The Role of p53 Serine 23 in Insulin Resistance

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Abstract

Ataxia Telangiectasia (A-T) patients exhibit several clinical pathologies, including Type-2 Diabetes. Several factors, such as increased Reactive Oxygen Species (ROS) levels, increased inflammatory signaling have been proposed to account for insulin resistance in Type 2 Diabetes. However, the major factors that contribute to insulin resistance in A-T patients due to the disruption of the ATM-p53 signaling pathway are still unclear. In this project, the mouse model p53Ser23Ala in which the p53 phosphorylation site at Serine 23 was mutated exhibited insulin resistance. The expression of p53-dependent antioxidant genes (Sestrin 1, 2 and 3) in the fibroblasts of the p53Ser23Ala mice was found to be significantly decreased, indicating the potential for increased oxidative stress. As p53 is the key downstream effector in the disrupted ATM-p53 signaling pathway, these data have implicated that p53 phosphorylation sites at Ser23 plays an important role in insulin resistance by regulating the expression of Sestrin genes.

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Background

Diabetes

Diabetes is a metabolic disease characterized by abnormally high blood glucose levels or hyperglycemia. As insulin is required to transport glucose from the bloodstream into the cells to produce energy, diabetes could result from the absence of insulin production by the pancreas or defects in insulin signaling pathway or both. There are two major types of diabetes: type 1 and type 2. In type 1 diabetes, the β cells of pancreas, which are responsible for insulin production, are mistakenly attacked by the body's immune system. The pancreas, therefore, could not produce insulin, leading to the state of hyperglycemia. In type 2 diabetes, the body does not respond to insulin properly, which is defined as insulin resistance. As a result, the pancreas has to produce extra insulin to make up for this defect at first. However, over time, the pancreas cannot keep up with it, causing insulin insufficiency (American Diabetes Association, 2014). When glucose accumulates in the bloodstream instead of being transported to the cells, the cells are starved for energy. Over time, high blood glucose levels cause damage to different parts of the body including the eyes, kidneys, nerves and heart (American Diabetes Association, 2014). Type 2 diabetes is the most common form of diabetes, being responsible for 90%- 95% of diabetes cases in the United States (Centers for Disease Control and Prevention, 2014). Though it is still not fully understood why some people develop insulin resistance, it has been found that obesity is the primary risk factor for type 2 diabetes. The cells are more likely to become insulin resistant when one has more fatty tissue (Mayo Clinic, 2013). In addition, certain groups, such as African Americans, Latinos, Native Americans, Asian Americans/Pacific Islanders as well as the aged population have higher risk for Type 2 diabetes than other groups (American Diabetes Association, 2013).

Insulin signaling pathway

The primary role of insulin is to increase the uptake of glucose in muscle and fat tissue. Insulin can also induce the uptake of fatty acids into adipose tissue and muscle. Insulin increases glycogen synthesis, which is the storage of glucose as glycogen in liver and muscle. The primary role of insulin in the liver is to suppress hepatic gluconeogenesis, the production of glucose from non-sugar substrates. Figure 1 below presents how the insulin signaling pathway regulates the uptake of glucose.

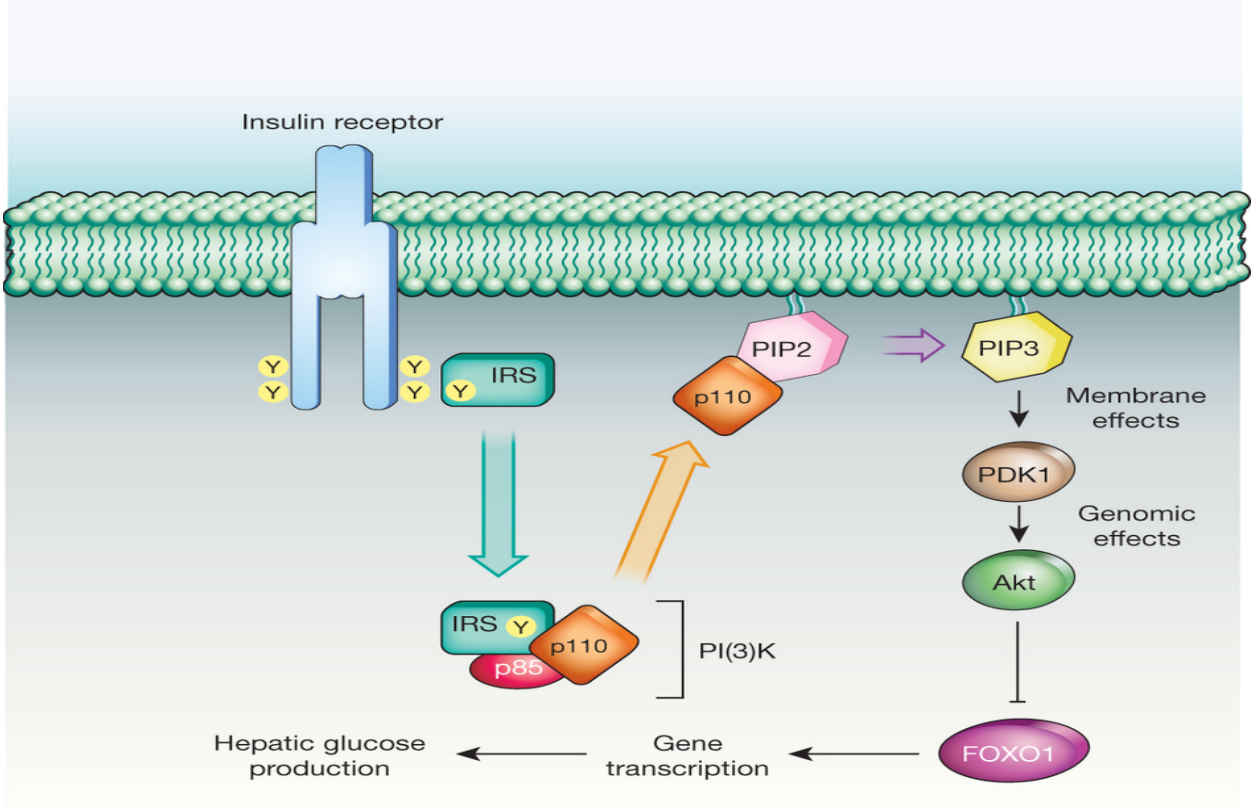

Figure 1: Insulin signal transduction pathway (Schwartz *et al*., 2013)

Regulation of glucose uptake and storage:

The insulin receptor consists of two extracellular α subunits and two transmembrane β subunits linked by disulphide bonds. When insulin binds to the α subunit of the receptor, a number of tyrosine residues in the β subunit undergo autophosphorylation and are then recognized by members of insulin receptor substrate (IRS) family (Figure 1) (Van Obberghen *et al.,* 2001, Saltiel and Kahn, 2001, Lizcano and Alessi, 2002, Hooper, n.d). As seen in Figure 1, the signal is then relayed by the binding of the regulatory subunit of PI3K to phosphorylated key tyrosine residues on IRS proteins and the phosphorylation of phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$) to $PI(3,4,5)P_3$. One key downstream effector of $PI(3,4,5)P_3$ is AKT, which, once activated, induces glycogen synthesis through inhibition of GSK-3 and promotes glucose storage through translocation of glucose transporter GLUT4 to the plasma membrane (Lizcano and Alessi, 2002; Saltiel and Kahn, 2001, Hooper, n.d). In addition to inducing glucose storage, insulin inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis, which also involves AKT (Saltiel and Kahn, 2001, Hooper, n.d).

Regulation of lipid synthesis:

In addition to maintain glucose homeostasis, insulin plays an important role in the uptake of fatty acids and the synthesis of lipid. It has been found that the transcription factor steroid regulatory element-binding protein is upregulated in lipid synthesis (SREBP)-1c (Shimomura *et al.,* 1999; Saltiel and Kahn, 2001, Hooper, n.d). However, the pathway of how insulin regulates SREBP-1c expression is still unknown. While promoting lipid storage, insulin also inhibits lipid metabolism through decreasing cellular concentrations of cAMP by activating a cAMP specific phosphodiesterase in adipocytes (Kitamura *et al.,* 1999, Saltiel and Kahn, 2001, Hooper, n.d).

Regulation of protein synthesis:

Likewise, insulin triggers amino acid uptake into cells, inhibits protein degradation and promotes protein synthesis (Saltiel and Kahn, 2001, Hooper, n.d). Once active, AKT activates mTOR and inactivates GSK-3, leading to the dephosphorylation and activation of eIF2B, both of which promote protein synthesis and the storage of amino acids (Lizcano and Alessi, 2002, Asnaghi *et al.,* 2004, Hooper, n.d).

Underlying Mechanisms

Many defects in the insulin signaling pathway have been proposed as the underlying mechanisms of insulin resistance (Saltiel and Kahn, 2001). As tyrosine phosphorylation of IRS is a key process in insulin signaling, it has been suggested that the decrease in tyrosine phosphorylation of IRS family members in a potential mechanism for insulin resistance (Morino *et al*., 2006; Zick, 2005). The activation of serine/threonine kinases that phosphorylate of IRS proteins at serine residues prevents the binding of IRS proteins with the insulin receptor, consequently decreasing the activation of PI3K (Zick, 2005). Activation of such kinases could be the consequence of increased pro-inflammatory signaling caused by excess circulating free fatty acids (FFAs). IκB kinase (IKK) and c-Jun N-terminal kinase (JNK), which phosphorylate IRS-1 at serine residues, are then activated and cause damage to the insulin signaling pathway. Increased circulating FFAs also trigger the production of cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) (Shi *et al*., 2006; Kim *et al*., 2007; Senn *et al*., 2006). The activation of serine kinases like IKK and JNK could also be attributed to endoplasmic reticulum (ER) stress (Kim *et al*., 2008) and excess reactive oxygen stress (ROS) caused by mitochondrial dysfunction (Nishikawa, 2007).

Ataxia Telangiectasia (A-T) Patients and Insulin Resistance

Ataxia Telangiectasia (A-T) is a rare, recessive genetic disorder caused by mutations in A-T mutated (ATM) gene. A-T is characterized by progressive cerebellar ataxia, telangiectasias, immunodeficiency with susceptibility to sinus and respiratory infections, and a high risk of cancer (Jozwiak *et al*., 2012). Normally, protein kinase ATM phosphorylates several critical substrates for DNA repair and cell cycle control in response to DNA damage. Though less common, about 25% of A-T patients are also diagnosed with insulin resistance, showing defective glucose homeostasis (Miles *et al*., 2007). Thus, this clinical phenotype represents a potential connection between tumor suppressor pathway and glucose homeostasis. Recent work by the Sluss lab has demonstrated in vivo that mice deficient in ATM and an ATM phosphorylation site on p53 develop insulin resistance (Armata *et al*., 2010).

An emerging role of p53 tumor suppressor and ATM/p53 signaling pathway

Encoded by the TP53 gene, the p53 tumor suppressor protein is potent a transcription factor that plays a critical role in the protection against tumor formation. Under normal conditions, p53 tumor suppressor is found at extremely low levels (Goodsell, 1999). However, when DNA damage is sensed, p53 levels rise and initiate protective processes by inducing cell cycle arrest mostly at G1 checkpoint, thus isolating the damaged genome. Also, p53 can trigger apoptosis to further remove the DNA damage (Goodsell, 1999). About 50% of all cases of human cancer can be attributed to mutations in p53 tumor suppressor. Such mutations abrogate the protective role of p53, thus facilitating carcinogenesis (Oren, 1999).

The p53 tumor suppressor, composed of four identical protein chains, can be divided into seven domains: the activation domain 1 or trans-activation domain at the N- terminus, the activation domain 2, the proline-rich domain, the site-specific DNA-binding domain, the nuclear

localization domain, the tetramerization domain, and the C-terminus involving the downregulation of DNA binding of the central domain (Figure 2) (Appella and Anderson, 2001). Many phosphorylation sites in p53 have been identified and it has been suggested that specific phosphorylation events are responsible for specific functions of p53. In A-T disorder, ATM that is normally activated when sensing DNA strand breaks caused by ionizing radiation, is mutated. As the ATM/p53 signaling pathway is disrupted, ATM cannot directly phosphorylate p53 directly at Serine 18 residue in mouse (Serine 15 residue in human) or activate Chk2 to phosphorylate p53 at Serine 23 residue in mouse (Serine 20 residue in human) to induce cell cycle arrest and apoptosis (Figure 2).

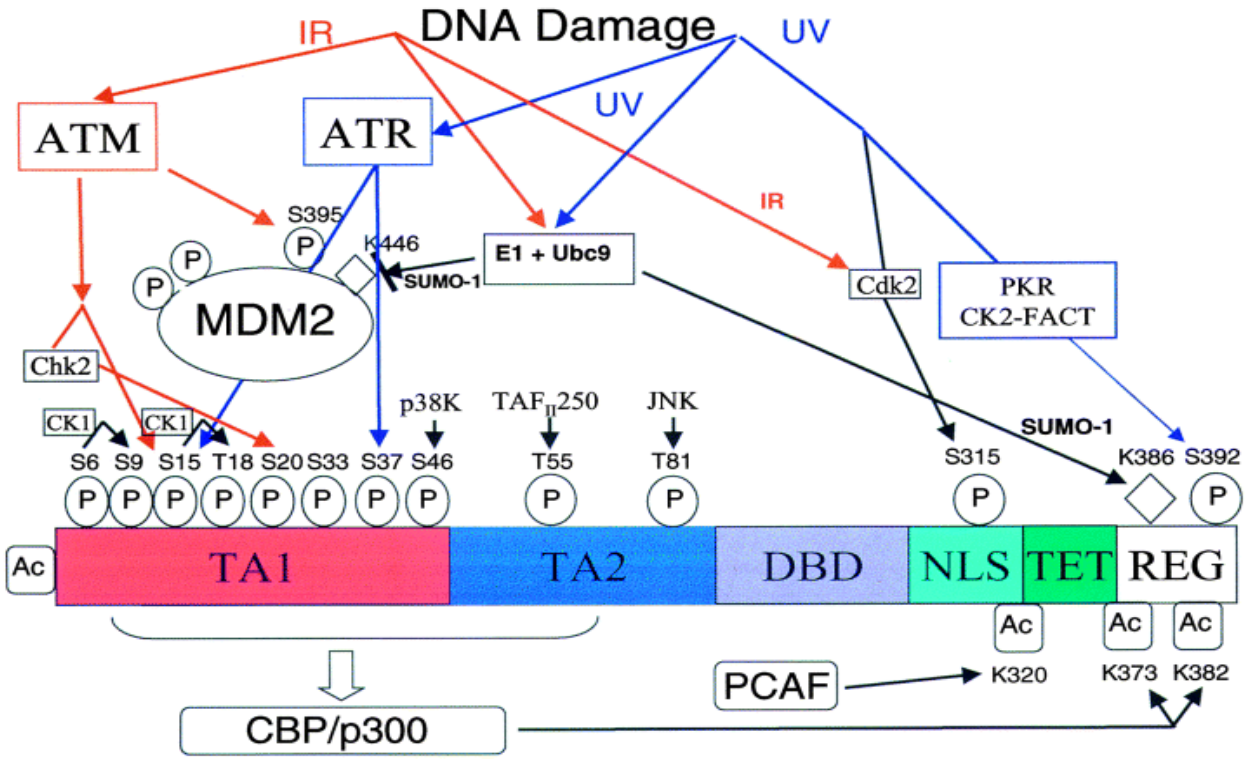

Figure 2: DNA damage-induced post-translational modifications to human p53 (Appella and Anderson, 2001)

In addition to its traditional role as a DNA damage sensor, ATM has been shown to play an important role in metabolic and cardiovascular diseases. Several studies have demonstrated that ATM could inhibit JNK, thus improving insulin sensitivity and cardiovascular effects (Shoelson, 2006). Also, there is a lot of growing evidence showing that p53 is an important regulator of metabolic pathways (Maddocks and Vousden, 2011). For example, as seen in Figure 3, p53 has been found to influence glycolysis by upregulating the expression of glucose transporter 1 and 4 (GLUT1, GLUT4), TP53-induced glycolysis and apoptosis regulator (TIGAR), and downregulating the expression of phosphoglycerate mutase (PGM) (Puzio-Kuter, 2011). Therefore, it is proposed that disrupted ATM-mediated phosphorylation sites of p53 in A-T patients could be related to their development of insulin resistance.

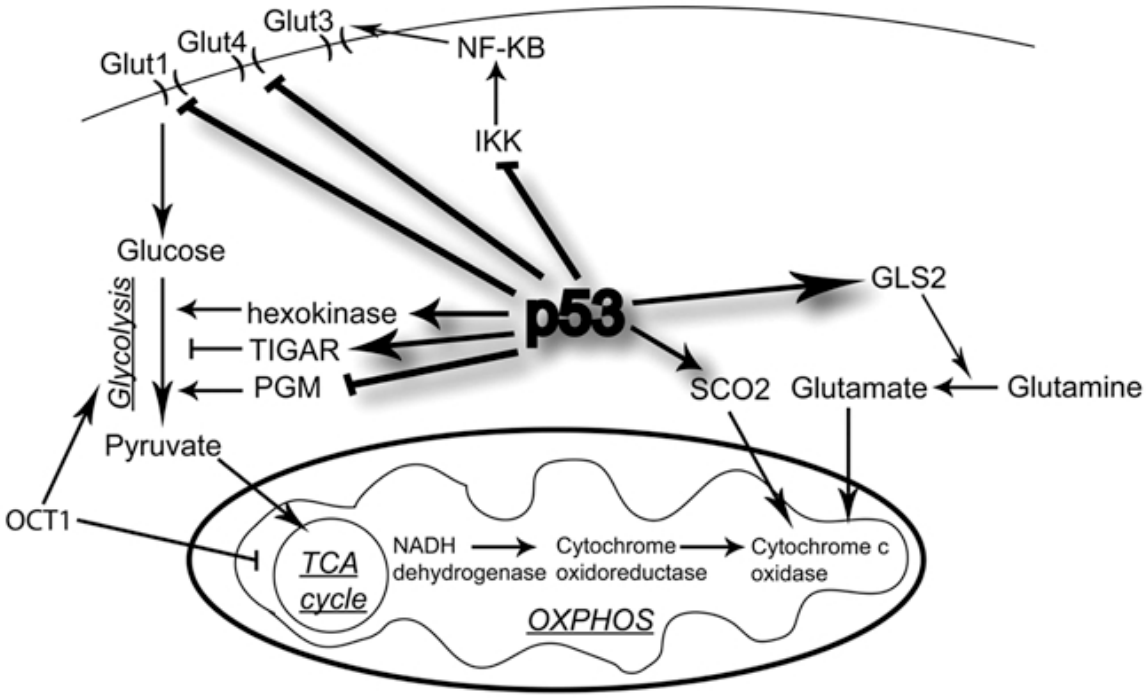

Figure 3: The role of p53 in regulating energy metabolism: glycolysis and oxidative phosphorylation (Puzio-Kuter, 2011)

Sluss Lab Studies on the role of ATM-mediated Phosphorylation Sites in Insulin Resistance

Therefore, it is proposed that disrupted ATM-mediated phosphorylation sites of p53 in A-T patients could be related to their development of insulin resistance.

p53Ser18Ala mouse model:

It has been previously found in Sluss Laboratory that ATM-mediated Serine 18 phosphorylation site is required to maintain glucose homeostasis (Armata *et al.*, 2010). A mouse model in which the p53 Serine 18 phosphorylation site in the trans-activation domain of p53 was replaced with Alanine (p53Ser18Ala) was used for the study. These mice were found to exhibit glucose intolerance and insulin resistance. Increased oxidative stress due to the downregulation of antioxidant gene expression was proposed to be the major contributor to the defective glucose homeostasis in p53Ser18Ala mice, as insulin sensitivity was restored with antioxidant treatment. Thus, it can be concluded that p53 Serine 18 phosphorylation plays a protective role against insulin resistance.

p53Ser23Ala mouse model:

In order to further define the role of p53 tumor suppressor pathway in glucose homeostasis, the Serine 23 phosphorylation site will be studied. Thus, a mouse model in which the Serine 23 residue in the trans-activation domain of p53 was replaced with Alanine was created (MacPherson *et al*., 2004). Mice on a regular chow diet were analyzed. Previous unpublished data from Sluss Lab have demonstrated that through a glucose tolerance test (GTT)

and Insulin tolerance test that these mice also develop insulin as presented in Figure 4 and 5 below

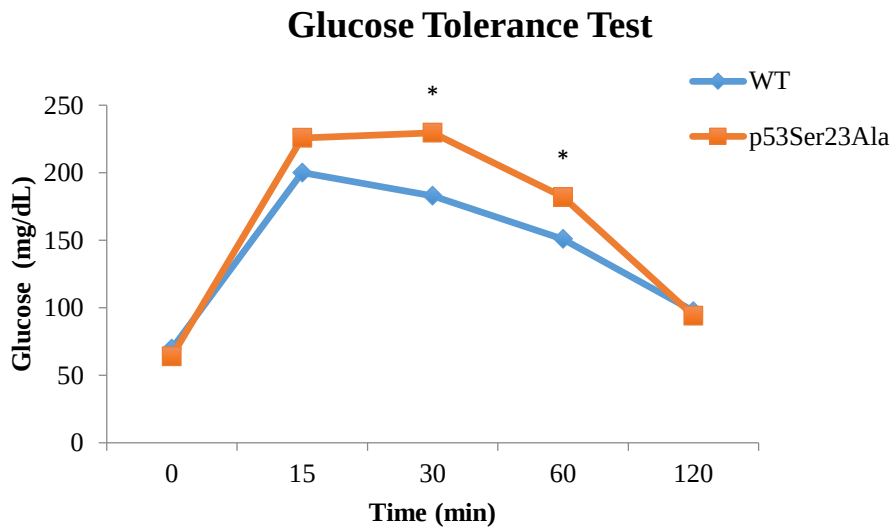

Figure 4: Glucose tolerance test (GTT). Mice fasted overnight were treated with glucose (1g/kg) by intraperitoneal injection. Blood glucose concentration was measured at the indicated times.

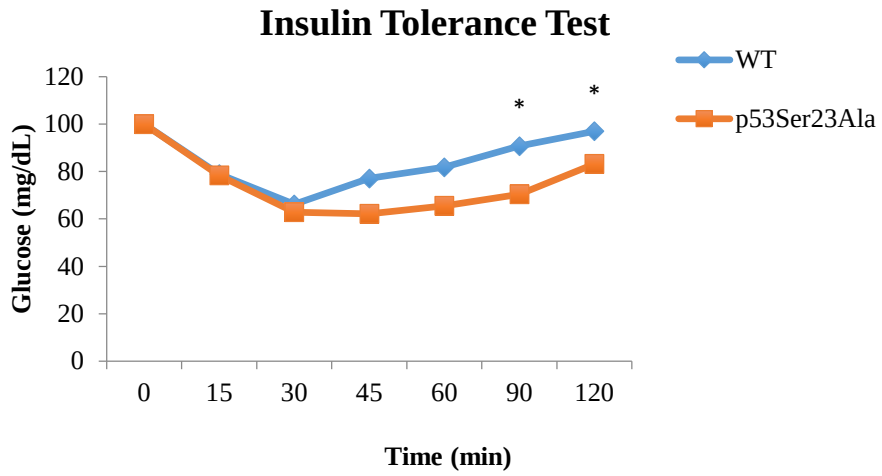

Figure 5: Insulin Tolerance Test (ITT). Mice fasted overnight were treated with insulin (0.75U/kg) by intraperitoneal injection. Blood glucose concentration was measured at the indicated times.

In this project, several factors including the expression of antioxidant genes and inflammatory genes will be examined to determine the significant factor contributing to insulin resistance in p53Ser23Ala mice. Also, AKT, one key downstream effector in the insulin signaling pathway in fibroblasts, liver and muscle tissues will be examined as evidence for disruption of this pathway. It is hypothesized that the disruption of ATM pathway would lead to the downregulation of antioxidant genes and upregulation of pro-inflammatory genes in mutant p53Ser23Ala mice, as excessive oxidative stress and increase in activation of inflammation signaling are proposed mechanisms for insulin resistance in Type-2 Diabetes.

Materials and Methods

Tissue Culture

Primary murine embryo fibroblasts (MEFs) were prepared from wild-type and p53S23A embryos. The MEFs were cultured in Dulbecco's modified Eagle's medium (Invitrogen, #11965-118) supplemented with 10% Fetal Bovine Serum (Thermo Scientific, SH30396.03), Antibiotic (Invitrogen, #15140-122) and Glutamax (Invitrogen, #35050-061). MEFs were at passage 2 for insulin response experiment. For insulin response analysis, cells were starved 15 to 18 h in Dulbecco's glucose free media (Invitrogen, #11966). Cells were then treated with insulin (Sigma, #I0516, Insulin Bovine Pancreas, 10 mg/mL) and harvested at the indicated time points including 0, 30 minute, 1 hour and 4 hour. The cell pellets were stored at -80^{o}C for Immunoblot Analysis.

Quantitative Real time PCR

RNA Purification

Total RNA was prepared with RNeasy Mini kits (Qiagen, #74104) by following the manufacturer's instructions. The first and second elution RNA concentrations were measured using Thermo Nanodrop 2000 Spectrophotometer. Purified RNA samples were frozen on dry ice and stored at -80^{o}C.

RNA Clean up

The purified RNA was subjected to an additional DNase treatment to remove contaminating genomic DNA prior to final column purification by RNeasy Mini kit. 20 µg of the purified RNA and nuclease-free water, in a total volume of 80 µl were added to 20 µl of a mix consisting of 2 µl DNAse, 10 µl 10X DNase I Buffer and 8 µl of nuclease-free water from the

DNA-free kit (Ambion, #AM1906). The samples were incubated for 20 minutes at 37ºC, and then an additional 1 µl of DNase from the kit was added to each sample. The samples were incubated for another 20 minutes at 37ºC, and then 10 µl of the DNase Inactivation Reagent from the kit was added to each sample and mixed for 2 minutes at room temperature. The samples were spun at max speed 13000 rpm for 2 minutes, and the supernatant was transferred to a new tube. The instructions of RNeasy Mini kit were followed again. Cleaned RNA samples were measured again using Thermo Nanodrop 2000 Spectrophotometer and stored at -80ºC.

cDNA synthesis

cDNA was prepared using iScript Reverse Transcription Supermix (Biorad, #170-8841) with 0.5 µg to 1 µg of RNA per tissue, following the manufacturer's instructions for First Strand Synthesis. cDNA samples were stored at -20ºC.

Quantitative Real-Time PCR

Quantitative Real-Time PCR was performed on a Bio-Rad iCycler using Sybr green master mix (Bio-Rad). The primer sequences for the murine genes were as follows: *Gapdh* (5-CTTCACCACCATGGAGAAGGC-3, 5-GGCATGGACTGTGGTCAT-3), *Sesn1* (5-GGCCAGGACGAGGAACTTG-3, 5- AAGGAGTCTGCAAATAACGCAT-3), *Sesn2* (5-TCCGAGTGCCATTCCGAGAT-3, 5- TCCGGGTGTAGACCCATCAC-3), *Sesn3* (5-GCGAGGAGAAGAACATTTGCC-3, 5- CCAAACATACAGTGAACATAGT-3), *mTNF* (5-ACAGAAAGCATGATCCGCG-3, 5-CTGGGCCATAGAACTGATG-3), *mIL6* (5-AAGAGACTTCCATCCAGTTGC-3, 5-CTCCGACTTGTGAAGTGGTAT-3), *mIL1*(5-AAAGCTCTCCACCTCAATGG-3, 5-TCTTCTTTGGGTATTGCTTGG-3), mGhrl (5-GCTGTCTTCAGGCACCATCT-3, 5- GTGGCTTCTTGGATTCCTTTC-3). All samples were

examined in triplicate, and values were normalized for baseline expression and for expression of Gapdh. Calculations of values were made using the ΔΔCT method. Statistical significance was calculated using threshold cycle (CT) values.

Immunoblots

Preparation of Protein Lysates:

Animal tissues and MEF pellets were lysed in 1X TLB Lysis Buffer (2X Triton Buffer, 50% Glycerol, Water, 1M β-glycerophosphate, 100 mM Sodium Orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 5 mg /mL Aprotinin and 10 mg/mL Leupeptin). Protein samples were snap-frozen into liquid nitrogen and stored at -80^{o}C.

BCA Protein Assay

Protein samples and standards were prepared using BCA Protein Assay Kit (Pierce, #23225) following the manufacturer‘s instructions. In each well of a 96-well plate, 2 µl of the protein lysate sample was added to 23 µl of sterile water, then 200 µl of the working reagent was added to each well and then mixed. The plate was incubated at 37^{o}C for 30 minutes and the absorbance was then read at 550 nm on a Thermo Multitask Ascent Plate Reader. The measured concentrations were then used to prepare loading samples for Western Blots. Each sample had 50 µg-100 µg of protein in a total volume of 50 µl; 10 µl of Laemmli’s SDS-sample buffer (6X, reducing) was added to the appropriate amount of protein and mixed well. 1X TLB Lysis Buffer was added to the sample to bring the final volume to 50 µl.

Immunoblot Analysis

Protein lysate extracts from tissues and MEFs (50-100 µg) were analyzed by Immunoblot analysis using primary antibodies to RNa Protein extracts were run on 10% acrylamide gel with 1X Running Buffer (10X Running Buffer, 10% SDS, and Water) and then transferred into

membrane using Transfer Buffer (10X Running Buffer, Methanol and Water). The membrane was blocked in 1X TBS-T 10% skim milk (10 mM Tris pH 8.0, 120 mM NaCl, 0.05% Tween 20) for at least 1 hour. Then primary antibody (8 µl of the primary antibody in 8 mL of the 1X TBS-T 10% milk) was added to each membrane. The membrane was incubated overnight at 4^{o}C. The membrane was washed three times with 10 minutes each time in 1X TBS-T and then incubated with secondary antibodies (1:20000 anti-mouse or anti-rabbit in 10% skim milk in TBS-T block solution) for one hour. The membrane was washed again three times with 10 minutes each time in 1X TBS-T and then developed with chemilumenescene techniques.

Results

ATM protein kinase is mutated in A-T patients, thus could not phosphorylate p53 at Serine 23 in mice in response to DNA damage by UV. Also, a number of A-T patients develop insulin resistance, suggesting a connection between the ATM signaling pathway and the insulin signaling pathway. The p53Ser23Ala mouse model with a mutation at ATM target phosphorylation site Serine 23 on the p53 protein in this project. The p53Ser23Ala mice were found to exhibit insulin resistance through GTT and ITT results, stressing the linkage between these two pathways. As excessive oxidative stress and increased pro-inflammatory signaling are proposed underlying mechanisms for insulin resistance, the role of these two factors will be examined using Real-Time PCR to analyze the relative expression of antioxidant genes and pro-inflammatory genes in the fibroblasts and livers of wild-type and p53Ser23Ala. Additionally, the activation of the downstream effector AKT in the insulin signaling pathway in fibroblasts, liver and muscle tissue of wild-type and p53Ser23Ala mice will be investigated using Immunoblots to obtain evidence for insulin resistance in this mouse model.

Expression of antioxidant genes and pro-inflammatory genes in wild-type and p53Ser23Ala mice

In order to investigate whether antioxidant gene expression contributes to insulin resistance in p53Ser23Ala mice, the expression of such genes including *Sestrin 1*, *2* and *3*, which are upregulated by the p53 protein upon DNA damage and oxidative stress (Budanov and Karin, 2008), was examined by Real-Time PCR. As seen in Figure 6A, the levels of Sestrin gene expression in the fibroblasts of p53Ser23Ala mice were significantly reduced compared to those in wild-type mice, indicating a decrease in the antioxidant defense of the mutant mice. Additionally, inflammation has been proposed to play an important role in insulin resistance in

Type 2 Diabetes. Therefore, the relative expression of inflammatory cytokines, such as TNF-α, IL-6, IL-1 in MEFs and livers of wild type and p53Ser23Ala mice was also examined using Real-Time PCR. As shown in Figure 6B the relative expression of IL-6 was significantly greater in p53Ser12Ala MEFs. Increased IL-6 expression indicates increased inflammatory signaling. Figure 7 showed significantly increased levels of IL-1 and increased expression of TNF-α and IL-6 in the livers of p53Ser23Ala mice. In addition, the gene expression of Ghrelin, a hormone that plays an important role in growth hormone secretion, appetite regulation and metabolism, was observed to be downregulated (Figure 6C), which has been reported to be associated with insulin resistance by several studies (Pöykkö *et al*., 2003, Dimaraki and Jaffe, 2006).

Insulin Signaling Pathway

From the GTT and ITT results, it has been found that p53Ser23Ala mice exhibited insulin resistance, suggesting defects in the insulin signaling pathway. Thus, in order to examine the insulin signaling pathway in these mutant mice and compare to that in the wild-type mice; the activation of AKT, one key downstream effector of the pathway was investigated in MEFs, liver and muscle tissue (Figure 8 and 9). Fibroblasts from wild-type mice and p53Ser23Ala mice were harvested at indicated time points after insulin treatment. Proteins were then extracted from the lysed fibroblasts. Similarly, extracts were obtained from the lysed liver and muscle tissues from wild type and p53Ser23Ala mice treated without and with insulin, and then examined by Immunoblot Analysis using anti-AKT, anti-pSer473 AKT, and anti-pThr308 AKT antibodies.

In the first and second panel of Figure 8A, the band intensity of wild type MEFs 0.5 hour after the insulin treatment increased, indicating that AKT was phosphorylated at Ser473 and Thr308 correspondingly. After 1 hour, the band intensities of wild type MEFs were stronger, showing that more AKT was activated. After 4 hours, the bands of wild type MEFs faded as

insulin signaling was terminated. The band intensities of p53Ser23Ala MEFs also increased 0.5 hour after the treatment. However, these bands were fainter than those of the wild-type MEFs, indicating that not as much AKT was activated. Also, the bands of p53Ser23Ala MEFs already faded at 1 hour after the treatment. The band intensities of p53Ser23Ala MEFs at 1 hour were shown to reduce compared to those of wild-type MEFs. The third panel demonstrates equal loading of AKT for both wild type and p53Ser23Ala fibroblasts. The quantification of Immunoblots for anti-pThr308 AKT as presented in Figure 8B demonstrated that the difference in the activation of AKT between wild-type and p53Ser23Ala MEFs 1 hour after the treatment was significantly greater compared to that of the control.

In the upper panels of Figure 9A, in the absence of insulin, AKT was not activated for both wild type and p53Ser23Ala liver tissues. However, in response to insulin treatment, the signals for wild type tissues were stronger than those of p53Ser23Ala tissues, which also indicates insulin resistance in the liver tissue. Equal loading of AKT was shown in the lower panels.

In contrast, there was no difference in AKT phosphorylation between the wild type and p53Ser23Ala muscle as shown in the upper and middle panels of Figure 9B. AKT was activated in the muscle of both genotypes in response to insulin. Likewise, equal loading of AKT was presented in the lower panels.

Together, these results indicate the insulin signaling pathway in p53Ser23Ala MEFs and liver was impaired, but the muscle tissue of the mutant mice can still maintain the normal insulin sensitivity.

Discussion

Type 2 Diabetes is one of the clinical pathologies exhibited in some A-T patients. Several factors including increased ROS levels and increased inflammatory signaling have been believed to contribute to insulin resistance in Type 2 Diabetes. However, the major contributors to insulin resistance in A-T patients caused by the disruption of their ATM-p53 signaling pathway are still not fully understood. In this project, the p53Ser23Ala mouse model in which ATM-p53 signaling pathway was interrupted at the p53 phosphorylation site Serine 23 was utilized to investigate the insulin signaling pathway.

Through Immunoblot results, the effect due to the interruption of the ATM-p53 on the insulin signaling pathway was demonstrated. It was found that the insulin signaling pathway was impaired in the fibroblasts and liver tissue of the p53Ser23Ala mice, however still remained active in their muscle tissue. Typically, Type 2 diabetes is characterized by insulin resistance in the certain types of tissue, including liver, adipose, and muscle tissue. Since the defect in the insulin transduction pathway was only observed in fibroblasts and liver tissue, it could be concluded that the mutation at the phosphorylation site Serine 23 just leads to tissue-specific insulin resistance.

As the results the Immunoblots implicated the evidence and characterization of insulin resistance in p53Ser23Ala mice, the factors leading to insulin resistance in the fibroblasts and liver tissue were then investigated using quantitative Real-Time PCR. The gene expression of pro-inflammatory cytokines, though was found to be elevated, but only significantly in the fibroblasts. Additionally, the expression of p53-dependent antioxidant genes (*Sesn 1*, *2*, and *3*) in the fibroblasts of p53Ser23Ala mice, one factor that is balanced against the ROS levels, has been

found to be significantly downregulated, indicating the potential for increased ROS levels in these mice. Increased ROS levels, which has been believed to primarily account for A-T patients' clinical pathologies (Barlow *et al*., 1999, Watter, 2003), have been also attributed as one of underlying mechanisms for insulin resistance in Type 2 diabetes. Furthermore, as p53 protein is the downstream effector in the ATM-p53 pathway, these results indicate that the p53-regulated Sestrin antioxidant gene expression is the main contributor to insulin resistance in p53Ser23Ala mice.

Previous study from Sluss Lab also found that loss of antioxidant gene expression in the p53Ser18Ala mouse model associates with insulin resistance. As it is the same factor that was found in both p53Ser18Ala and p53Ser23Ala mouse models, it is believed that antioxidant therapy could be the potential treatment to restore the insulin sensitivity in A-T patients. Further experiments should include Immunoblots on p53Ser23 mice on antioxidant diet to check whether their insulin signaling pathway is restored or not to confirm the conclusion. The expression of antioxidant genes on the liver tissue should also be examined. Other factors that might lead to excess ROS levels or have been proposed as underlying mechanism for insulin resistance in Type 2 Diabetes should also be monitored.

In conclusion, these findings implicate that the both of ATM-mediated phosphorylation sites on p53 at Serine18 and Serine 23 play an important role in insulin resistance by regulating the expression of antioxidant genes. They also highlight the emerging role of p53 in metabolic diseases, not just their traditional role as the tumor suppressor protein.

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Figures

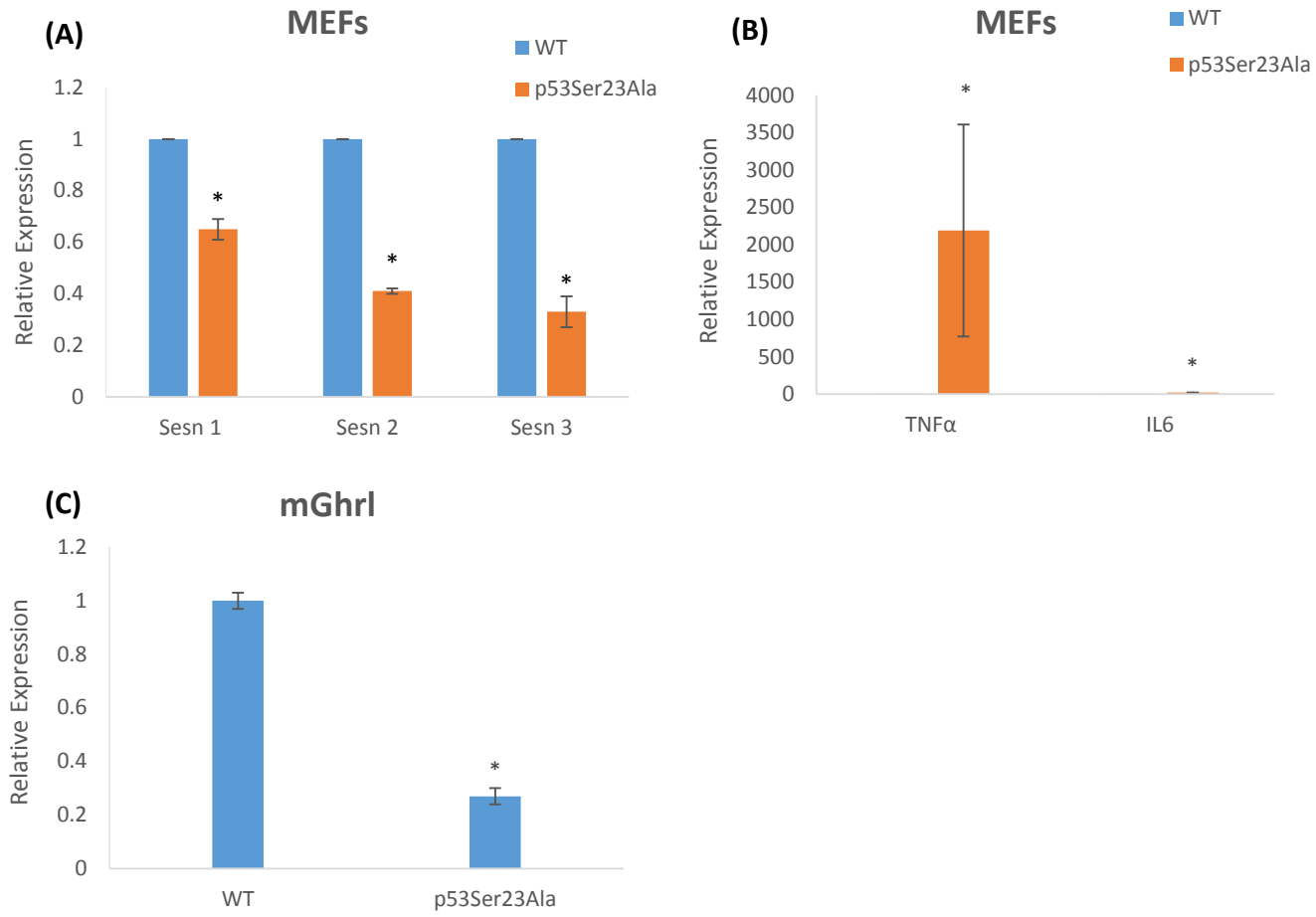


Figure 6: **The relative expression in WT and p53Ser23Ala MEFs of *Sesn1, 2,* and *3* (A); *TNF-α* and *IL-6* (B); *mGhrl* (C)**. Relative expression between WT vs. p53Ser23Ala mice was measured by quantitative Real-Time PCR analysis. The amount of *Gapdh* mRNA in each sample was used to calculate relative mRNA expression (means ± SEM, n=3). Statistically significant differences between WT and p53Ser23Ala mice are indicated (*, $p < 0.05$). **(A)** The levels of Sestrin antioxidant gene expression in p53Ser23Ala mice were significantly reduced compared to those of WT mice **(B)** The levels of inflammatory gene expression, including TNF-α and IL-6 were significantly increased in the MEFs of p53Ser23Ala mice compared to those of WT mice **(C)** The gene expression of Ghrl, a growth-hormone stimulator, was significantly downregulated in the MEFs of p53Ser23 mice compared to that of WT mice.

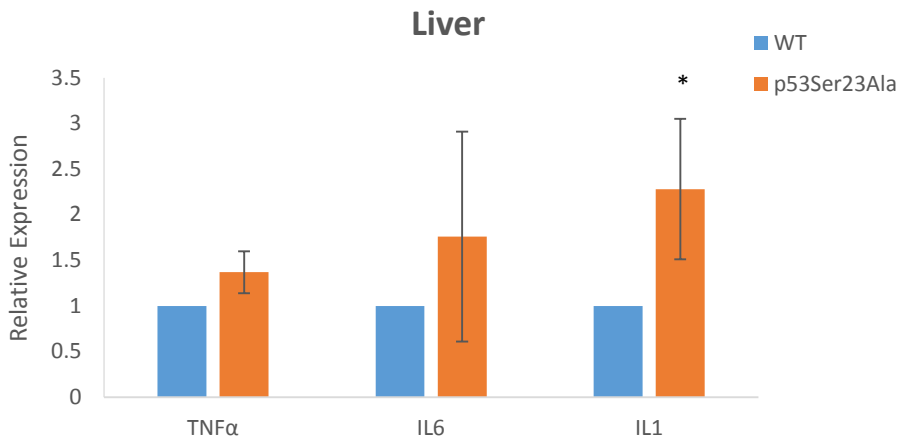
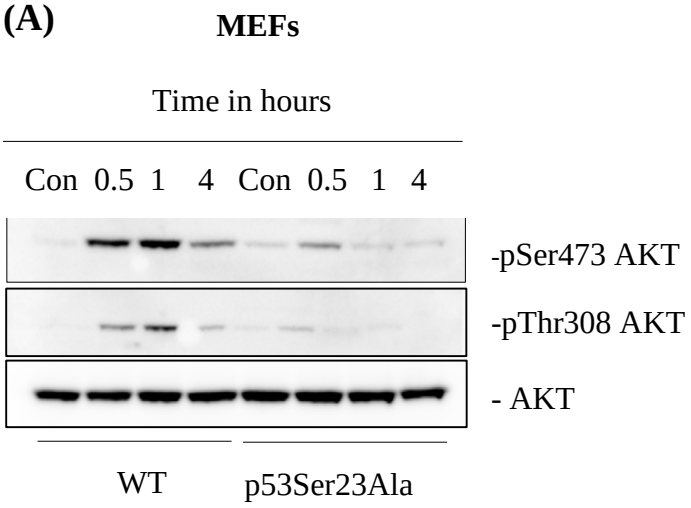


Figure 7: **The relative expression in WT and p53Ser23Ala livers of *TNF-α, IL-6, and IL-1*** Relative expression between WT vs. p53Ser23Ala mice was measured by quantitative Real-Time PCR analysis. The amount of *Gapdh* mRNA in each sample was used to calculate relative mRNA expression (means ± SEM, n=9). Statistically significant differences between WT and p53Ser23Ala mice are indicated (*, $p < 0.05$). The levels of inflammatory gene expression, including TNF-α and IL-6 were significantly increased in the p53Ser23Ala mice compared to WT mice, indicating increased inflammatory signaling in p53Ser23Ala liver.

(A)



(B)

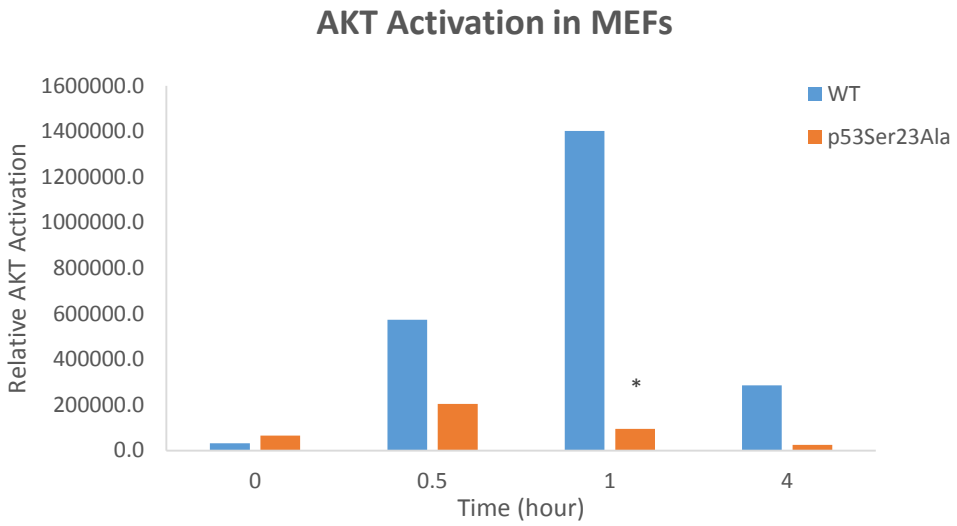


Figure 8: **(A) Immunoblots for MEFs Time Course Insulin Treatment examining investigating AKT phosphorylation in WT and p53Ser23Ala mice using anti-pSer473 AKT, anti-pThr308 AKT, anti AKT**. The first and second panels showed the defect in the insulin signaling pathway. The bands of p53Ser23Ala mice at 0.5 hour and 1 hour time points were fainter compared to WT mice. The third panel presented equal loading. **(B) Quantification of AKT Activation from Immunoblots using anti-pThr308 AKT**. Not as much AKT was activated in p53Ser23Ala mice compared to WT mice. The difference in the activation of AKT between WT and p53Ser23Ala MEFs at 1 hour time point the treatment was significantly greater compared to the difference in the activation of AKT between WT and p53Ser23Ala mice at 0 hour time point (*, $p<0.05$)

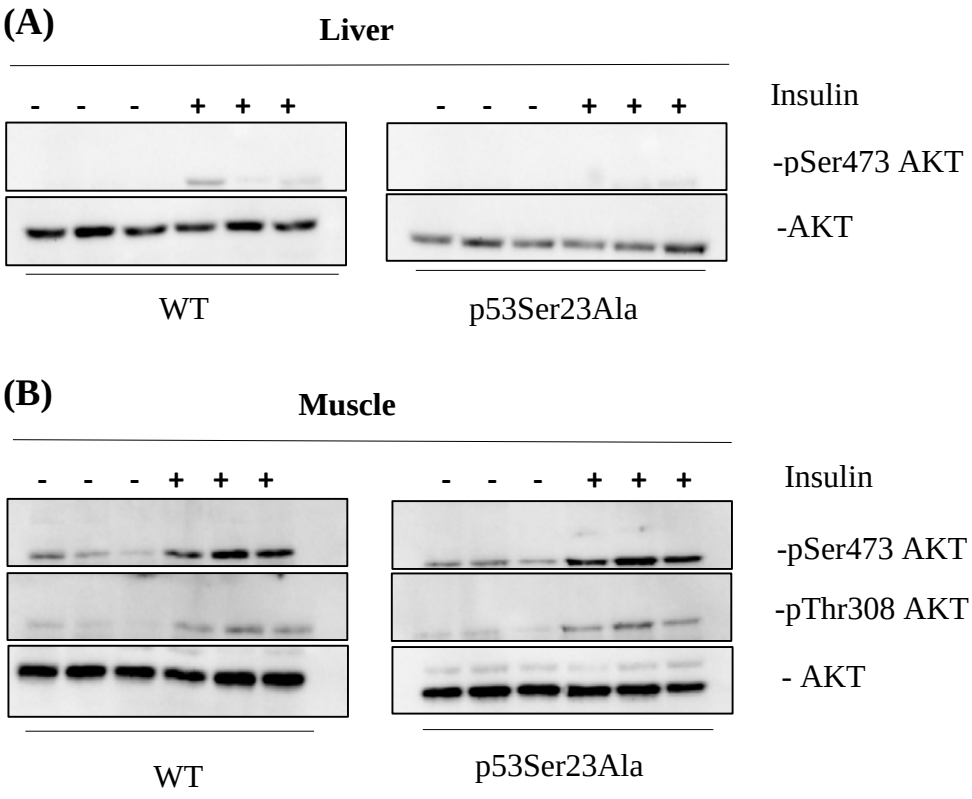


Figure 9: **Immunoblots for Liver (A) and Muscle (B) investigating AKT phosphorylation in WT and p53Ser23Ala mice.** Extracts from livers and muscle of wild-type and p53Ser23Ala mice were examined by Immunoblot analysis using anti-pSer473 AKT, anti-pThr308 AKT, and anti-AKT antibodies. **(A)** In the upper panels, in the absence of insulin, AKT was not activated for both wild type and p53Ser23Ala liver tissues. However, in response to insulin treatment, the signals for wild type tissues were stronger than those of p53Ser23Ala tissues, indicating insulin resistance in the liver tissue. Equal loading of AKT was shown in the lower panels. **(B)** The upper and middle panels demonstrated that there was no difference in AKT phosphorylation between the wild type and p53Ser23Ala muscle tissue. AKT was activated in the muscle of both genotypes in response to insulin. Equal loading of AKT was presented in the lower panels.