Cell PRESS
Developmental Cell
Short Article

p38MAPK Controls Expression of Multiple Cell Cycle Inhibitors and Islet Proliferation with Advancing Age

Esther Sook Miin Wong,[1,4] Xavier Le Guezennec,[1,4] Oleg N. Demidov,[1] Nicolette Theresa Marshall,[1] Siew Tein Wang,[2] Janakiraman Krishnamurthy,[3] Norman E. Sharpless,[3] N. Ray Dunn,[2] and Dmitry V. Bulavin[1,*]
[1]Institute of Molecular and Cell Biology, 61 Biopolis Drive, Proteos 138673, Singapore
[2]Institute of Medical Biology, 8A Biomedical Grove, Immunos 138648, Singapore
[3]Departments of Medicine and Genetics, The Lineberger Comprehensive Cancer Center, The University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA
[4]These authors contributed equally to this work
*Correspondence: dvbulavin@imcb.a-star.edu.sg
DOI 10.1016/j.devcel.2009.05.009

SUMMARY

Aging is a complex organismal process that is controlled by genetic, environmental, and behavioral factors. Accumulating evidence supports a role for different cell cycle inhibitors in mammalian aging. Little is known, however, about the upstream signals that induce their expression. Here, we explore the role of p38MAPK by generating a dominant-negative allele (*p38^AF^*) in which activating phosphorylation sites Thr180 and Tyr182 are mutated. Heterozygous *p38^AF^* mice show a marked attenuation of p38-dependent signaling and age-induced expression of multiple cell cycle inhibitors in different organs, including pancreatic islets. As a result, aged *p38^AF/+^* mice show enhanced proliferation and regeneration of islets when compared to wild-type littermates. We further find an age-related reduction in expression of the p38-specific phosphatase Wip1. *Wip1*-deficient mice demonstrate decreased islet proliferation, while *Wip1* overexpression rescues aging-related decline in proliferation and regenerative capacity. We propose that modulation of p38MAPK activity may provide new avenues for treating certain age-related degenerative diseases.

INTRODUCTION

A major aspect of mammalian aging is an age-associated decline in functional competence of certain self-renewing cell types, including adult stem cells, early proliferative progenitors, and more differentiated cell types (Molofsky et al., 2006; Krishnamurthy et al., 2006; Janzen et al., 2006). While several molecular pathways have been implicated in the regulation of age-related phenotypes, recent studies have focused on the products of the *Cdkn2a* tumor suppressor locus, Ink4a and Arf, that inhibit proliferation and promote senescence. Expression of $p16^{Ink4a}$ and $p19^{Arf}$ increases markedly with aging in several murine tissues and is modulated by anti-aging maneuvers such as caloric restriction (Zindy et al., 1997; Krishnamurthy et al., 2004). More recent work with hematopoietic stem cells, neural stem cells, and pancreatic islets has shown $p16^{Ink4a}$ levels not only correlate with aging, but also contribute to age-induced replicative failure in these tissues (Molofsky et al., 2006; Krishnamurthy et al., 2006; Janzen et al., 2006). While $p16^{Ink4a}$ appears to serve as a biomarker and effector of aging, the molecular basis for its induction with aging is poorly understood.

The most extensively characterized regulators of $p16^{Ink4a}$ expression are those that are activated in response to oncogenes. For example, in human fibroblasts, Ras-Raf activation results in increased $p16^{Ink4a}$ expression through Erk-mediated activation of Ets1/2 transcription factors (Ohtani et al., 2001). Additionally, a few repressors of *Cdkn2a* transcription have been identified. These include the T-box proteins and the polycomb group (PcG) genes (*Bmi1*, *Ezh2*, *Cbx7*, *Mel18*) (Jacobs et al., 1999; Jacobs et al., 2000; Gil et al., 2004; Kranc et al., 2003).

Several lines of evidence support a role for p38MAPK in the regulation of $p16^{Ink4a}$ and $p19^{Arf}$ expression. Overexpression of active MKK3 or MKK6, which lie upstream of p38MAPK, results in activation of $p16^{Ink4a}$ transcription under certain conditions (Wang et al., 2002). Activation of p38MAPK in cells that are deficient in the p38 negative regulator, Wip1 phosphatase, also contributes to $p16^{Ink4a}$ and $p19^{Arf}$ upregulation in some, but not all, cell types and could explain why Wip1-deficient MEFs exit the cell cycle prematurely (Bulavin et al., 2004). This pathway could also be critical in rendering *Wip1*-deficient MEFs resistant to oncogene-induced transformation. Finally, several candidate regulators of $p16^{Ink4a}$ and $p19^{Arf}$ expression, including Bmi1, have been identified that p38MAPK may regulate in some in vitro systems (Voncken et al., 2005). Whether p38MAPK signaling modulates expression of *Ink4a/Arf* and other inhibitors of cell cycle progression with aging in vivo, however, is largely unknown.

RESULTS

No Apparent Phenotype in Mutant Mice with Attenuated p38MAPK Signaling

To investigate the role of p38MAPK in vivo, we generated $p38\alpha$ mutant mice in which we substituted Thr180 with Ala and

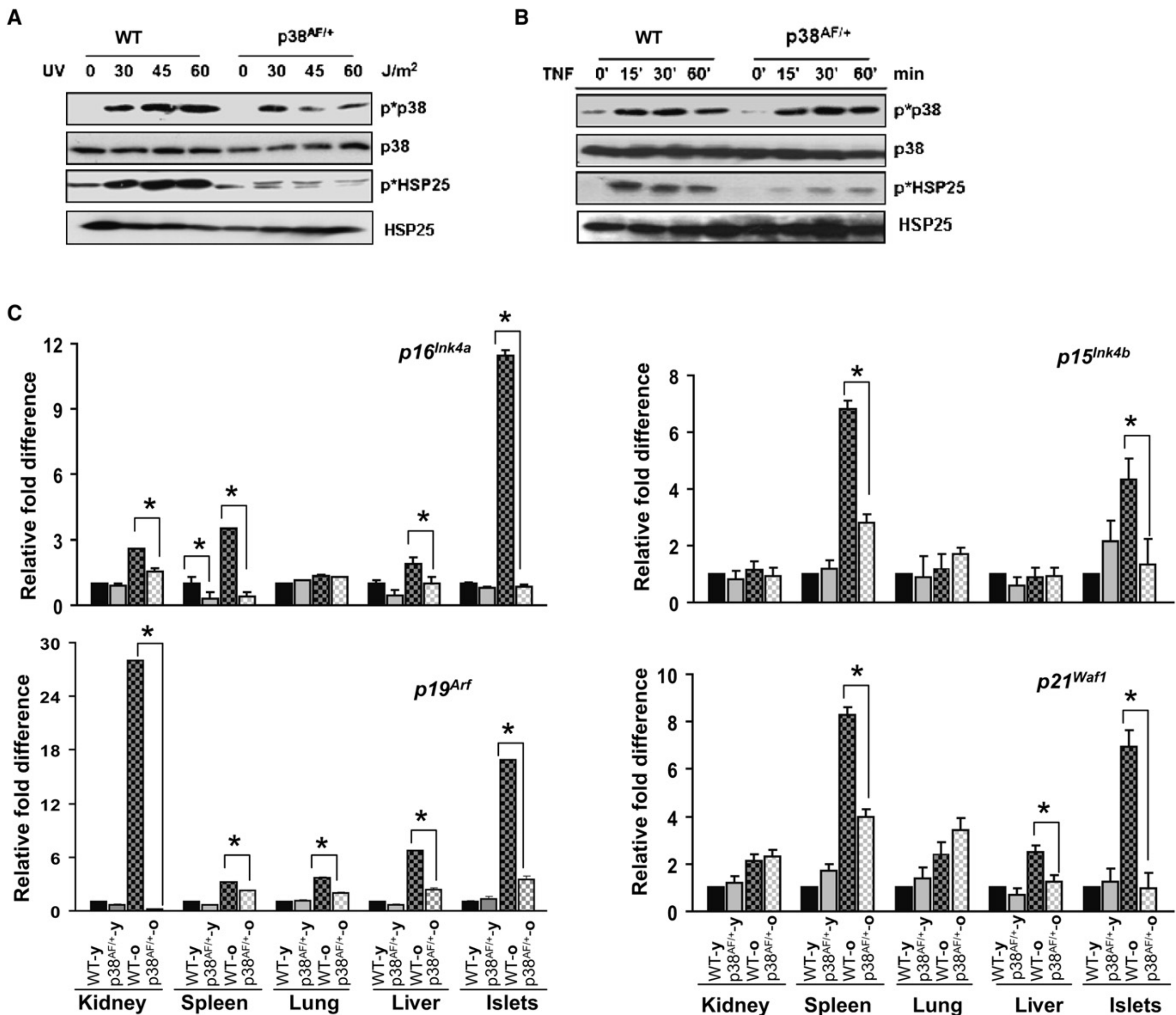

Figure 1. Activation of p38MAPK Is Required for Induction of Inhibitors of Cyclin-Dependent Kinases with Age

(A) Activation of p38 pathway was analyzed 30min after different doses of UVC treatment in MEFs obtained from WT and *p38*$^{AF/+}$ mice. Analysis of HSP25 phosphorylation was used as a read out for p38MAPK activation.

(B) Activation of p38 pathway was analyzed in TNFα (10 ng/ml)-treated MEFs obtained from WT and *p38*$^{AF/+}$ mice.

(C) Analysis of gene expression in young (2–3 months old, Y) and old (22–25 months old, O) WT and *p38*$^{AF/+}$ mice was carried out in different organs using RT-PCR. At least six mice for each genotype were used for analysis. $^*p < 0.05$.

Tyr182 with Phe (see Figures S1A–S1D available online). These phosphorylation sites are essential for p38MAPK activation, and we expectedly found that homozygous *p38*$^{AF/AF}$ mutant embryos die around day 11.5 with placental and heart defects (Figures S1E and S1F) similar to that observed in *Mkk3/6*-double mutant and *p38*α-deficient mice (Adams et al., 2000; Brancho et al., 2003). As mutation of these phosphorylation sites in vitro generates a dominant negative p38 isoform (Raingeaud et al., 1996), we therefore investigated the *p38*^{AF} allele for this possibility in vivo. *p38*$^{AF/+}$ mice were intercrossed, and mouse embryo fibroblasts (MEFs) were derived from day 10.5 embryos of three genotypes: wild-type, *p38*$^{AF/+}$, and *p38*$^{AF/AF}$. The MAPK superfamily consists of three major signaling pathways, Erk, Jnk, and p38. We found that the activation of Erk and Jnk in *p38*$^{AF/+}$ and *p38*$^{AF/AF}$ MEFs was similar to wild-type cells after treatment with UVC and TNFα at different doses and time points (Figures 1A, 1B, and S2). In contrast, phosphorylation of the p38MAPK downstream target Hsp25 was abrogated in *p38*$^{AF/AF}$ and substantially reduced in *p38*$^{AF/+}$ MEFs (Figures 1 and S2). Thus, deregulation of p38MAPK signaling either by loss of the upstream kinases, Mkk3 and Mkk6 (Brancho et al., 2003), or by mutation of activating phosphorylation sites (present study), specifically attenuates p38MAPK signaling without an effect on other MAPK pathways. In addition, our finding confirms that

the *p38*^{AF} allele encodes a dominant-negative isoform, with only one mutant allele sufficient to suppress p38MAPK signaling in MEFs.

p38MAPK plays an important role in numerous cellular processes and was proposed to be a tumor suppressor (reviewed in Bulavin and Fornace, 2004). To understand whether attenuation of p38MAPK in our mouse model contributes to tumorigenesis, we analyzed *p38*$^{AF/+}$ mice for predisposition to spontaneous tumors. A careful examination of old mice revealed no apparent difference between wild-type and *p38*$^{AF/+}$ littermates in formation of spontaneous tumors (data not shown). We further crossed *p38*$^{AF/+}$ mice with *E*μ*-myc* transgenics and *APC*^{Min} mice to investigate whether p38MAPK contributes to lymphoid and epithelial cancers, the most common types of tumors in mice. No difference was found in the onset of B cell lymphomas or the number of polyps between different genotypes (Figure S3). Thus, reduction of p38 signaling as seen in *p38*$^{AF/+}$ mice does not contribute to spontaneous, *myc*- or *APC*^{Min}-induced tumorigenesis.

p38MAPK Plays a Major Role in Regulating Expression of Multiple Cell Cycle Inhibitors

To investigate whether p38MAPK regulates *p16*^{Ink4a} and *p19*^{Arf} expression with age, we next analyzed gene expression in different organs obtained from young (2-3 months) and old (22–25 months) wild-type and *p38*$^{AF/+}$ littermates (Figure 1C). Consistent with previous data (Zindy et al., 1997; Krishnamurthy et al., 2004), quantitative PCR analysis reveals an increase in *p16*^{Ink4a} and *p19*^{Arf} mRNA levels in multiple organs in wild-type mice of advanced age. In striking contrast, *p16*^{Ink4a} and *p19*^{Arf} levels remain low in old *p38*$^{AF/+}$ mice at levels comparable to young mice (Figure 1C, left panels). This finding suggests that p38MAPK activity is required for full *Ink4a/Arf* induction with aging in several tissues.

We next extended our analysis to two other inhibitors of cyclin-dependent kinases, *Cdkn2b* (*p15*^{Ink4b}) and *p21*^{Waf1}. *Cdkn2b* is located in close physical proximity to the *Cdkn2a* locus and its expression is coordinately regulated in some settings (e.g., *Bmi1* loss; Jacobs et al., 1999; Gonzalez et al., 2006), while *p21*^{Waf1} is a downstream target of p19^{Arf}-p53 signaling pathway. The expression of these two genes is also increased in multiple tissues harvested from old wild-type mice, although to a lesser extent than *p16*^{Ink4a} and *p19*^{Arf} (Figure 1C, right panels). As with *Ink4a/Arf* expression, however, *p15*^{Ink4b} and *p21*^{Waf1} expression levels in old *p38*$^{AF/+}$ mice were generally lower than wild-type mice and instead were comparable to expression in young mice.

p38MAPK Controls Age-Related Decline in Islet Proliferation and Regenerative Capacity

Given that islet replication is known to decline with age in humans (Butler et al., 2003) and mice (Teta et al., 2005; Krishnamurthy et al., 2006) and given the large effects on gene expression we observed in isolated *p38*$^{AF/+}$ islets (Figure 1C), we chose to further investigate the impact of compromised p38MAPK signaling on islet physiology and proliferation. First, we analyzed the level of Hsp25 phosphorylation, a readout for p38MAPK signaling. Hsp25 phosphorylation was significantly increased in the pancreas of old wild-type mice (Figure 2A). In contrast, no detectable change was observed in old versus young *p38*$^{AF/+}$ mice. This observation suggests that age-related changes in islets result in activation of p38MAPK (Figure 2A), which in turn upregulates *p16*^{Ink4a} and *p19*^{Arf} (Figure 1C).

As p16^{Ink4a} limits proliferation and regenerative capacity of β cells and is induced with advancing age in wild-type, but not *p38*$^{AF/+}$ mice (Figure 1C), we tested whether decreased *p16*^{Ink4a} expression in *p38*$^{AF/+}$ mice influenced islet physiology. Plasma blood glucose measurements following intraperitoneal glucose tolerance tests (IPGTT) and insulin tolerance tests (ITT) revealed neither compromised insulin sensitivity nor evidence for insulin resistance in *p38*$^{AF/+}$ mice (data not shown). Furthermore, overall pancreatic morphology, islet size, shape, and number were similar between wild-type and *p38*$^{AF/+}$ littermates of different age groups (data not shown). Therefore, in unstressed animals, attenuated p38MAPK signaling did not overtly enhance islet function, even in old mice.

To determine the effects of p38MAPK with aging, islets in young and old wild-type and *p38*$^{AF/+}$ littermates were analyzed. No evidence of pancreatic neoplasia was noted in *p38*$^{AF/+}$ animals (data not shown). Islet proliferation measured by calculating the Ki67^{+} proliferation index was significantly decreased with aging in wild-type mice (Figure 2B). Attenuation of p38 signaling as in *p38*$^{AF/+}$ mice did not affect islet proliferation in young mice; however, the age-induced decrease in islet proliferation in 22- to 24-month-old *p38*$^{AF/+}$ mice was partially rescued when compared to wild-type counterparts. We believe that incomplete rescue of islet proliferation noted in *p38*$^{AF/+}$ mice is most likely the result of *Ink4a/Arf*-independent mechanisms of aging as was previously described for *p16*^{Ink4a}-deficient mice (Krishnamurthy et al., 2006).

To study the effect of attenuated p38MAPK signaling on islet physiology under stressed conditions, we utilized a well-characterized model of islet regeneration after treatment with streptozocin (STZ). STZ is a specific β cell toxin that induces rapid hyperglycemia, simulating type 1 diabetes, when given as a single dose. Importantly, the ability to control islet proliferation and glucose levels as well as the survival of STZ-treated mice decline with advancing age and are enhanced in animals lacking *p16*^{Ink4a} (Krishnamurthy et al., 2006). We could not use mice older than 14 months for this study due to prohibitively high mortality rate (data not shown). Mice of different ages were injected with STZ and glucose levels were monitored every week for 12 weeks (Figure 2C). Blood glucose measurements after STZ treatment were not different between young wild-type and *p38*$^{AF/+}$ mice (Figure 2C, top panel). However, significant differences emerged between the two genotypes in older mice after 11 weeks in 4- to 6 month old mice (Figure 2C, middle panel) and after 7 weeks in 9- to 14 month old mice (Figure 2C, bottom panel).

To understand whether the differences in glucose levels between wild-type and *p38*$^{AF/+}$ mice after STZ treatment were reflective of differences in islet proliferation, we carried out Ki67 staining in 10- to 12-month-old mice. We found that attenuation of p38MAPK signaling in *p38*$^{AF/+}$ mice resulted in a significant increase in islet proliferation when compared to wild-type mice both at 3 and 30 days after STZ treatment (Figure 2D). Taken together, these data support the conclusion that recovery from STZ treatment, which is dependent upon *p16*^{Ink4a}

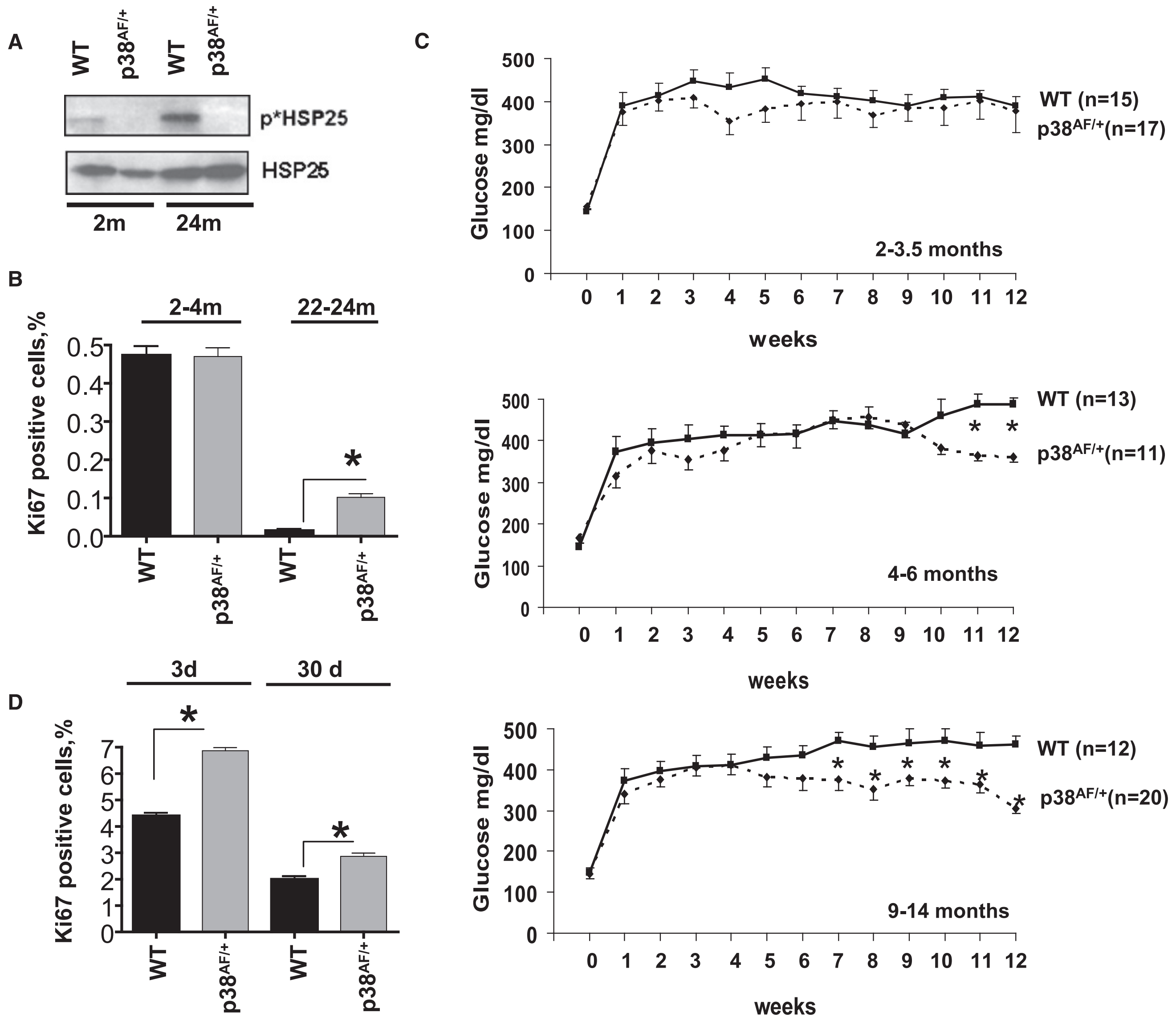

Figure 2. p38MAPK Attenuates Islet Proliferation and Regeneration with Aging

(A) Activation of p38 MAPK was analyzed in the pancreas obtained from young and old WT and *p38*$^{AF/+}$ mice. Analysis of HSP25 phosphorylation was used as a readout.

(B) Islet proliferation was measured by Ki67 staining, and was performed on young (2–4 months) and old (22–24 months) wild-type and *p38*$^{AF/+}$ mice.

(C) WT and *p38*$^{AF/+}$ mice of different age (as indicated in the panel) were treated with STZ and their blood glucose levels determined weekly. All mice that died within 2 weeks following injection were excluded from analysis. Wild-type mice are depicted with a solid and *p38*$^{AF/+}$ with a dashed line.

(D) Islet proliferation based on Ki67 staining was analyzed in 10- to 12-month-old wild-type and p38$^{AF/+}$ mice at 3 and 30 days after STZ injection. *$p < 0.05$.

regulation of islet proliferation (Krishnamurthy et al., 2006), is hastened in aged *p38*$^{AF/+}$ mice compared to their wild-type counterparts.

Wip1 Phosphatase Is a Candidate Regulator of p38MAPK with Aging

The mechanisms that regulate p38MAPK in old wild-type mice (Figure 2A) are largely uncharacterized. Candidates include the p38 phosphatases such as Wip1. As aging results in reduced proliferation of stem cells and early progenitors (Molofsky et al., 2006; Krishnamurthy et al., 2006; Janzen et al., 2006), p38-dependent regulation of *Cdkn2a* expression through regulation of Wip1 levels is of particular interest. Wip1 is abundant in adult stem cells, and loss of *Wip1* results in increased *Ink4a/Arf* expression and tumor resistance in multiple mouse models (Bulavin et al., 2004; Shreeram et al., 2006; Demidov et al., 2007). We therefore analyzed *Wip1* expression in enzymatically isolated islets and found a significant decrease ($p < 0.05$) in *Wip1* mRNA levels in islets of old versus young wild-type mice, while the difference was insignificant in *p38*$^{AF/+}$ mice (Figure 3A). Because downregulation of *Wip1* may contribute to activation of p38MAPK with age, we next took advantage of

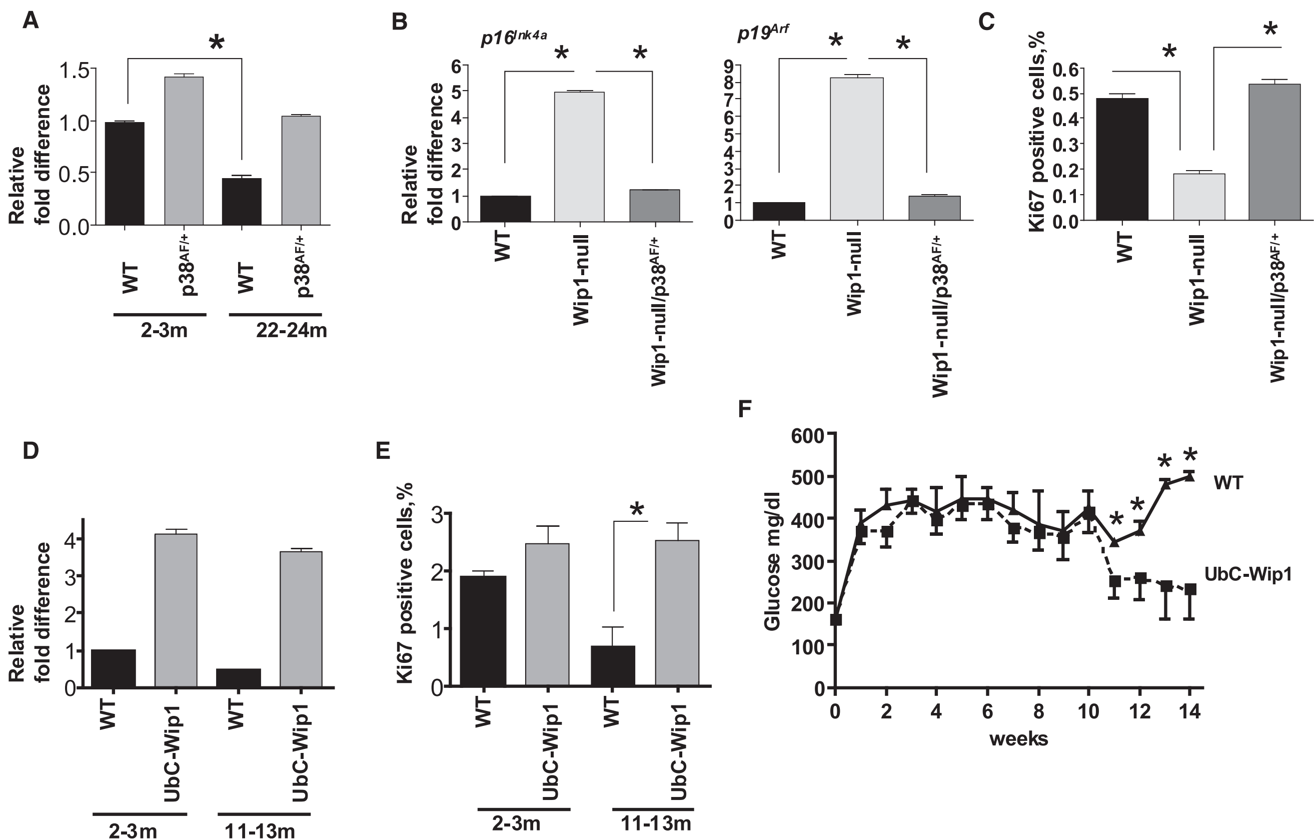

Figure 3. Dose-Dependent Effect of Wip1 on Islet Proliferation and Regeneration with Aging

(A) Downregulation of *Wip1* mRNA levels with age in WT mice. The level of *Wip1* was analyzed in young (2–3 months) and old (22–24 months) WT and $p38^{AF/+}$ mice by RT-PCR. The level of *Wip1* mRNA in young wild-type mice was assumed as 1.

(B) Analysis of *Cdkn2a* gene expression in 4- to 6-month-old mice of different genotypes.

(C) Analysis of Ki67-positive cells in islets of 4- to 6-month-old mice of different genotypes.

(D) The level of *Wip1* mRNA levels in islets from young (2–3 months) and aged (11–12 months) WT and *UbC-Wip1* mice. The level of *Wip1* mRNA in young wild-type mice was assumed as 1.

(E) Islet proliferation based on Ki67 staining was analyzed in young (2–3 months) and aged (11–13 months) WT and *UbC-Wip1* transgenic mice.

(F) Six- to eight-month-old WT and *UbC-Wip1* mice were injected with STZ and glucose levels were monitored every week. *$p < 0.05$.

Wip1-decifient mice to investigate *Cdkn2a* expression in islets. *Wip1*-deficient mice display heightened p38MAPK activity (Bulavin et al., 2004), thus allowing us to address the question whether forced activation of p38 is sufficient to induce *Cdkn2a* expression. First, we demonstrated that the level of p38 phosphorylation in *Wip1*-deficient islets is efficiently reduced to wild-type levels by introducing the $p38^{AF}$ allele (Figure S4A). Furthermore, Wip1 deficiency resulted in upregulation of *Cdkn2a* genes in a p38MAPK-dependent manner (Figure 3B). Lastly, islet proliferation in *Wip1*-deficient mice was reduced in a p38MAPK-dependent manner (Figure 3C). We further observed impaired glucose tolerance in *Wip1*-deficient mice following IPGTT (Figure S4B). This effect was totally reversed to wild-type situation in *Wip1*-deficient mice carrying the $p38^{AF}$ allele.

While *Cdkn2a* expression in islets, impaired proliferation and glucose intolerance in *Wip1*-deficient mice show dependence on p38MAPK, these effects could be indirect acting through imposed non-physiological stress, which in turn cause β-cell failure via p38-dependent mechanisms, such as hyperglycemia. To rule out such a possibility, we chose to use transgenic mice in which *Wip1* expression is driven throughout all tissues by the *ubiquitin C* (*UbC*) promoter (Le Guezennec et al., personal communication). The *UbC* promoter allows only a mild (~4-fold) upregulation of *Wip1* (Figure 3D), which falls within the normal range of transcriptional activation of *Wip1* by assorted stimuli (Fiscella et al., 1997). In turn, this level of *Wip1* overexpression was sufficient to abolish age-induced activation of $p16^{Ink4a}$ and $p19^{Arf}$ in islets (data not shown). We next examined whether an increase in *Wip1* levels would be sufficient to modulate islet proliferation with aging. Young (2–3 months old) and aged (11–13 months old) wild-type and *UbC-Wip1* littermates were sacrificed and analyzed for islet proliferation based on Ki67 staining. While we observed ~3-fold drop in proliferation of wild-type FVB/N mice between 2 and 12 months, *UbC-Wip1* FVB/N mice retained their high proliferative potential (Figure 3E). Thus, overexpression of *Wip1* is sufficient to overcome the age-related decline in islet proliferation.

We next analyzed the regenerative capacity of islets under conditions of *Wip1* overexpression. As young $p16^{Ink4a}$-deficient and $p38^{AF/+}$ mice do not show any attenuation in STZ response

(Krishnamurthy et al., 2006; Figure 2C), for this set of experiments we used only 6- to 10-month-old mice. Mice were injected with STZ, and their survival, weight, and glucose levels were monitored every week for 14 weeks (Figures 3F and S5). As these mice were maintained in a pure FVB/N background, we observed a significant difference ($p = 0.042$) in the survival and a consistent change in the weight of injected wild-type and *UbC-Wip1* mice (Figure S5). Significant differences in glucose levels also emerged between the two genotypes after 11 weeks (Figure 3F), supporting the idea that *Wip1* overexpression is sufficient to reduce age-related decline in regenerative capacity of islets. Taken together, our genetic data suggest that modulation of Wip1 levels (Figure 3) is sufficient to regulate islet proliferation and their regenerative capacity with advancing age.

Bmi1 Binding to the Ink4a Promoter Is Regulated in a p38-Dependent Manner with Aging

Several candidate regulators of $p16^{Ink4a}$ and $p19^{Arf}$ expression have been identified and some of them, including Bmi1, have been found to be regulated by p38MAPK in some in vitro systems. It has been shown that MK3, a downstream target of p38, can directly phosphorylate Bmi1 and reduce its abundance on chromatin (Voncken et al., 2005), a finding that we have reproduced in vitro (Figure S6). To directly assess the *Ink4a* promoter occupancy by Bmi1 with aging, we next carried out a modified chromatin immunoprecipitation (ChIP) assay. We could not perform ChIP on islets due to insufficient amount of material; therefore, we used spleen as an alternative since it shows a similar p38-dependent upregulation of cell cycle inhibitors with aging (Figure 1C). While we did not see a significant difference in *Bmi1* mRNA levels between different genotypes of different age, we found that Bmi1 abundance on the *Ink4a* promoter was reduced ~5-fold in splenocytes obtained from old when compared to young wild-type mice. In turn, there was only an ~2.5-fold decrease for age-matched $p38^{AF/+}$ mice (Figure 4). Thus, Bmi1 binding to the *Ink4a* promoter is regulated in a p38-dependent manner with aging.

DISCUSSION

To investigate the role of p38α MAPK in vivo, we established a new mouse genetic model in which activating phosphorylation sites Thr180 and Tyr182 were mutated to alanine and phenylalanine, respectively. Embryos homozygous for the manipulated $p38^{AF}$ allele die during mid-gestation due to placental and heart defects (Figures S1E and S1F) similar to *p38* knockout mice (Adams et al., 2000; Tamura et al., 2000). In accordance with previously published data using *p38* null and *Mkk3/6*-double null MEFs (Bulavin et al., 2002; Brancho et al., 2003), $p38^{AF/AF}$ MEFs grew into large tumors when explanted into nude mice (data not shown). These results are consistent with the role of p38 as a negative regulator of tumorigenesis (Bulavin et al., 2002; Brancho et al., 2003). However, partial inactivation of p38, as seen in $p38^{AF/+}$ cells, did not result in accelerated tumorigenesis, and $p38^{AF/+}$ mice crossed into two tumor-prone genetic backgrounds show tumorigenic potential comparable to wild-type (Figure S3). In turn, partial inactivation of p38MAPK is sufficient to prevent aging-induced activation of multiple cell cycle inhibitors, including *Cdkn2a* (Figure 1C), and

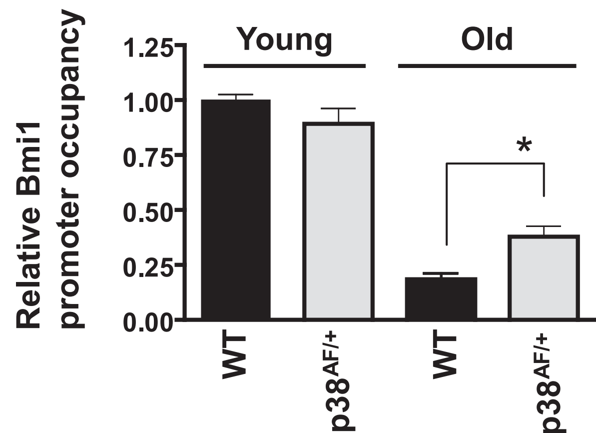

Figure 4. p38-Dependent Regulation of Bmi1 Binding to the Ink4a Promoter with Aging
Analysis of Bmi1 binding to the *Ink4a* promoter was analyzed by ChIP in splenocytes obtained from young (2–3 months old) and old (22–25 months old) WT and $p38^{AF/+}$ mice. At least six mice for each genotype were used for analysis. $^{*}p < 0.05$.

improves proliferation and regeneration in the islets of old $p38^{AF/+}$ mice (Figures 2B and 2D).

Cdkn2a genes play a major role in regulating the decline in proliferation and regenerative capacity in certain organs as they age (Molofsky et al., 2006; Krishnamurthy et al., 2006; Janzen et al., 2006) and, consequently, are major tumor suppressors (Sherr, 2001). Attenuation of *Cdkn2a* expression (by either gene knockout or by overexpression of the negative regulator *Bmi1*) ameliorates the effects of aging, but may also serve as a double-edged sword by enhancing tumorigenesis. Our data provide important insight into the level at which activation of *Cdkn2a* genes can be disrupted in order to improve age-induced phenotypes without promoting tumorigenesis. In this respect, partial inactivation of p38 is sufficient to reduce aging-dependent activation of cell cycle inhibitors $p16^{Ink4a}$, $p19^{Arf}$, $p15^{Ink4b}$, and $p21^{Waf1}$ in multiple tissues without increasing cancer predisposition (Figures 1C and S3).

p38MAPK has been implicated in the upstream regulation of *Cdkn2a* expression in vitro through several signaling pathways that either directly or indirectly modulate $p16^{Ink4a}$ and $p19^{Arf}$ expression. A recently identified link between p38MAPK and Bmi1 (Voncken et al., 2005) is of particular interest as Bmi1 has been shown to regulate stem cell behavior in part through its ability to modulate *Cdkn2a* expression (Molofsky et al., 2003). It has been shown that MK3, a downstream target of p38, can directly phosphorylate Bmi1 and reduce its abundance on chromatin (Voncken et al., 2005), a finding that we have reproduced both in vitro and in vivo (Figures S6 and 4). Given this connection, we favor a p38-Bmi1 link in the regulation of *Cdkn2a* expression in aging tissues.

The mechanism of p38MAPK activation with advanced age remains unclear (Figure 2A). Here, we show that it coincides with downregulation of a potent regulator of p38, Wip1 phosphatase (Figure 3A). *Wip1* deficiency results in a p38-dependent upregulation of *Cdkn2a* genes and impairs islet proliferation (Figure 3B,C). In turn, *Wip1* overexpression averts the age-related decline in proliferation and regenerative potential of islets

(Figures 3D–3F). Thus, Wip1 may serve as a potent physiological regulator of p38 signaling with aging.

In summary, we propose that in a situation when a premature aging phenotype or any aging-related degenerative condition is dependent upon p38MAPK, attenuation of its signaling could be beneficial in ameliorating such conditions. As this can be accomplished pharmacologically through the use of small molecule inhibitors of p38 kinase activity now entering human clinical trials, our genetic data suggest an approach to attenuate some aspects of mammalian aging.

EXPERIMENTAL PROCEDURES

Targeted Mutation of p38MAPK in ES Cells

The original BAC clone RP23-83F4 of *Mus musculus* strain C57BL6/J (NCI Intramural Sequencing center) was used to generate all targeting vectors. Targeting vectors were constructed that contained the equivalent of 4.5 kb of mouse DNA with an exon containing the Thr180/Tyr182 sites of p38 alpha. These sites were subsequently mutated to alanine and phenylalanine by site-directed mutagenesis. An additional BamH1 site was incorporated 500 bp outside the Thr180/Tyr182 sites. A PGK-neo cassette (in either of two orientations) flanked by two LoxP sites was inserted into Nco1 site. The entire vector was placed into pTK plasmid, which contained two flanking thymidine kinase genes. The two targeting vectors were electroporated into B6 ES cells (kindly provided by Dr. Colin Stewart [NIH, Frederick]). For genotyping by genomic Southern-blot analysis, we probed BamH1-digested DNA with a 500 bp fragment corresponding to a region in genomic DNA immediately 5′ to that in the targeting vector. Southern blot analysis of ES cell clones yielded an approximately 9 kb hybridizing fragment corresponding to the endogenous p38, whereas the mutated AF allele was seen as 5.9kb hybridizing band.

Statistical Analysis

To assess the statistical significance of the results, each experiment was repeated at least three times, means and standard deviations between different groups were calculated, and error bars were included where necessary. Student's t test was performed for paired analysis. Kaplan-Meier analysis of survival was performed with the assistance of PRISM4 statistical analysis software.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00211-1.

ACKNOWLEDGMENTS

N.R.D. and D.V.B. were supported by the Agency for Science, Technology and Research (A*STAR, Singapore). We are grateful to K. Helin for his kind gift of Bmi1 antibody.

Received: October 23, 2008
Revised: April 7, 2009
Accepted: May 14, 2009
Published: July 20, 2009

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