A. SPECIFIC AIMS

The CDC estimates that approximately 32 percent of Americans are obese, even more, 66 percent are overweight or obese. Factors that control obesity via dietary intake or supplemental control are of much interest. Unsaturated fatty acids, especially polyunsaturated fatty acids (PUFAs), have been shown in clinical and animal studies to be useful in controlling lipid storage and regulating body weight and obesity in mammals (1). The molecular mechanisms behind what controls these actions are not well understood. Diseases such as Cushing Syndrome, obesity, type 2 diabetes, cardiovascular disease and the Metabolic Syndrome have been linked to the actions of glucocorticoids on the body (2). Alterations to the glucocorticoid receptor complex, either positively or negatively, has been shown by our laboratory to be useful in studying these diseases.

Our laboratory has recently uncovered a promising approach that involves regulation of GR by tetratricopeptide repeat (TPR) proteins, such as FKBP52, FKBP51, Cyp40 and PP5. For example, we have generated FKBP52-deficient mice (3, 4), which are viable at birth and apparently normal into adulthood (except for infertility). Yet, cells derived from FKBP52 KO mice have reduced GR activity. Thus, FKBP52 is not a global regulator of GR, as such an effect, like GR KO mice, should result in peri-natal lethality (5). Strikingly, our FKBP52 heterozygous mice, when fed a high fat diet, acquire symptoms similar to the Metabolic Syndrome; in which they develop hyperglycemia, hyperlipidemia, hyperinsulinemia and weight gain. On the other hand, our most recent data suggest that both FKBP51 and PP5 have similar modulatory effects on GR. We have found that FKBP51-deficient mice, when fed high-fat diets, have significantly lower triglyceride plasma levels and lack the ability to store fatty acids in the visceral adipose tissue, possibly due to increased GR activity resulting from loss of inhibitory FKBP51. The loss of PP5 also results in increased GR transcriptional activity, as well as, augmented phosphorylation of GR (6). The effect that FKBP51 and FKBP52 may have on phosphorylation of GR is unknown. Furthermore, when PP5 is bound by fatty acids (e.g., arachidonic acid) the phosphatase activity of PP5 increases approximately 10 fold (7). In an adipogenesis study, PP5 KO and FKBP51 KO MEF cells accumulated less fatty acids compared to WT MEF cells. This suggested that TPR proteins play major roles in storage and accumulation of intracellular lipids and is a possible method of controlling GR actions in the body.

Based on the above, we propose a **hypothesis** in which TPR proteins may participate in lipid storage, export or metabolism by binding fatty acids, and possibly leading to activation of PP5 phosphatase activity and subsequent inhibit binding to the GR complex. As well, fatty acids may “prime” GR by inhibiting TPR proteins, such as FKBP51 or PP5, to bind to the GR complex, which may result in enhanced GR phosphorylation and induced gene activity upon elevation of glucocorticoids, either by exercise or treatment. As a corollary, we further predict that PP5-deficient mice in response to high-fat diets should be highly insensitive to development of visceral obesity and perhaps overall obesity, allowing us to test for susceptibility to diabetes and cardiovascular disease in follow-up studies.

Aim 1) Determine role of fatty acids in regulation of GR activity. This study will evaluate saturated fatty acids, MUFAs and PUFAs to determine their effects on TPR proteins and succeeding actions on GR from fatty acid treatment. Co-immunoadsorption of GR complexes will be performed with and without fatty acid treatment to determine if any TPR protein is inhibited from binding the GR complex. Also, to ascertain if any fatty acids “prime” GR by inhibiting TPR proteins, such as FKBP51 or PP5, from binding to the GR complex, reporter genes and PCR analysis of endogenous genes will be used to assay for GR activity. Phosphorylation status of GR with fatty acid and glucocorticoid treatment will be assayed in FKBP51 KO, FKBP52 KO, PP5 KO and WT MEF cells, as well as, primary adipocytes. Phosphorylation will be assayed using phospho-specific antibodies that recognize the ligand-regulated serines at positions 212, 220 and 234.

Aim 2) Determine role of TPR proteins in adipose differentiation in MEF and 3T3-L1 cells. This study will use TPR KO and WT MEF cells, and, 3T3-L1 cells in an adipose differentiation assay. TPR protein levels will be measured in differentiated cells by semi-quantitative Western blot. Stably transfected reporter genes and PCR analysis of endogenous genes will be used to assay for GR activity. Free fatty acids, triglycerides and glycerol will be measured after adipogenesis to determine the affect on lipogenesis and lipolysis.

Aim 3) Determine role of PP5 in lipid and carbohydrate metabolism in response to fasting and high fat diet. The goal of this study is to examine the effects of high-fat and fasted-state diets in PP5 deficient and wild type mice. This is a preliminary investigation, and only select tests of lipid and sugar metabolism will be done. The results should form basis for more complete investigations. The loss of PP5 has shown to increase the

ability of GR to induce GR regulated gene expression. Thus, PP5 deficient mice may experience exacerbated lypolysis and may be less susceptible to cardiovascular disease, obesity, and diabetes.

B. BACKGROUND AND SIGNIFICANCE

B.1. Introduction

Obesity is an ongoing epidemic that needs immediate attention. The development of lean tissues in mammals, that is to reduce the amount of body fat, has been linked to a significant decrease in diseases such as type 2 diabetes mellitus, cardiovascular disease, atherosclerosis and some cancers (2). Increases in these diseases have been associated with sedentary lifestyle and a diet that is high in fat (1, 2). Controlling dietary intake and exercise have been shown to give the best results in significantly decreasing body fat percentage (1). The focus of this proposal is to study the different affects that specific fatty acids such as saturated fatty acids, monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), as well as, comparison of Omega-3 and Omega-6 unsaturated fatty acids have in specific tissues of mammals.

In humans, obesity often results from alterations to carbohydrate and lipid metabolism, processes that are principally controlled by insulin and glucocorticoids (8). In the fasted state and during exercise, glucocorticoids stimulate several processes that collectively serve to increase and maintain normal concentrations of glucose in blood (8, 9). Stimulation of gluconeogenesis, particularly in the liver, results in synthesis of glucose from non-hexose substrates such as amino acids and lipids (8, 9). As well, glucocorticoids stimulate fat breakdown in adipose tissue and inhibit glucose uptake in muscle and adipose tissues (9). Thus, glucocorticoids are best categorized as physiologic antagonists to insulin, which promotes glucose uptake and storage of lipids. Chronic glucocorticoid elevation leads to insulin resistance and glucose intolerance, which may result in type II diabetes mellitus and possibly the Metabolic Syndrome. Although these properties of glucocorticoids should make them effective agents in the treatment of various metabolic conditions, glucocorticoid agonists and antagonists have proven ineffective, largely due to their global actions on many tissues, leading to undesirable side effects, especially immunosuppression (2). Thus, new targets that control the tissue- or metabolic-specific actions of glucocorticoid receptor (GR) are needed.

As a steroid receptor, there are important early steps required for GR in generation and activation of the receptor. Some of these early steps include: generation of hormone-competent receptors through a HSP90-based chaperone system, the hormone-binding event, hyperphosphorylation upon hormone binding, dissociation of the GR complexes, translocation to the nucleus and binding of glucocorticoid response elements (GREs) at gene promoters (2, 10). Our laboratory has extensive experience characterizing the mechanisms controlling GR localization, activation, translocation, gene regulation and the physiological effects of altered forms of steroid receptor complexes. GR is a hormone-regulated transcription factor that forms heterocomplexes with HSP90 that, in turn, serves as the binding site for one of several tetratricopeptide repeat (TPR) proteins. The TPR proteins known to interact with GR include PP5, FKBP51, FKBP52, and Cyp40 (Figure 1). Although the contribution of FKBP52 and FKBP51 to GR cellular function has been much investigated, very little is known of PP5 actions on GR or any other steroid receptor. It is widely speculated that PP5 interacts with most members of the steroid receptor family; however, direct evidence only exists for the estrogen receptors (ER) (11) and GR (12). In both cases, interaction with PP5 is mediated by HSP90 (10). PP5 may regulate the intrinsic ability of GR to bind glucocorticoids, as GR complexes containing PP5 have higher binding affinity for glucocorticoids compared to complexes containing FKBP51 (13). Furthermore, PP5 may control nuclear translocation of GR due to its ability to interact with the motor protein dynein via its PPIase-like domain (14).

B.2. Glucocorticoid receptor heteromeric complexes

Mature forms of the GR heteromeric complex contain one molecule of receptor, two molecules of HSP90, and one molecule of a stabilizing protein - p23 (Figure 1) (10). The HSP90 dimer generates a single binding cleft for one TPR protein, termed TPR-binding domain (TBD). There are four known TPR proteins that bind to mature GR heteromeric complexes; FK506-binding protein 51 (FKBP51), FK506-binding protein 52 (FKBP52; also known as HSP56), cyclophilin-40 (Cyp40), and protein phosphatase 5 (PP5). Recently, our lab has demonstrated that when GR is bound to agonist an exchange of FKBP51 for FKBP52 occurs that coincides with translocation of GR to the nucleus (13, 15). The ability of FKBP51/FKBP52 and Cyp40 to bind the

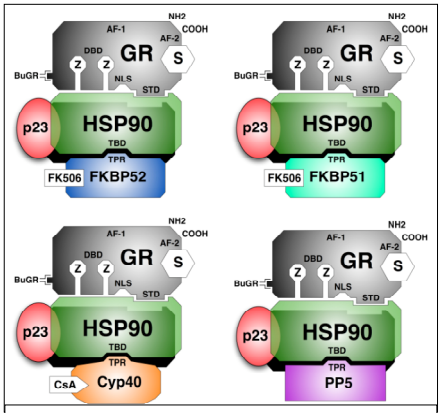

Figure 1. Mature GR heteromeric

immunosuppressive drug FK506 and cyclosporine A (CsA) has served to categorize these proteins as immunophilins. However, it is because all of these proteins, including PP5, contain TPR domains that we refer to them as TPR proteins.

B.3.a. Structure and function of FKBP51, FKBP52, Cyp40, and PP5 Figure 2 illustrates the functional domain structures of FKBP52, FKBP51, Cyp40 and PP5. Binding by these proteins to the TBD of HSP90 is via the three consecutive TPR domains located in the N-terminal region of PP5 and C terminus of the other TPR proteins. TPR domains are imperfect 34 amino acid sequences that mediate protein-protein interactions (16). PP5 is unique among phosphatases because it contains TPR domains and a peptidyl-prolyl isomerase (PPIase)-like domain. Both FKBP52 and FKBP51 have similar PPIase-like domain structures. However, an additional PPIase domain has been observed within their structure that has been shown to bind to the immunosuppressive drug FK506; this domain is also within the structure of Cyp40 and is bound by CsA. Additionally, FKBP52 and Cyp40 contain one other domain, a putative binding site for calmodulin (CaM) that has not been revealed in FKBP51 or PP5.

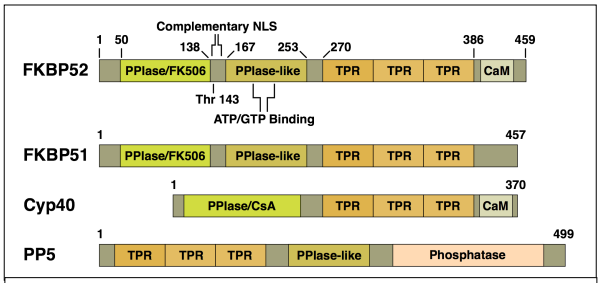

Figure 2. TPR protein map – see text.

B.3.b. Structure and function of PP5 and lipid activation via TPR domains A diagram showing pertinent features of PP5 and its isoforms are seen in Figure 3. The phosphatase domain resides in the C-terminal region and contains all the relevant motifs of the PPP family of phosphatases (17, 18, 19). PP5, unlike other phosphatases, displays low phosphatase activity under normal conditions (18) that may result from an inhibitory interaction between the C-terminus (residues 490-499) and the TPR domain (7). Residues E76 in the second TPR motif and Q495 in the C-terminus are critical to the inhibitory interaction (7). (19). Such intrachain interactions may allow the TPR domain to mediate phosphatase activity by binding and shielding it from activation. The phosphatase domain can be activated when the TPR domains are bound or truncated from PP5 (18, 19). It is for this reason that stimulation of PP5 can occur with fatty acids, such as arachidonic acid (20), which bind the TPR domain of PP5 to initiate phosphatase activity (21). Interestingly, saturated fatty acids and fatty acid esters did not stimulate phosphatase activity in this study (18) (21). TPR proteins may aid in FA metabolism, export and/or storage. A study in *Drosophila melanogaster* demonstrated that the *adp* gene knockout resulted in significant increase of FA storage (22). The *adp* gene encodes a protein that contains three consecutive TPR domains that can bind long chain fatty acids, similar to PP5 (22). The loss of this gene resulted in adipocyte hypertrophy and obesity (22). This suggest a possible role of TPR proteins in aiding in lipid storage or metabolism.

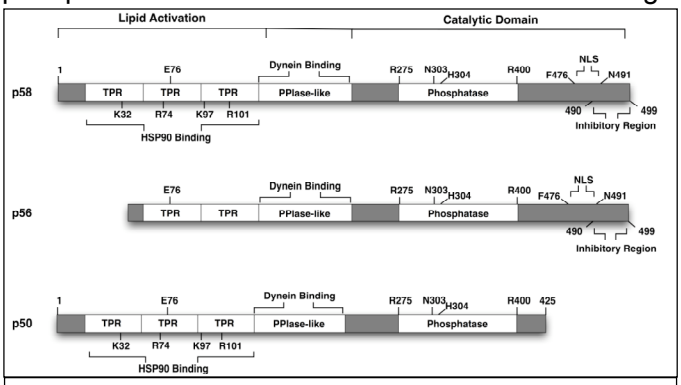

Figure 3. PP5 isoforms Key residues in the phosphatase domain responsible for binding the phosphate moeity of substrates are R275, N303, H304 and R400 (19, 20).

B.4. Development of adipose tissue and storage of fatty acids

Adipose tissue allows storage of fatty acids and triaclyglycerol in periods of energy excess and the consequent use of triaclyglycerol stores during energy deprivation. Possible causes of obesity are two fold: 1) an increase in the adipose cell precursor population, or 2) alteration of the lipogenic/lipolytic equilibrium that results in adipocyte hypertrophy (1, 23). We are focusing on the later by investigating the role TPR proteins have in lipid storage, export or metabolism during adipogenesis and lipogenesis. It has been well documented that differentiation from early preadipocytes can be performed with a cocktail of insulin, isobutylmethylxanthine (IBMX), and glucocorticoid treatment (24). The

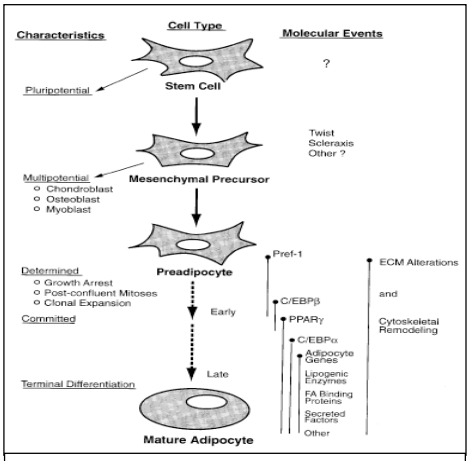

Figure 4. Adipocyte differentiation. This illustration was adapted from Gregoire et al., 1998, Physiol Rev, 78, 783-809.

induction conditions and media vary according to the cell lines. Treatment of cells with IBMX activates the transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) (Figure 4) (24, 25). IBMX inhibits soluble cyclic nucleotide phosphodiesterases and results in increase intracellular cAMP levels. At the nuclear level, treatment with glucocorticoids results in activation of the related transcription factor C/EBPδ (24). C/EBPβ and δ in turn induce transcription of C/EBPα and PPARγ. Thus, C/EBPα and PPARγ are key regulators of the complex cascade of transcriptional regulators of adipocyte differentiation (Figure 4) (24). Within 3 days of exposure to the inducer cocktail, the cells undergo two rounds of mitosis, termed mitotic clonal expansion, which is required for differentiation. High concentrations of insulin have been used in combination with these inducing agents. Insulin promotes adipocyte differentiation by activating PI3-kinase and Akt activity. Modulation of the activity of the forkhead transcription factor Foxo1 appears to be necessary for insulin to promote adipocyte differentiation (24). C/EBPα and PPARγ direct the final phase of adipogenesis by activating adipocyte-specific genes, mostly those involved in *De novo* fatty acid synthesis and storage (lipogenesis), such as perilipins, fatty acid synthetase, lipoprotein lipase (LPL), leptin, SREBP-1c and LXRalpha (24, 26). The perilipins belong to a family of hydrophobic lipid droplet-associated phosphoproteins that are phosphorylated by PKA in multiple residues (27). Perilipins are an adipose specific protein that is located on the surface of the lipid droplet and functions to prevent lipolysis of triglycerides (TGs) during basal conditions (27). On the other hand, GR has been shown to upregulate the expression of genes involved in lipolysis, such as hormone sensitive lipase (HSL), adipocyte lipid binding protein (ALBP), DGAT1, GILZ and ANGPTL4 (28, 29). HSL and ALBP have been shown to be two critical genes that regulate the release of TGs during lipolysis, via perilipin interaction (27). HSL and perlipin are phophorylated by PKA and this leads to catalysis of TG and diglyceride breakdown, producing a subsequent release of free fatty acids (FFA) and glycerol (27). The involvement of TPR proteins during adipogenesis and lipogenesis and their actions on GR after differentiation into adipocyte has not been documented. However, one study demonstrated that FKBP51 is upregulated during adipogenesis, and GR and FKBP52 downregulated (30) (31). We therefore speculate that FKBP51 may be involved in storage or metabolism of fatty acids (lipogenesis), most likely via TPR domains, and FKBP52 in lipolysis. **A major goal of this proposal is to use fatty acids that bind to the TPR proteins, via TPR domains, to intensify levels of glucocorticoid induced fatty acid metabolism and accelerate lipolysis, perhaps via HSL, after adipocyte differentiation. Such a role should provide important insights into the protection and etiology of obesity and early development of diabetes, cardiovascular disease and some cancers.**

B.5. SIGNIFICANCE

Obesity and insulin resistance are strong indicators for risk of developing cardiovascular disease and diabetes. TPR proteins as lipid sensors and the PP5-deficient mouse model of amplified lypolysis presented in this application provides a novel approach through which the pathogenesis of these diseases may be studied, as well as potential new treatments and therapies. Should we find that TPR proteins are lipid sensors and that PP5 deficient mice have a lower predisposition to obesity and/or diabetes, we can compare our in vitro studies in which we attempt to regulate TPR proteins via MUFAs and PUFAs, followed by analysis of metabolic and cardiovascular phenotypes.

C. PRELIMINARY DATA:

The development of PP5, FKBP51 and FKBP52 deficient mice has given our laboratory the opportunity to study the impact that the loss of these TPR proteins have within the cell. We have already documented the affects from the loss of FKBP52. This study emphasizes the impact that the loss of PP5 has on GR localization, translocation, phosphorylation, and activity, as well as, affects of TPR proteins in lipid storage, metabolism and/or export and adipogenesis.

C.1. GR and FKBP51 expression is lower in PP5KO MEF cells (unpublished). First, we profiled the TPR proteins in WT, PP5 heterozygous, and PP5 KO MEF cells from whole cell lysates by quantitative Western blotting (Figure 5). Interestingly, GR and FKBP51 levels decline with a reduction and absence of PP5. No noticeable changes in FKBP52 and Cyp40 levels were observed. Since there was a difference in FKBP51 levels in PP5KO MEF cells, we next wanted to analyze how this affected the GR complex.

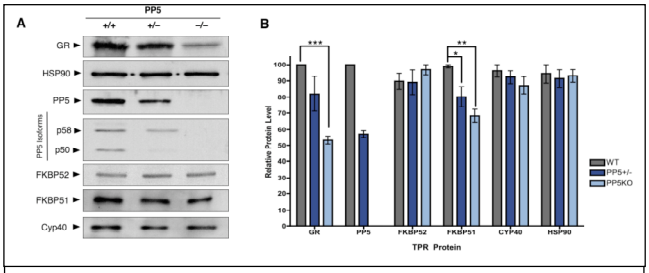

Figure 5. TPR profile. (**A**) Western blot demonstration of presence (+/+), (+/-) and absence (–/–) of PP5 in MEF cell lines. (**B**) Densitometric analysis of results from Western blots, n=3.

C.2. Preferential interaction of GR with PP5 and

FKBP51/Loss of PP5 does not cause compensation by other TPRs (unpublished). We used a co-immunoadsorption assay to confirm the presence of PP5 in hormone-free GR complexes and to test whether loss of PP5 affected GR complex composition. Immunoprecipation of GR was performed from cytosolic lysates of equal protein content. In WT cells, GR was found to preferentially bind PP5 and FKBP51, as seen for GR in other cell lines (data not shown). Interestingly, GR complexes in PP5 KO cells had the same FKBP51 levels (Figure 6), and the levels of other TPRs did not increase, showing that compensation was not occurring. Also of interest, the p50 isoform of PP5 was found in the complex; this hasn't been previously reported to be in the GR heteromeric complex. At this point, we do not know if the p50 interaction is unique to MEF cells or common to GR in all cell types.

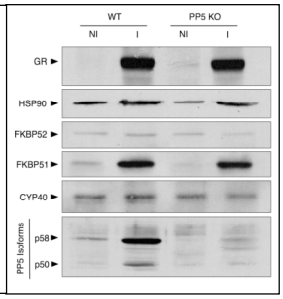

Figure 6. GR was immunoadsorbed to protein-A sepharose beads with a GR specific antibody. Samples were resolved on denaturing SDS gels, transferred to PVDF membranes, then immonspecific antibodies were used to detect proteins of interest. NI, non-immune antibody. I, immune FiGR antibody.

C.3. Hyperphosphorylation in the AF-1 of GR in PP5KO Cells (unpublished). Phospho specific antibodies for serines 212, 220 and 234 in the AF-1 region of GR were used to determine the phosphorylation status of GR in WT and PP5 KO MEF cells (Figure 7). One hour hormone treatment resulted in hyperphosphorylation of GR at all three serines in PP5KO cells. No increase of basal phosphorylation in PP5KO cells. The increase in phosphorylation of GR in the AF-1 may result in more GR induced gene activity and as a result we took the study one step further and analyzed the transcriptional activity of GR in a reporter gene assay.

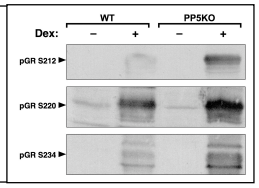

Figure 7. Phospho specific antibodies for serines 212, 220 and 234 in the AF-1 region of GR. One hour hormone treatment in PP5 KO and WT cells

C.4. PP5 is a negative regulator of GR induced reporter gene activity (unpublished). WT and PP5KO MEF cells were transiently transfected with a minimal reporter, GRE2E1B-luc, composed of two synthetic GRE elements and the E1B TATA-box driving expression of luciferase enzyme. In Figure 8A, both WT and PP5KO cells were simultaneously transfected with pSV2Wrec plasmid encoding mouse GR. We realized that the GR activity could possibly be a result of the GR expression level. Therefore, in figure 8B & 8C we transfected only the PP5KO cells with pSV2Wrec plasmid to compensate for the GR differences. In these studies, cells were treated with or without $1x10^{-6}$ M dexamethasone for 24 hours. When the GR levels were normalized the results show a dramatic increase of Dex-induced GR activity in the PP5 KO cells (Figure 8B & 8C).

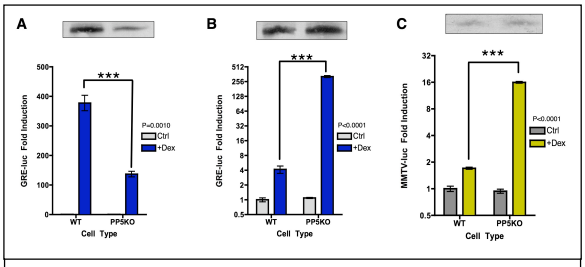

Figure 8. Luciferase activity of WT and PP5KO MEF cells. Transfected with reporter, GRE2E1B-luc (A & B) and MMTV-luc (C).

C.5. FKBP51 and PP5 are negative regulators of GR activity (unpublished). WT, FKBP52 KO and FKBP51 KO MEF cells were transiently transfected with a minimal reporter, GRE2E1B-luc, as stated above. The results in Figure 9 indicate that FKBP52 is a positive regulator and FKBP51 is a negative regulator of GR induced gene activity.

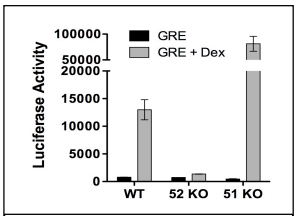

Figure 9. Luciferase activity of WT FKBP52 KO and FKBP51 KO MEF cells. Transfected with a minimal reporter, GRE2E1B-luc

C.6. Real-time PCR Confirms PP5 is a negative regulator of GR activity (unpublished). Endogenous genes were measured via Real-time PCR in WT, PP5KO and 52KO MEF cells with 100 nM dexamethasone treatment for two hours. The data agree with the GRE-luc and MMTV-luc assays, that PP5 is a negative regulator of GR induce gene activity and that FKBP52 is a positive regulator. Also, there are gene specific differences that may be controlled by each TPR protein when comparing these four genes, SGK, FKBP51, p21 and GILZ (Figure 10). This suggest that TPR proteins may control specific GR regulated genes. With the understanding that FKBP51 and PP5 are most likely a

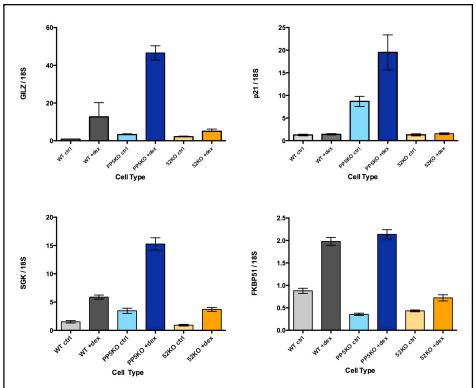

Figure 10. Realt-time PCR results for WT, PP5 KO and 52 KO cells. Cells were treated with 100nM dex for 2 hours.

negative regulators of GR induced gene activity, along with PP5 possibly aiding in fatty acid storage, export or metabolism, we next analyzed the impact the two would have in an adipose differentiation and fatty acid accumulation, because GR most likely assist in adipose differentiation.

C.7. PP5 KO and FKBP51 KO MEF cells accumulate less fatty acids during adipose differentiation compared to WT cells. A major goal of this proposal is to test the role of TPR proteins in GR-mediated metabolism. Because PP5 can be regulated by lipids (20) (21), and because we now have shown that PP5 and FKBP51 are inhibitory to GR activity (Figures 8 & 9), we next tested the impact the loss of TPR proteins would have in a Dex-induced adipose differentiation assay (Figure 11). In this assay, MEF cells were induced to undergo adipocyte differentiation by treatment with insulin, isobutylmethylxanthine (IBMX), and Dexamethasone. Oil red O staining was used to measure fatty acid accumulation. PP5KO and FKBP51KO cells accumulated less intracellular lipids compared to WT cells. The lower accumulation of lipids in the PP5 and FKBP51 KO MEFs may be a result of more GR activity and increased lipolysis, possibly via HSL. We speculate that PP5 deficient mice should accumulate less fatty acids in tissues similar to this study. As a comparison, we had already begun our studies in FKBP51 deficient mice.

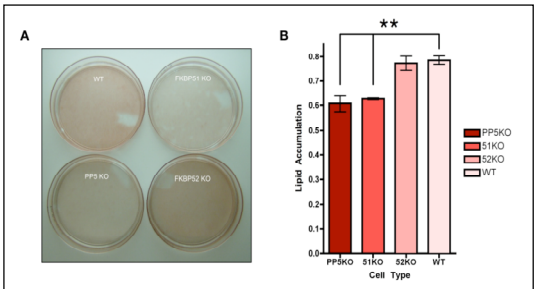

Figure 11. Accumulation of fatty acids in wild type and PP5 KO MEF cells in a MEF cell differentiation study using insulin, IBMX and dexamethasone to induce adipocyte diffentiation. (A) The results from Red Oil O stain. (B) Isoproponal removal of the Red Oil O stain and reading at 500 nm, n=3.

C.8. TPR proteins regulate obesity in mice (manuscript in publication). The mice in this study were fed high-fat diets for 4 weeks and the triglyceride, insulin and glucose plasma levels were measured. At the end of the study, the liver and visceral tissues were collected and analyzed for lipid accumulation. The FKBP51 knock out mice had a decrease in the amount of triglycerides in plasma and visceral tissues, with similar accumulation of lipids in the liver, compared to WT mice (Figure 12 A—F). The lack of lipid accumulation correlated with a markedly reduced ability of FKBP51 KO animals to gain weight following high-fat diet (data not shown). Lipid accumulation in the FKBP51 KO mice was also comparable to the adipose differentiation study in Figure 11 with fewer lipids being stored after adipogenesis. We found this to be exciting results, as it validates our hypothesis that TPR proteins are involved in metabolic processes, most likely through increasing GR activity in select tissues. Further validation has been found in FKBP52 heterozygous mice in which they acquire symptoms similar to the Metabolic Syndrome, in which they gain weight, have hyperlipidemia, hyperglycemia and insulin resistance when fed a high fat diet, compared to WT animals (Figure 12 G—L). Thus, it is likely that PP5 deficient animals will also have a unique set of altered metabolic phenotypes, which should lead to a better understanding of the tissue- and pathway-specific factors controlling obesity, diabetes and cardiovascular disease.

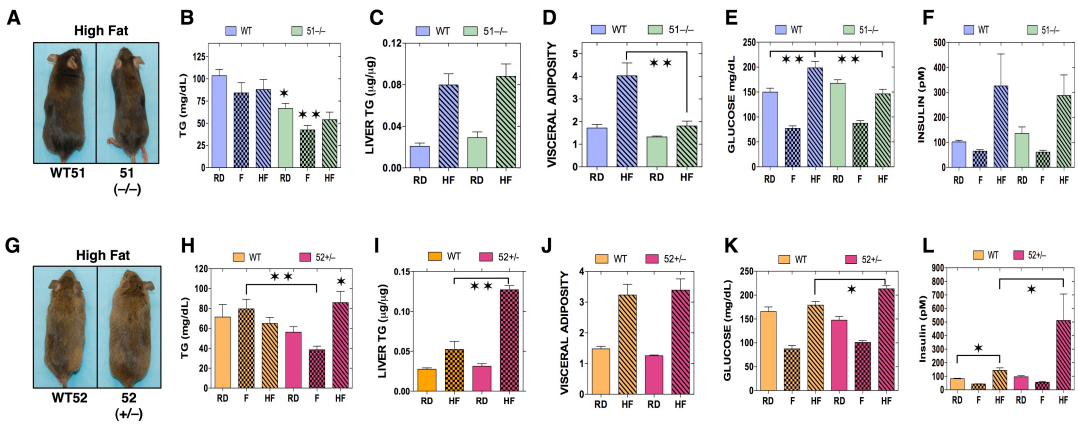

Figure 12. A—F represents the data collected from WT 51 and FKBP51 deficient mice. G—L represents the data collected from WT 52 and FKBP52 heterozygous mice.

D. RESEARCH DESIGN AND METHODS

D.1. Aim 1. Determine role of fatty acids in regulation of GR activity.

D.1.a. Hypothesis: We hypothesize that TPR proteins may participate as lipid sensors by binding fatty acids, and possibly aiding in lipid storage, export or metabolism. We also speculate that long chain unsaturated fatty acid treatment will result in activation of PP5 phosphatase activity, most likely by derepressing the TPR domain

from the C-terminus, and as a corollary inhibit binding to the TPR binding domain (TBD) of the GR complex. As well, long chain unsaturated fatty acids may "prime" GR by inhibiting TPR proteins, such as FKBP51 or PP5, to bind to the GR complex, via TBD, which may result in enhanced GR regulated gene activity or augmented phosphorylation at serines 212, 220 and 234 upon glucocorticoid treatment, similar to PP5 KO cells (Figure 7).

D.1.b. Rationale: Preliminary data from our laboratory shows that PP5 and FKBP51 are important negative regulators of GR transcriptional activity, and that both bind the GR complex to suppress activity under basal conditions (Figure 6). These data are in good agreement with published results showing that GR is a phosphoprotein that becomes hyperphosphorylated when bound to glucocorticoids (6), that phosphorylation is critical to GR activity (32), and that long chain fatty acids, such as arachidonic acid, bind to the TPR domains of PP5 and this may inhibit binding of PP5 to the GR complex and similar result may be seen as in PP5 KO (21). Thus, it is highly likely that FKBP51 and PP5 will be uncovered as important regulators of lipid storage or metabolism.

D.1.c. Proposed Experiments: The proposed experiments focus on treatment of cells with various fatty acids, such as saturated fatty acids, MUFAs and PUFAs, as well as, comparison of Omega-3 and Omega-6 unsaturated fatty acids. And, how these treatments affect TPR proteins within the GR heteromeric complex, phosphorylation of GR, and regulate GR controlled gene expression in PP5 KO, FKBP51 KO, FKBP52 KO and WT cells. We propose to isolate primary adipocytes from WT and PP5 KO mice, in addition to using our already established MEF cell lines. Using phospho-specific antibodies, we will characterize the phosphorylation status of GR in these cell types. Lastly, we will analyze gene expression via reporter genes and Real-time PCR analysis of endogenous genes with various FA and glucocorticoid treatments. FA will be treated prior to glucocorticoid treatment at different time intervals to determine the optimal effect on GR activity. Taken as a whole, these approaches should lead to a good understanding of how TPR proteins respond to fatty acids and how this affects GR activity.

D.1.d. Methods:

D.1.d.i. Treatment of cells with fatty acids. Fatty acids that will be used in this study are pure compounds and include: *Saturated fatty acid kit:* Arachidic acid (C18:0), Behenic acid (C22:0), Decanoic acid (C10:0), Dodecanoic acid (C12:0), Hexanoic acid (C6:0), Lignoceric acid (C24:0), Myristic acid (C14:0), Octanoic acid (C8:0), Palmitic acid (C16:0) and Stearic acid (C18:0) (Fatty Acid Kit, Sigma-Aldrich, Product No: EC10A-1KT). *Unsaturated fatty acid kit:* Arachidonic acid (C20:4, n-6), Linoleic acid (C18:2, n-6), Linolenic acid (C18:2, n-6), alpha-linolenic acid (C18:3, n-3), cis-4,7,10,13,16,19-Docosahexaenoic acid (C22:6, n-3), Elaidic acid (C18:1, n-9), Erucic acid (22:1, n-9), Nervonic acid (C24:1, n-9), Oleic acid (C18:1, n-9), Palmitoleic acid (C16:1, n-7), Petroselinic acid (C18:1, n-12) (Fatty Acid Unsaturated Kit, Sigma-Aldrich, Product No: UN10-1KT).

Fatty acid stocks will be prepared by dissolving fatty acids in ethanol to a final concentration of 100 mM fatty acid. The fatty acid stock solutions will be diluted to 50 to 100 μM in serum-free medium containing 10 to 20 μM charcoal-treated, solvent-extracted, fatty acid–free bovine serum albumin as a fatty acid carrier.

D.1.d.ii. Co-Immunoprecipation of GR complex following fatty acid treatment. WT cells will be treated with various FA treatments and Co-Immunoprecipation of GR complex will be performed (as described in C.2) to determine if basal TPR proteins, PP5 and FKBP51, are still bound at the same levels, or if there is recruitment of any other TPR protein. FA may bind the TPR domain of PP5 and FKBP51, and inhibit binding to the GR complex, and as a result increase GR activity upon glucocorticoid treatment.

D.1.d.iii. Compare phosphorylation of GR in WT and TPR KO cells. We will use WT and TPR KO MEF cells already generated, as well as isolate primary adipocytes from WT and PP5 deficient mice. The first test will be to treat each cell type with or without Dex to determine responsiveness of GR to hormone-induced phosphorylation at serines 212, 220 and 234. An initial dose dependence for Dex will be done in WT cells to determine the optimal concentration needed for induction of phosphorylation. Whole cell extracts for each cell type will be prepared, followed by assay for protein levels, and SDS-PAGE and Western blotting utilizing phospho-specific antibodies for serines 212, 220 and 234, as well as measure total GR. The Garabedian laboratory has already generously provided us with the GR phospho-specific antibodies for residues S203, S211 and S226 of human GR (6), which do react with analogous sites in rat and mouse GR (serines 212, 220 and 234). We will analyze the data by densitometry using ImageJ software. Statistical analysis of the data will be performed to compare any variations; *P* values less than 0.05 will be considered to be statistically significant. We expect to see Dex-induced phosphorylation at serines 212, 220 and 234 for GR of WT cells. In PP5 KO cells, the overall expectation is Dex-induced phosphorylation of one or more of the three phospho-

serines. The affects of FKBP51 and FKBP52 on phosphorylation of GR is unknown, however, we expect that FKBP51 will have similar effects as PP5 given that they have similar reporter gene activity. We also speculate that FKBP52 will positively effect GR phosphorylation and the loss will result hypophosphorylation of GR.

The second test will be to determine the effects of FA treatment on TPR proteins and on FA treatment prior to Dex-induced GR phosphorylation. This will be achieved by repeating the experiments described above, but in the presence and absence of FA (as described in D1.d.i.). As above, a dose dependence for each of the FA will be done in WT cells to determine the optimal concentration needed for affecting GR phosphorylation. It is possible that MUFA or PUFA treatment, may inhibit PP5 or FKBP51 from binding the GR complex. Thus, making GR more sensitive to glucocorticoid treatment and increasing the ability of GR to induce gene activity. Also, as above, we may find cell-type specific differences in this inhibition (to be explored below).

D.1.d.iv. Does TPR proteins control gene specific activity of GR? We predict that FKBP51, FKBP52 and PP5 may modify GR phosphorylation status and this in turn will affect the gene-specific activity of GR, and that this effect will vary across cell types. This will be tested by measuring gene-specific expression via Real-Time PCR in WT and FKBP51 KO, FKBP52 KO and PP5 KO cells treated with FA prior to dexamethasone. We will measure GR regulated genes via Real-time PCR: SGK, p21, GILZ, and FKBP51 (see Figure 10 for preliminary Real-time PCR results). Characterizing the expression of these genes in the various cell types with glucocorticoid and FA treatment will allow us to correlate GR gene activity with the results of the phosphorylation status (see above).

Pitfalls and Alternatives: Because the Garabedian laboratory has already demonstrated the efficacy of the phospho-antibodies, and because we have already demonstrated feasibility for all of the fundamental assays proposed (Western-blotting and PCR), there are no serious technological pitfalls associated with this proposal. Instead, we may simply find that one or more of our expected biological outcomes may not be seen. Alternative approaches will depend on the unforeseen results, but can include the following. If we find no differences in GR-mediated gene expression, it could be because we chose the wrong genes. Thus, others can be selected for analysis. If GR phosphorylation is altered by loss of PP5, FKBP51 or FKBP52 in all cell types and in the same way, we will simply conclude that these TPR proteins serve a global function on this property of GR.

D.2. Aim 2. Determine role of TPR proteins in adipocyte differentiation in MEF and 3T3-L1 cells.

D.2.a. Hypothesis: We hypothesize that TPR proteins may participate in adipogenesis and lipogenesis, possibly through lipid storage, export or metabolism by binding FA via TPR domains. We further propose that FA may bind TPR proteins, and inhibit binding to the GR complex, which may result in increased GR activity. As a result, there may be accelerated lipolysis possibly by elevated HSL expression and this should affect the amount of lipid accumulation in PP5 KO and FKBP51 KO cells.

D.2.b. Rationale: Preliminary data from our laboratory shows that TPR proteins regulate GR transcriptional activity. We also show that PP5 and FKBP51 can affect the ability of MEF cells to undergo adipocyte differentiation and lipid accumulation. TPR proteins may be uncovered as an important differential regulator of GR phosphorylation, specific gene activity, with direct consequences on GR-controlled metabolic pathways, such as adipocyte differentiation and lipid metabolism.

D.2.c. Proposed Experiments: The proposed experiments focus on treatment of cells with the adipocyte differentiation cocktail, with emphasizes on changes within GR complex, and how these changes either by TPR protein or phosphorylation affect GR controlled gene expression. With comparison of the final result of measuring the lipid accumulation in PP5 KO, FKBP51 KO, FKBP52 KO and WT cells. We will analyze gene expression via stable transfected GRE-luc, PPARγ-luc and CEBP-luc reporter genes and PCR analysis of endogenous genes such as: PPARγ, PPARα, fatty acid synthase, C/EBPα, β and δ, and adiponectin (and genes listed under lipogenesis and lipolysis in section B.4). Taken as a whole, these approaches should lead to a good understanding of what role TPR proteins have in adipogenesis and lipogenesis.

D.2.d. Methods:

D.2.d.i. Role of TPRs in MEF cell adipogenesis. It is not known whether TPR proteins can affect adipogenesis in fibroblasts. We will perform the adipogenesis as described in the preliminary data study of C.7, using FKBP51 KO, FKBP52 KO, PP5 KO and WT MEF cells. We will use the GR phospho-specific antibodies to characterize the phosphorylation status of GR during a time-course of adipocyte differentiation. This will help us determine what role the different TPR proteins have in controlling GR during adipogenesis. We will perform the experiments and analyze the data as described above. Because of the preliminary data in C.4—C.8, we

expect to find that PP5 KO and FKBP51 KO MEF cells should accumulate fewer lipids, possibly by increased HSL expression. On the other hand, FKBP52 KO MEF cells should have similar levels of lipid accumulated compared to WT MEF cells (Figure 11). We will also measure free fatty acids (FFA), TGs and free glycerol after adipocyte differentiation. This will aid in comparing gene expression and fatty acid metabolism or storage. Studying the mechanism of why these cells accumulate less or more lipids, by means of phosphorylation of GR, GR regulated gene, genes involved in adipogenesis and fatty acid metabolism or storage, will give more specific answers to causes of diabetes and obesity.

D.2.d.ii. Does TPR proteins control gene specific activity of GR during adipogenesis?. We predict that GR phosphorylation status will affect the gene-specific activity of GR, and that this effect will vary across cell types. This will be tested by measuring gene-specific expression via Real-Time PCR in WT, FKBP51 KO, FKBP52 KO and PP5 KO cells over an eight-day time scale during adipogenesis and lipogenesis. Genes that will be measured are listed above (D.2.c.). Characterizing the expression of these genes in the various cell types will allow us to correlate GR gene activity with the results of the phosphorylation and lipogenesis (see above).

D.2.d.iii. Lipid accumulation in wild type and TPR knock out cells. WT and TPR deficient cells will be treated with the adipose differentiation cocktail (insulin, IBMX and dexamethasone) to determine if there are any differences in fatty acid accumulation in these cells. After differentiation, the cells will be stained with Red Oil O to measure total lipid accumulation, pictures will be taken of the cells, followed by extraction of stained lipids with isopropyl alcohol. Lipid content in the isopropyl alcohol extracts will be measured at 500 nm in a spectrophotometer to analyze the difference in lipid accumulation between the cell types and treatments. To measure fatty acid metabolism, export, and/or storage we will use Free Fatty Acid Quantification, Triglyceride Quantification and Free Glycerol Assay kits (BioVision, Mountain View, California). This as whole should give more specific answers to how GR and TPR proteins are involved in adipogenesis and lipogenesis.

Expected Results: With respect to fatty acid accumulation and adipogenesis, we expect that FKBP51 and PP5 KO cells should accumulate less intracellular fatty acids based on the preliminary data. Lastly, analysis of GR gene activity by PCR will allow us to correlate altered gene expression with the observed effects on phoshorylation and adipogenesis. Our decision to focus on metabolic genes should aid in this analysis.

D.3. Aim 3. Determine role of PP5 in lipid and carbohydrate metabolism in response to fasting and high fat diet.

D.3.a. Hypothesis: We hypothesize that amplified GR activity in PP5 deficient mice will result in reduced accumulation of lipids in visceral adipose tissue in response to high-fat diet, which should correlate with the FKBP51 KO mice. If the loss of PP5 also affects hepatic GR activity, an increase of gluconeogenesis should be seen, leading to high plasma levels of fasting glucose in PP5 KO mice compared to WT.

D.3.b. Rationale: We have shown that loss of PP5 leads to increased GR activity in a reporter gene assay. We also showed that loss of PP5 inhibits the ability of MEF cells to accumulate fatty acids during an in vitro Dex-induced adipogenesis assay. These data, combined with the well-known ability of glucocorticoids to control visceral adiposity, suggest that PP5 loss should make mice less susceptible to diet-induced visceral obesity.

D.3.c. Proposed Experiments: We have designed this aim to serve as a screen of select GR-mediated metabolic processes in vivo, not as an comprehensive test of all metabolic pathways possibly affected by PP5. The reasons for this are twofold. First, this will be the first study of PP5 and its role in metabolism, so clearly the first priority should be to document one or more effects before proceeding. Second, animal costs are very expensive, and are not covered by the fellowship. Thus, targeted choices must be made. Those choices, outlined below, stand a good chance of providing key observations that will then serve as starting points for more extensive investigations. The overall plan is to expose WT and PP5 KO mice to high fat and fasting diets, followed by select assay for metabolic endpoints. Adult male, 8-week old littermates will be used for each genotype. Animals will be derived from double heterozygote [PP5 (+/–)] matings.

D.3.d. Methods.

D.3.d.i. High fat diet and metabolic measurements: Age-matched WT and PP5 KO males will be fed a high fat diet (45% Kcal from fat) and regular diet for 4 weeks. Blood will be collected, followed by euthanasia, measurement of body weight, and the harvesting of select organs. The following assays will be performed (as detailed below): plasma triglyceride, free fatty acids, glucose and corticosterone; liver, visceral adipose and soleus muscle weights; histological staining for lipid content in liver, adipose and muscle.

D.3.d.ii. Plasma constituent measurements: Blood will be collected from fasting mice by retro-orbital bleeding following anesthesia. Fasting plasma FFA will be measured with the NEFA C kit (Wako), triglycerides will be measured with the Infinity Triglycerides reagent (Sigma) and glucose will be measured with a glucometer. Lastly, since we are expecting effects on GR mediated processes, we will also measure plasma corticosterone levels via ELISA. This last measurement will determine if adrenal corticosterone secretion is compensating for gain of GR activity in PP5 KO mice.

D.3.d.iii. Histological staining: Liver, muscle and visceral adipose tissues will be removed and weighed. The liver will be sharply split open longitudinally and placed under a dissection scope to analyze for lipid accumulation. The severity of any lipid accumulation will be mapped and scored. We will then stain a portion of the fresh tissue with Sudan red to accentuate potential areas of fatty streaks. The major portion of tissue will be fixed in 10% formalin. This tissue will be selectively cut, processed, and embedded on edge in paraffin for hematoxylin and eosin-stained (H&E) sections. We will examine the tissue for the development of lipid accumulation. The last portion of tissue will be simultaneously snap-frozen with OCT compound for later cutting on a cryostat into 4-8μm sections. To detect neutral lipid, these 4μm sections will be stained with Oil red O and counterstained with hematoxylin. Similar procedures will be performed on muscle.

D.3.d.iv. Fasting and metabolic measurements: Age-matched WT and PP5 KO males will be fasted for 18 h overnight by complete removal of food and replacement of bedding to remove residual food debris. Water will be supplied. Blood will be collected and plasma glucose levels will be measured.

Expected Results: We expect that PP5 KO mice in contrast to WT will have underdeveloped adipose tissue, with reduced lipid content, in response to high fat diet. Preliminary data in C.7 indicates that PP5 promotes lipid metabolism and storage in MEF cells. Thus, an alteration to visceral adipose phenotype in high fat fed PP5 KO mice is a reasonable expectation. If adipose cannot properly store lipids in PP5 KO mice fed high fat, it is likely that the excess lipid will be directed to liver or muscle. Also, because GR promotes lipolysis in both adipose and muscle, increased GR activity in these organs should accelerate lipolysis and export. The net effect of these processes should therefore be a large increase of lipid accumulation in the liver (steatosis) of PP5 KO animals following high fat diet. We also expect to find higher than normal levels of plasma triglycerides and free fatty acids in the PP5 KO mice. The results to fasting are somewhat harder to predict. Typically, lipids are mobilized from adipose and directed to liver for conversion to glucose during fasting, resulting in transient hepatic steatosis. But increased GR activity due to PP5 loss at gluconeogenic pathways in the liver may result in exacerbated hyperglycemia. This is the most likely result. Should this be seen, future experimentation to determine GR activity at hepatic gluconeogenic enzymes will be considered.

Pitfalls and Alternatives: We have recently developed expertise in all of the animal husbandry and metabolic assays proposed in this application. Manuscripts using these assays on FKBP51 KO and FKBP52 heterozygous animals are in preparation. Moreover, our laboratory is part of the Center for Diabetes and Endocrine Research, from which we draw much experience and advice on metabolic processes, particularly from the laboratory of Dr. Sonia Najjar, an accomplished researcher of diabetes using mouse models.

The only biological pitfall that we can foresee is a lack of phenotype in the fasting model. We proposed an overnight (18 h) fast, as this is typical for the field of diabetic research and as this condition requires less oversight by the regulatory IACUC process. However, if 18 h fasting shows no difference between PP5 KO and WT, we will propose to use longer fasting intervals (36 to 48 h) in order to maximize a response. Indeed, typical fasting conditions for induction of GR-mediated gluconeogenic pathways in the liver are on this order and would therefore be justified.

Time Table:

Aim #	Year 1	Year
1	Isolation of primary adipocytes and characterization of phosphorylation of GR in WT and TPR KO cells with glucocorticoid and fatty acid treatment.	
2	Adipogenesis study in FKBP51 KO, FKBP52 KO, PP5 KO and WT MEF cells. Characterization of genes involved in adipogenesis and phosphorylation of GR.	
3		Feed WT and PP5 deficient mice HFD for 90 days, followed by lipid accumulation characterization.

E. ETHICAL ASPECTS OF THE PROPOSED RESEARCH

We have no plans to use human subjects and justification for animal subjects is given below in Section F.

F. VERTEBRATE ANIMAL SUBJECTS

F1. A detailed description of the proposed use of the animals:

As detailed in the Section D.2 of the proposed studies, we will evaluate the effect of PP5 on fat metabolism via glucocorticoids and lipolysis. For these studies, we need 10 mice (*Mus musculus*) per mutant and control group each for mRNA extraction, protein isolation, and histological analysis. Blood collection can be performed prior to organ harvest. Thus, we need 30 mice per each mutant and control group at each age. The blood pressure measurements can be performed on the same animals prior to sacrifice and organ harvest, requiring no additional mice. The total number of mice required for Aim 1 is thus 20 mice.

In Aim 2, we plan to investigate whether PP5 deficient mice (Mus musculus) develop accumulation of lipids in tissue and obesity when given a high fat diet. These studies will require 10 mice per mutant and control group each for mRNA extraction, protein isolation, and histological analysis. Blood collection can be performed prior to organ harvest. The blood pressure measurements can be performed on the same animals prior to sacrifice and organ harvest, requiring no additional mice. We will begin the 90 day high fat feeding for longitudinal studies at 1 and 4 months of age for age matched comparisons at 4, and 7 months of age. We expect to need 90 mice for Specific Aim 2.

We will perform the procedures described in the Research Plan and according to the Institutional Animal Care and Use Committee (IACUC) approval. Briefly, the following will be performed:

• Adult tissues will be collected for analysis of gene expression, pathological examination and to assay for lipid composition. To obtain tissues, animals will be euthanized as specified below.
• Measurement of metabolites in serum: blood will be removed from the retroorbital sinuses following anesthesis, and the serum extracted to measure FFA, glucose, and triglycerides levels.
• Blood Pressure: Blood pressure will be measured using a non-invasive tail-cuff monitor (NIBP-8, Columbus Instruments, USA). Animals will be adapted to the apparatus over 4 x 40 minutes-sessions the week prior to testing, and the average of 10 consecutive measurements will be taken as a representative pressure for each animal.

F.2. Justification for animal use:

F.2.a. *Rationale for animal and species use:* An important component of this proposal is use of genetically altered mouse lines. The mouse (*Mus musculus*) offers considerable advantages for investigating human diseases, in that its regulation is well understood, and many complex assays and procedures can be standardized and carried out without significant technical hurdles. While we are fully aware that mice and humans are different and that lessons derived from laboratory mice may not be applicable directly to humans, we should also consider that genetic analysis in mice has had a profound impact on our understanding of human genetics.

F.2.b. *Rationale for number of mice requested*: Justification of the number of mice used has been described in F.1 above. The number of animals required for each experiment has been derived as follows: Adipose study and experiments (including periodically sampling blood glucose and triglyceride levels, histology, RT-PCR, and western blotting) require a colony of at least 10 PP5 knock out mice and an equal number of controls from each group. We routinely set up crosses from a large breeder pool (at least 10-20 individuals of each sex) to circumvent the problem of inbreeding. Control animals are derived from the same cross, and not from parallel matings of wild-type animals, to ensure as close a genetic match as possible to the test animals. Each colony is genotyped, and informative animals are housed (two to three per cage) for various lengths of time. Each cross requires a minimum of 50 cages/year.

F.3. General Veterinary Care at the University of Toledo:

The UT HSC animal care program uses the ILAR publication, *The Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academy Press, Washington, D.C., 1996), as a

basis for operation. The program is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALACI) and is in compliance with all municipal, state and federal laws and regulations governing animal research. The Institutional Animal Care and Use Committee (IACUC) is constituted with regard to and is operated in accordance with the USDA and HHS standards and policies. The animal care facility is a centralized resource called the Department of Laboratory Animal Resources (DLAR). It is directed by a full-time veterinarian, Philip Robinson, D.V.M., who is a member of the American College of Zoological Medicine. DLAR management and staff are responsible for care, husbandry, and veterinary medicine for all teaching and research animals housed within the unit.

The DLAR facilities are centralized in the Health Education Building with an additional small core of rooms located in the Block Health Science Building. DLAR maintains rooms, equipment and trained personnel for the maintenance of most common laboratory animal species. Cagewashing, postmortem, and storage areas are available. Specialized facilities for survival surgery, intensive care and biohazard containment are available. Microbiological barrier facilities are available for hazard containment and for protection of rodents from murine pathogens.

UT HSC ANIMAL HEALTH PROGRAM - GENERAL VETERINARY CARE

Clinical veterinary medicine is an integral aspect of the UT HSC animal resource program. The program is lead by an ACZM Board Certified veterinarian. All animals are observed daily by investigators, research technicians and the animal resources staff. Ill animals are managed, treated or euthanized as needed to promote animal welfare and the research goal. Preventive medicine is emphasized in the clinical medicine program. The intent is to provide healthy animals for research and to avoid clinical illness. Where feasible, only animals specifically bred for research are procured. These are from reputable commercial vendors maintaining quality control programs. Animal health protocols have been written for quarantining and conditioning of all species and these are formulated with consideration of the vendor source quality. Limited clinical diagnostic capability is maintained within the animal care department but the UT HSC hospital and outside commercial laboratories are used to augment them as necessary.

Rodents are typically acquired as specific pathogen free (SPF) animals. They are examined on arrival for clinical appearance and consistency with specifications. Quarantining and conditioning generally consists of a physiological stabilization period for SPF animals but can be extensive for rodents procured from non-commercial sources. The facility contains both conventional housing and barrier housing systems as determined by user needs. All rodent rooms are monitored for key bacterial and viral pathogens through a quarterly surveillance program. Testing is done through serological antibody monitoring by well-known commercial testing companies.

F.4. Procedures to Ensure Limited Discomfort, Distress and Pain to Animal: Anesthetic:

Animals are anesthetized with an intraperitoneal injection of Nembutal Sodium (Pentobarbital, 40 mg/kg body weight). We will test the animal's awareness by applying pressure on hind limb to gauge responsiveness. We will also make sure that the mice lose their corneal reflex (about 5-10 minutes).

F.5. Euthanasia:

Animals are anesthetized with an intraperitoneal injection of Nembutal Sodium (Pentobarbital, 40 mg/kg body weight). Blood is removed from the retroorbital sinus as part of the bleeding process to measure FFA, TG and glucose, and the animals euthanized by cervical dislocation.

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