5 μM KN-93 on Day 0, then refreshed every 2 days with drug-containing media (KK2 + KN-93). Images were acquired at original magnification x 630 (n = 2). (C) Immunoblot analysis of phospho- and total CaMKII in 32D cells that were either untreated (U) or treated with vehicle alone (DMSO), ionomycin (Iono) [2 uM] for 5 min, or pre-treated with KN-93 [2.5 or 5 uM] for 1 h prior to ionomycin treatment. (D) Quantification of (C), showing fold chage of phospho-CaMKII levels normalized by total CaMKII (n = 3; *$p<0.05$).

function requires CaMKK2 activity as the kinase-inactive CaMKK2^{D311A} mutant failed to impede granulopoiesis.

As KN-93 treatment of 32D-KK2 cells did not rescue granulocyte differentiation and because CaMKIV was undetectable in 32D cells (Figure 24A), these data suggest that CaMKI and CaMKIV are unlikely to be relevant downstream substrates of CaMKK2 during granulopoiesis. We next investigated whether AMPK, the third well-established substrate of CaMKK2 (Hawley, Pan et al. 2005; Hurley, Anderson et al. 2005; Witters, Kemp et al. 2006), was involved in blocking granulocyte development downstream of CaMKK2. To this end, we first evaluated phosphorylated AMPK (pAMPK) in 32D-KK2, 32D-D311A and control cells, and found no significant differences in pAMPK in any of the cell lines (Figure 25A, B). In addition, we evaluated the amount of CaMKK2 in these cells, and found it to be over-expressed approximately 12-18-fold in 32D-KK2 and -D311A lines relative to the control level (Figure 25C). Next, we knocked down CaMKK2 by short hairpin RNA-interference (sh-KK2) in 32D cells and also found pAMPK unchanged (Figure 25D, E). To further examine whether Ca^{2+}-signaling in these cells regulates phosphorylation of AMPK, we treated 32D cells with ionomycin to increase intracellular calcium. This induced increase of intracellular Ca^{2+} in 32D cells did not change the levels of pAMPK (Figure 25F, G). Taken together, these results suggest that the phosphorylation of AMPK is unlikely to depend on CaMKK2 in 32D myeloid progenitor cells. Thus, although we cannot identify the substrate responsible, our

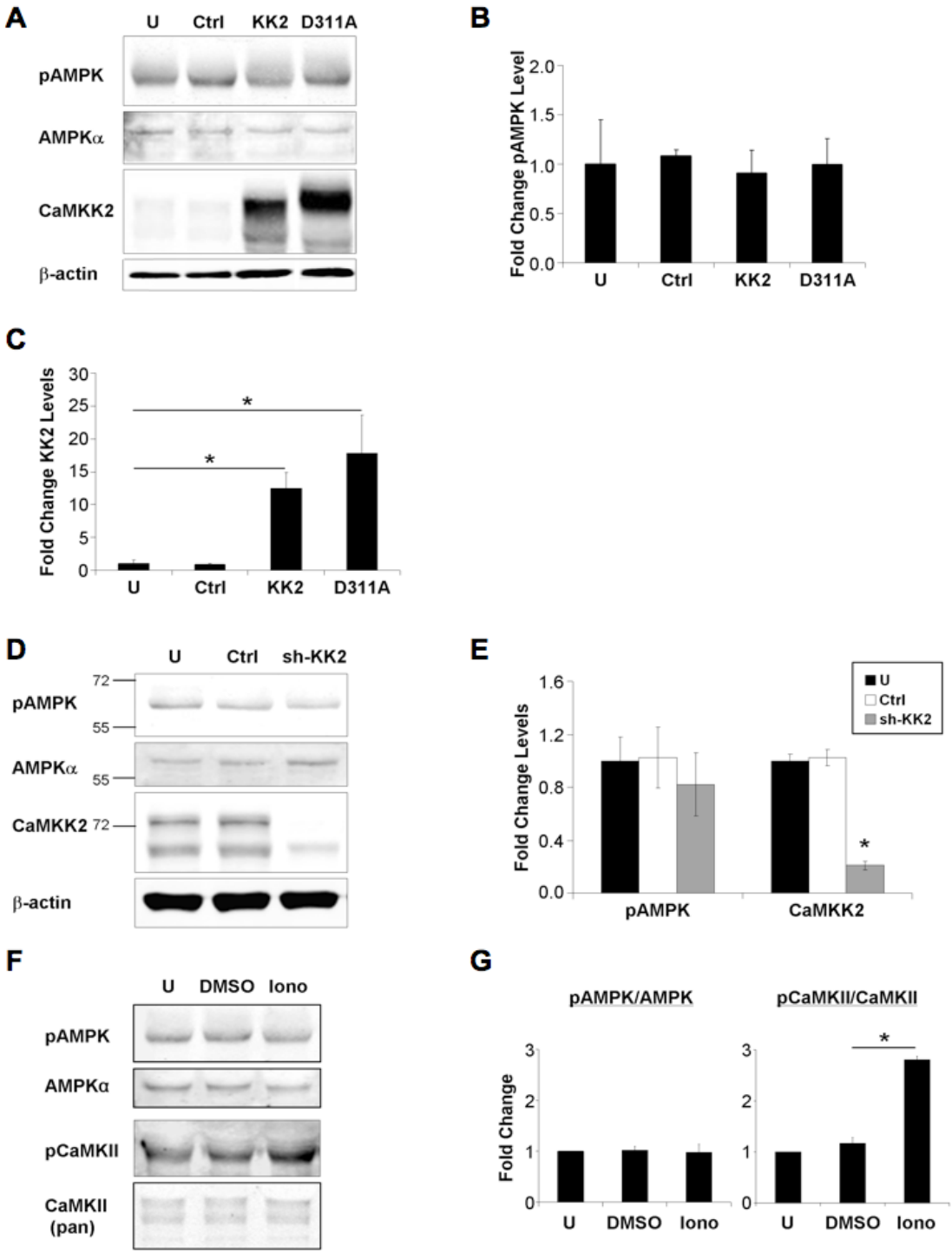

Figure 25: Neither alteration of CaMKK2 levels nor increased Ca^{2+}-signaling influence AMPK phosphorylation in 32D cells

(A) Undifferentiated cell lines as shown in Figure 22A were analyzed by immunoblot for levels of phosphorylated AMPKα (pAMPK), total AMPKα, CaMKK2, and β-actin. (B, C) Quantification of (A) showing fold change of (B) pAMPK levels normalized by total AMPKα, and (C) CaMKK2 levels normalized by β-actin in transduced cells relative to 32D-U (n = 3; *$p<0.05$;). (D) Immunoblot analysis of pAMPKα and CaMKK2 in CaMKK2-knock-down (sh-KK2) 32D cells compared to 32D-U and -Ctrl cells. (E) Quantification of (D) showing fold change of pAMPK and CaMKK2 levels relative to 32D-U, normalized as in (B, C) (n = 3; *$p<0.001$). (F) Immunoblot analysis of phospho- and total AMPK, as well as phospho- and total CaMKII (as controls) in 32D cells treated with ionomycin as in Figure 23C. (G) Quantification of (F), showing phospho-proteins normalized by respective total protein amounts (n = 3; *$p<0.05$).

collective data support the notion that CaMKK2 plays a cell-intrinsic role in the restriction of granulocyte differentiation in myeloid progenitor cells of homeostatic BM.

4.3 Conclusions

Granulocytes serve a critical function in the innate immune system by maintaining a frontline defense against bacterial and fungal pathogens. However, precise control of granulocyte number is important to provide an appropriate balance between effective immunity versus immunosuppression or inflammation (Nakamura, Ueki et al. 2000; Boxer and Dale 2002). In this study, we identify a role for CaMKK2 in the repression of granulocytic fate commitment and differentiation during early granulopoiesis. Furthermore, we also show that unlike CaMKIV, CaMKK2 is not an essential regulator of HSC homeostasis. These results, taken together with the myeloid defects shown here in *Camkk2*$^{-/-}$ mice that are not present in *Camk4*$^{-/-}$ mice (Kitsos, Sankar

et al. 2005), suggest that the CaMKK2-CaMKIV signaling cascade is not functionally relevant in the biology of early hematopoietic cells. Rather, our findings demonstrate that CaMKK2 functions as a critical regulator of myeloid cell fate.

The absence of CaMKK2 in mice results in increased CMPs, decreased GMPs, and increased granulocytes in the BM under basal conditions. Similarly, when *Camkk2*$^{-/-}$ HSCs are transplanted into lethally irradiated recipient mice, an expansion of granulocytes occurs both in the BM and peripheral blood. These results demonstrate that in both basal and stressed conditions (i.e. bone marrow transplantation), the loss of CaMKK2 leads to an aberrant increase in granulopoiesis *in vivo*. Based on our results, we suggest that the accelerated differentiation of *Camkk2*$^{-/-}$ GMPs into granulocytes results in the reduction of the GMP population and concomitant increase in CMPs in the null mice possibly as a feed-forward mechanism to compensate for a paracrine-detected deficiency of GMPs.

Indeed, cytological examination of immature granulocytes (Gr1^{lo}Mac1$^{lo/+}$) from *Camkk2*$^{-/-}$ mice revealed robust, accelerated granulocyte differentiation compared to WT. Moreover, *Camkk2*$^{-/-}$ GMPs formed significantly more CFU-G *in vitro*, and *Camkk2*$^{-/-}$ CMPs differentiated into more Gr1^{+}Mac1^{+} cells compared to WT CMPs. When CaMKK2 was ectopically re-expressed in *Camkk2*$^{-/-}$ CMPs, this was sufficient to rescue the enhanced granulopoiesis phenotype, indicating that the differentiation effect in CaMKK2 null cells was specifically due to the loss of CaMKK2. Altogether, these data

support a cell autonomous role for CaMKK2 in restricting granulocytic commitment and differentiation during early granulopoiesis.

In correlation with our conclusions that CaMKK2 plays an inhibitory role in granulocyte commitment and differentiation, we found that the mRNA levels of C/EBPα and PU.1, two transcription factors essential for granulocyte differentiation, were significantly elevated in *Camkk2*$^{-/-}$ CMPs, suggesting enhanced granulocyte-lineage commitment at the transcriptional level of CaMKK2 null CMPs. We also found that while CaMKK2 mRNA is present in primary myeloid progenitors, it is very lowly if at all expressed in terminally differentiated granulocytes. Similarly, our analysis of CaMKK2 protein levels in granulocytes also suggested that it is not expressed in these cells, and in our studies of 32D cells during granulocyte differentiation, we found that CaMKK2 expression was down-regulated as a function of time.

Furthermore, the over-expression of CaMKK2 in 32D cells was able to significantly repress granulocyte differentiation in a kinase-dependent manner, as over-expression of the inactive CaMKK2 mutant failed to inhibit differentiation. We additionally tested whether the three known targets of CaMKK2, namely CaMKI, CaMKIV, and AMPK, were involved in CaMKK2-mediated regulation of granulocyte differentiation. Utilization of the selective pharmacological CaMK inhibitor, KN-93, failed to rescue the differentiation inhibition resulting from over-expression of CaMKK2, which suggested that CaMKI and CaMKIV did not function downstream of CaMKK2 in

this process. Moreover, neither the depletion nor over-expression of CaMKK2, nor increase in intracellular calcium levels altered levels of phospho-AMPK, suggesting that AMPK is likely not regulated by CaMKK2 in myeloid progenitors. Thus based on our studies, while it seems that none of the currently known targets of CaMKK2 play a role in regulating granulopoiesis, our collective results indeed suggest that CaMKK2 is inhibited during granulocyte differentiation and plays an antagonistic role toward granulocyte fate commitment and granulopoiesis.

5. Discussion and Future Directions

Longevity in higher-level organisms is dependent on the maintenance of tissue homeostasis that is in part determined by the integrity of tissue-specific stem cells. There are several adult tissue compartments in mammalian systems that are highly reliant on stem cells for their maintenance and propagation, including skin, gastrointestinal epithelium, and blood. In such systems, stem cells persist for the life of the organism and give rise to committed progenitors that in turn give rise to terminally differentiated effector cells. Blood is the product of hematopoiesis, a process that is regulated by a dynamic balance between hematopoietic stem cell (HSC) self-renewal and differentiation, and maintenance of this balance is of critical importance, as too little self-renewal or too much differentiation may jeopardize the ability to sustain hematopoiesis throughout life, whereas excessive self-renewal and/or aberrant differentiation may result in leukemogenesis. While the signaling mechanisms that regulate HSC homeostasis and function are not well understood, herein we have identified novel effectors of calcium/CaM signaling that may play an important role in this process.

In Chapter 3, we identified *Gfer* as a gene whose expression appeared to be absent in CaMKIV-null HSCs based on comparative microarray analysis with wild-type HSCs, and thus seemed a potential target of CaMKIV. In order to determine if loss of

Gfer function might play a role in CaMKIV-null HSC phenotypes, we used RNAi to deplete Gfer from HSCs. Similar to *Camk4*$^{-/-}$ HSCs, Gfer-deficient HSCs exhibited compromised *in vivo* engraftment potential and triggered a hyper-proliferative response that led to HSC exhaustion. Despite Gfer's known roles in mitochondrial regulation (Todd, Damin et al. 2010a; Todd, Gomathinayagam et al. 2010b), the knock-down of Gfer in HSCs did not elicit a significant alteration of mitochondrial morphology or loss of cell viability. However, using immunocytochemistry techniques we determined that these cells possessed significantly reduced levels of the cyclin-dependent kinase inhibitor (CDKI) p27^{kip1}. Conversely, Gfer over-expression in wild-type HSCs resulted in significantly elevated total and nuclear p27^{kip1}. Of significant note, decreased levels of p27^{kip1} are also observed in *Camk4*$^{-/-}$ HSCs, which can be rescued by the restoration of Gfer (Sankar and Means 2011).

To investigate the mechanism by which differential levels of Gfer influence p27^{kip1} levels, we hypothesized that Gfer might affect the interaction between p27^{kip1} and its inhibitor Jab1. Indeed, depletion of Gfer resulted in enhanced binding of p27^{kip1} to Jab1. In contrast, over-expression of Gfer resulted in its enhanced binding to Jab1 and inhibition of the Jab1-p27^{kip1} interaction. Furthermore, normalization of p27^{kip1} in Gfer-KD HSCs rescues their *in vitro* proliferation deficits. Taken together, these data demonstrate the presence of a novel Gfer-Jab1-p27^{kip1} pathway in HSCs that functions to restrict abnormal proliferation.

An important question remains: how does CaMKIV fit into the Gfer-Jab1-p27^{kip1} pathway? There are certainly many similarities between *Camk4*$^{-/-}$ and Gfer-KD HSCs. Although Gfer is absent in *Camk4*$^{-/-}$ HSCs, our current data do not support a role for CaMKIV in the regulation of Gfer levels. Indeed, the restoration of CaMKIV in these cells does not rescue Gfer levels[1], even though it rescues the hyper-proliferative phenotype. However, the fact that Gfer over-expression can complement the CaMKIV-null phenotype suggests the possibility of functional overlap between the two proteins. One explanation could be that CaMKIV regulates p27^{kip1} levels through another pathway. We have previously shown that CaMKIV regulates phospho-CREB and Bcl-2 levels (Kitsos, Sankar et al. 2005) and Bcl-2 has been shown to up-regulate p27^{kip1} (Greider, Chattopadhyay et al. 2002). However, it is also conceivable that CaMKIV may regulate Jab1 due to the fact that CaMKs are involved in IκB kinase (IKK) activation, which may lead to Jab1 ubiquitination (Chen and Lin 2001; Orel, Neumeier et al. 2010). Hence, CaMKIV plays an important role in hematopoiesis, but much more work is required to elucidate its precise functions in HSCs.

Given the critical role of CaMKIV in HSCs and hematopoietic development, we next focused our research on CaMKIV's upstream activator CaMKK2. These studies were largely based on the usage of mice genetically ablated for the *Camkk2* gene in the germline. In Chapter 4, we identified a role for CaMKK2 in the restriction of

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[1] Unpublished observations: Uma Sankar, Ellen Teng, and Anthony Means.

granulocytic fate commitment and differentiation of myeloid progenitor cells. Following bone marrow transplantation, engraftment by *Camkk2*$^{-/-}$ donor cells resulted in the increased production of mature granulocytes in the bone marrow and peripheral blood. Similarly, *Camkk2*$^{-/-}$ mice possessed elevated numbers of common myeloid progenitor cells, and exhibited an accelerated granulopoietic phenotype in the bone marrow.

Consistent with these findings, our results also demonstrated that C/EBPα and PU.1 levels are significantly increased in *Camkk2*$^{-/-}$ CMPs. Studies have shown that under homeostatic conditions, C/EBPα is required for the transition from CMP to GMP (Zhang, Iwasaki-Arai et al. 2004) as its genetic ablation in neonatal mice resulted in the complete absence of neutrophils and eosinophils while other hematopoietic lineages including monocytes were not affected (Zhang, Zhang et al. 1997) and, during hematopoiesis, it is predominantly expressed in the granulocyte and monocyte lineages (Scott, Civin et al. 1992; Radomska, Huettner et al. 1998). Additionally, expression of low levels of PU.1 in PU.1 (-/-) myeloprogenitor cells induces granulopoiesis, while high levels can induce monopoiesis (Dahl, Walsh et al. 2003; Laslo, Spooner et al. 2006). Moreover, it has been well documented that PU.1 physically interacts with and represses GATA-1, a transcription factor that promotes erythroid differentiation, thereby inhibiting the erythroid differentiation program during lineage commitment decisions in myeloerythroid cells (Zhang, Zhang et al. 2000; Rhodes, Hagen et al. 2005; Stopka,

Amanatullah et al. 2005). In light of these findings, our data suggest that the depletion of CaMKK2 leads to altered fate commitment in progenitor cells as early as CMPs, to favor differentiation of CMPs into granulocyte/monocyte precursor cells instead of megakaryocyte/erythrocyte lineage cells.

Our studies reveal that CaMKK2 is not expressed in terminally differentiated granulocytes, and CaMKK2 expression is progressively down-regulated during granulocytic differentiation. Conversely, when CaMKK2 is over-expressed in *Camkk2*$^{-/-}$ CMP cells or undifferentiated 32D myeloprogenitor cells, it is sufficient to block granulocyte differentiation, and at least in 32D cells, does so in a manner that requires its kinase activity. Together these data support a role for CaMKK2 as a selective inhibitor of early granulopoiesis.

However, our results suggest that this effect may not be mediated by any of the three known targets of CaMKK2, viz. CaMKIV, CaMKI, or AMPK. In the analysis of *Camkk2*$^{-/-}$ mice, we identified myeloid progenitor and granulopoiesis phenotypes that are not exhibited by *Camk4*$^{-/-}$ mice (Kitsos, Sankar et al. 2005). Moreover, CaMKIV was not detected in 32D cells, suggesting that it may not be expressed in myeloid progenitor cells. Although a specific isoform of CaMKI, CKLiK, has previously been implicated in late neutrophil maturation and function (Verploegen, Lammers et al. 2000; Verploegen, Ulfman et al. 2005; Laiosa, Stadtfeld et al. 2006; Gaines, Lamoureux et al. 2008), it does not seem a likely target of CaMKK2 in early granulopoiesis, as our CaMK-inhibition

studies using KN-93 showed that KN-93 treatment of CaMKK2-over-expressing cells did not abrogate the CaMKK2-mediated inhibition of granulocyte differentiation. These results suggest that neither CaMKI nor CaMKIV is the potential effector substrate of CaMKK2 in the signaling pathway that represses granulocyte differentiation.

Although AMPK can also be phosphorylated by CaMKK2, reduction of CaMKK2 expression with shRNA and over-expression of CaMKK2 did not alter the phosphorylation status of AMPK. We believe that this could be for a number of reasons. In other studies, we've found that there is relatively little CaMKK2 expressed in a number of non-neuronal cell types compared to the amount of AMPK present, and CaMKK2 binds to and interacts with only a small pool of the AMPK in the cell. Furthermore, several other kinases in addition to CaMKK2 also function as AMPK kinases (AMPKK) including LKB1, transforming growth factor-β-activated kinase 1 (TAK1), and Ataxia telangiectasia mutated (ATM) (Jensen, Wojtaszewski et al. 2009). Thus, given these factors and the appreciation that many pathways involve and cross-regulate AMPK, it is not surprising that alteration of CaMKK2 expression in myeloid progenitor fails to affect the level of phosphorylated AMPK. Collectively, these results suggest that CaMKK2 may regulate early granulopoiesis through yet to be identified targets.

Based on the information presented so far, it would seem that CaMKK1/2 should have more than just three substrates, and there are a couple candidates. Indeed,

PKB/Akt and PKC have activation loop amino acid sequences similar to those of CaMKIV and AMPK (Pullen, Dennis et al. 1998). However, whether CaMKK1 can phosphorylate Akt or not is controversial (Pullen, Dennis et al. 1998; Yano, Tokumitsu et al. 1998), and no reports thus far have shown CaMKK1/2 to phosphorylate PKC. Interestingly, PKC phosphorylates SHP1/2 (Strack, Krutzfeldt et al. 2002; Liu, Kruhlak et al. 2007), which have been recently implicated in granulocyte differentiation (Zhang and Friedman 2011). Similar to CaMKK2 over-expression, the knock-down of SHP2 in 32Dcl3 cells inhibits G-CSF-induced granulocyte differentiation and C/EBPα expression (Zhang and Friedman 2011). Since PKC-mediated phosphorylation of SHP1/2 may be inhibitory (Liu, Kruhlak et al. 2007), it is certainly worth investigating a potential role for CaMKK2 in this signaling pathway.

How do CaMKK signaling pathways regulate early and late stages of granulopoiesis? Although CaMKK2 is down-regulated in developing granulocytes, CaMKK1-CKLiK signaling may become important in modulating late-stage neutrophil development and effector functions, given that we detected CaMKK1 expression in mature BM granulocytes (Figure 21B), a finding that confirms a previous study (Gaines, Lamoureux et al. 2008). Taken together, these observations suggest an important role for the known and yet to be discovered CaMKK cascade members in the regulation of appropriate granulocyte production and function throughout granulopoiesis.

Implications for Therapy

In this dissertation, we identify two novel regulators of hematopoiesis: (1) Gfer in HSC function and (2) CaMKK2 in restricting granulopoiesis. In addition, each protein represents a unique opportunity for therapeutic intervention. In the case of Gfer, we find that the inhibition of Gfer results in hyper-proliferation of HSCs with minimal effects on differentiation, apoptosis, or mitochondrial function. Thus, one might use reversible inhibitors of the Gfer-Jab1 interaction to expand stem cells *in vitro* prior to transplantation, which might improve the success of engraftment. Alternatively, one might consider Gfer up-regulation in malignancies to increase $p27^{kip1}$ levels and hence restrict cell proliferation.

Highly elevated Jab1 and low or cytoplasmic $p27^{kip1}$ are indicators of poor prognosis in multiple types of cancers including chronic myeloid leukemia (CML) (Tomoda, Kato et al. 2005). Transgenic expression of a stable form of Jab1 significantly enhanced the proliferative potential of hematopoietic progenitors in the mouse bone marrow (Mori, Yoneda-Kato et al. 2008). Jab1 is a multifunctional protein that modulates the stability of a plethora of cell regulatory proteins. While it stabilizes c-jun, hypoxia-inducible factor 1 α subunit and Mdm-2, Jab1 also directly promotes the nuclear export of several key regulators of cellular homeostasis including p53 and $p27^{kip1}$ (Wei, Serino et al. 2008; Kato and Yoneda-Kato 2009; Schwechheimer and Isono 2010). Our data indicating a direct interaction of Gfer with Jab1 to restrict Jab1-mediated de-

stabilization of $p27^{kip1}$ (Figure 11) underscores the importance of a Gfer-Jab1-$p27^{kip1}$ pathway not only in the functional maintenance of normal HSCs, but also in the prevention and/or treatment of malignancies. Controlled modulation of Gfer expression to inhibit Jab1 and thereby elevate $p27^{kip1}$ could be a relevant therapeutic strategy in the treatment of CML and other myelo-proliferative diseases. Next, we will discuss granulopoiesis and the potential opportunity for targeting CaMKK2.

In humans, granulocyte production can be impaired by immunosuppressive chemotherapy or severe congenital disorders (Panopoulos and Watowich 2008). The lack of neutrophils, or neutropenia, can cause extreme susceptibility to life-threatening infections. Hence G-CSF, which stimulates granulocyte production, has become a primary therapeutic agent to treat neutropenia. However in some cases of severe congenital neutropenia, individuals possess a mutant G-CSF receptor (G-CSFR) allele leading to neutrophil maturation arrest at the promyelocyte/myelocyte stage of development, a correlated onset of high-risk myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), and reduced responsiveness to G-CSF therapy (Germeshausen, Ballmaier et al. 2007; Panopoulos and Watowich 2008). Our results may be relevant to such conditions, as a potentially useful treatment option could be the combined therapy of G-CSF with a specific CaMKK2 inhibitor to serve as a more effective, postreceptor means of stimulating neutrophil production in these patients. Moreover, the specific inhibition of CaMKK2 would not interfere with the biological

function of fully mature neutrophils, as one would predict to occur with the use of KN-93. The combination treatment might also decrease the risks of purported G-CSF-induced leukemia (Panopoulos and Watowich 2008), since smaller doses of G-CSF could be used and cell differentiation would be accelerated.

Finally, the combination of G-CSF and a CaMKK2 inhibitor might also be useful for the treatment of AML, in which the blocked differentiation of myeloid precursors is a hallmark of the disease. Interestingly, recent studies suggest CaMKK2 to be a potentially relevant target in prostate cancer cells, where CaMKK2 is expressed in an androgen-dependent manner and is required for invasiveness (Frigo, Howe et al. 2011). Thus, further research is warranted to ascertain the role of CaMKK2 in hematologic malignancies, as its deregulated activity in myeloprogenitor cells may conceivably promote a maturation defect in this population, leading to increased risk of additional genetic mutations and leukemic transformation.

References

Adams, G. B., K. T. Chabner, et al. (2005). "Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor." Nature **439**(7076): 599-603.

Ahn, S., D. D. Ginty, et al. (1999). "A late phase of cerebellar long-term depression requires activation of CaMKIV and CREB." Neuron **23**(3): 559-568.

Akashi, K., D. Traver, et al. (2000). "A clonogenic common myeloid progenitor that gives rise to all myeloid lineages." Nature **404**: 193-197.

Anderson, K. A. and A. R. Means (2002). "Defective signaling in a subpopulation of CD4+ T Cells in the absence of Ca2+/calmodulin-dependent protein kinase IV." Mol Cell Biol. **22**(1): 23-29.

Anderson, K. A., R. L. Means, et al. (1998). "Components of a calmodulin-dependent protein kinase cascade." J Biol Chem. **273**(48): 31880-31889.

Anderson, K. A., T. J. Ribar, et al. (1997). "Defective survival and activation of thymocytes in transgenic mice expressing a catalytically inactive form of Ca2+/calmodulin-dependent protein kinase IV." Mol Endocrinol **11**(6): 725-737.

Anderson, K. A., T. J. Ribar, et al. (2008). "Hypothalamic CaMKK2 contributes to the regulation of energy balance." Cell Metab **7**(5): 377-388.

Anderson, K. A., T. J. Ribar, et al. (2008). "Hypothalamic CaMKK2 contributes to the regulation of energy balance." Cell Metab. **7**(5): 377-388.

Bainton, D. F., J. L. Ullyot, et al. (1971). "The development of neutrophilic polymorphonuclear leukocytes in human bone marrow." J Exp Med. **134**(4): 907-934.

Becher, D., J. Kricke, et al. (1999). "A mutant for the yeast *scERV1* gene displays a new defect in mitochondrial morphology and distribution." Yeast **15**(12): 1171-1181.

Boxer, L. and D. Dale (2002). "Neutropenia: causes and consequences." Semin Hematol. **39**(2): 75-81.

Braun, A. P. and H. Schulman (1995). "The multifunctional calcium/calmodulin-dependent protein kinase: from form to function." Annu Rev Physiol **57**(1): 417-445.

Bystrykh, L., E. Weersing, et al. (2005). "Uncovering regulatory pathways that affect hematopoietic stem cell function using 'genetical genomics'." Nat Genet **37**(3): 225-232.

Chen, B. C. and W. W. Lin (2001). "PKC and ERK dependent activation of I B kinase by lipopolysaccharide in macrophages: enhancement by P2Y receptor mediated CaMK activation." Br J Pharmacol **134**(5): 1055-1065.

Cheng, T., N. Rodrigues, et al. (2000). "Stem cell repopulation efficiency but not pool size is governed by p27kip1." Nat Med **6**(11): 1235-1240.

Chin, D. and A. R. Means (2000). "Calmodulin: a prototypical calcium sensor." Trends Cell Biol **10**(8): 322-328.

Chow, F. A., K. A. Anderson, et al. (2005). "The autonomous activity of calcium/calmodulin-dependent protein kinase IV is required for its role in transcription." J Biol Chem **280**(21): 20530-20538.

Chow, F. A. and A. R. Means (2007). The calcium/calmodulin-dependent protein kinase cascades. Calcium: a matter of life or death. J. Krebs and M. Michalak. Amsterdam, The Netherlands, Elsevier. **41:** 345-364.

Chow, F. A. and A. R. Means (2007). "The calcium/calmodulin-dependent protein kinase cascades." Calcium: a matter of life or death **41**: 345-364.

Christensen, J. L. and I. L. Weissman (2001). "Flk-2 is a marker in hematopoietic stem cell differentiation: A simple method to isolate long-term stem cells." Proc Natl Acad Sci U S A **98**(25): 14541-14546.

Colomer-Font, J. and A. R. Means (2007). Physiological roles of the Ca2+/CaM-dependent protein kinase cascade in health and disease. Calcium signaling and disease. E. Carafoli and M. Brini. Dordrecht, The Netherlands, Springer Science. **45:** 169-214.

Corcoran, E. E. and A. R. Means (2001). "Defining Ca2+/calmodulin-dependent protein kinase cascades in transcriptional regulation." J Biol Chem **276**(5): 2975-2978.

Dahl, R., J. C. Walsh, et al. (2003). "Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor." Nat Immunol. **4**(10): 1029-1036.

DeKoter, R. P., J. C. Walsh, et al. (1998). "PU. 1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors." EMBO J. **17**(15): 4456-4468.

Denburg, J. A., S. Telizyn, et al. (1985). "Heterogeneity of human peripheral blood eosinophil-type colonies: evidence for a common basophil-eosinophil progenitor." Blood **66**(2): 312-318.

Duncan, A. W., F. M. Rattis, et al. (2005). "Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance." Nat Immunol **6**(3): 314-322.

Edelman, A. M., K. I. Mitchelhill, et al. (1996). "Multiple Ca-Calmodulin-dependent Protein Kinase Kinases from Rat Brain." J Biol Chem **271**(18): 10806-10810.

Enver, T., C. M. Heyworth, et al. (1998). "Do stem cells play dice?" Blood **92**(2): 348-351.

Frigo, D. E., M. K. Howe, et al. (2011). "CaM Kinase Kinase -Mediated Activation of the Growth Regulatory Kinase AMPK Is Required for Androgen-Dependent Migration of Prostate Cancer Cells." Cancer Research **71**(2): 528-537.

Gaines, P., J. Lamoureux, et al. (2008). "A cascade of Ca2+/calmodulin-dependent protein kinases regulates the differentiation and functional activation of murine neutrophils." Exp Hematol **36**(7): 832-844.

Gatzidou, E., G. Kouraklis, et al. (2006). "Insights on augmenter of liver regeneration cloning and function." World J Gastroenterol **12**(31): 4951-4958.

Germeshausen, M., M. Ballmaier, et al. (2007). "Incidence of CSF3R mutations in severe congenital neutropenia and relevance for leukemogenesis: results of a long-term survey." Blood **109**(1): 93-99.

Goldberg, J., A. C. Nairn, et al. (1996). "Structural basis for the autoinhibition of calcium/calmodulin-dependent protein kinase I." Cell **84**(6): 875-887.

Greider, C., A. Chattopadhyay, et al. (2002). "BCL-x (L) and BCL2 delay Myc-induced cell cycle entry through elevation of p27 and inhibition of G1 cyclin-dependent kinases." Oncogene **21**(51): 7765-7775.

Hardie, D. G., J. W. Scott, et al. (2003). "Management of cellular energy by the AMP-activated protein kinase system." FEBS Lett **546**(1): 113-120.

Haribabu, B., S. Hook, et al. (1995). "Human calcium-calmodulin dependent protein kinase I: cDNA cloning, domain structure and activation by phosphorylation at threonine-177 by calcium-calmodulin dependent protein kinase I kinase." EMBO J **14**(15): 3679-3686.

Haurie, C., D. C. Dale, et al. (1998). "Cyclical neutropenia and other periodic hematological disorders: a review of mechanisms and mathematical models." Blood **92**(8): 2629-2640

Hawley, S. A., D. A. Pan, et al. (2005). "Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase." Cell Metab. **2**(1): 9-19.

Hestdal, K., F. Ruscetti, et al. (1991). "Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells." J Immunol. **147**(1): 22-28.

Ho, N., J. A. Liauw, et al. (2000). "Impaired synaptic plasticity and cAMP response element-binding protein activation in Ca2+/calmodulin-dependent protein kinase type IV/Gr-deficient mice." J Neurosci **20**(17): 6459-6472.

Hong, S. P., M. Momcilovic, et al. (2005). "Function of mammalian LKB1 and Ca2+/calmodulin-dependent protein kinase kinase as Snf1-activating kinases in yeast." J Biol Chem **280**(23): 21804-21809.

Hook, S. S. and A. R. Means (2001). "Ca(2+)/CaM-dependent kinases: from activation to function." Annu Rev Pharmacol Toxicol. **41**: 471-505.

Hoyer-Hansen, M., L. Bastholm, et al. (2007). "Control of Macroautophagy by Calcium, Calmodulin-Dependent Kinase Kinase-[beta], and Bcl-2." Mol Cell **25**(2): 193-205.

Hsu, L. S., G. D. Chen, et al. (2001). "Human Ca2+/Calmodulin-dependent Protein Kinase Kinase Gene Encodes Multiple Isoforms That Display Distinct Kinase Activity." J Biol Chem **276**(33): 31113-31123.

Hudmon, A. and H. Schulman (2002). "Neuronal Ca2+/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function." Annu Rev Biochem **71**(1): 473-510.

Hurley, R. L., K. A. Anderson, et al. (2005). "The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases." J Biol Chem. **280**(32): 29060-29066.

Illario, M., M. L. Giardino-Torchia, et al. (2008). "Calmodulin-dependent kinase IV links Toll-like receptor 4 signaling with survival pathway of activated dendritic cells." Blood. **111**(2): 723-731.

Ishikawa, Y., H. Tokumitsu, et al. (2003). "Identification and characterization of novel components of a Ca2+/calmodulin-dependent protein kinase cascade in HeLa cells." FEBS Lett **550**(1-3): 57-63.

Ito, K., A. Hirao, et al. (2006). "Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells." Nat Med **12**(4): 446-451.

Ivanova, N. B., J. T. Dimos, et al. (2002). "A stem cell molecular signature." Science **298**(5593): 601-604.

Iwasaki, H., S. Mizuno, et al. (2005). "Identification of eosinophil lineage-committed progenitors in the murine bone marrow." J Exp Med **201**(12): 1891-1897.

Jankovic, V., A. Ciarrocchi, et al. (2007). "Id1 restrains myeloid commitment, maintaining the self-renewal capacity of hematopoietic stem cells." Proc Natl Acad Sci U S A **104**(4): 1260-1265.

Jensen, T., J. Wojtaszewski, et al. (2009). "AMP activated protein kinase in contraction regulation of skeletal muscle metabolism: necessary and/or sufficient?" Acta Physiol **196**(1): 155-174.

Kahl, C. R. and A. R. Means (2004). "Regulation of cyclin D1/Cdk4 complexes by calcium/calmodulin-dependent protein kinase I." J Biol Chem **279**(15): 15411-15419.

Kahn, B. B., T. Alquier, et al. (2005). "AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism." Cell Metab **1**(1): 15-25.

Kane, C. D. and A. R. Means (2000). "Activation of orphan receptor-mediated transcription by Ca2+/calmodulin-dependent protein kinase IV." EMBO J **19**(4): 691-701.

Kang, H., L. D. Sun, et al. (2001). "An important role of neural activity-dependent CaMKIV signaling in the consolidation of long-term memory." Cell **106**(6): 771-783.

Kasahara, J., K. Fukunaga, et al. (2001). "Activation of calcium/calmodulin-dependent protein kinase IV in long term potentiation in the rat hippocampal CA1 region." J Biol Chem **276**(26): 24044-24050.

Kastner, P., H. Lawrence, et al. (2001). "Positive and negative regulation of granulopoiesis by endogenous RARalpha." Blood. **97**(5): 1314-1320.

Katayama, N., M. Nishikawa, et al. (1990). "A role for calmodulin in the growth of human hematopoietic progenitor cells." Blood **75**(7): 1446-1454.

Kato, J. and N. Yoneda-Kato (2009). "Mammalian COP9 signalosome." Genes Cells **14**(11): 1209-1225.

Kiel, M. J., H. Yilmaz, et al. (2005). "SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells." Cell **121**(7): 1109-1121.

Kitsos, C., U. Sankar, et al. (2005). "Calmodulin-dependent protein kinase IV regulates hematopoietic stem cell maintenance." J. Biol. Chem **280**(39): 33101-33108.

Kitsos, C., U. Sankar, et al. (2005). "Calmodulin-dependent protein kinase IV regulates hematopoietic stem cell maintenance." J Biol Chem **280**(39): 33101-33108.

Klebes, A., A. Sustar, et al. (2005). "Regulation of cellular plasticity in Drosophila imaginal disc cells by the Polycomb group, trithorax group and lama genes." Development **132**(16): 3753-3765.

Klissenbauer, M., S. Winters, et al. (2002). "Accumulation of the mitochondrial form of the sulphydryl oxidase Ervlp/Alrp during the early stages of spermatogenesis." J Exp Biol **205**(14): 1979-1986.

Kokubo, M., M. Nishio, et al. (2009). "BDNF-mediated cerebellar granule cell development is impaired in mice null for CaMKK2 or CaMKIV." J. Neurosci. **29**(28): 8901-8913.

Kokubo, M., M. Nishio, et al. (2009). "BDNF-mediated cerebellar granule cell development is impaired in mice null for CaMKK2 or CaMKIV." J Neurosci **29**(28): 8901-8913.

Kondo, M., I. L. Weissman, et al. (1997). "Identification of clonogenic common lymphoid progenitors in mouse bone marrow." Cell **91**(5): 661-672.

Laiosa, C. V., M. Stadtfeld, et al. (2006). "Determinants of lymphoid-myeloid lineage diversification." Annu Rev Immunol **24**: 705-738.

Lange, H., T. Lisowsky, et al. (2001). "An essential function of the mitochondrial sulfhydryl oxidase Erv1p/ALR in the maturation of cytosolic Fe/S proteins." EMBO reports **2**(8): 715-720.

Laslo, P., C. J. Spooner, et al. (2006). "Multilineage transcriptional priming and determination of alternate hematopoietic cell fates." Cell. **126**(4): 755-766.

Lisowsky, T., J. E. Lee, et al. (2001). "Mammalian augmenter of liver regeneration protein is a sulfhydryl oxidase." Dig Liver Dis **33**(2): 173-180.

Liu, Y., M. J. Kruhlak, et al. (2007). "Rapid T cell receptor-mediated SHP-1 S591 phosphorylation regulates SHP-1 cellular localization and phosphatase activity." J Leukoc Biol **82**(3): 742-751.

Lonze, B. E. and D. D. Ginty (2002). "Function and regulation of CREB family transcription factors in the nervous system." Neuron **35**(4): 605-623.

Lu, C., Y. Li, et al. (2002). "Intracrine hepatopoietin potentiates AP-1 activity through JAB1 independent of MAPK pathway." FASEB J **16**(1): 90-92.

Macrez, N. and J. Mironneau (2004). "Local Ca2+ signals in cellular signalling." Curr Mol Med **4**(3): 263-275.

Mantel, C., S. Messina-Graham, et al. (2010). "Upregulation of nascent mitochondrial biogenesis in mouse hematopoietic stem cells parallels upregulation of CD34 and loss of pluripotency: A potential strategy for reducing oxidative risk in stem cells." Cell cycle **9**(10): 2008-2017.

Matsushita, M. and A. C. Nairn (1998). "Characterization of the mechanism of regulation of Ca2+/calmodulin-dependent protein kinase I by calmodulin and by Ca2+/calmodulin-dependent protein kinase kinase." J Biol Chem **273**(34): 21473-21481.

Matthews, R. P., C. R. Guthrie, et al. (1994). "Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression." Mol Cell Biol **14**(9): 6107-6116.

Means, A. R. (2000). "Regulatory cascades involving calmodulin-dependent protein kinases." Mol Endocrinol **14**(1): 4-13.

Means, A. R. (2008). "Commentary: the year in basic science: calmodulin kinase cascades." Mol Endocrinol **22**(12): 2759-2765.

Means, A. R., T. J. Ribar, et al. (1997). "Regulation and properties of the rat Ca2+/calmodulin-dependent protein kinase IV gene and its protein products." Recent Prog Horm Res **52**: 389-406.

Mesecke, N., N. Terziyska, et al. (2005). "A disulfide relay system in the intermembrane space of mitochondria that mediates protein import." Cell **121**(7): 1059-1069.

Metcalf, D. (1998). "Lineage commitment and maturation in hematopoietic cells: the case for extrinsic regulation." Blood **92**(2): 345-347.

Metcalf, D. (2007). "Concise review: hematopoietic stem cells and tissue stem cells: current concepts and unanswered questions." Stem Cells **25**(10): 2390-2395.

Miyamoto, K., K. Y. Araki, et al. (2007). "Foxo3a is essential for maintenance of the hematopoietic stem cell pool." Cell Stem Cell **1**(1): 101-112.

Miyamoto, T., H. Iwasaki, et al. (2002). "Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment." Dev Cell **3**(1): 137-147.

Mori, M., N. Yoneda-Kato, et al. (2008). "Stable form of JAB1 enhances proliferation and maintenance of hematopoietic progenitors." J Biol Chem **283**(43): 29011-29021.

Morrison, S. J., H. D. Hemmati, et al. (1995). "The purification and characterization of fetal liver hematopoietic stem cells." Proc Natl Acad Sci U S A **92**(22): 10302-10306.

Morrison, S. J. and I. L. Weissman (1994). "The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype." Immunity **1**(8): 661-673.

Naito, Y., Y. Watanabe, et al. (1997). "Isoform-specific activation and structural diversity of calmodulin kinase I." J Biol Chem **272**(51): 32704-32708.

Nakajima, H. and J. N. Ihle (2001). "Granulocyte colony-stimulating factor regulates myeloid differentiation through CCAAT/enhancer-binding protein {epsilon}." Blood **98**(4): 897-905.

Nakamura, H., Y. Ueki, et al. (2000). "High serum and synovial fluid granulocyte colony stimulating factor (G-CSF) concentrations in patients with rheumatoid arthritis." Clin Exp Rheumatol. **18**(6): 713-718.

Nakamura, Y., S. Okuno, et al. (2001). "Immunohistochemical localization of Ca2+/calmodulin-dependent protein kinase kinase [beta] in the rat central nervous system." Neurosci Res **39**(2): 175-188.

Nishimura, H., H. Sakagami, et al. (2003). "Cloning, characterization and expression of two alternatively splicing isoforms of Ca2+/calmodulin dependent protein kinase I in the rat brain." J Neurochem **85**(5): 1216-1227.

O'Riordan, M. and R. Grosschedl (1999). "Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A." Immunity **11**(1): 21-31.

Orel, L., H. Neumeier, et al. (2010). "Crosstalk between the NF B activating IKK complex and the CSN signalosome." J Cell Mol Med **14**(6b): 1555-1568.

Osawa, M., K. Hanada, et al. (1996). "Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell." Science **273**(5272): 242-245.

Panopoulos, A. D. and S. S. Watowich (2008). "Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis." Cytokine **42**(3): 277-288.

Passier, R., H. Zeng, et al. (2000). "CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo." J Clin Invest **105**(10): 1395-1406.

Peters, M., K. Mizuno, et al. (2003). "Loss of Ca2+/calmodulin kinase kinase affects the formation of some, but not all, types of hippocampus-dependent long-term memory." J Neurosci **23**(30): 9752-9760.

Picciotto, M., A. Czernik, et al. (1993). "Calcium/calmodulin-dependent protein kinase I. cDNA cloning and identification of autophosphorylation site." J Biol Chem **268**(35): 26512-26521.

Picciotto, M. R., M. Zoli, et al. (1995). "Immunochemical localization of calcium/calmodulin dependent protein kinase I." Synapse **20**(1): 75-84.

Polimeno, L., T. Lisowsky, et al. (1999). "From yeast to man--from mitochondria to liver regeneration: a new essential gene family." Ital J Gastroenterol Hepatol **31**(6): 494-500.

Pullen, N., P. B. Dennis, et al. (1998). "Phosphorylation and activation of p70s6k by PDK1." Science **279**(5351): 707-710.

Qin, X. F., D. S. An, et al. (2003). "Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5." Proc Natl Acad Sci U S A **100**(1): 183-188.

Radomska, H. S., C. S. Huettner, et al. (1998). "CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors." Mol Cell Biol. **18**(7): 4301-4314.

Ramalho-Santos, M., S. Yoon, et al. (2002). "" Stemness": transcriptional profiling of embryonic and adult stem cells." Science **298**(5593): 597-600.

Ramalho-Santos, M., S. Yoon, et al. (2002). ""Stemness": transcriptional profiling of embryonic and adult stem cells." Science **298**(5593): 597-600.

Reya, T., S. J. Morrison, et al. (2001). "Stem cells, cancer, and cancer stem cells." Nature **414**(6859): 105-111.

Rhodes, J., A. Hagen, et al. (2005). "Interplay of PU. 1 and Gata1 determines myelo-erythroid progenitor cell fate in zebrafish." Dev Cell. **8**(1): 97-108.

Ribar, T. J., R. M. Rodriguiz, et al. (2000). "Cerebellar defects in Ca2+/calmodulin kinase IV-deficient mice." J Neurosci **20**(22): RC107.

Sakagami, H., A. Kamata, et al. (2005). "Prominent expression and activity dependent nuclear translocation of Ca2+/calmodulin dependent protein kinase I in hippocampal neurons." Eur J Neurosci **22**(11): 2697-2707.

Sakagami, H., S. Saito, et al. (1998). "Localization of the mRNAs for two isoforms of Ca2+/calmodulin-dependent protein kinase kinases in the adult rat brain." Mol Brain Res **54**(2): 311-315.

Sakagami, H., M. Umemiya, et al. (1999). "Immunological evidence that the isoform of Ca2+/calmodulin dependent protein kinase IV is a cerebellar granule cell specific product of the CaM kinase IV gene." Eur J Neurosci **11**(7): 2531-2536.

Sakagami, H., M. Umemiya, et al. (2000). "Distinct immunohistochemical localization of two isoforms of Ca2+/calmodulin dependent protein kinase kinases in the adult rat brain." Eur J Neurosci **12**(1): 89-99.

Sakamoto, K., A. McCarthy, et al. (2005). "Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction." EMBO J **24**(10): 1810-1820.

Saneyoshi, T., G. Wayman, et al. (2008). "Activity-Dependent Synaptogenesis: Regulation by a CaM-Kinase Kinase/CaM-Kinase I/[beta] PIX Signaling Complex." Neuron **57**(1): 94-107.

Sankar, U. and A. R. Means (2011). "Gfer is a critical regulator of HSC proliferation." Cell Cycle **10**(14): 2263-2268.

Sawamura, Y., H. Sakagami, et al. (1996). "Localization of mRNA for Ca2+/calmodulin-dependent protein kinase I in the brain of developing and mature rats." Brain Res **706**(2): 259-266.

Schwechheimer, C. and E. Isono (2010). "The COP9 signalosome and its role in plant development." Eur J Cell Biol **89**(2-3): 157-162.

Scott, E., M. Simon, et al. (1994). "Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages." Science. **265**: 1573-1577.

Scott, L. M., C. I. Civin, et al. (1992). "A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells." Blood. **80**(7): 1725-1735.

See, V., A. L. Boutillier, et al. (2001). "Calcium/calmodulin-dependent protein kinase type IV (CaMKIV) inhibits apoptosis induced by potassium deprivation in cerebellar granule neurons." FASEB J **15**(1): 134-144.

Shaw, R. J., M. Kosmatka, et al. (2004). "The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress." Proc Natl Acad Sci U S A **101**(10): 3329-3335.

Shaw, R. J., K. A. Lamia, et al. (2005). "The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin." Science **310**(5754): 1642-1646.

Soderling, T. R. (1999). "The Ca2+-calmodulin-dependent protein kinase cascade." Trends Biochem Sci **24**(6): 232-236.

Spangrude, G. J., S. Heimfeld, et al. (1988). "Purification and characterization of mouse hematopoietic stem cells." Science **241**(4861): 58-62.

Spangrude, G. J., S. Heimfeld, et al. (1988). "Purification and characterization of mouse hematopoietic stem cells." Science **241**: 58-62.

Stopka, T., D. F. Amanatullah, et al. (2005). "PU. 1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure." EMBO J. **24**(21): 3712-3723.

Strack, V., J. Krutzfeldt, et al. (2002). "The protein-tyrosine-phosphatase SHP2 is phosphorylated on serine residues 576 and 591 by protein kinase C isoforms , 1, 2, and." Biochemistry **41**(2): 603-608.

Sun, Z., R. L. Means, et al. (1995). "Organization and analysis of the complete rat calmodulin-dependent protein kinase IV gene." J Biol Chem **270**(49): 29507-29514.

Takemoto-Kimura, S., N. Ageta-Ishihara, et al. (2007). "Regulation of Dendritogenesis via a Lipid-Raft-Associated Ca2+/Calmodulin-Dependent Protein Kinase CLICK-III/CaMKI [gamma]." Neuron **54**(5): 755-770.

Takemoto-Kimura, S., H. Terai, et al. (2003). "Molecular Cloning and Characterization of CLICK-III/CaMKI , a Novel Membrane-anchored Neuronal Ca2+/Calmodulin-dependent Protein Kinase (CaMK)." J Biol Chem **278**(20): 18597-18605.

Teng, E. C., L. R. Todd, et al. (2011). "Gfer inhibits Jab1-mediated degradation of p27kip1 to restrict proliferation of hematopoietic stem cells." Mol Biol Cell **22**(8): 1312-1320.

Thomas, A., G. Bird, et al. (1996). "Spatial and temporal aspects of cellular calcium signaling." FASEB J **10**(13): 1505-1517.

Till, J. E. and E. A. McCulloch (1961). "A direct measurement of the radiation sensitivity of normal mouse bone marrow cells." Radiat Res. **14**(2): 213-222.

Todd, L. R., M. N. Damin, et al. (2010a). "Growth factor erv1-like modulates Drp1 to preserve mitochondrial dynamics and function in mouse embryonic stem cells." Mol Biol Cell **21**(7): 1225-1236.

Todd, L. R., R. Gomathinayagam, et al. (2010b). "A novel Gfer-Drp1 link in preserving mitochondrial dynamics and function in pluripotent stem cells." Autophagy **6**(6): 821-822.

Tokumitsu, H., H. Enslen, et al. (1995). "Characterization of a Ca2+/calmodulin-dependent protein kinase cascade. Molecular cloning and expression of calcium/calmodulin-dependent protein kinase kinase." J Biol Chem **270**(33): 19320-19324.

Tomoda, K., J. Kato, et al. (2005). "The Jab1/COP9 signalosome subcomplex is a downstream mediator of Bcr-Abl kinase activity and facilitates cell-cycle progression." Blood **105**(2): 775-783.

Tomoda, K., Y. Kubota, et al. (2002). "The cytoplasmic shuttling and subsequent degradation of p27Kip1 mediated by Jab1/CSN5 and the COP9 signalosome complex." J Biol Chem **277**(3): 2302-2310.

Tomoda, K., Y. Kubota, et al. (1999). "Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1." Nature **398**(6723): 160-165.

Tremper-Wells, B., A. Mathur, et al. (2002). "Trophic agents that prevent neuronal apoptosis activate calpain and down regulate CaMKIV." J Neurochem **81**(2): 314-324.

Tsai, S., S. Bartelmez, et al. (1994). "Lymphohematopoietic progenitors immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor can recapitulate lymphoid, myeloid, and erythroid development." Genes Dev **8**(23): 2831-2841.

Ueda, S., M. Mizuki, et al. (2002). "Critical roles of c-Kit tyrosine residues 567 and 719 in stem cell factor-induced chemotaxis: contribution of src family kinase and PI3-kinase on calcium mobilization and cell migration." Blood **99**(9): 3342-3349.

Ueda, T., H. Sakagami, et al. (1999). "Distribution and intracellular localization of a mouse homologue of Ca2/calmodulin-dependent protein kinase I 2 in the nervous system." J Neurochem **73**: 2119-2129.

Urbanek, P., Z. Q. Wang, et al. (1994). "Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP." Cell **79**(5): 901-912.

Valtieri, M., D. Tweardy, et al. (1987). "Cytokine-dependent granulocytic differentiation. Regulation of proliferative and differentiative responses in a murine progenitor cell line." J Immunol **138**(11): 3829-3835.

Verploegen, S., J. W. J. Lammers, et al. (2000). "Identification and characterization of CKLiK, a novel granulocyte Ca++/calmodulin-dependent kinase." Blood **96**(9): 3215-3223.

Verploegen, S., L. Ulfman, et al. (2005). "Characterization of the role of CaMKI-like kinase (CKLiK) in human granulocyte function." Blood **106**(3): 1076-1083.

Viatour, P., T. C. Somervaille, et al. (2008). "Hematopoietic stem cell quiescence is maintained by compound contributions of the retinoblastoma gene family." Cell Stem Cell **3**(4): 416-428.

Wang, S. L., T. J. Ribar, et al. (2001). "Expression of Ca (2+)/calmodulin-dependent protein kinase IV (caMKIV) messenger RNA during murine embryogenesis." Cell Growth Differ **12**(7): 351-361.

Wang, X., E. Scott, et al. (1999). "C/EBPalpha bypasses granulocyte colony-stimulating factor signals to rapidly induce PU. 1 gene expression, stimulate granulocytic differentiation, and limit proliferation in 32D cl3 myeloblasts." Blood **94**(2): 560-571.

Wang, Y., C. Lu, et al. (2004). "Hepatopoietin interacts directly with COP9 signalosome and regulates AP-1 activity." FEBS Lett **572**(1-3): 85-91.

Wayman, G. A., S. Kaech, et al. (2004). "Regulation of axonal extension and growth cone motility by calmodulin-dependent protein kinase I." J Neurosci **24**(15): 3786-3794.

Wei, F., C. S. Qiu, et al. (2002). "Calcium-calmodulin-dependent protein kinase IV is required for fear memory." Nat Neurosci **5**(6): 573-579.

Wei, N., G. Serino, et al. (2008). "The COP9 signalosome: more than a protease." Trends Biochem Sci **33**(12): 592-600.

Weissman, I. L. and J. A. Shizuru (2008). "The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases." Blood **112**(9): 3543-3553.

Wilson, A., E. Laurenti, et al. (2008). "Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair." Cell **135**(6): 1118-1129.

Wilson, A. and A. Trumpp (2006). "Bone-marrow haematopoietic-stem-cell niches." Nat Rev Immunol **6**(2): 93-106.

Witters, L. A., B. E. Kemp, et al. (2006). "Chutes and Ladders: the search for protein kinases that act on AMPK." Trend Biochem Sci. **31**(1): 13-16.

Woods, A., K. Dickerson, et al. (2005). "Ca2+/calmodulin-dependent protein kinase kinase-[beta] acts upstream of AMP-activated protein kinase in mammalian cells." Cell Metab **2**(1): 21-33.

Wu, G. Y., K. Deisseroth, et al. (2001). "Activity-dependent CREB phosphorylation: convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway." Proc Natl Acad Sci U S A **98**(5): 2808-2813.

Wu, J. Y., I. J. Gonzalez-Robayna, et al. (2000). "Female fertility is reduced in mice lacking Ca2+/calmodulin-dependent protein kinase IV." Endocrinology **141**(12): 4777-4783.

Yang, L., D. Bryder, et al. (2005). "Identification of Lin-Sca1+ kit+ CD34+ Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients." Blood **105**(7): 2717-2723.

Yano, S., H. Tokumitsu, et al. (1998). "Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway." Nature **396**(6711): 584-587.

Yu, C. T., H. Shih, et al. (2001). "Multiple signals required for cyclic AMP-responsive element binding protein (CREB) binding protein interaction induced by CD3/CD28 costimulation." J Immunol **166**(1): 284-292.

Zhang, D. E., P. Zhang, et al. (1997). "Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein-deficient mice." Proc Natl Acad Sci U S A **94**(2): 569-574.

Zhang, L. and A. Friedman (2011). "SHP2 tyrosine phosphatase stimulates CEBPA gene expression to mediate cytokine-dependent granulopoiesis." Blood: DOI 10.1182/blood-2011-1101-331157.

Zhang, P., J. Iwasaki-Arai, et al. (2004). "Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBPalpha." Immunity. **21**(6): 853-863.

Zhang, P., X. Zhang, et al. (2000). "PU. 1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding." Blood. **96**(8): 2641-2648.

Zhou, L., L. Li, et al. (2008). "Notch-dependent control of myelopoiesis is regulated by fucosylation." Blood **112**: 308-319.

Zhou, W., Q. Yang, et al. (2009). "Pin1 catalyzes conformational changes of Thr-187 in p27Kip1 and mediates its stability through a polyubiquitination process." J Biol Chem **284**(36): 23980-23988.

Biography

Ellen Chao Teng was born in Fairfax, VA in the spring of 1982, and mostly raised in South Florida. After graduating high school, Ellen attended Washington University in St. Louis, where she was first exposed to laboratory research. Under the mentorship of Nicholas Davidson, she began her research experiences in biomedical research and during her two years in the Davidson laboratory received an American Gastroenterological Association Student Research Fellowship Award. In 2004, Ellen graduated cum laude with a Bachelor of Arts in Biology and a minor in Chinese Language and Culture. She also received a National Institutes of Health (NIH) Postbaccalaureate Intramural Research Training Award which provided her a one-year research fellowship at the NIH (Division of Diabetes, Digestive and Kidney Diseases) in the laboratory of Jonathan Hanover. In 2005, Ellen enrolled in the Cell and Molecular Biology Program and subsequently the Molecular Cancer Biology Program at Duke University where she joined the laboratory of Tony Means. Her efforts in the lab helped lead to a publication in Molecular Biology of the Cell, entitled "Gfer inhibits Jab1-mediated degradation of $p27^{kip1}$ to restrict proliferation of hematopoietic stem cells", as well as a second publication in the Journal of Leukocyte Biology, entitled " A cell-intrinsic role for CaMKK2 in granulocyte lineage-commitment and differentiation".