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IN VIVO ANALYSIS OF GENE EXPRESSION IN LONG-LIVED MICE LACKING THE PREGNANCY-ASSOCIATED PLASMA PROTEIN A (*PAPPA*) GENE

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Abstract

Mice lacking the pregnancy-associated plasma protein A (*PappA*) gene exhibit diminished localized IGF-1 bioavailability and a 30% increase in mean life span. However, it is uncertain which tissues exhibit reduced IGF-1 signals in the *PappA*($-/-$) mouse, and whether effects of this mutation parallel those of mutations that diminish IGF-1 in serum. Across a panel of 21 tissues, we used RT-PCR to evaluate the effects of the *PappA*($-/-$) mutation on expression of *Igfbp5*, which served as an *in vivo* indicator of IGF-1 signaling. Among these tissues, expression of *Igfbp5* was significantly reduced by *PappA*($-/-$) only in kidney. A broader survey of IGF-associated genes in six organs identified five other genes responsive to *PappA*($-/-$) in kidney, with stronger effects in this organ relative to other tissues. Renal expression of *Irs1* and *Mt1* was increased by *PappA*($-/-$) as well as by mutations that reduce IGF-1 in serum (i.e., *Ghr*($-/-$), *Pit1*(*dw/dw*) and *Prop1*(*df/df*)), and we demonstrate that expression of these genes is regulated by growth hormone-treatment and calorie restriction. These results provide *in vivo* data on an important new model of mammalian aging, and characterize both similar and contrasting expression patterns between long-lived mice with reduced local IGF-1 availability and diminished IGF-1 in serum.

Keywords

aging; dwarf; growth hormone; insulin-like growth factor; lifespan; longevity

1. Introduction

Mice that lack the gene encoding pregnancy-associated plasma protein A (*PappA*) exhibit a substantial increase in lifespan and a delayed rate of age-associated thymic atrophy (Conover et al., 2007; Vallejo et al., 2009). The *PappA*($-/-$) mutation was observed to increase mouse lifespan by 22 – 43%, which is a larger effect than has been observed for any other pro-longevity mutation recently identified (Conover et al., 2007; Swindell, 2009).

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This strong lifespan effect arises from a narrow endocrine deficiency that involves localized reductions of IGF-1 bioavailability, without significant decline in serum GH or IGF-1 levels (Conover et al., 2007, 2008). This localized endocrine effect contrasts with other long-lived dwarf mouse models, such as the *Ghr(-/-)*, *Pit1(dw/dw)* and *Prop1(df/df)* mice, in which circulating IGF-1 levels are diminished, along with other endocrine factors in some cases (e.g., GH, thyroid stimulating hormone, prolactin) (Berryman et al., 2008). The narrow endocrine deficiency associated with the *PappA(-/-)* mutation, combined with its large effect on lifespan, make the *PappA(-/-)* mouse an attractive model system from the standpoint of basic aging research. In particular, because the *PappA(-/-)* mouse lacks any known secondary endocrine deficiency resulting from reduced IGF-1 bioavailability within tissues, dissecting out the relationship between the effects of this mutation and overall lifespan may prove more tractable than is the case for other varieties of long-lived dwarf mice. Moreover, since localized IGF-1 synthesis is GH-dependent in both hepatic and extrahepatic tissues (Phillips et al., 1990), reduced IGF-1 availability within certain tissues could be a feature that *PappA(-/-)* mice share with long-lived mutants for which GH and/or IGF-1 levels are reduced in circulation (Conover et al., 2008).

The *PappA(-/-)* mutation regulates localized IGF-1 bioavailability through a mechanism involving the PAPP-A protease and its interaction with insulin-like growth factor binding protein 4 (i.e., IGFBP-4) (Boldt et al., 2007). Within an *in vitro* context, the PAPP-A protease has been shown to cleave IGFBP-4 in a reaction that requires the presence of either IGF-1 or IGF-2 as cofactors (Lawrence et al., 1999). Because IGFBP-4 binds IGF-1/2 and prevents binding of these IGFs to their receptors, this cleavage reaction should effectively govern the *in vivo* availability of IGF-1 and 2 within the local tissue environment (Boldt et al., 2007). In the *PappA(-/-)* mouse, therefore, absence of the PAPP-A protease is believed to promote over-accumulation of IGFBP-4, resulting in reduced bioavailability of IGF-1/2 and diminished signals downstream of IGF-1/2. This model has been supported by the observation that *PappA(-/-)* mice are, at birth, only 60% the body weight of wild-type littermates, consistent with reduced availability of at least IGF-2 during the course of embryonic development (Conover et al., 2004). Additionally, *PappA(-/-)* mice have reduced femoral bone mineral density and diminished vascular cell proliferation in response to injury, and both of these effects are consistent with reduced *in vivo* growth factor availability (Resch et al., 2006; Tanner et al., 2008). The effects of *PappA(-/-)* on lifespan also appear consistent with previous work, in worms, flies and mice, showing that depression of insulin / insulin-like signals has positive effects on aging and longevity (Berryman et al., 2008; Taguchi et al., 2008). In *PappA(-/-)* mice, increased survivorship is accompanied by delayed thymic atrophy with age and increased spontaneous physical activity (Conover et al., 2008; Vallejo et al., 2009). Surprisingly, however, metabolic parameters of the *PappA(-/-)* mice are normal in many respects, and *PappA(-/-)* mice do not differ from wild type littermates in terms of food intake, total or resting energy expenditure, glucose levels, insulin levels, or sensitivity to glucose and insulin (Conover et al., 2008). At present, specific pathologies influenced by the *PappA(-/-)* mutation are not completely established, but necropsy of older *PappA(-/-)* mice has revealed reduced tumor burden (Conover et al., 2007; Vallejo et al., 2009).

The influence of the *PappA(-/-)* mutation on IGF-1 availability and signaling may vary among cell types and organs. In some tissues, cellular IGF-1 synthesis may be weak, such that localized over-abundance of IGFBP-4 would have little influence on signals associated with activation of the IGF-1 receptor. Additionally, some cell types may express low levels of the PAPP-A protease, or the IGFBP-4 binding protein, or cofactors required for the cleavage of IGFBP-4 by PAPP-A (e.g., IGF-1 and 2). Low expression of any of these components would alter the degree to which *PappA(-/-)* influences IGFBP-4 over-abundance, IGF-1 availability and IGF-1 signaling. Among all tissues, therefore, the

influence of the *PappA*($-/-$) mutation may be heterogeneous, with IGF-1 signaling strongly attenuated in some tissues and weakly attenuated in others. Previous studies have shown that the *PappA*($-/-$) mutation has *in vivo* effects in bone (femur), pancreas, vascular tissue, and within the thymus and associated immune cell populations (Conover et al., 2004; Tanner et al., 2008; Vallejo et al., 2009). These *in vivo* effects, however, may or may not reflect loss of IGF-1 signals in the tissue type examined, and could stem from modified IGF-1 signaling in unrelated tissues. Ultimately, establishing the relative effects of the *PappA*($-/-$) mutation on IGF-1 signals across organ systems will advance this model as a tool for exploring the local effects of IGF signals, and for distinguishing between tissue types for which loss of IGF signals has favorable versus deleterious effects on aging and longevity. In the central nervous system, for example, enhanced IGF-1 action has favorable effects on neuronal survival (Chen et al., 2005; Frago et al., 2002; Serbedzija et al., 2009; Subramaniam et al., 2005), and likewise, over-expression of IGF-1 in cardiac tissue was shown to increase mouse survivorship (Li et al., 2007). *In vivo* effects of the *PappA*($-/-$) mutation may thus, to some degree, be restricted to certain tissues for which loss of localized IGF-1 availability has a favorable influence on longevity. Potentially, such tissues may be similarly affected by other pro-longevity mutations that reduce GH / IGF-1 in serum (Berryman et al., 2008).

The goals of this study were to evaluate *in vivo* effects of the *PappA*($-/-$) mutation on the expression of IGF-associated genes across a range of mouse tissues, and to evaluate the association between these effects and those observed in long-lived dwarf mice with reduced IGF-1 levels in serum (i.e., *Ghr*($-/-$), *Pit1*(*dw/dw*) and *Prop1*(*df/df*) mice). With respect to 21 mouse tissues, we used RT-PCR to examine the expression of insulin-like binding protein 5 (*Igfbp5*) in *PappA*($-/-$) mice and their wild type littermates. The expression of *Igfbp5* is regulated by IGF-1 availability and previous studies have used expression of this gene as an indicator of IGF-1 signaling *in vivo* (Adamo et al., 2006; Bach et al., 1991; Backeljauw et al., 1993; Bartlett et al., 1991; Bondy et al., 1993; Duan et al., 1999; Harrington et al., 2007; Resch et al., 2006; Tanner et al., 2008; Ye et al., 1998). A more comprehensive evaluation of genes encoding IGF system components was also carried out with respect to six major organs (kidney, liver, heart, muscle, lung and neocortex), and in these organs we evaluate whether significant effects of *PappA*($-/-$) on gene expression are also associated with the *Ghr*($-/-$), *Pit1*(*dw/dw*) and *Prop1*(*df/df*) mutations. These results provide *in vivo* data on an important model of mouse aging and longevity, and identify gene expression patterns associated with diminished IGF-1 in tissues, in serum, or in both serum and the local tissue environment.

2. Materials and methods

2.1. Mice

All *PappA*($-/-$) and *Pit1*(*dw/dw*) mice used in this study were generated from breeding colonies at the University of Michigan, while all *Ghr*($-/-$) and *Prop1*(*df/df*) mice were generated from colonies at Southern Illinois University. The colony of *PappA*($-/-$) mice at University of Michigan was initiated from a breeding pair generously provided by Dr. Cheryl A. Conover. The generation of these *PappA*($-/-$) mice using targeted disruption of the *PappA* gene (exon 4) has been described previously (Conover et al., 2004). The *PappA*($-/-$) and *Pit1*(*dw/dw*) mutations were generated on mixed C57BL/6-129SV/E and DW/J *Pit1*^{dw} × C3H/HeJ *Pit1*^{dw-J} genetic backgrounds, respectively. The *Ghr*($-/-$) mutation was generated on a mixed background generated by crossing mice of 1290la, BALB/c, C57BL/6 and C3H strains, as described by Panici et al. (2009). The *Prop1*(*df/df*) mice are also maintained on a heterogeneous genetic background (Dhahbi et al., 2007). Genotypes of *PappA*($-/-$) mice and wild-type littermates were confirmed by PCR analysis of DNA extracted from tail snip biopsies at the time of euthanasia. All experimental animals were raised in specific-pathogen free facilities and provided *ad libitum* access to water. The

pathogen-free status of colonies was verified by exposing sentinel mice to bedding and evaluating sentinels for antibodies and parasites, with no positive test results obtained during the study period. All procedures were approved by animal care and use boards at University of Michigan and Southern Illinois University.

2.2. Total RNA extraction and RT-PCR

Tissues were dissected from euthanized mice and immediately flash-frozen in liquid nitrogen or submerged in RNAlater solution (Ambion cat. no. 7020). Tissues stored in RNAlater were placed at 4°C for at least 24 hours prior to extraction of total RNA (or extended storage at -20°C). Flash-frozen tissues were initially stored at -80°C and later transitioned to -20°C with incubation in RNAlater ICE solution (Ambion cat. no. AM7030). Immediately prior to extraction of total RNA, tissues were placed in small vessels containing RLT buffer and homogenized using a rotor-stator apparatus. The extraction of RNA was then carried out using columns provided in Qiagen RNeasy kits (Qiagen cat. no. 74104), with on-column DNase digestion to limit the possibility of DNA contamination (Qiagen cat. no. 79254). Extracted RNA was diluted in 60 – 100 µL of RNAase-free water and quantification was carried out by dispensing 2 µL of extracted RNA onto the optical pedestal of a NanoDrop spectrophotometer. Working RNA dilutions of 2 – 4 ng/µl were later prepared for use in one-step RT-PCR reactions. All reactions were set up on ice in 0.1 mL strip tubes at a volume of 20 µl with 4 – 8 ng of template RNA per reaction. Master and RT mix reagents used in RT-PCR reactions were obtained from Quantitect SYBR Green RT-PCR kits (Qiagen cat. no. 204243). Thermocycling was performed using the Rotor-Gene 3000 (Corbett) with samples arranged in a 72-well block that rotates throughout the reaction procedure, which limits block effects and ensures uniformity of temperature among samples. Melting curve analyses were performed following the completion of each run and we inspected log-derivative plots to ensure specific amplification of target and reference genes. All genes evaluated in this analysis were amplified using bioinformatically validated primer assays (Qiagen; see Supplemental Data File).

2.4. GH-treatment of ad lib and CR-fed mice

Mice of the (Ames) *Prop1(df/df)* genotype and their wild-type littermates (approximately 7 months of age) were maintained on a caloric restriction (CR) or *ad lib* diet and treated with daily injections of growth hormone (GH) or saline. The caloric restriction regime corresponded to a 30% decrease in calories relative to the *ad lib-fed* group and was maintained for a period of 8 weeks. The GH-treatments were carried out over a six week period at a dose of 4 µg/gram of body weight per day (porcine GH, Sigma, St. Louis, MO). Over the six week treatment period, we observed an average body weight increase in GH-treated *Prop1(df/df)* mice of 30%, with an average increase of only 5% in saline-treated *Prop1(df/df)* mice. Additionally, we observed significant responses in blood chemistry measures during the treatment period (insulin, IGF-1, glucose, triglycerides, FFA; data not shown).

2.4. Statistical Analysis

The ratio of gene expression in mutant mice relative to wild-type littermates was calculated using the $\Delta\Delta C_t$ method with expression of either 18S ribosomal RNA (*Rn18s*) or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as an internal reference gene. All experiments were based upon at least 6 biological replicates per treatment, with 2–3 technical replicates performed for each biological replicate. For each biological replicate, ΔC_t values were calculated as the difference in the mean C_t value obtained for the target gene and the mean C_t value obtained for the reference gene. Statistical analyses were based upon ΔC_t values, corresponding to relative expression on a logarithmic scale. For two-treatment comparisons involving mutant and wild-type littermates, a two-sample t-test (two-

sided) was used to evaluate whether the average ΔC_t value among mutant mice differed significantly from the average ΔC_t value among wild-type littermates. In a small number of cases, one or more outlying observations was present, and we applied a non-parametric Kolmogorov-Smirnov test, which evaluates the null hypothesis that ΔC_t values associated with experimental and control mice are drawn from the same continuous distribution.

For experiments evaluating expression of insulin receptor substrate 1 (*Irs1*), metallothionein 1 (*Mt1*) and insulin-like growth factor binding protein acid labile subunit (*Igfals*) in response to GH-treatment within *ad lib* and CR-fed mice, five sets of RT-PCR reactions were performed, where each set included target and reference gene reactions for $n = 2$ mice from each of the eight experimental groups. We calculated ΔC_t values for each set of reactions, and adjusted these ΔC_t values to have a mean of zero in each of the five sets ($n = 10$ per treatment in total). A three-factor analysis of variance was applied to adjusted ΔC_t values, with genotype (*Prop1*(*df/df*) or wild-type), diet (*ad lib* or CR) and treatment (GH or saline-injections) as fixed effects. *Post-hoc* comparisons among treatments were performed using the Tukey honest significant difference method, or Dunnett's approach for multiple comparisons to a common control treatment.

3. Results

3.1. In vivo effects of *PappA*(*-/-*) on the expression of IGF-associated genes

The expression of *Igfbp5* is positively regulated by IGF-1 signals and *Igfbp5* expression has previously been used as an *in vivo* indicator of IGF-1 signaling (Adamo et al., 2006; Bach et al., 1991; Backeljauw et al., 1993; Bartlett et al., 1991; Bondy et al., 1993; Duan et al., 1999; Harrington et al., 2007; Resch et al., 2006; Tanner et al., 2008; Ye et al., 1998). To identify tissues in which IGF-1 signals may be attenuated, therefore, we evaluated the expression of *Igfbp5* in *PappA*(*-/-*) mice and wild type littermates across a panel of 21 tissues (males, 3 – 6 months of age, $n = 6$ per treatment). It was expected that *Igfbp5* expression would be reduced in *PappA*(*-/-*) mice relative to wild-type littermates, and for 13 of the 21 tissues, *Igfbp5* expression was indeed lower among *PappA*(*-/-*) mice relative to controls (Fig. 1) ($P = 0.047$; sign-test with one-sided alternative hypothesis). However, in most cases, effects of *PappA*(*-/-*) were weak, and on average among all tissues, the average reduction of *Igfbp5* expression was only 5%. Additionally, the influence of *PappA*(*-/-*) on *Igfbp5* expression was heterogeneous among tissues, and in contrast to the expectation, *Igfbp5* expression was increased in bone ($P = 0.022$) and cerebellum ($P < 0.001$) (Fig. 1). The expected decrease in *Igfbp5* expression was only observed to a significant degree in kidney ($P = 0.024$), although the expected trend was also evident in intestine ($P = 0.125$), pancreas ($P = 0.070$) and trachea ($P = 0.101$) (Fig. 1). The effect of *PappA*(*-/-*) on *Igfbp5* expression thus varied continuously and was heterogeneous among tissues, but was consistent with localized reduction of IGF-1 signals in the kidney of *PappA*(*-/-*) mice.

The effects of *PappA*(*-/-*) on the expression of other IGF-associated genes was evaluated in six major organ types (i.e., kidney, liver, muscle, heart, neocortex and lung). We examined genes that encoded components of the IGF-1 system (i.e., *Igfbp1*, *Igfbp2*, *Igfbp3*, *Igfbp4*, *Igfbp5*, *Igfbp6*, *Igfbp7*, *Igf1*, *Igf1r*, *Igf2*, *Irs1*, *Irs2*, *Igfals*, *Pten*), as well as genes that do not encode components of the IGF-1 system, but were viewed as possible targets of the IGF-1 pathway (i.e., *Sgk1*, *Ccnd1*, *Hbegf*, *Mt1*, *Lifr* and *Fmo3*) (see Supplemental Data File 1). These analyses again suggested that IGF-1 signals were most strongly perturbed with respect to the *PappA*(*-/-*) kidney (Fig. 2). In kidney, *PappA*(*-/-*) significantly increased the expression of *Igfbp2*, *Igfals*, *Irs1*, *Mt1*, and *Ccnd1*, and had marginally significant effects on the expression of several other genes (i.e., *Igfbp1*, *Igfbp3*, *Pten* and *Lifr*) (Fig. 2). Relative to kidney, effects of *PappA*(*-/-*) on gene expression were weaker in other tissues. In cardiac tissue, *PappA*(*-/-*) significantly increased expression of *Igf1*, *Igf2* and *Igfbp6*,

while in liver, *PappA*($-/-$) increased expression of *Igfbp2* and decreased expression of *Igfbp7* and *Igf2* (Fig. 2). In muscle, *PappA*($-/-$) increased expression of *Igfbp1* and *Sgk1* (Fig. 2), and with respect to lung and neocortex, only one examined gene was significantly altered by the *PappA*($-/-$) mutation (Fig. 2). Taken together, these data are consistent with the pattern of *Igfbp5* expression and point to the kidney as an organ in which the *PappA*($-/-$) mutation has strong effects relative to other tissues.

3.2. Relationship between *PappA*($-/-$) and mutations that reduce IGF-1 in serum

A previous analysis of microarray experiments identified genes for which expression in liver tissue was altered, in a similar direction, by multiple mutations that promote dwarfism and longevity, including the (GHRKO) *Ghr*($-/-$) and (Snell) *Pit1(dw/dw)* mutations (Swindell, 2007). Both of these mutations are associated with decreased IGF-1 in serum (Berryman et al., 2008). In the case of *Ghr*($-/-$), decreased IGF-1 in circulation is due to GH-insensitivity (Coschigano et al., 2003), and with respect to *Pit1(dw/dw)*, serum IGF-1 levels are diminished as a secondary consequence of reduced GH levels (Flurkey et al., 2001). In both mutants, low serum IGF-1 reflects decreased synthesis of IGF-1 in liver, with localized reduction of IGF-1 within at least some liver cells possessing endocrine function. We therefore considered whether genes characteristically altered in liver tissue of *Ghr*($-/-$) and *Pit1(dw/dw)* mice were correspondingly influenced by the *PappA*($-/-$) mutation (Table 1). The genes examined included some encoding IGF-system components (e.g., *Igf1*, *Igfals*), along with others for which an association to GH / IGF-1 signals was primarily based upon prior whole-genome array data (e.g., *Lifr*, *Keg1*, *Fmo3*) (Swindell, 2007). Our RT-PCR analyses confirmed, for nearly all genes, the expected expression patterns for selected genes in *Ghr*($-/-$) and *Pit1(dw/dw)* mice (Table 1) (Swindell, 2007). However, we observed little or no association between these expression patterns and those from *PappA*($-/-$) mice. In 4 of 12 cases, *PappA*($-/-$) had a marginally significant effect on hepatic expression, but in three of these cases (*Igfals*, *Lifr*, *Hao3*) the weak effect of *PappA*($-/-$) was opposite to that associated with the *Ghr*($-/-$) and *Pit1(dw/dw)* mutations. The one similar effect among all three mutations was decreased expression of kidney expressed gene 1 (*Keg1*), which was markedly observed in *Ghr*($-/-$) and *Pit1(dw/dw)* mice ($P < 0.001$; Table 1), and observed to a much lesser degree in *PappA*($-/-$) mice ($P = 0.062$, two-sided t-test; $P = 0.031$, one-sided t-test; Table 1).

Our evaluation of kidney, liver, heart, muscle, neocortex and lung in *PappA*($-/-$) mice identified 16 significant effects of the *PappA*($-/-$) mutation (Fig. 2), and we evaluated whether these effects were shared by the *Ghr*($-/-$) and *Pit1(dw/dw)* mutations (Table 2). In heart, lung, muscle and neocortex, genes for which expression was altered in *PappA*($-/-$) mice were not similarly altered *Ghr*($-/-$) or *Pit1(dw/dw)* mutants (Table 2). In muscle, *PappA*($-/-$) increased expression of *Sgk1* ($P = 0.028$), while the opposite effect was observed in *Pit1(dw/dw)* mice ($P = 0.041$). In cardiac tissue, *PappA*($-/-$) and *Pit1(dw/dw)* had similar effects on the expression of *Igf2* ($P < 0.026$), but contrasting effects on the expression of *Igf1* ($P < 0.045$), with no significant effect of the *Ghr*($-/-$) mutation on expression of either *Igf1* or *Igf2* ($P > 0.393$) (Table 2). Interestingly, while both *PappA*($-/-$) and *Pit1(dw/dw)* had similar effects on *Igf2* expression in heart, these mutations had contrasting effects on *Igf2* expression in liver, with *PappA*($-/-$) decreasing hepatic *Igf2* expression and *Pit1(dw/dw)* increasing *Igf2* expression (Table 2). All three mutations, *PappA*($-/-$), *Gir1*($-/-$) and *Pit1(dw/dw)*, increased *Igfbp2* expression in liver, although the effects of *Ghr*($-/-$) were marginally significant ($P = 0.051$) (Table 2).

The strongest correspondence among the three mutations was observed with respect to certain genes for which expression was modified by *PappA*($-/-$) in kidney (Table 2). In this organ, the expression of *Mt1* and *Irs1* was increased by *PappA*($-/-$), *Ghr*($-/-$) and *Pit1(dw/dw)* ($P < 0.04$), and each of these mutations also tended to increase renal *Igfbp2* expression

as well ($P < 0.213$). In contrast to these points of consistency, however, *PappA*($-/-$) increased expression of *Igfals* in kidney ($P < 0.001$), while both *Ghr*($-/-$) and *Pit1(dw/dw)* decreased expression of this gene ($P < 0.001$).

3.3. Transcriptional regulation of *Irs1*, *Mt1* and *Igfals* in kidney by circulating GH / IGF-1 levels

Comparison of expression patterns among long-lived mutants revealed that, in kidney, expression of insulin receptor substrate 1 (*Irs1*), metallothionein 1 (*Mt1*) and insulin-like growth factor acid labile subunit (*Igfals*) is altered by each of three mutations affecting GH / IGF-1 signaling (i.e., *PappA*($-/-$), *Ghr*($-/-$) and *Pit1(dw/dw)*). This suggested that expression of these genes was regulated by local IGF-1 availability or plasma GH / IGF-1 levels. To further evaluate the role of GH / IGF-1 in transcriptional regulation, we treated GH-deficient (Ames)*Prop1(df/df)* and wild-type littermates with GH or saline for a period of six weeks, and evaluated expression of *Irs1*, *Mt1* and *Igfals* in kidney. It was expected that expression of these genes would be altered in *Prop1(df/df)* mice relative to wild-type littermates, and that such effects of *Prop1(df/df)* would be countered in GH-treated *Prop1(df/df)* mice. The experiment was carried out using both *ad lib* mice and mice provided a calorie restriction (CR) diet (6 weeks; 30% CR), because CR reduces serum IGF-1 in mice and also because effects of the *Prop1(df/df)* mutation on longevity are diet-dependent (Garcia et al., 2008). Additionally, previous analyses of whole-genome expression data have indicated that expression of *Mt1* is sensitive to CR in several organs (Swindell, 2008a).

Renal expression of *Irs1* was regulated by circulating GH / IGF-1 levels in *ad lib* fed *Prop1(df/df)* mice, as expected, but was also decreased by CR, and the effect of CR interfered with the influence of GH-treatment on *Irs1* expression (Fig. 3A). Our analysis of long-lived mutants had indicated that expression of *Irs1* was significantly increased in kidney by the *PappA*($-/-$), *Ghr*($-/-$) and *Pit1(dw/dw)* mutations ($P \leq 0.02$) (Table 2). Consistent with these results, *Irs1* expression was also significantly increased in kidney tissue from *ad lib-fed Prop1(df/df)* mice ($P < 0.001$; Dunnett *post-hoc* comparison) (Fig. 3A). Following GH-treatment of *Prop1(df/df)* mice on the *ad lib* diet, expression of *Irs1* was decreased and comparable to that observed in wild-type mice ($P = 0.002$, two-sample t-test; $P = 0.051$, Tukey *post-hoc* comparison) (Fig. 3A). Interestingly, in contrast to observed effects of GH / IGF-1 mutations, the effect of CR was to decrease *Irs1* expression ($P = 0.007$; analysis of variance; see Table 3). Additionally, whereas GH-treatment of *ad lib* fed mice reduced *Irs1* expression, this effect was not observed in CR-fed mice ($P = 0.562$; two-sample t-test) (Fig. 3A). Overall, no significant diet \times treatment interaction was observed ($P = 0.319$; Table 3), although this interaction effect was marginally significant when analyses were limited to the *Prop1(df/df)* mice ($P = 0.09$).

The expression of *Mt1* was also regulated by circulating GH / IGF-1 levels in *ad lib* fed *Prop1(df/df)* mice (Fig. 3B). Interestingly, *Mt1* expression was increased by CR only in GH-injected *Prop1(df/df)* mice, and CR also interfered with the influence of GH-treatment on *Mt1* expression in *Prop1(df/df)* mice (Fig. 3B). Among mutations we examined, expression of *Mt1* in kidney was increased by *PappA*($-/-$), *Ghr*($-/-$) and *Pit1(dw/dw)* (Table 2), and this was also true of the *Prop1(df/df)* mutation ($P < 0.001$; Dunnett *post-hoc* comparison) (Fig. 3B). Expression of *Mt1* was GH-sensitive in *ad lib-fed Prop1(df/df)* mice, and decreased significantly following GH-treatment ($P = 0.007$, Tukey *post-hoc* comparison) (Fig. 3B). However, the effect of GH-treatment was weaker and non-significant in CR-fed mice ($P = 0.174$, two-sample t-test) (Fig. 3B). There was a significant diet \times treatment interaction effect, with CR having no significant effect in wild-type mice, but increasing *Mt1* expression in *Prop1(df/df)* mice. For instance, CR increased *Mt1* expression of in GH-treated *Prop1(df/df)* mice ($P = 0.004$, Tukey *post-hoc* comparison), but this effect was not

observed in GH-treated wild-type mice ($P = 0.489$, two-sample t-test; $P = 0.998$, Tukey *post-hoc* comparison). Across the same treatment groups, *Mt2* exhibited a very similar pattern of gene expression (data not shown), which was expected because *Mt1* and *Mt2* are co-expressed in most tissues (Swindell, 2008b).

The expression of *Igfals* in kidney was regulated by circulating GH / IGF-1 levels in *ad lib* fed *Prop1(df/df)* mice, but there was little effect of CR on expression of this gene (Fig. 3C). Both *Ghr(-/-)* and *Pit1(dw/dw)* significantly decreased *Igfals* expression, while the *PappA(-/-)* mutation had an opposite effect (Table 2). Consistent with effects of *Ghr(-/-)* and *Pit1(dw/dw)*, expression of *Igfals* was decreased in *Prop1(df/df)* mice ($P < 0.001$; Tukey *post-hoc* comparison) (Fig. 3C). This appeared to be a consequence of circulating GH levels, since GH-treatment restored normal levels of *Igfals* expression in *Prop1(df/df)* mice ($P = 0.021$; Tukey *post-hoc* test). As with *Irs1* and *Mt1*, this effect was weakened in CR-fed mice, but was nonetheless marginally significant ($P = 0.060$, two-sample t-test; 0.163 , Tukey *post-hoc* comparison) (Fig. 3C).

4. Discussion

Mice lacking the pregnancy associated plasma protein A (*PappA*) gene exhibit a considerable increase in lifespan that arises from a narrow endocrine deficiency involving localized reductions of IGF-1 availability. These characteristics make the *PappA(-/-)* mouse an important model for the study of mammalian aging and a valuable tool for exploring local effects of IGF-1 availability independently of GH / IGF-1 levels in serum. This study provided an *in vivo* characterization of IGF-associated gene expression patterns in multiple tissues from *PappA(-/-)* mice and wild-type littermates. Among 21 mouse tissues, *PappA(-/-)* had weak and variable effects on the expression of insulin-like growth factor binding protein 5 (*Igfbp5*), which served as an *in vivo* indicator of IGF-1 signals in our analysis (Adamo et al., 2006; Bach et al., 1991; Bäckeljauw et al., 1993; Bartlett et al., 1991; Bondy et al., 1993; Duan et al., 1999; Harrington et al., 2007; Resch et al., 2006; Tanner et al., 2008; Ye et al., 1998). Expression of *Igfbp5* was decreased by *PappA(-/-)* only kidney, and we identified several other IGF-associated genes for which expression was modified by *PappA(-/-)* in this organ (e.g., *Igfbp2*, *Igfals*, *Irs1*, *Ccnd1*, *Mt1*). This result highlights the kidney as an organ of potential importance in future investigations of the *PappA(-/-)* mutation, and as a site at which loss of IGF signals could have favorable effects on mouse longevity. Overlap between effects of *PappA(-/-)* and GH / IGF-1 endocrine mutations on gene expression was limited, but was most marked with respect to kidney. Renal expression of *Irs1* and *Mt1* was elevated in *PappA(-/-)*, *Ghr(-/-)*, *Pit1(dw/dw)* and *Prop1(df/df)* mice, and we show that expression of these genes is regulated by GH and also sensitive to caloric restriction. These expression patterns represent a common feature among four mutations that increase mouse lifespan by an IGF-mediated mechanism, and warrant further investigation as downstream elements of pathways sensitive to both loss of localized IGF-1 availability and depression of the GH/IGF-1 endocrine axis.

Reduction or loss of insulin / insulin-like signals (IIS) increases lifespan in worms, flies and mice and thus the IIS pathway appears to have conserved effects on longevity across species (Berryman et al., 2008; Taguchi et al., 2008). In *C. elegans*, there have been attempts to identify cell types that mediate effects of IIS inhibition on survival (Libina et al., 2003; Wolkow et al., 2000). For instance, in long-lived *daf-2* mutant worms, it was found that restoration of *daf-2* signals in neurons abolished increased lifespan, whereas this was not the case with respect to muscle or intestine (Wolkow et al., 2000). However, a later study indicated that, in short-lived *daf-2(-)*; *daf-16(-)* mutants, restoration of *daf-16* activity in adipose tissue (also intestine of *C. elegans*) increased lifespan, but such effects were not observed with respect to neurons or muscle (Libina et al., 2003). These results conflict, but

have highlighted a possible role for nervous, intestinal and adipose tissues as sites for which loss of IIS could have favorable effects, and indeed, insulin receptor knockout mutations specific to brain and adipose tissue were shown to increase mouse lifespan by a small margin (Blüher et al., 2003; Taguchi et al., 2007). In this study, *PappA*($-/-$) decreased *Igfbp5* expression in intestine and fat by 56% and 30%, respectively, but in both cases the effect was non-significant ($P > 0.126$, $n = 6$ per treatment). There was no significant indication, therefore, that *PappA*($-/-$) altered IGF-1 signals in either fat or intestine, although we cannot rule out that significant effects would have been observed based upon a larger sample size, or that other indicators of IGF-1 signaling might have yielded significant results (e.g., phosphorylation of the IGF-1 receptor, expression of other IGF-sensitive genes). With regard to the nervous system, we evaluated the effects of *PappA*($-/-$) in three brain regions (i.e., neocortex, cerebellum and pons) as well as in an element of the peripheral nervous system (i.e., sciatic nerve), but found that *PappA*($-/-$) did not decrease *Igfbp5* expression significantly in any of these tissues. Hypothalamus was not included among the brain components evaluated in our analysis, although this may be an informative target for future work, given the potential role of this gland in the regulation of GH secretion. Interestingly, *PappA*($-/-$) significantly increased *Igfbp5* expression in cerebellum, which is suggestive of elevated IGF-1 signals in this region of the central nervous system (Ye et al., 1998). Evidence of increased IGF-1 signaling within the central nervous system (hippocampus) has also been reported in long-lived (Ames) *Prop1*(*df/df*) mice that lack GH in circulation (Sun et al., 2005), and this counterintuitive feature of GH/IGF-1-deficient mice could be neuroprotective in some respects (Chen et al., 2005; Frago et al., 2002; Serbedzija et al., 2009; Subramaniam et al., 2005), but might also augment accumulation of betaamyloid (Freude et al., 2009).

The *PappA*($-/-$) mutation had the strongest effects on IGF-associated gene expression patterns in the kidney and led to a significant (31%) decline in *Igfbp5* expression in this organ. There is, at present, no direct evidence to indicate that loss of insulin or IGF-1 signals in the kidney can increase mammalian lifespan, and several potentially important phenotypic characteristics of *PappA*($-/-$) mice are unlikely to be explained in terms of renal IGF-1 signaling alone. For instance, the *PappA*($-/-$) mice exhibit increased locomotion and are reported to have reduced tumor burden in kidney but also several other organ systems (liver, lung and colon) (Conover and Bale, 2007). Additionally, although expression of *Igfbp5* may be responsive to localized IGF availability, it is unknown whether expression of this gene coincides with activation of key pathways downstream of IGF that mediate beneficial effects of the *PappA*($-/-$) mutation on longevity. Nevertheless, it is clear that, in both mice and rats, genetic manipulations that alter GH / IGF-1 levels in circulation have large effects on kidney pathology, particularly renal lesions associated with progression of glomerulosclerosis (Chen et al., 1995; Doi et al., 1988, 1990, 2001; Doublier et al., 2000; Gay et al., 1997; Shimokawa et al., 2002; Zha et al., 2006, 2008), and it has been postulated that these effects are indeed consequential for survivorship (Razzaque, 2007). In particular, severity of glomerulosclerosis is enhanced with elevated serum GH / IGF-1 levels (Doublier et al., 2000), and is alleviated with declines in serum GH / IGF-1 levels (Zha et al., 2006, 2008). While the role of plasma IGF-1 levels in these effects is not disputed, circulating GH levels can also have direct effects of local IGF-1 availability within the kidney (Jacobs et al., 1997; Flyvbjerg et al., 1992), which raises the possibility that effects of the GH / IGF-1 endocrine axis on kidney pathology are mediated by both localized and circulating IGF-1 (Doi et al., 1990). In support of this notion, oral administration of rapamycin, a compound that increases mouse lifespan and inhibits mTOR signals downstream of IGF-1 (Harrison et al., 2009), has been shown to inhibit progression of anti-thy1-induced glomerulosclerosis in rats (Krämer et al., 2008). These effects of rapamycin on glomerulosclerosis, and other forms of kidney pathology (Bonegio et al., 2005; Diekmann et al., 2007; Esposito et al., 2009), appear related to inhibition of the IGF-1 / mTOR pathway at the local tissue level,

without alternation of endocrine GH / IGF-1 signals. Taken together, these observations suggest that glomerulosclerosis could be among age-associated pathologies attenuated in *PappA(-/-)* mice, and that such effects may be shared among long-lived mutants carrying GH / IGF-1 endocrine mutations, and possibly rapamycin-treated mice as well. Future biochemical and histological investigations of renal tissue from young and old *PappA(-/-)* mice may thus provide a useful evaluation of localized effects of IGF-1 availability on glomerulosclerosis, apart from circulating GH / IGF-1 levels.

Our results show that the kidney expression of insulin receptor substrate 1 (*Irs1*), metallothionein 1 (*Mt1*) and insulin-like growth factor acid labile subunit (*Igfals*) are regulated by the GH / IGF-1 endocrine axis. In the case of *Irs1* and *Mt1*, renal expression was inversely related to serum GH / IGF-1 levels, and expression was increased by the *PappA(-/-)*, *Ghr(-/-)*, *Pit1(dw/dw)* and *Prop1(df/df)* mutations. Expression of these genes was also sensitive to caloric restriction (CR), although effects of CR on *Irs1* expression opposed those of the dwarf mutations, while effects of CR on *Mt1* expression were only observed in *Prop1(df/df)* mice treated with growth hormone. Collectively, these data suggest that several factors combine to modulate the expression of *Irs1* and *Mt1* in kidney, including serum GH / IGF-1 levels, caloric intake, and possibly localized IGF-1 availability as well. It remains unclear whether regulation of renal *Irs1* and *Mt1* expression by GH/IGF-1 is direct, or if the effect is indirect and dependent upon certain metabolic consequences of reduced or elevated levels of GH / IGF-1 in circulation (e.g., altered adipose tissue mass and associated cytokine levels). Understanding the transcriptional regulation of these genes will shed light on the *in vivo* molecular events associated with genetic and environmental manipulations that influence the aging process and increase mouse lifespan. It is of considerable interest, for example, that kidney expression of *Mt1*, and hepatic expression of both *Mt1* and *Mt2*, was elevated in multiple long-lived dwarf models, since the expression of these genes is also increased in many tissues of CR-fed mice (Swindell, 2008a). The metallothioneins have anti-oxidant properties and their elevated expression could serve to combat accumulation of oxidative stress damage in both dwarf mice and CR-fed mice (Bokov et al., 2009). The influence of GH / IGF-1 signals and CR on *Mt1/2* expression could reflect a conserved effect associated with manipulations that promote longevity, since both CR and loss of insulin / insulin-like signals (IIS) in *C. elegans* have been shown to increase expression of *mtl-1* and *mtl-2* (Barysytte et al., 2001; Szewczyk et al., 2006), which are orthologous to the mouse *Mt1* and *Mt2* genes.

The identification of mutations that increase mouse lifespan has considerably bolstered efforts to understand the genetic basis of mammalian aging (Berryman et al., 2008; Taguchi et al., 2008). In recent years, however, the trend has been towards a rapidly increasing number of new pro-longevity mutations, but with relatively small effects on mouse lifespan in nearly every case (Swindell, 2009). The *PappA(-/-)* mutation represents a notable exception to this trend, with a large effect on lifespan that rivals that of the *Pit1(dw/dw)* and *Prop1(df/df)* hypo-pituitary mutations, but with a much narrower endocrine deficiency. There remain substantial questions regarding mechanisms by which *PappA(-/-)* has *in vivo* effects, and foremost among these are whether some effects of *PappA(-/-)* are IGFBP4-independent. This is a possibility because the PAPP-A protease can also degrade both IGFBP-2 and IGFBP-5 (Laursen et al., 2001, 2002; Monget et al., 2003; Rivera et al., 2003), which could have compounded *in vivo* effects in *PappA(-/-)* mice, since both IGFBP-2 and -5 have cellular effects that are IGF-independent (Miyakoshi et al., 2001). Moreover, notwithstanding IGFBP4-independent effects of the PAPP-A protease, the view of IGFBP-4 as strictly an inhibitor of IGF signals *in vivo* requires further study, since mice lacking the *Igfbp4* gene exhibit a dwarf phenotype, possibly due to a disruption of IGF-2 action that is mediated by IGFBP-4 during development (Ning et al., 2008). Nevertheless, the long-lived *PappA(-/-)* mouse may ultimately provide impetus for a conceptual shift, involving

increased attention to the role played by localized IGF-1 availability as a regulator of age-associated pathology in mice, with endocrine GH / IGF-1 levels serving primarily to mediate these tissue-specific effects (Conover et al., 2008). This development may advance understanding of the diverse effects of IGF action within different cell types, and contribute towards a more detailed model of mechanisms by which insulin / insulin-like signaling controls mammalian aging and longevity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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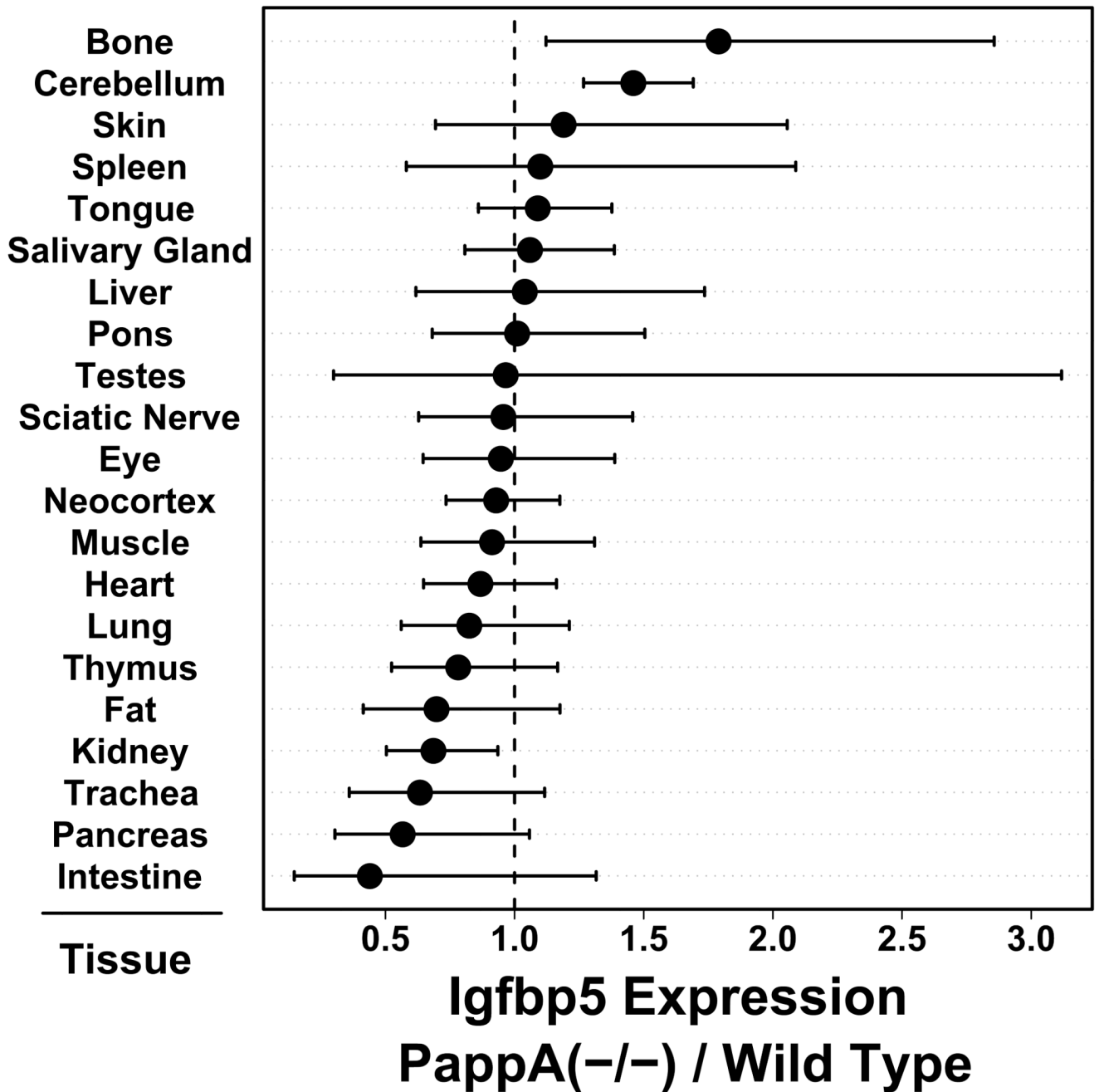


Figure 1.

Insulin-like growth factor binding protein 5 (*Igfbp5*) expression in 21 tissues of *PappA*(-/-) mice and wild type littermates. The chart shows 95% confidence intervals associated with the estimated ratio of expression in *PappA*(-/-) mice relative to wild type littermates ($n = 6$ mice per tissue; RT-PCR). Estimates larger than one indicate increased expression of *Igfbp5* in *PappA*(-/-) mice relative to controls, while estimates less than one indicate decreased *Igfbp5* expression in *PappA*(-/-) mice relative to controls. Significant effects are indicated by confidence intervals that do not overlap with the dotted vertical line, which corresponds to an estimated ratio of one (i.e., equal expression in *PappA*(-/-) and control mice).

Calculations were performed using the $\Delta\Delta C_t$ method and expression of either 18S ribosomal

RNA (*Rn18s*) or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an internal reference gene.

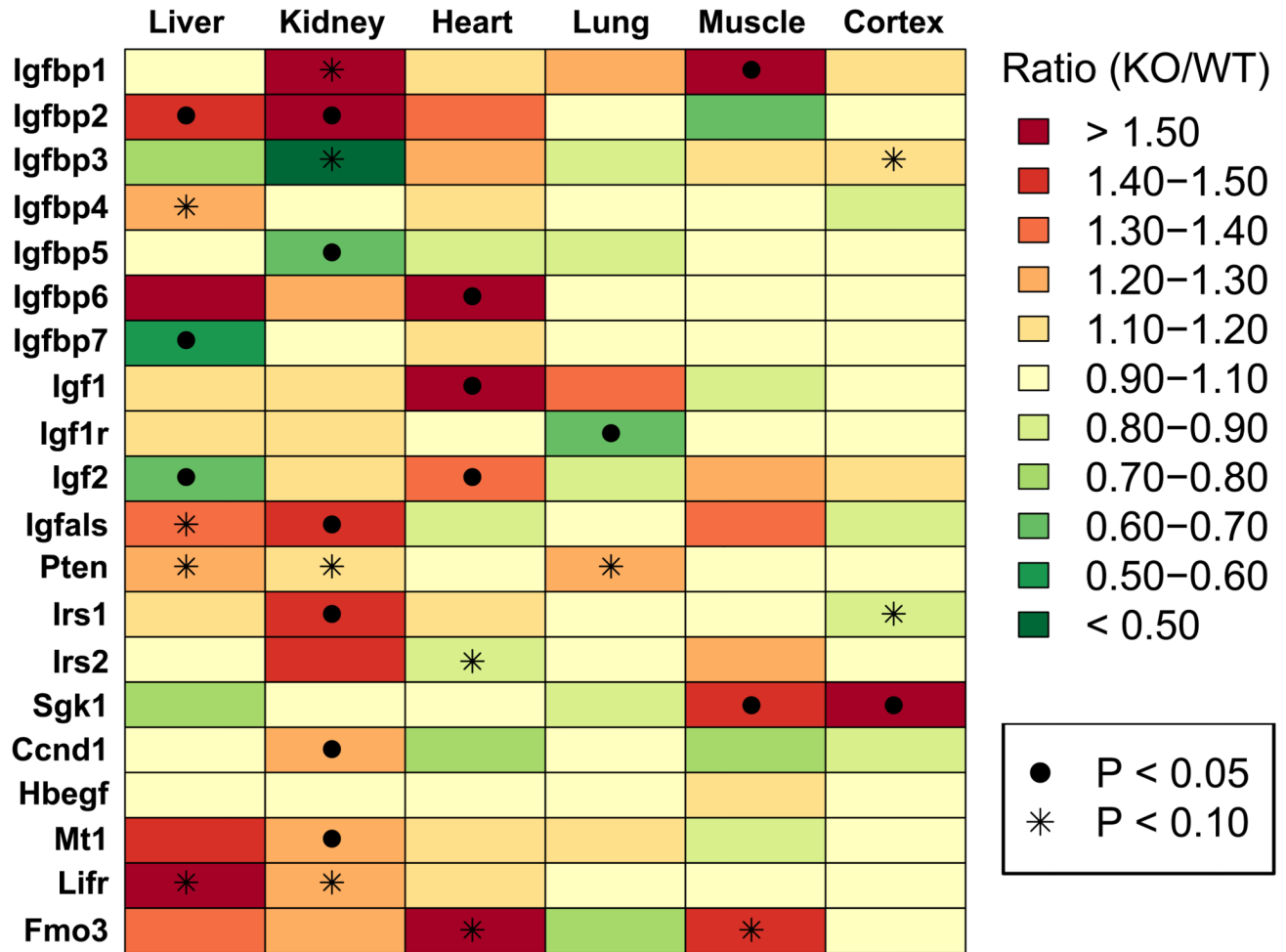
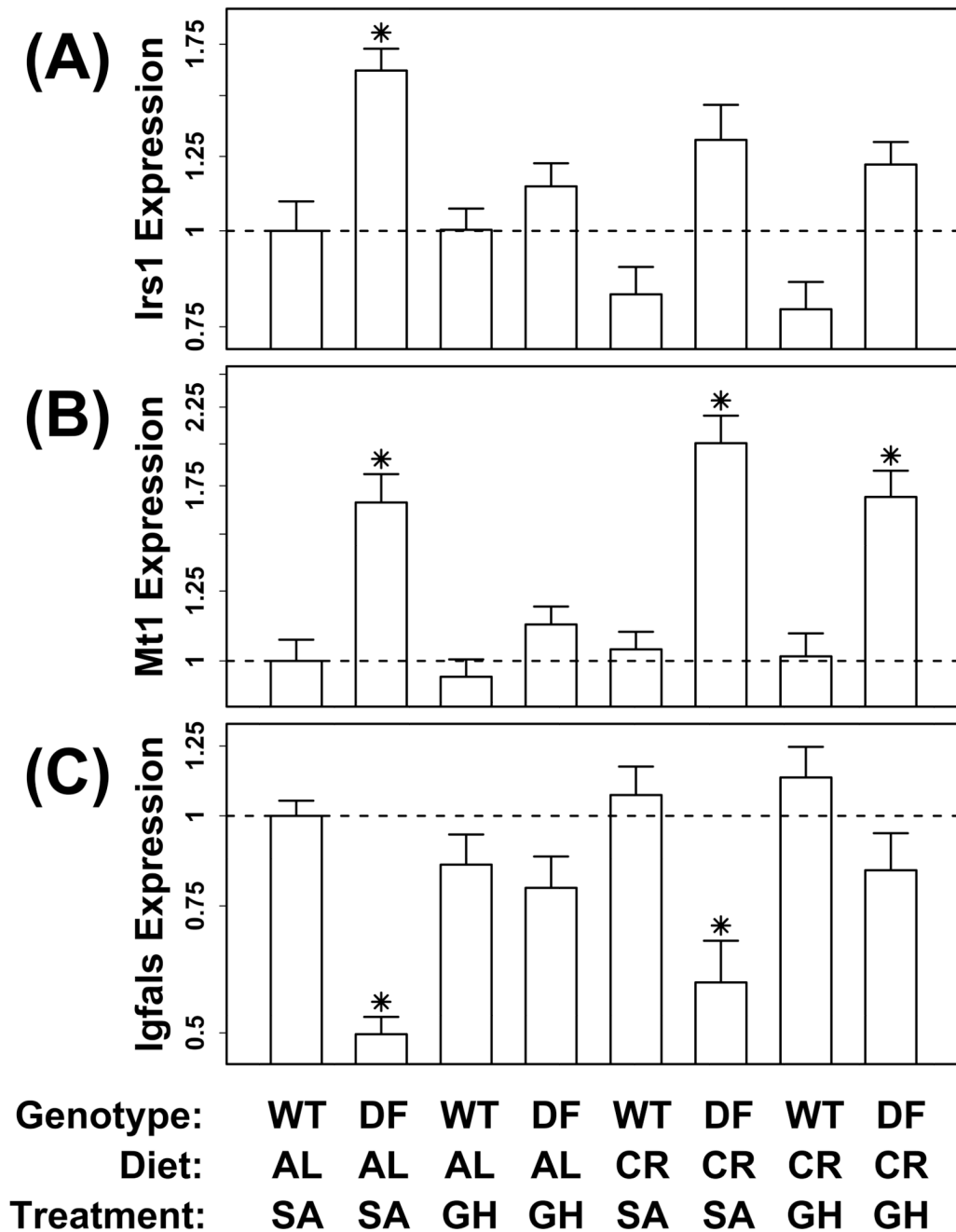


Figure 2.

Relative expression of IGF-associated genes in *PappA*($-/-$) mice and wild-type littermates with respect to six organs (liver, heart, kidney, muscle, lung and neocortex) (RT-PCR). Colors correspond to the estimated ratio of expression in *PappA*($-/-$) relative to wild type littermates ($n = 6$ mice per treatment). Red colors correspond to increased expression in *PappA*($-/-$) mice relative to controls, while green colors correspond to decreased expression in *PappA*($-/-$) mice relative to controls (see color chart). Significant effects of the *PappA*($-/-$) mutation are indicated by the ● symbol ($P < 0.05$; two-sample t-test or rank-sum test). Marginally significant effects of the *PappA*($-/-$) mutation are indicated by the * symbol ($0.05 < P < 0.10$; two-sample t-test or rank-sum test). Calculations were performed using the $\Delta\Delta C_t$ method with expression of either 18S ribosomal RNA (*Rn18s*) or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) used as an internal reference gene. The full gene name associated with each gene symbol is provided in Supplemental Data File 1.

**Figure 3.**

Transcriptional regulation of insulin receptor substrate 1 (*Irs1*), metallothionein 1 (*Mt1*) and insulin-like growth factor binding protein acid labile subunit (*Igfals*) by growth hormone (GH) and/or calorie restriction (CR) in kidney. GH-deficient (*Ames Prop1(df/df)*) and wild-type mice were injected with either GH or saline for a six-week period. The experiment was performed with mice maintained on either a *ad lib* or 30% CR diet. The chart shows the relative expression of each gene in each of the eight experimental treatments ($n = 10$ mice per treatment). The asterisk (*) symbol is used to denote treatments that differ significantly from the baseline treatment (i.e., *ad lib* fed wild-type mice; $P < 0.05$, Dunnett post-hoc

comparison). Calculations were performed using the $\Delta\Delta C_t$ method with expression of 18S ribosomal RNA (*Rn18s*) used as an internal reference gene.

Table 1

Hepatic gene expression patterns in *PappA(-/-)*, *Ghr(-/-)* and *Pit1(dw/dw)* mice relative to wild-type controls. RT-PCR was used to evaluate relative expression of genes associated with loss of GH / IGF-1 signals, which had been identified in a prior meta-analysis of microarray experiments (Swindell, 2007). The table lists estimated ratios of expression in mutant mice relative to wild-type littermates ($n = 6$ per mice treatment), with values larger than one indicating increased expression in mutants. Calculations were performed using the $\Delta\Delta C_t$ method with expression of 18S ribosomal RNA (*Rn18s*) as an internal reference gene. P-values in parentheses are based upon results from two-sample t-tests applied to the estimated ΔC_t values. Significant results are shown in bold-faced type. The full gene name associated with each gene symbol is provided in Supplemental Data File 1.

Gene	<i>PappA(-/-)</i> / WT (P-value)	<i>Ghr(-/-)</i> / WT (P-Value)	<i>Pit1(dw/dw)</i> / WT (P-Value)
<i>Igf1</i>	1.16 (0.319)	0.01 (< 0.001)	0.02 (< 0.001)
<i>Igfals</i>	1.35 (0.073)	0.03 (< 0.001)	0.04 (< 0.001)
<i>Socs2</i>	0.96 (0.927)	0.25 (0.002)	0.12 (< 0.001)
<i>Lifr</i>	1.59 (0.070)	0.74 (0.147)	0.32 (< 0.001)
<i>Egfr</i>	0.87 (0.358)	0.11 (< 0.001)	0.08 (< 0.001)
<i>Keg1</i>	0.76 (0.062)	0.03 (< 0.001)	0.05 (< 0.001)
<i>Mup3</i>	1.08 (0.627)	0.09 (< 0.001)	0.00 (< 0.001)
<i>Sult2a2</i>	0.92 (0.686)	2070 (< 0.001)	696 (< 0.001)
<i>Spink3</i>	1.92 (0.224)	104 (< 0.001)	187 (< 0.001)
<i>Fmo3</i>	1.34 (0.401)	28.0 (< 0.001)	921 (< 0.001)
<i>Mt1</i>	1.42 (0.451)	4.91 (0.071)	30.1 (< 0.001)
<i>Mt2</i>	1.12 (0.818)	7.46 (0.080)	40.3 (< 0.001)
<i>Hao3</i>	0.59 (0.069)	8.84 (0.003)	18.9 (< 0.001)
<i>Cyp2a4</i>	0.937 (0.795)	5.27 (< 0.001)	2.74 (< 0.001)

Table 2

Comparison between significant effects of the *PappA*($-/-$) mutation in six organs and gene expression patterns in *Ghr*($-/-$) and *Pit1*(*dw/dw*) mice. Our analysis of IGF-associated genes in six organs from the *PappA*($-/-$) mouse identified 16 significant effects of the *PappA*($-/-$) mutation. For each gene and tissue in which a significant effect was found, we evaluated effects of the *Ghr*($-/-$) and *Pit1*(*dw/dw*) mutations. The table lists estimated ratios of expression in mutant mice relative to wild-type littermates ($n = 6$ per mice treatment), with values larger than one indicating increased expression in mutants. Calculations were performed using the $\Delta\Delta C_t$ method with expression of either 18S ribosomal RNA (*Rn18s*) or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) used as an internal reference gene. Unless otherwise indicated, p-values in parentheses are based upon results from two-sample t-tests applied to the estimated ΔC_t values. Significant results are shown in bold-faced type. The full gene name associated with each gene symbol is provided in Supplemental Data File 1.

Tissue	Gene	<i>PappA</i> ($-/-$) / WT (P-value)	<i>Ghr</i> ($-/-$) / WT (P-Value)	<i>Pit1</i> (<i>dw/dw</i>) / WT (P-Value)
Kidney	<i>Igfbp2</i>	1.65 (0.026[†])	1.76 (0.114)	2.66 (0.213)
Kidney	<i>Igfbp5</i>	0.69 (0.024)	0.78 (0.311)	1.19 (0.389)
Kidney	<i>Igfals</i>	1.46 (0.001)	0.29 (< 0.001)	0.16 (< 0.001)
Kidney	<i>Irs1</i>	1.42 (0.020)	1.90 (0.004)	3.23 (< 0.001)
Kidney	<i>Ccnd1</i>	1.23 (0.017)	0.80 (0.154)	0.69 (0.275)
Kidney	<i>Mt1</i>	1.23 (0.040)	1.69 (0.002)	2.72 (0.024)
Liver	<i>Igf2</i>	0.64 (0.021)	1.57 (0.129)	6.76 (< 0.001)
Liver	<i>Igfbp2</i>	1.48 (0.026[†])	3.65 (0.051)	6.24 (< 0.001)
Liver	<i>Igfbp7</i>	0.57 (0.032)	1.51 (0.022)	0.74 (0.095)
Heart	<i>Igfbp6</i>	1.77 (0.003)	1.53 (0.061)	1.17 (0.548)
Heart	<i>Igf1</i>	1.62 (0.009)	0.97 (0.932)	0.37 (0.045)
Heart	<i>Igf2</i>	1.40 (0.026[†])	0.76 (0.393)	2.66 (0.009)
Lung	<i>Igf1r</i>	0.66 (0.011)	0.90 (0.776)	2.21 (< 0.001)
Muscle	<i>Igfbp1</i>	1.54 (0.003)	0.99 (0.954)	0.75 (0.575)
Muscle	<i>Sgk1</i>	1.47 (0.028)	0.88 (0.638)	0.55 (0.041)
Cortex	<i>Sgk1</i>	1.57 (0.038)	0.80 (0.154)	1.09 (0.583)

[†]P-value generated from non-parametric Kolmogorov-Smirnov test

Table 3

Regulation of *Irs1*, *Mt1* and *Igfals* in kidney by growth hormone (GH) and/or calorie restriction (CR). The tables lists p-values generated from an analysis of variance applied to the expression data shown in Figure 3. The analysis of variance included three factors, including genotype (*Prop1(df/df)* or wild-type), diet (CR or *ad lib*) and treatment (GH or saline injection). For each of the three genes examined, the three-way interaction between genotype, diet and treatment was non-significant, and was therefore excluded from each model. Significant p-values are shown in bold-faced type.

Effect	P-value (<i>Irs1</i>)	P-Value (<i>Mt1</i>)	P-Value (<i>Igfals</i>)
Genotype (G)	< 0.001	< 0.001	< 0.001
Diet (D)	0.007	0.907	0.219
Treatment (T)	0.430	0.275	0.525
G*D	0.208	0.019	0.654
G*T	0.096	0.020	0.001
D*T	0.319	0.236	0.708