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The p53 Codon 72 Polymorphism Modifies the Cellular Response to Inflammatory Challenge in the Liver

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Abstract

The p53 protein is a critical stress-response mediator and signal coordinator in cellular metabolism and environmental exposure to deleterious agents. In human populations, the p53 gene contains a common single nucleotide polymorphism (SNP) affecting codon 72 that determines whether a proline (P72) or an arginine (R72) is present at this amino acid position of the polypeptide. Previous studies carried out using human populations, mouse models, and cell culture analyses have provided evidence that this amino acid difference can alter p53 functional activities, and potentially also can affect clinical presentation of disease. The clinical presentation associated with many forms of liver disease is variable, but few of the responsible underlying genetic factors or molecular pathways have been identified. The aim of the present study was to investigate whether the p53 codon 72 polymorphism influences the cellular response to hepatic stresses. A humanized p53 knock-in (Hupki) mouse model was used to address this issue. Mice expressing either the P72 or R72 normal variation of p53 were given an acute-, intermittent- or a chronic challenge, associated with exposure to lipopolysaccharide, D-galactosamine, or a high-fat diet. The results reveal that the livers of the P72 and R72 mice exhibit notable differences in inflammatory and apoptotic response to these distinct forms of stress. Interestingly the influence of this polymorphism on the response to stress is context dependent, with P72 showing increased response to liver toxins (lipopolysaccharide and D-galactosamine), but R72 showing increased response to metabolic stress (high fat diet). When taken together, these data point to the p53 codon 72 polymorphism as an important molecular mediator of events contributing to hepatic inflammation and metabolic homeostasis.

Keywords: Liver; Gene polymorphism; p53 codon 72 polymorphism; Hepatic inflammation; Apoptosis; Hupki

Abbreviations: LPS: Lipopolysaccharide; GalN: D-galactosamine; HFD: High-Fat Diet; MCP1: Monocyte Chemo-attractant Protein-1; MDM2: Murine Double Minute; IGFBP1: Insulin-like Growth Factor Binding Protein-1; HNF4α: Hepatocyte Nuclear Factor 4α; AMPK: Adenosine Monophosphate-activated Protein Kinase; ACC: Acetyl-CoA Carboxylase; mTOR: Mammalian Target of Rapamycin; AKT: Serine/threonine Kinase also known as Protein Kinase B

Introduction

The p53 tumor suppressor protein is a critical signaling integrator. Its actions help to orchestrate the particular cellular response to a broad range of stresses, including oncogenic, genotoxic and nutritional insults. Stress-activated p53 functions as a nuclear transcription factor that modulates the expression of a large and diverse array of genes, and p53 also has a direct apoptotic role at mitochondria under some conditions [1-4]. The physiologic outcome of a p53-mediated stress response is context-dependent; it can be growth-inhibitory, apoptotic, or even pro-survival [1-4]. A number of factors influence the outcome, including the nature of the stress, its severity, and whether the affected cells are normal or transformed. While most of what is understood about the regulation and actions of p53 center on its activities in tumorigenesis, recent investigations underscore that this protein also has important but much less well understood functions in diverse physiologic processes, including energy metabolism and anti-oxidant defense [1-4]. Thus, understanding the critical parameters mediating the actions of wild type forms of p53 in metabolic stress has potential significant clinical implications. In human populations, the normal p53 gene contains a common non-synonymous single nucleotide polymorphism (SNP) affecting codon 72 [5-7]. This functional polymorphism leads to incorporation of either a proline (CCC, P72) or arginine (CGC, R72) in the amino acid sequence.

Interestingly, the R72 and P72 allele frequencies vary in different ethnic populations and geographic regions. The P72 allele is more common in populations near the equator, while R72 is more common in those living at a distance from the equator [5-7]. For example, approximately 64% of African-Americans, and 58% of Hispanic-Americans, express the P72 variant. In contrast, about 32% of Caucasian-Americans express the P72 variant, and only about 10% are homozygous for this allele. This observation has prompted suggestions that the p53 codon 72 polymorphism may contribute to the ethnic differences noted among individuals in response to different stresses and/or their susceptibility to particular disorders. Efforts to evaluate the effect of this polymorphism in individuals or populations using epidemiologic analyses have tended to focus on cancer risk or therapy. However, such studies have led to conflicting results, presumably due to small sample sizes, genetic heterogeneity of human populations, and environmental influences that complicate this approach. In other investigations employing cultured cell lines, we and others have obtained consistent evidence that this SNP does impact the function of p53, in part by modulating the protein's ability to induce transcriptional, apoptotic, or senescence responses to different stimuli [5-12].

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The recent development of genetically engineered mouse models for the human codon 72 variants provides a unique opportunity for functional analysis of these p53 forms in different tissues, under diverse conditions, and in the context of an intact organism [13-15]. As part of our efforts to investigate p53 biology and examine the potential physiologic impact of the codon 72 SNP in different normal tissues we utilized a humanized knock-in model for p53 (humanized p53 knock-in, or Hupki) [13,14]. The Hupki mouse model was generated by replacing both copies of the murine p53 gene from exon 4 through exon 9 with the corresponding wild-type human segment, which includes codon 72. This generates p53 proteins differing only in the presence of either proline (Hupki-P72) or arginine (Hupki-R72). Importantly, the Hupki mice retain normal abundance and regulation of the p53 protein. Initial investigations have confirmed that Hupki p53 proteins are fully functional and responsive to different forms of stress [13,14]. Notably, no differences in the survival and tumor spectrum have been noted between P72 and R72 mice [13,14].

We and others have shown that mouse models for the codon 72 polymorphism of p53 accurately recapitulate the phenotypes associated with this polymorphism in human populations. For example, as in human populations we found that P72 variant shows superior transactivation of the p21/waf1 cyclin-dependent kinase inhibitor, along with increased ability to induce senescence [13]. Additionally, as reported in human, we found that the Hupki R72 variant shows enhanced ability to transactivate LIF1, which plays a positive role in fecundity [16,17]. In a previous functional analysis of potential differences in the p53 variants using the Hupki model we showed that, not only does the codon 72 polymorphism impact the apoptotic function of p53, it does so in a tissue-specific manner [13,14]. Also, microarray analyses of gamma-irradiated mice revealed that, in the thymus of the P72 and the R72 mice, the majority of p53-induced genes were regulated identically, as expected. However, a small subset of genes showed increased expression in the thymus of the P72 mice relative to this tissue in R72 samples. Interestingly, the affected genes included several with roles in innate immunity and inflammation [13]. Such data underscore the need to better understand the contributions of p53 and this common polymorphism to stresses associated with normal physiology.

The liver has a key role in metabolizing almost all ingested substances, including toxins, yet few studies have addressed the potential role of this critical stress-response mediator in contributing to the etiology or pathology of liver disease. Here we have used the Hupki mouse model to help better understand the potential influence of the p53 codon 72 polymorphic variants, in the cellular response to different forms of liver injury. The combined data provide new evidence that the p53 polymorphism can influence the outcome following an acute-, intermittent-, or a chronic challenge to liver metabolism.

Materials and Methods

Mice

Hupki-R72 mice were purchased from the Jackson Laboratory. The Hupki-P72 model was generated as reported previously [13] and intercrossed with Hupki-R72 mice for thirteen generations. Mice were housed in plastic cages and maintained at 22°C with a 12-hour dark/12- hour light cycle and had free access to food. All experiments have been performed on males. For the lipopolysaccharide (LPS) and D-galactosamine (GalN) experiments, the mice were provided with a standard chow (LabDiet 5001: 28.507% protein, 57.996% carbohydrate, 13.496% fat). For the high fat diet (HFD) studies, three to four week old mice were fed either a control standard diet (Research Diet, D12450B: 20% protein, 70% carbohydrate, and 10% fat) or high fat diet (Research Diet, D12492: 20% protein, 20% carbohydrate, and 60% fat) for 4 weeks or 40 weeks, as indicated. Glucose concentrations were monitored by tail bleeding, using a glucometer. All experiments with mice conformed to the guidelines of The Institutional Animal Care and Use Committee of the Perelman School of Medicine at the University of Pennsylvania, which approved all animal work according to federal and institutional policies and regulations.

p53 codon 72 genotyping

Animals were genotyped by polymerase chain reaction (PCR) using the following primers: 5′-CCGTCCCAAGCAATGGATGATTTGATGCTG-3′ and 5′-CTTGGCTGTCCCAGAATGCAAGAAGCCCAG-3′). The PCR condition was 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min; and 2 cycles of 72°C for 2 min. The PCR product was purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare 28-9034-70) and digested for 1 h or overnight at 37°C using BstUI (or Bsh1236I, Fermentas Life Sciences #ER0921). The fragments were separated by electrophoresis on 2.5% agarose gel, and then stained with ethidium bromide. The Arg/Arg homozygote was cleaved by BstUI, yielding 108 bp and 150 bp bands. The Pro/Pro homozygote was not cleaved by BstUI and was represented by a single 258 bp band; whereas the Arg/Pro heterozygote contained all three bands (108, 150, and 258 bp) following restriction digestion. Some samples were confirmed by direct sequencing of PCR products to verify the accuracy of the genotyping.

Western blot, immunohistochemical analyses, antibodies, and statistical analysis

The livers were homogenized in PEN-Buffer (pH 7.4) containing 2.67 mM KCl, 1.47 mM KH_2PO4, 137.9 mM NaCl, 8.06 mM Na_2HPO_4-$7H_2O$, 2 mM EDTA, 0.5% NP-40. Protein concentrations of liver extracts were determined using the Bradford Reagent (Rio-Rad Laboratories). The following primary antibodies were used in this work: anti-p53 (Santa Cruz, sc-65226 and sc- 56182), anti-p53 (Leica Biosystems Newcastle Ltd., NCL-p53-505), anti-Sestrin-1 (Abgent AP7650b), anti-PTEN (Cell Signaling, 9552), anti-MDM2 (Calbiochem OP144), anti-NFκB p65 (ABCAM ab7970), anti-NFκB p65 (Santa Cruz, sc-109), anti-cleaved lamin A (Cell Signaling, 2035), anti-cleaved caspase-3 (Cell Signaling, 9661), anti-F4/80 (ABCAM ab6640), anti-Ki67 (Leica Biosystems Newcastle Ltd., NCL-Ki67p), anti-p-NF-κB p65-Ser276 (Cell Signaling, 3037), anti-CD3 (LabVision RM-9107-S1), anti-CD45R/B220 (PharMingen #550286), anti-LBP (Santa Cruz, sc-14668), anti-SirT1 (Cell Signaling, 3931), anti-Histone H2A (Cell Signaling, 3636 and 2578), anti-p19ARF (GeneTex GTX20080) and anti-H2B (Cell Signaling 2934). The peroxidase conjugated secondary antibodies (i.e. donkey anti-rabbit, donkey anti-mouse, donkey anti-rat and donkey anti-goat) were from the Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The biotinylated secondary antibodies for tissue staining were from the Vector Laboratories (Burlingame, CA). The VECTASTAIN Elite ABC Kit (PK-6100), Avidin/Biotin Blocking Kit (SP-2001), and DAB Peroxidase Substrate Kit, 3,3'-diaminobenzidine (SK- 4100) were purchased from the Vector Laboratories (Burlingame, CA). Results are presented as mean+SD. Statistical analysis of differences between groups was performed by unpaired Student's t test and P-values<0.05 was considered statistically significant.

Results

Hupki mouse phenotype and initial characterization

The Hupki-P72 and Hupki-R72 mice (herein referred to as P72 and R72 mice) were intercrossed for at least thirteen generations. Heterozygous offspring were then bred to homozygosity, as confirmed by PCR analyses. Genotype analyses of offspring from heterozygous matings were consistent with Mendelian inheritance. Adult mice homozygous for the P72 or R72 alleles were phenotypically indistinguishable from each other as well as their heterozygous littermates. No embryonic lethality or significant developmental defects were observed. We did note that in homozygous matings, 8-20 week old R72 mice generally produced a larger litter size (~8-10 pups per female) than age-matched P72 mice (~3-6 pups per female); this may be due to increased ability of the R72 variant to transactivate LIF1, which is required for blastocyst implantation [16,17].

Differential LPS-signaling in the R72 and P72 livers

LPS is a major outer membrane component of gram-negative bacteria, and it elicits a strong inflammatory reaction [18,19]. This endotoxin is removed by macrophages through scavenger receptors that are highly expressed in the liver; the severity of LPS-mediated liver injury closely parallels the severity of systemic inflammatory response syndrome. We previously reported an enhanced response to LPS challenge in P72 thymocytes [13]. Therefore, we extended our studies to examine the liver. Because several important hepatic factors related to acute LPS-toxicity have been identified, we used western blot analysis of liver proteins to examine the expression of several of these genes with previously identified roles in LPS-induced signaling pathways. As shown in figure 1A, the protein levels of CD14, Lipopolysaccharide Binding Protein (LBP), TNFα, Monocyte Chemoattractant Protein-1 (MCP1), Hepatocyte Nuclear Factor 4α (HNF4α) and activated STAT3 (p-STAT3-Y705) increased to a greater degree in the P72 liver compared to those of the R72 mice. In contrast, there was no detectable difference in Toll Like Receptor-4 (TLR4) or p53 protein abundance when comparing the P72 or R72 samples (Figure 1A). The latter observation is consistent with our previous findings, that following gamma-irradiation of Hupki mice, p53 levels in the thymus did not differ between P72 and R72 mice. Also, our initial studies indicate no difference in the localization of these variants to mitochondria or cytosol, following either LPS-treatment or gamma-irradiation [13]. Both HNF4α and MCP1 are known p53 target genes [20-22]. HNF4α is a well-studied liver enriched transcriptional factor that transcriptionally regulates more than 1500 liver-specific genes in hepatocytes, many of which are pro-inflammatory and pro-apoptotic [22]. Increased production of MCP1 has been reported to promote macrophage infiltration, inflammation and apoptotic cell death. Elevated expression of MCP1 has also been linked to hepatic failure, in both humans and rodents following sepsis as well as LPS exposure [23]. Our findings (Figure 1) therefore suggest that the P72 mice treated with LPS may exhibit enhanced inflammatory liver injury relative to the LPS-treated R72 mice. Indeed, immunohistochemical staining revealed an approximately 30% and 185% increase in the number of macrophages (F4/80 positive) and T lymphocytes (CD3 positive), respectively, in the LPS-treated P72 liver relative to the untreated control P72 mice. In contrast, we found no significant increase in either of these markers in the livers of the R72 mice, suggestive of lower inflammation and apoptotic injury (Figures 1B and 1C). For the immunohistochemical studies, we also used an antibody that recognizes the active caspase-3 p17 subunit, as an indicator for apoptosis. The results revealed a four-fold increase in caspase-3 associated apoptosis in the LPS-exposed P72 hepatocytes relative to LPS-treated R72 livers. This enhanced apoptotic phenotype in the P72 livers acutely exposed to LPS correlates positively with increased expression of histone H2A and histone H2B. Enhanced expression of such soluble forms of H2A and H2B also are indicative of apoptosis and associate with multiple organ dysfunction and failure in both rodents and baboons [24]. In contrast, a significant decrease in caspase-11 p20 expression was noted in the LPS-treated Hupki R72 liver. Caspase-11 (also known as caspase-4) is a pro-inflammatory

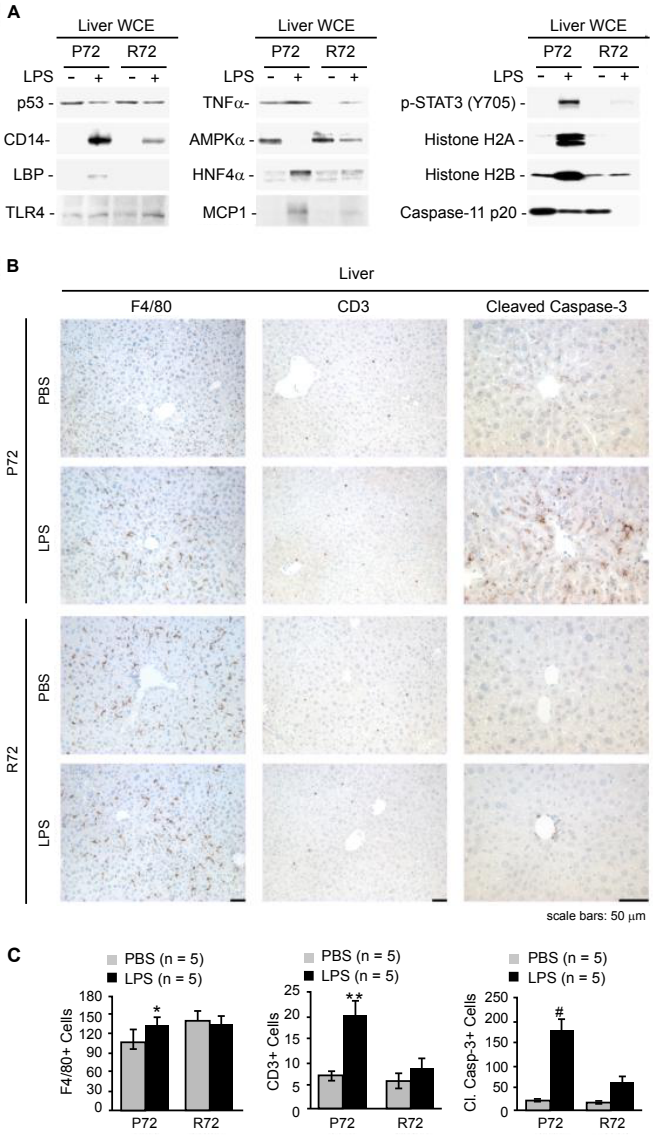

Figure 1: P72 mice demonstrate an enhanced response to LPS. (A) P72 and R72 mice were intraperitoneally injected with PBS (control) or 20 mg/kg of LPS for 19 h. Liver whole cell extracts (WCEs) were prepared and examined for the expression of proteins indicated. (B) Immunohistochemical staining of control P72 liver, P72 liver 19 h after 20 mg/kg LPS injection, control R72 liver, and R72 liver 19 h after LPS challenge, using anti-F4/80, anti-CD3 and anticleaved caspase-3 antibodies, as indicated. The brown staining indicates positive staining. Photomicrographs are representative of 5 mice/treatment group. Bar: 50 μm. (C) Quantification of F4/80 ($P<0.003$), CD3 ($P<0.007$) and cleaved caspase-3 (p17 subunit, $P<0.001$) positive cells in the control and LPS-treated P72 and R72 livers. The data depicted are representative of ten random 10X fields ± SD. *$P<0.003$, **$P<0.007$ and #$P<0.001$ compared to LPS challenged R72 mice using Student's *t* test.

caspase that has been reported to have a key role in LPS-induced lethality; indeed, caspase-11 knockout mice are protected from a lethal dose of LPS [25]. Thus, the marked reduction in caspase-11 expression observed in the LPS-treated R72 liver could contribute to a reduced LPS-mediated mortality previously seen in the R72 mice [13]. Our findings suggest that the P72 mice are more susceptible to LPS-induced septic shock, both macroscopically and microscopically. Of note, racial disparities in sepsis incidence and outcomes have been reported [26-29].

Taken together, our results provide evidence that the liver of P72 mice respond differently to the acute toxic effects of LPS than those of the R72 mice; this outcome is associated with greater production of septic mediators and evidence of cell death in livers of the P72 mice following an LPS-induced endotoxic shock. The combined data also suggest that the p53 codon 72 polymorphism has an important influence on LPS mediated hepatotoxicity.

Response to GalN-modulated liver damage

Among experimental hepatotoxic agents, the amino sugar D-galactosamine (GalN) has been shown to induce metabolic and morphological changes similar to those observed in human viral hepatitis and liver fibrosis [30-32]. GalN induce hepatotoxicity is characterized by macrophage infiltration, increased cellular proliferation, enhanced apoptotic cell death and abnormal collagen accumulation. Thus, to study the role of the p53 codon 72 polymorphism in a progressive liver disease, the Hupki mice were injected i.p. with 1.5 mg/kg GalN every 48 hours for four weeks. We choose this intermittent regimen, sometimes used in rat studies, in part because the Hupki mice treated singularly with GalN showed no obvious phenotypic hepatic abnormalities (data not shown) and because GalN has a half-life of approximately 20 h. Following administration of the GalN, livers of the P72 and R72 mice were examined by immunohistochemical staining. We found that the P72 livers exhibited evidence of greater damage than the R72 tissue (Figures 2A and 2B). This injury was associated with a higher percentage of cells positive for Ki67, cleaved caspase-3 (p17), and cleaved Lamin A; these are markers for cell proliferation, apoptosis and nuclear disassembly/cell death, respectively. The immunohistochemical analyses also revealed that the P72 livers exhibited increased and enlarged intrasinusoidal macrophages (F4/80 positive cells) as well as enhanced collagen deposition, an indicator of fibrosis (Figure 2). These findings support the conclusion that the response to GalN-mediated acute inflammatory challenge and liver damage, similar to LPS-induced hepatotoxicity, is influenced by the particular p53 codon 72 SNP that is expressed in the liver; specifically in this case the P72 mice exhibited evidence of a greater inflammatory response and resulting tissue damage.

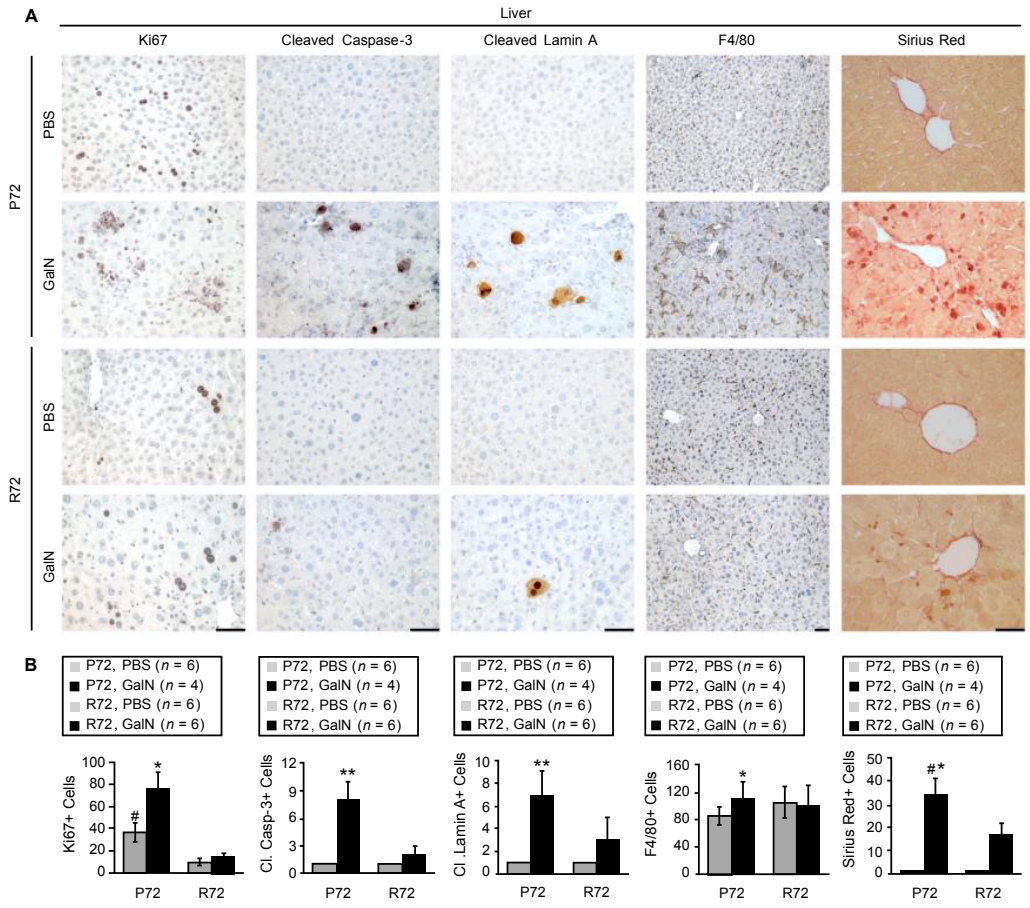

Figure 2: Enhanced sensitization of P72 mice to GalN-mediated liver injuries. (A) Histochemical staining for Ki67, cleaved caspase-3, cleaved lamin A, F4/80, and Sirius Red, as indicated, in P72 and R72 mice intraperitoneally injected with PBS (control) or 1.5 mg/kg GalN every 48 hours for four weeks. (B) Quantification of Ki67 (ten random 20X fields ± SD), cleaved caspase-3 (ten random 20X fields ± SD), cleaved lamin A (ten random 20X fields ± SD), F4/80 (ten random 10X fields ± SD), and Sirius Red (ten random 20X fields ± SD) positive cells in the control and GalN-treated P72 and R72 livers. #P<0.05 compared to R72 mice treated with PBS; *P<0.03, **P<0.001 or #*P<0.005 compared to GalN-treated R72 mice.

Preferential accumulation of the P72 protein in response to a high fat diet (HFD)

p53 has been implicated to play a role in both alcoholic and non-alcoholic fatty liver disease [33-40]. p53 has also been reported to be upregulated in the livers of mice fed a high fat diet (HFD) as well as in mouse models of fatty liver disease [38,39]. Accordingly, we initiated studies to compare the phenotypes of the P72 and R72 mice resulting from a physiologically relevant chronic stress of HFD. As shown in table 1, all cohorts exhibited a comparable increase in body weight, following either four weeks of normal chow or HFD. However, western blot analyses of liver protein from the mice showed greater accumulation of the P72 variant relative to the R72 form following exposure to 4 weeks of HFD (Figures 3A and 3B). The higher levels of the P72 protein also correlated with increased expression levels of the proteins MDM2 (Figures 3A and 3B) and IGFBP1 (Figure 3C). Interestingly, both of these proteins are encoded by p53 target genes, and both can antagonize pro-apoptotic p53 activities. In contrast, we saw no difference in expression levels of *Sestrin1*, *PTEN, and p21*, three other p53 transcriptional targets (Figure 3A). Also, there was no change in the basal expression levels of the p65 subunit of nuclear factor kappa B (NFκB), STAT3 or activated STAT3 (p-STAT3-Y705), genes often implicated in mediating cellular inflammatory response (Figure 3B).

HFD-induced inflammatory liver injury in the Hupki mice

It is recognized that, in response to some conditions of physiologic stress, p53 can induce the expression of prosurvival factors that help to reduce cell damage and evade cell death [1-4]. The IGFBP1 protein is an early response, hepatoprotective, liver factor that is rapidly upregulated in response to metabolic stress and liver damaging agents; the diverse actions of this protein are thought to help restore hepatic homeostasis and reduce liver injury [40-42]. We previously reported that p53 activation leads to induction of IGFBP1 in liver; in turn, IGFBP1 acts intracellularly to antagonize the prodeath and pro-inflammatory functions of p53 in this organ [42]. As noted above, livers of the P72 mice subjected to HFD exhibit higher levels of IGFBP1 than the R72 mice, which may correlate with a difference in a diet-induced inflammatory response in this tissue. Histopathological examination of H&E-stained sections supported this idea, and revealed a variable degree of multifocal, mixed, aggregated inflammatory cell infiltration in the livers of the HFD-fed R72 mice that were present to a lesser degree in the HFD-fed P72 samples (Figure 4A). Also, eosinophilic hepatocytes with shrunken/pyknotic nuclei were frequently noted in the HFD-fed R72 livers. Immuno-histochemical studies showed that the R72 livers from animals exposed to four weeks of the HFD contained a higher degree of hepatic infiltrates that were positive for CD45R ($P<0.002$), CD3 ($P<0.008$) and F4/80 ($P<0.002$) (Figures 4A and 4B), which are markers of B cells, T cells, and activated Kupffer cells/macrophages, respectively. Recent evidence suggests that enhanced activation of NFκB via phosphorylation of Ser276 in the p65 subunit (p-NFκB p65-Ser276) can promote hepatic inflammation and induce hepatocyte cell death [43]; therefore we chose to analyze this protein. Compared to the HFD-fed P72 mice, the livers of the R72 mice showed a significantly higher degree of staining for p-NFκB (p65-Ser276, $P<0.000006$), as well as for the apoptosis markers cleaved lamin A ($P<0.005$) and cleaved caspase-3 (p17 subunit, $P<0.01$) (Figure 5A and 5B). In contrast, this feeding regimen had little detectable effect on p-NFκB (p65-Ser276) expression levels in the kidneys and spleens of mice carrying either p53 variant (Figure 6). Overall, the data provide evidence that the livers in R72 mice are more sensitive to HFD-induced liver inflammation and apoptotic injury when compared to those in the P72 cohorts.

	P72, 10% fat (*n*=21)	P72, 60% fat (*n*=43)	R72, 10% fat (*n*=23)	R72, 60% fat (*n*=50)
Week 1	8.3 ± 1.7	8.1 ± 1.5	8.6 ± 1.0	9.4 ± 1.8
Week 2	19.0 ± 0.8	22.4 ± 2.2	21.1 ± 2.6	22.4 ± 3.4
Week 3	21.1 ± 1.2	25.0 ± 2.5	22.6 ± 2.7	24.1 ± 3.8
Week 4	24.1 ± 2.1	25.9 ± 2.9	25.3 ± 2.8	25.8 ± 2.5

Table 1: Four-week old P72 and R72 mice were fed the control chow or the HFD for four weeks. The weight of each mouse within each group was measured weekly; the average and variance of the weights are shown.

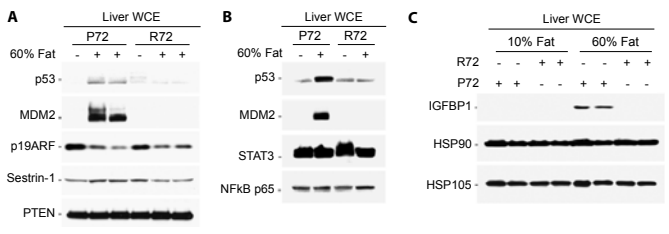

Figure 3: Activation of the P72 variant of p53 in response to a high fat diet. (A-C) Liver whole cell protein extracts (WCE) prepared from P72 or R72 mice, following either 4 weeks of control diet (10% kcal% fat) or high fat diet (HFD, 60% kcal% fat) for 4 weeks, were examined for the proteins indicated. The results shown are representative of at least three mice for each dietary condition and genotype, as indicated.

Serum adipokine levels in Hupki mice subjected to a HFD

Because the release of adipokines following HFD-induced obesity has been shown to increase the risk for certain metabolic-stress related complications, we analyzed serum levels of several adipokines in both chow-fed and HFD-fed P72 and R72 mice. Surprisingly, circulating levels of resistin, leptin, and insulin were significantly higher in the chow-fed R72 mice relative to the diet-matched P72 mice (Tables 2 and 3). However, comparable levels of TNFα and IL-6 were noted in the chow-fed P72 and R72 mice. As expected, the mice on the HFD exhibited an increase in circulating insulin and leptin levels (Tables 2 and 3). Overall, the expression levels for the factors examined were either comparable between the R72 mice and P72 mice (IL-6, TNFα), or they were somewhat higher in the R72 animals. As an example of the latter, the level of serum MCP1 was higher in the R72 mice, compared to the P72 animals, both on the control chow ($P<0.02$) and especially on the HFD ($P<0.000005$). Notably, the 23% increase in circulating adiponectin level noted in the HFD-fed R72 mice correlated with increased phosphorylation and activation of adenosine monophosphate (AMP)-activated protein kinase (p-AMPK-T172) as well as its downstream target acetyl-CoA carboxylase (p-ACC-S79) (Figure 7A). The 22% increase in circulating insulin level in the HFD-fed R72 mice also varied directly with increased activation of AKT (p-AKTS473) and the mammalian target of rapamycin (p-mTOR-S2448) (Figure 7A). For comparison, the serum levels of these factors in the HFD-fed P72 mice closely approximated those in HFD-fed wild type mice; the latter harbor the endogenous mouse p53 (data not shown). In the mouse, codon 72 is an alanine and no polymorphism at this site has been reported.

We extended these studies to investigate the impact of forty weeks of continuous HFD feeding regimen on forty-four week old P72 and R72 mice, with an average lifespan of approximately two years. As shown (Figure 7B), both P72 and R72 mice exhibited a similar extent of obesity when fed a HFD (57.7 ± 5.6 grams and 58.1 ± 4 grams for HFD-fed P72 and R72 mice, respectively). The fasting blood glucose levels were about 30% higher in the R72 mice fed a HFD, compared

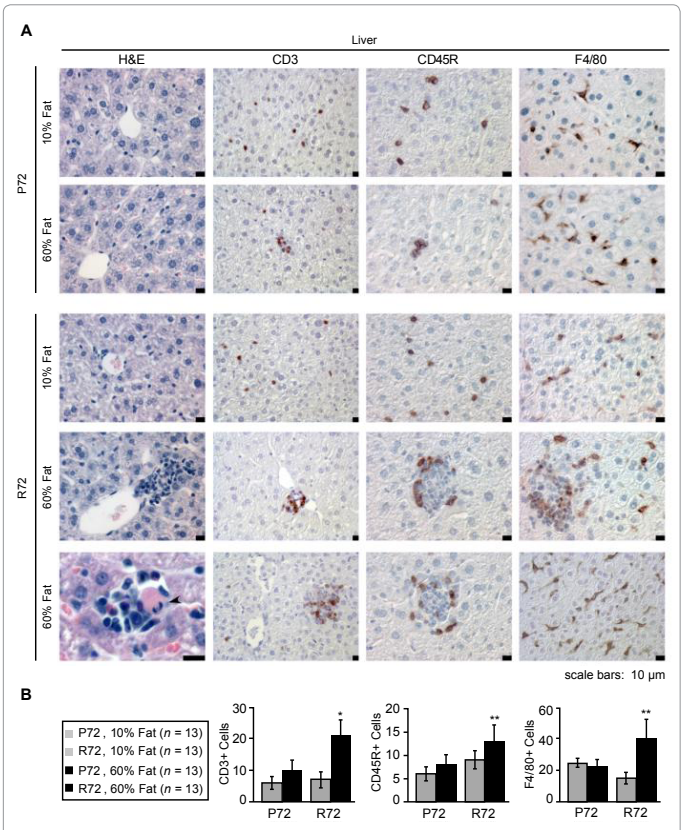

Figure 4: Histologic phenotype of the Hupki livers following a high fat diet. (A) Representative Hemotoxylin and Eosin (H&E)-, CD3-, CD45R-, and F4/80-stained liver sections, Bars: 10 µm (B) Quantification of CD3 (ten random 20X fields ± SD), CD45R (ten random 20X fields ± SD) and F4/80 (ten random 40X fields ± SD) positive cells in the control and HFD-fed P72 and R72 livers. *P<0.008 or **P<0.002 compared to diet-matched P72 mice.

to the P72 mice (107 ± 18 mg/dl and 139 ± 14 mg/dl for HFD-fed P72 and R72 mice, respectively; p<0.0027). Since fasting blood sugar of >126 mg/dl suggests a risk for type 2 diabetes, the forty-four week old HFD-fed R72 mice may exhibit impaired glucose clearance following a glucose tolerance test. Indeed, when challenged with 2 g/kg glucose, the HFD-fed R72 mice correspondingly exhibited a slightly greater glucose intolerance and delayed clearance of glucose, as evidenced by the 28.6% and 26.3% higher blood glucose concentration after injection at 15 min (p<0.007) and 120 min (p<0.0027), respectively (Figure 7B). In contrast, the blood glucose concentrations after overnight fast and during the glucose tolerance tests were comparable in the chow-fed P72 and R72 mice (data not shown). It is of interest that P72 homozygosity has been found to protect against increases in glucose levels during oral glucose tolerance test in one study of human subjects [44]. Also, the p53 codon 72 polymorphism has been associated with risk for type 2 diabetes, based on recent epidemiologic studies [45-49]. The combined data demonstrate that the livers of R72 mice exhibit increased macrophage infiltration and inflammatory signaling following a HFD.

HFD-induced alterations in pancreas and adipose tissue in the Hupki mice

A HFD has been shown to affect white adipose tissue (WAT) as well as the pancreas. An increase in circulating MCP1 level, as noted in the HFD-fed R72 mice (Tables 2 and 3), has also been linked to increased inflammatory infiltrates in both the WAT and pancreas. Indeed, there was a greater abundance of inflammatory infiltrates in both WAT and pancreas of the HFD-fed R72 mice, relative to both the R72 mice on control chow and the HFD-fed P72 mice. This outcome correlated with an approximately eleven-fold increase (P<0.0000005) and two-fold increase (P<0.0003) in F4/80 positive cells in the pancreas and WAT tissue, respectively, of HFD-fed R72 mice (Figures 8A and 8B). The F4/80 positive cells were generally found to surround and/or infiltrate the degenerating pancreata and, to a lesser extent, the smaller islets in HFD-fed R72 mice. In contrast, F4/80 staining was comparable in the kidneys of the R72 and P72 mice (P<0.2).

Histopathological examination of H&E-stained pancreas sections revealed an increase in islet mass in the HFD-fed P72 mice; this is similar to what has previously been described as a normal pancreatic response in mice to HFD stress. In contrast, in the HFD-fed R72 pancreas, the islets were smaller (Figure 9). The results provided evidence of possible pancreatic injury, such as necrosis, in the HFD-fed R72 mice (Figure

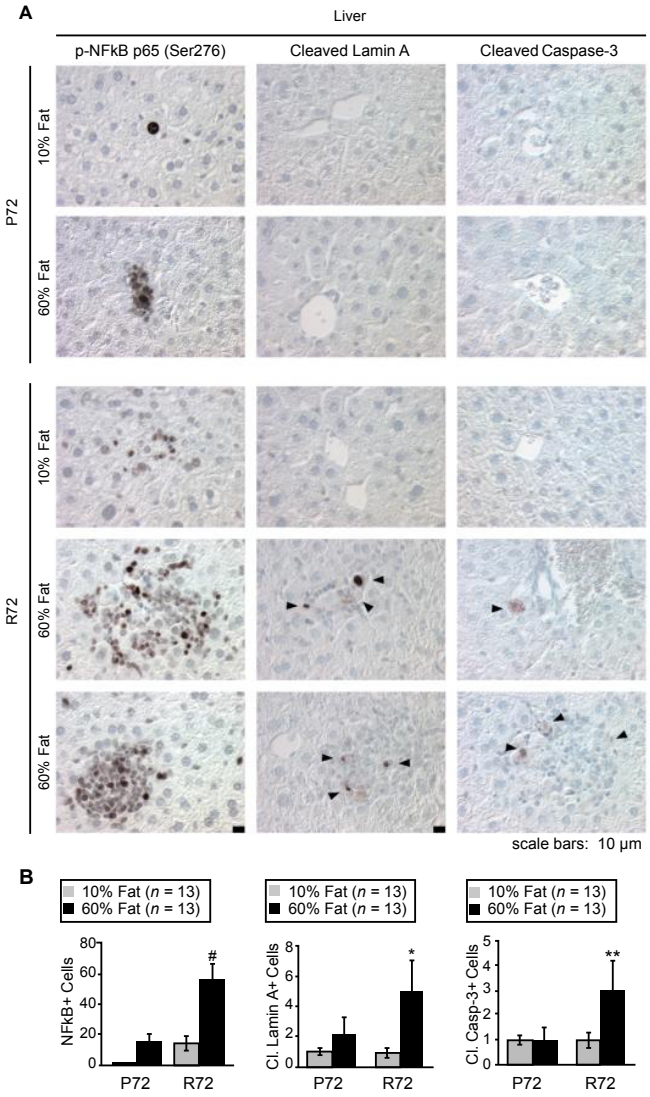

Figure 5: Increased apoptosis and inflammation in livers from R72 mice fed a high fat diet. (A) Histology after staining for p-NFκB p65-Ser276, cleaved lamin A and cleaved caspase-3, as indicated, Bars: 10 µm (B) Quantification of immunohistochemical staining in the indicated groups (A), which were fed the control chow or HFD diet for 4 weeks. The data depicted are representative of ten random 20X fields ± SD. #P<0.000006, *P<0.005 or **P<0.01 compared to diet-matched P72 mice.

9); the latter of which could be attributed to increased macrophage infiltration (Figures 8A and 8B). Although the underlying mechanism leading to a higher accumulation of macrophages in the pancreas of HFD-fed R72 mice remains to be determined, it is of interest that a similar infiltration pattern has been noted in the Non-obese Diabetic

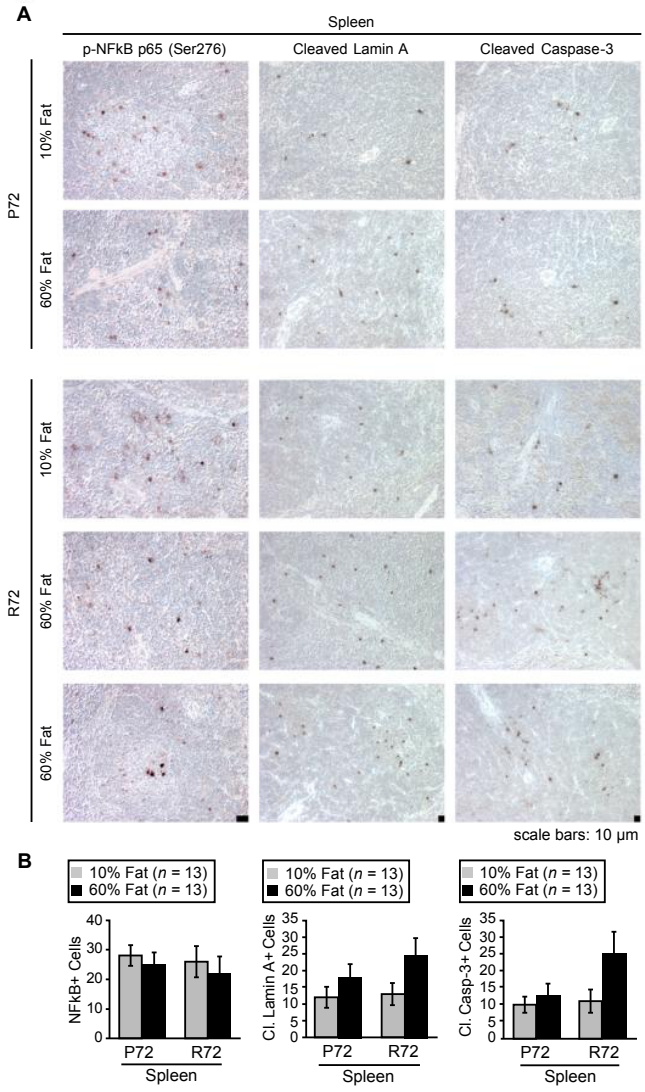

Figure 6: Spleens from Hupki mice fed a high fat diet. (A) Histology after staining for p-NFκB p65-Ser276, cleaved lamin A and cleaved caspase-3, as indicated. Bars: 10 µm. (B) Quantification of immunohistochemical staining in the indicated groups (A), which were fed the control chow or HFD diet for 4 weeks. The data depicted are representative of ten random 20X fields ± SD.

	P72, 10% fat (*n*=13)	P72, 60% fat (*n*=13)	R72, 10% fat (*n*=13)	R72, 60% fat (*n*=13)
Resistin (pg/ml)	1868 ± 527	2733 ± 839	3043 ± 950	3282 ± 832
Leptin (pg/ml)	1365 ± 356	5933 ± 1235	2183 ± 667	6888 ± 1345
Insulin (pg/ml)	791 ± 205	2204 ± 801	1449 ± 517	2680 ± 845
Adiponectin (pg/ml)	9.4 ± 1.0	12.3 ± 2.4	12.8 ± 2.4	15.1 ± 3.0
IL-6 (pg/ml)	12.1 ± 0.2	13.7 ± 2.7	13.3 ± 2.7	12.3 ± 0.6
TNFα (pg/ml)	12.2 ± 0.1	12.2 ± 0.1	12.2 ± 0.2	12.2 ± 0.1
MCP1 (pg/ml)	14.0 ± 3.5	14.6 ± 5.7	19.8 ± 5.1	36.1 ± 4.9

Table 2: Elisa analysis of serum adipokine levels in chow-fed and HFD-fed P72 and R72 mice.

	P72, 10% fat vs. P72, 60% fat	R72, 10% fat vs. R72, 60% fat
Resistin (pg/ml)	P<0.001	P<0.3
Leptin (pg/ml)	$P<1.0\times10^{-9}$	$P<1.0\times10^{-10}$
Insulin (pg/ml)	$P<1.0\times10^{-8}$	$P<1.0\times10^{-4}$
Adiponectin (pg/ml)	$P<2.0\times10^{-5}$	P<0.05
IL-6 (pg/ml)	P<0.02	P<0.06
TNFα (pg/ml)	P<0.2	P<0.3
MCP1 (pg/ml)	P<0.4	$P<1.0\times10^{-5}$

Table 3: P-value for serum adipokine analysis.

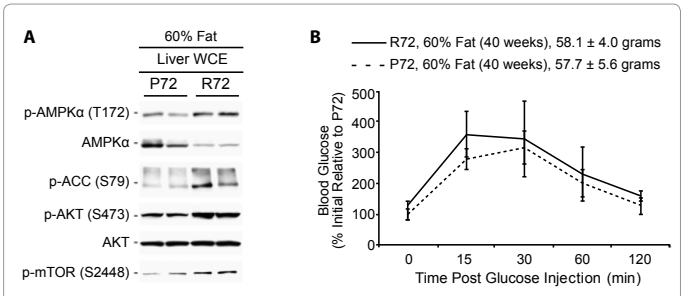

Figure 7: R72 mice demonstrate an enhanced response to HFD. (A) Liver whole cell protein extracts (WCE) prepared from P72 or R72 mice, following 4 weeks of HFD, were examined for the proteins indicated. The results shown are representative of at least three mice for each dietary condition and genotype, as indicated. (B) Blood glucose concentrations during the intraperitoneal glucose tolerance test (2 g/kg) following overnight fasting in conscious P72 and R72 mice fed a HFD for 40 weeks. Blood glucose was measured with a portable glucometer. Results are means ± SD.

(NOD) mouse model with autoimmune diabetes typified by β-cell destructive insulitis [50-53] and in the IGFBP1 knockout mouse model [40,41]. These data indicate that the p53 polymorphism can alter the course and extent of such diet-induced proinflammatory tissue injury.

Discussion

As the primary site of toxin exposure and metabolism, liver is a major target for injury and disease development. Adverse consequences can include fatty liver, hepatitis, fibrosis, cirrhosis, and even hepatocellular carcinoma. Such disorders typically are poorly resolved, with few successful therapies, and they represent a growing public health issue. The appearance and presentation of such liver disorders is believed to be influenced by complex interactions of both environmental and genetic factors, but few of the responsible underlying molecular markers or pathways are known. Moreover, defining how particular human sequence variations (polymorphisms) may influence the response to environmental or metabolic stresses is poorly understood. Thus, there is a need for new models and experimental systems to improve understanding of critical molecular pathways, as well as to identify potential new targets for risk assessment and improved treatment options. As a critical stress-response protein, p53 has emerged as having a central role in the pathogenesis of metabolic disorders, including alcoholic and non-alcoholic fatty liver disease, liver fibrosis, cirrhosis and diabetes [33-40,45-49]. It is important to better understand the role of p53 proteins in disease initiation and progression. The p53 codon 72 polymorphism has been recognized for more than twenty years. This naturally occurring single amino acid difference leads to a change in the electrophoretic mobility of the p53 protein, suggestive of a conformational alteration that could modify one or more aspects of the protein's regulation and function [5-7]. However, very little is understood about how this common genetic variation might influence p53 signaling events, or about its potential to modify an individual's

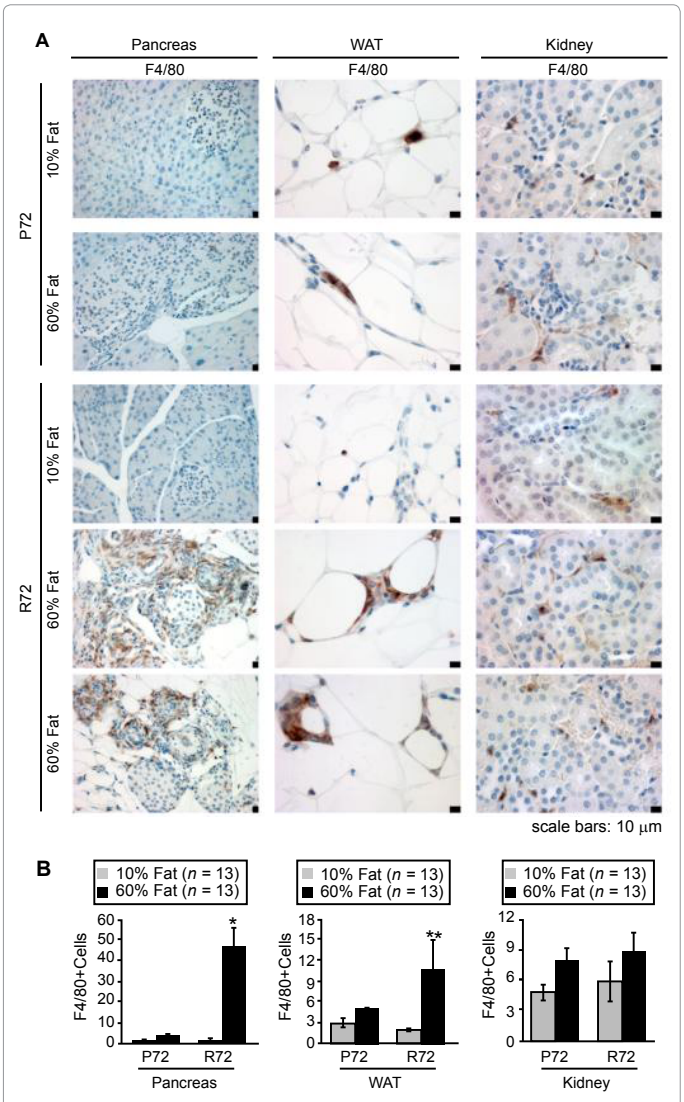

Figure 8: Increased macrophage infiltrations in pancreases and WAT from R72 mice fed a high fat diet. (A) Immunohistochemically stained WAT and pancreas samples are representative of the indicated groups. Bars: 10 μm (B) Quantification of F4/80 staining (A), in at least ten random 40X fields ± SD. *P<0.0000005 or **P<0.0003 compared to diet-matched P72 mice.

risk for a particular disorder. Recent efforts to better understand the potential functional differences between these naturally occurring forms of the p53 protein have utilized newly developed mouse models for this polymorphism. In addition to the Hupki model that we have used in our analyses, Zhu et al. employed a separate p53 codon 72 mouse model [15]. They reported increased apoptotic potential associated with the R72 variant following gamma irradiation in the skin epidermis and small intestine. Consistent with this outcome, we found that the Hupki-R72 mice also exhibit increased apoptosis in these same tissues following exposure to gamma irradiation [13,14]. Interestingly, however, the Hupki mice exhibited decreased apoptosis in the thymus under the same conditions, while the spleens in the R72 and P72 mice showed comparable responses to this acute stress. Moreover, the R72 and P72 variants exhibited a differential ability to transactivate a subset of target genes in the thymus that include those encoding both innate and acquired immune cytokines. These previous observations, together with the data presented here, reinforce the conclusion that the influence of the p53 polymorphism on the response to stress is context dependent; it is affected not only by tissue type and presence of interacting factors, but also by the particular type of stress, and according to our findings, whether that stress is chronic or acute.

The observations reported here demonstrate that the p53 codon 72 polymorphism affects the inflammatory response resulting from a chronic HFD, at least in the liver, white adipose tissue, and pancreas of the treated mice. The p53 codon 72 polymorphism also impacts LPS-mediated liver injury as well as GalN-sensitized liver fibrosis, but the p53 variants respond differently to the acute and chronic stresses used in these studies. The molecular changes related to these observations are expected to be multifaceted, and defining the critical mediators requires further investigations. At least one potential liver factor that may play a role is IGFBP1. Our investigations have shown that, in response to a HFD, expression of this liver protein is increased to a greater extent in the P72 livers relative to the R72 samples. The higher IGFBP1 expression in the P72 samples correlated with lower levels of liver inflammation and apoptotic injury when compared to those in the R72 cohorts. Altered IGFBP1 expression has been linked to several metabolic and/or hepatic disorders. For example, in a study of middle-aged Swedish males, reduced IGFBP1 expression was associated with the development of abnormal glucose regulation and development of diabetes [54]. Also, in a mouse model, elevated IGFBP1 expression had favorable effects on insulin sensitivity and atherosclerosis [55]. Future studies will explore the potential role of the p53 variants in modifying IGFBP1 expression, as well as the role of the latter protein in contributing to the differential effects of a HFD noted in the Hupki mice. Future studies will correspondingly investigate the underlying mechanisms leading to preferential stabilization of the P72 variant of

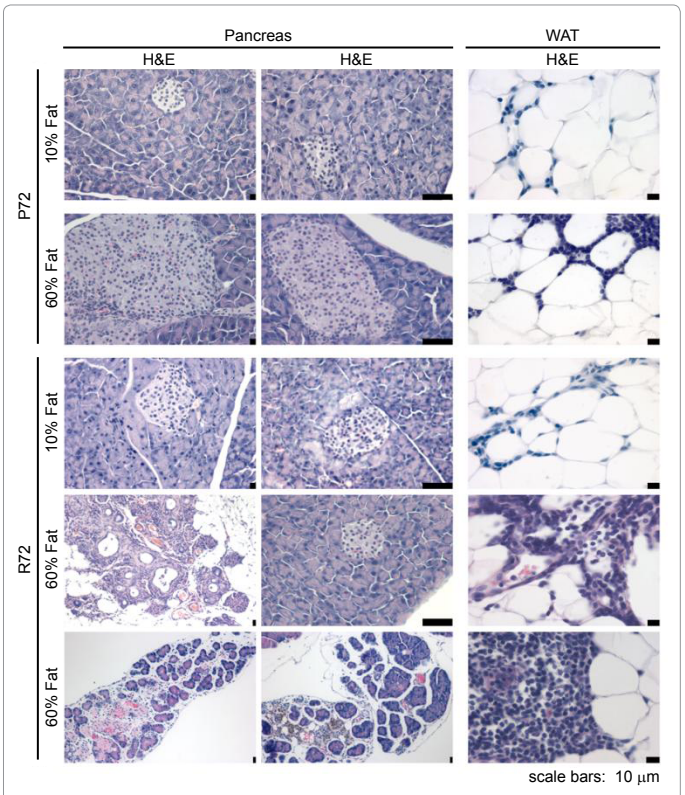

Figure 9: Increased infiltrations in pancreases and WAT from R72 mice fed a HFD. H&E stained WAT and pancreas samples are representative of the indicated groups, Bars: 10 μm.

p53 following HFD. We previously determined that stabilization of p53 correlated positively with upregulation of IGFBP1 mRNA and protein expression, both *in vitro* and *in vivo* [42]. There are several potential mechanisms that could contribute to preferentially stabilization of P72 relative to R72 in the Hupki mice after the HFD. The P72 variant of p53 protein could be differentially modified [1-4]. Another possibility would center on the p53 regulator, MDM2. For example, a HFD promotes phosphorylation of Ataxia Telangiectasia Mutated (p-ATM) in the livers of wild-type mice [56]. p-ATM subsequently phosphorylates MDM2 on multiple sites near its RING domain; these modifications attenuate the ability of MDM2 to poly-ubiquitinate p53, thereby promoting p53 stabilization [57].

An important role for p53 in contributing to antiviral immunity and inflammation has been established [58-61], and previous investigations point to an association between the p53 codon 72 polymorphism and certain human disorders associated with inflammation. As an example, several studies suggest that the p53 codon 72 polymorphism can influence the clinical course of some liver diseases, ulcerative colitis or development of systemic lupus erythematosus [62-67]. Interestingly, an association has been reported between p53 codon 72 polymorphism and both type 1- and type 2-diabetes, at least in a few sampled populations [45-49,68]. In each of these cases, the underlying molecular foundations remain to be identified. The distinctions noted among various populations may reflect differences in genetic makeup combined with exposure to distinct environmental factors, with an impact on the actions of the p53 proteins. The context dependent effect of this p53 polymorphism, as we report here and previously, adds to the experimental complexity and challenge of ongoing efforts to clarify the contribution of this genetic alteration to clinically-relevant stresses and disease risk. Thus, modeling this common genetic variation in a relevant mouse model now provides a valuable experimental system to begin to address at least some of these important issues, and contribute to the goal of improving treatment outcomes.

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