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Insights into STAT5 Biology

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By

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Introduction

I: Adipose Tissue Background

As the prevalence of both obesity and type 2 diabetes (T2DM) continue to escalate, it is becoming increasingly crucial to study the mechanisms that control adipocyte differentiation as they can be associated with the pathways that lead to metabolic diseases (Stephens 2012). Today, metabolic syndrome is one of the leading causes of death in the world (Escande et al. 2015). Metabolic syndrome is a collection of conditions including high blood pressure, high blood sugar, excess abdominal tissue, and high cholesterol. Individuals with excess abdominal adipose tissue are predisposed to developing chronic diseases including not only T2DM, but also cardiovascular disease and hypertension (Frayn 2001). Adipose tissue is a biologically active organ that is primarily composed of adipocytes, fat cells, but also contains other cell types as well (Cristancho and Lazar 2011, Sarjeant and Stephens 2012). Adipogenesis is the differentiation of a preadipocyte into a mature adipocyte. Adipocytes have three main functions which include insulin sensitivity, lipid storage in the form of triacylglycerol, and endocrine functions (Ronti et al. 2006). The accumulation of adipose tissue alone is not necessarily detrimental, as it was once considered. Metabolic disease results when adipocytes falter on at least one of their three main functions (Pellegrinelli et al. 2016). As long as adipocytes are allowed to differentiate properly and maintain their three main functions, adipose tissue expansion leads to a "healthy obese" phenotype (Stefan et al. 2013). When adipocytes do not properly store lipids, lipids must accumulate in other tissues. This lipid accumulation leads to ectopic lipid deposits in the liver and skeletal muscles which have been linked to adipose tissue dysfunction leading to insulin resistance (VanHerpen and Schrauwen-Hinderling 2008). The exact mechanism of this insulin resistance is not currently understood.

Adipocytes regulate appetite, insulin sensitivity, inflammation, and energy homeostasis by synthesizing and secreting many cytokines and hormones. Many of these secretions are capable of activating the JAK-STAT pathway. Adipocytes exist as both white adipocytes and brown adipocytes, each possessing distinct functions. White adipocytes perform the three main functions of adipocytes: they store lipid, uptake glucose when stimulated by insulin, and secrete hormones (Richard and Stephens 2014). Insulin stimulation of adipocytes causes a signaling cascade that is responsible for localizing glucose transporter-4 (GLUT4) to the plasma membrane. Once GLUT4 is present in the membrane, it can transport glucose into the adipocyte, therefore preventing hyperglycemia (Bruno et al. 2016). Brown adipocytes on the other hand play a more prominent role in energy expenditure. White adipocytes are responsible for a larger uptake of glucose compared to brown adipocytes (Chechi et al. 2017). Adipocytes are not simply derived from other adipocytes. Instead, they arise from differentiation of certain precursor cells (Stephens 2012). White adipocytes and brown adipocytes arise from different mesenchymal precursor cells (Cristancho and Lazar 2011, Sarjeant and Stephens 2012).

As stated earlier, adipose tissue is not solely comprised of adipocytes. In addition to adipocytes, adipose tissue is also comprised of endothelial cells, connective tissue, blood cells, fibroblasts, pericytes, pre-adipocytes, macrophages, and other immune cells (Richard and Stephens 2011, Sarjeant and Stephens 2012). The specific function of these immune cells in relation to adipocytes is not currently fully understood (Richard and Stephens 2014).

II: JAK-STAT Background

To date, there are 4 Janus Kinases (JAKs) and there are seven Signal Transducers and activators of Transcription (STATs) (Darnell 1997, Schindler et al 2007). The four JAKs are Jak1, Jak2, Jak3, and Tyk2 and the seven STATs are STAT1, STAT 2, STAT3, STAT4,

STAT5a, STAT5b, and STAT6 (Schindler et al. 2007). The JAK-STAT intracellular signal transduction pathway occurs in all cell types, but the distribution and function of each STAT protein is different between tissue types (Heim MH 1996, Richard and Stephens 2014). This specificity allows for the regulation of tissue specific genes (Richard and Stephens 2011).

JAKs associate with a plasma membrane bound receptor at the proline-rich, membrane-proximal box1/box1 domain found on the receptor. When STATs are inactive, they are largely present in the cytoplasm as homodimers. When a ligand binds this membrane bound receptor, the JAKs present on the receptor become activated by trans-phosphorylation (Schindler et al. 2007). The activated JAKs phosphorylate the receptor on tyrosine residues. The phosphorylation of specific tyrosine residues leads to the recruitment of specific STAT proteins. STATs bind the activated membrane bound receptor with their SH2, Src homology 2, domains and are phosphorylated by the JAKs on specific tyrosine residues near their C terminus (Darnell 1997). Once activated by JAKs, the STATs can form either anti-parallel homodimers or anti-parallel heterodimers which then translocate to the nucleus (Rawlings et al. 2004). Once present in the nucleus, these STAT dimers can bind to STAT consensus sequences and regulate the transcription of their specific target genes (Richard and Stephens 2014). Activated STAT proteins can rapidly accumulate in the nucleus but their regulatory effects are short lived and they are quickly re-exported back into the cytosol (Schindler et al. 2007).

The expression of several STATs increases during adipocyte differentiation (Harp et al. 2001). This suggests that STATs play a role in adipogenesis (Stephens et al. 1996). STATs have been shown to facilitate adipocyte maturation (Harris 2014). Many studies support that STAT5 is the specific STAT that is responsible for regulating adipocyte differentiation in both mice and humans (Richard and Stephens 2014). Abnormal adipose tissue resulted from either

STAT5A or STAT5B knockout (Teglund et al. 1998). As of now, there are no studies proving that JAKs have a significant effect on adipogenesis that is independent of STAT protein function (Richard and Stephens 2014).

III: STAT5 Background

STAT5A and STAT5B in vertebrates share a high degree of homology with STAT5A and STAT5B in invertebrates. STAT5A and STAT5B have a substantial degree of functional redundancy and have both previously been shown to play a role in both erythropoiesis, the production of red blood cells, and lymphopoiesis, the generation of lymphocytes (Schindler et al 2007). STAT5 proteins are capable of regulating adipogenesis *in vivo* (Stewart et al. 2011). During adipocyte differentiation in both mice and humans, the levels of both STAT5A and STAT5B are increased (Richard and Stephens 2014). To date, growth hormone and prolactin are the only known activators of STAT5A and STAT5B under physiological conditions in adipocytes (Smit et al. 1996, Zhoa and Stephens 2013). STAT5 proteins are activated by phosphorylation shortly after the onset of adipogenesis. STAT5A plays a more prominent role in adipogenesis than STAT5B does (Richard and Stephens 2014). Studies have shown that cells with constitutively active STAT5s no longer require growth hormone for adipogenesis (Shang and Waters 2003).

STAT5 interacting partners have become an intensely studied topic. STAT5 is capable of acting as either an activator or a repressor depending on the gene of interest and the conditions of the cell (Richard and Stephens 2014). In preadipocytes, STAT5 plays an adipogenic role, while in mature adipocytes, it plays an anti-lipogenic role (Richard and Stephens 2011). STAT5 proteins regulate the expression of genes that are involved in adipocyte development, insulin sensitivity, and fat and carbohydrate metabolism (Kaltenecker et al. 2016).

While many of STAT5's target genes function during adipocyte differentiation, STAT5's association with the PPARy promoter is absolutely crucial for adipogenesis (Stewart et al. 1999). Research shows that STAT5 binds the promoter of acyl CoA oxidase (AOX), which is the rate limiting enzyme for fatty acid ß-oxidation (Coulter and Stephens 2006, Richard and Stephens 2014). STAT5 also regulates the expression of aP2, which is a lipid binding protein in fat cells (Richter 2003). Fatty acid synthase (FAS) is responsible for synthesizing long chain fatty acids and its expression is also regulated by STAT5 binding to its promoter (Hogan and Stephens 2005). STAT5A represses the expression of both FAS and aP2 which leads to an increase in lipid release and a decrease in lipid storage (Richard and Stephens 2011). STAT5 is also capable of binding to and activating the promoter of pyruvate dehydrogenase kinase 4 (PDK4), which is responsible for regulating glycolysis. When PDK4 is induced at high levels, the adipocyte becomes insulin resistant (White et al. 2007). STAT5 proteins also regulate the expression of adiponectin (White et al. 2016), which is a hormone that is responsible for modulating glucose and lipid metabolism as well as playing a cardioprotective role in both mice and humans. In mice that are fed a high fat diet, overexpression of adiponectin resulted in obese mice that remained insulin sensitive (Escande et al. 2015). These examples are by no means an exhaustive list of genes that are regulated by STAT5, but they encompass the fact that STAT5 regulates genes that are responsible for the three main functions of adipocytes. The knockout of both STAT5A and STAT5B results in the accumulation of fat in the liver leading to fatty livers and liver steatosis (Barclay et al. 2011).

IV: DBC1 Background

DBC1, deleted in breast cancer 1, is a nuclear protein that was originally identified in 1967 when it was localized to a region of chromosome 8p21 during breast cancer research (Kim

and Sung 2009). This specific region is homozygously deleted in certain forms of breast cancer (Ha et al. 2016). DBC1 is also known as CCAR2, p30 DBC, and KIAA1967 (Mareno-Navarrete 2015). CCAR2 stands for cell cycle and apoptosis regulator 2 which has recently become this proteins most prominent name. Despite being named deleted in breast cancer 1, some studies have actually shown an increase of DBC1 mRNA levels in certain breast cancers (Kim and Sung 2009). DBC1 is expressed in breast cancer tissue (Kim and Kim 2013), T-cells, kidney cells (Giguere et al. 2016), gallbladder tissue (Won et al. 2015), hepatocellular carcinoma tissue (Ha et al. 2016), adipose tissue (Escande et al. 2015), as well as many other types of tissue. Although DBC1 is implicated in many different types of cancer, it does not play a simple overall encompassing role in cancer as a whole (Kim and Sung 2015).

DBC1 plays a role in the regulation of cell survival and death. When a cell is damaged, DBC1 promotes apoptosis by inhibiting the Sirtuin 1 (Sirt1) deacetylase activity. DBC1 plays a role in the stability of the genome when exposed to UV-induced stress (Kim and Kim 2013). DBC1 is also known to be both an inhibitor of histone deacetylase 3, HDAC3, and a nuclear hormone receptor transcriptional cofactor. By monitoring DBC1 mRNA levels in T cells and kidney cells, it was discovered that DBC1 plays a conserved regulatory role in both of these types of cells. DBC1 was shown to regulate gene expression and chromatin organization by its interaction with HDAC3 and Sirt1, as well as regulating the cell cycle progression leading to apoptosis. Cells that had deficient levels of DBC1 also showed lower levels of JNK activation (Giguere et al. 2016). JNKs, Jun N-terminal kinases, play an important role in apoptotic pathways. JNKs up-regulate pro-apoptotic genes and therefore, lower levels of JNK will lead to lower levels of apoptosis which can lead to cancer (Dhanasekaran and Premkumar 2008).

DBC1 plays a role in regulating an adipocyte's capacity to store lipid. When DBC1 is knocked out, adipocyte differentiation and capacity of lipid storage both increase. If adipocytes are capable of storing larger quantities of fat, then there will be less fatty acid spillover into surrounding tissues. This increase in storage capacity and decrease in ectopic fat due to DBC1 knockout results in a "healthy obese" phenotype in which individuals remain insulin sensitive. DBC1 knockout mice gained more weight on a high fat diet compared to wild type mice did, but despite their greater weight gain the DBC1 knockout mice remained insulin sensitive (Escande et al. 2015). DBC1 might be the link between increased free fatty acids and metabolic disease because the knockout of DBC1 seems to protect against adipose tissue dysfunction (Escande et al. 2010). DBC1 inhibits SIRT 1 and this interaction frequency is increased when mice were fed a high fat diet (Escande et al. 2015, Moreno-Navarrete et al. 2015). SIRT1 activity remains high when DBC1 is knocked out. Escande et al. produced results showing that DBC1 definitively plays a role in the development of metabolic syndrome, however the mechanisms behind how DBC1 causes these effects in adipocytes was not clear (Escande et al. 2015).

Knockdown of DBC1 also leads to a decreased level of expression of many inflammatory genes in mature adipocytes. Inflammation of adipose tissue leads to a decreased ability of the tissue to store lipids (Moreno-Navarrete et al. 2015). As previously stated, one of the primary functions of DBC1 is that it can interact with and inhibit SIRT1 (Escande et al. 2010). DBC1 inhibits SIRT1 by interacting with the SIRT1 catalytic core. SIRT1 is responsible for preventing adipose tissue inflammation, which is driven by nuclear factor Kappa B (Chalkiadaki and Guarente 2012, Moreno-Nacarrete et al. 2015). Nuclear factor Kappa B reduces an adipocyte's adipogenic capacity to store fatty acids in the form of triacylglycerols, and it increases adipose tissue inflammation. NF-κB promotes the expression of pro-inflammatory genes in adipose

tissue. DBC1 also up regulates NF-κB driven inflammation by interacting with and inhibiting IKK-B. IKK-B is responsible for inhibiting the NF-κB pathway, which leads to cellular senescence by inhibiting the activity of histone deacetylase 3, HDAC3. This ultimately leads to fatty acid spillover because the adipocytes have a reduced ability to store fatty acids. Fatty acid spillover leads to insulin resistance and metabolic syndrome phenotype. When DBC1 was knocked down, adipose tissue was protected against inflammation and senescence (Moreno-Navarrete et al. 2015). DBC1 up-regulates apoptosis, limits the lipid storing capacity of adipocytes, and increases the expression of inflammatory genes in adipocytes (Escande et al. 2015, Kim and Kim 2013, Moreno-Navarrete et al. 2015).

V: Brief CD36 Background

Cluster of differentiation 36, CD36, which is also known as fatty acid translocase (FAT), is a transmembrane protein that is responsible for transporting fatty acids across the plasma membrane (Coburn et al. 2000, Glatz and Luiken 2016). CD36 is the primary protein that is responsible for fatty acid uptake into intestinal enterocytes, adipocytes, cardiac myocytes, and skeletal myocytes. Coburn et al. found that CD36 null mice have a 60-70% decrease in lipid uptake (Coburn et al. 2000). Dysfunction of CD36 has been linked to metabolic dysfunction including high fat diet-induced insulin resistance (Hosui 2016).

VI: Brief HDAC5 Background

Histone deacetylase 5, HDAC5, acts as a transcriptional co-repressor of GLUT4 by interacting with GEF, the GLUT4 enhancer factor, and inhibiting the transcription of GLUT4. GLUT4 is responsible for the uptake of glucose into the cell following insulin stimulation and dysfunction of this transporter can lead to metabolic disease (Sparling et al. 2008). Although

HDACs are usually involved with transcriptional repression, studies have shown that HDACs are required as co-activators of STAT5 (Schindler et al. 2007).

Materials and Methods

I: Western Blotting

Western blots were performed according to standard western blotting protocol, using SDS-PAGE and then transferring the proteins to nitrocellulose. SDS-PAGE stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins are run on a polyacrylamide gel containing SDS which will linearize the proteins and coat them with a negative charge. After loading the samples into the wells of the gel, an electric current is run through the gel so that the negatively charged proteins are attracted to the positive node located at the bottom of the gel. SDS-PAGE separates proteins based on molecular weight because smaller proteins will pass through the gel more quickly than larger proteins. The blots were probed using standard antibody probing technique and then imaged using either the Odyssey Imaging System by LI-COR or by film development. All DBC1 blots were imaged using the Odyssey Imaging System. The blots for liver and iWAT tissues were imaged using film development. A list of all antibodies that were used is provided in Materials and Methods section IV. The exact amount of protein loaded in each well is indicated under the appropriate figure.

II: Immunoprecipitation

Both immunoprecipitations (IP) were performed following standard immunoprecipitation protocol. Although IPs can be utilized for multiple purposes, these IPs were performed to validate the interaction between two proteins, STAT5A and DBC1. This is referred to as a pull-down assay. In short, an IP works by using an antibody against your protein of interest which

leads to the formation of an antibody/protein complex. Then agarose beads are used to bind the complex and many washes are performed to rid the beads of all proteins other than the protein of interest and whatever other proteins the protein of interest interacts with. Then the complex of proteins is separated from the beads by applying heat and the proteins are run on a western blot (Lal et al. 2006). The western blot of the immunoprecipitation of STAT5A was probed for the pull down of DBC1. The western blot of the immunoprecipitation of DBC1 was probed for the pull down of STAT5A.

III: Adipocyte specific STAT5 Knockout

All animal studies were performed according to the ethical procedures outlined by Pennington Biomedical Research Center. Animals were housed in 12 on/12 off cycled light and they had access to food and water *ad lib*. Liver and iWAT tissues were collected from mice lacking STAT5 in adipose tissue. These mice were generated by crossing mice with floxed STAT5 genes with Adiponectin-Cre mice. Studies not shown confirm that STAT5 was knocked out specifically in adipose tissue. Briefly, the Cre (cyclization recombination) protein is a recombinase from bacteriophage P1 that can catalyze the recombination between two *loxP* sites. *loxP* sites are inserted by homologous recombination on both sides of the target DNA and then Cre is delivered in order to splice out the gene that is targeted for knock out (Metzger and Feil 1999).

IV: List of Antibodies

- **STAT5A:** Stat5a 1:10000, Rabbit polyclonal, Santa Cruz 1081
- **DBC1:** DBC1 antibody, Rabbit polyclonal, Cell Signaling 5693
- **CD36:** CD36 (H-300), Rabbit polyclonal, Santa Cruz 9154
- **ERK1/2:** ERK 1 (c-16), Rabbit polyclonal, Santa Cruz 93

- **HDAC5:** HDAC(DIJ7V), Rabbit monoclonal, Cell Signaling 20458
- **STAT5B:** Anti-STAT5B, Rabbit polyclonal, Upstate Cell Signaling Solutions 06-554

Results and Discussion

DBC1 as a STAT5A interacting partner:

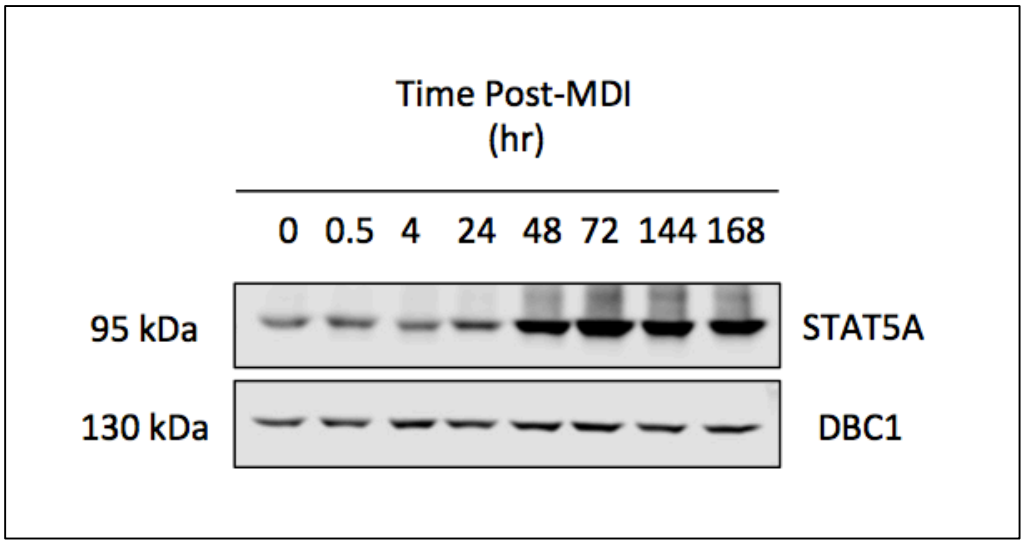

Figure 1: DBC1 protein expression is unaffected during adipocyte differentiation. Adipogenesis time course using 3T3-L1 preadipocytes. Cells were harvested with 1X IP buffer and protease and phosphatase inhibitors at each of the time points given. A BCA assay was performed, 50 µg of protein was loaded into each well, and gel electrophoresis was performed on a 7.5% polyacrylamide gel. After the gel ran at 50V overnight, it was taken down and the proteins were transferred to nitrocellulose. The nitrocellulose membrane was blocked with 4% nonfat milk for one hour and then incubated in primary antibodies (1:1000) (Stat5a(L-20) and DBC1 antibody) overnight at 4ºC. The membrane was then washed three times with 1X TBS-T and then probed with secondary antibody (1:10,000) (anti-rabbit secondary antibody) for one hour. The membrane was washed three more times with 1X TBS-T and then visualized using the Odyssey Imaging System.

As stated previously, the fact that many STAT proteins are expressed at higher levels during adipogenesis suggests that STATs play a role in adipocyte differentiation (Harp et al. 2001). As shown in Figure 1, STAT5A protein expression is induced during adipogenesis (Fig. 1). On the other hand, DBC1 expression remains constant throughout adipogenesis (Fig. 1).

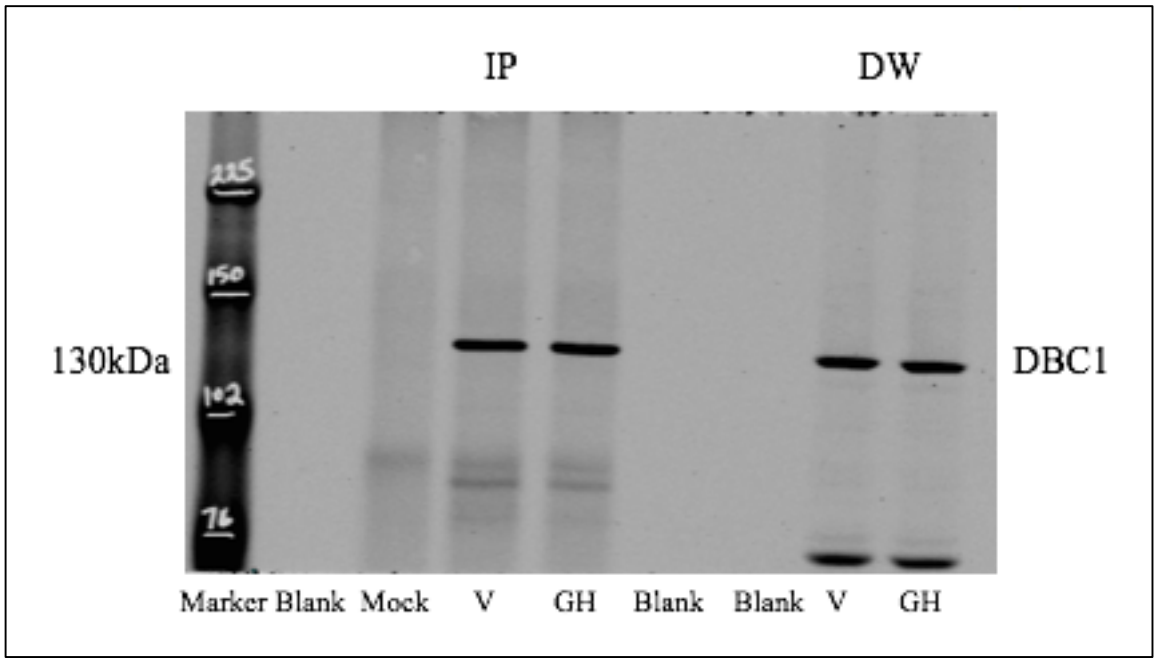

Figure 2: The physical association of STAT5A and DBC1 is not dependent on GH induced STAT activation. 3T3-L1 mature adipocytes were harvested with IP buffer and protease and phosphatase inhibitors. A BCA assay was performed, 300 µg of protein was loaded into each well, and gel electrophoresis was performed on a 15% polyacrylamide gel. After the gel ran at 50V overnight, it was taken down and the proteins were transferred to nitrocellulose. The nitrocellulose membrane was blocked with 4% nonfat milk for one hour and then incubated in primary antibody (1:1000) (DBC1 antibody) overnight at 4ºC. The membrane was washed three times with 1X TBS-T and then probed with secondary antibody (1:10,000) (anti-rabbit secondary antibody) for one hour. The membrane was then washed three more times with 1X TBS-T and then visualized using the Odyssey Imaging System.

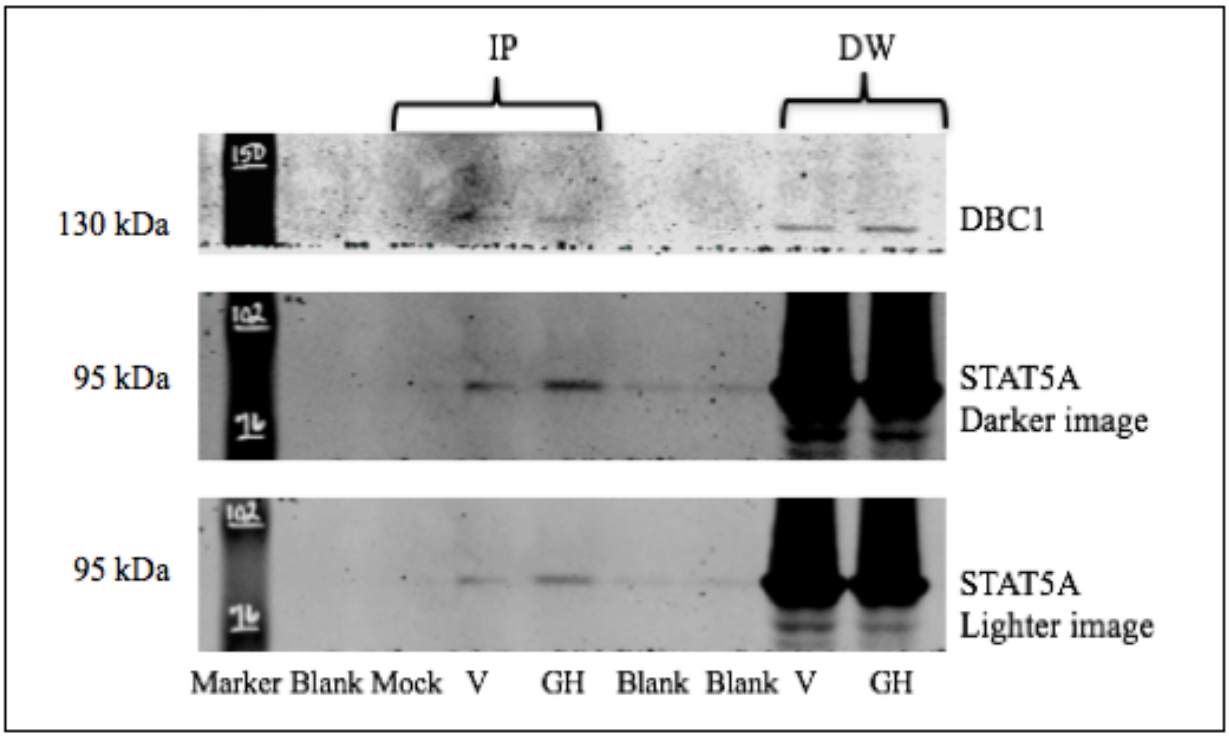

Figure 3: The physical interaction between DBC1 and STAT5A was confirmed by performing immunoprecipitations with DBC1. 3T3-L1 mature adipocytes were harvested with 1 X IP buffer and protease and phosphatase inhibitors. A BCA assay was performed, 75 µg of protein was loaded into each well, and gel electrophoresis was performed on a 15% polyacrylamide gel. After the gel ran at 50V overnight, it was taken down and the proteins were transferred to nitrocellulose. The nitrocellulose membrane was blocked with 4% nonfat milk for one hour and then incubated in primary antibodies (1:1000) (Stat5a(L-20) and DBC1 antibody) overnight at 4ºC. The membrane was then washed three times with 1X TBS-T and then probed with secondary antibody (1:10,000) (anti-rabbit secondary antibody) for one hour. The membrane was washed three more times with 1X TBS-T and then visualized using the Odyssey Imaging System.

These two blots show the results of two different IPs that were both tested with vehicle treatment and GH treatment as well as direct westerns to validate the interaction between STAT5A and DBC1. STAT5A has a molecular weight of 95 kDa and DBC1 has a molecular weight of 130 kDa. Figure 2 shows the IP of STAT5A and the pull down of DBC1. This IP validated that STAT5A does interact with DBC1, however the interaction was not GH dependent (Fig. 2). The reverse IP of DBC1 showed that DBC1 is capable of pulling down STAT5A (Fig. 3). This experiment confirmed the interaction between DBC1 and STAT5A a second time and again found that the interaction was not GH dependent. The weaker bands for the DBC1 IP are suspected to be due to the DBC1 antibody not being as ideal for immunoprecipitations as the STAT5A antibody is, even though its use is validated for this purpose.

Since STAT5 is activated when GH binds to the membrane bound receptor leading to activation of the JAK-STAT pathway (Schindler et al. 2007), we had hypothesized that the DBC1/STAT5A interaction would only be present when GH was present. This would mean that DBC1 and STAT5A only interact when STAT5 is activated and therefore, potentially plays a role in regulating the three main functions of adipocytes: insulin sensitivity, endocrine function, and lipid storage (Ronti et al. 2006). This interaction would then mean that DBC1 could play a role in regulating how activated STAT5A contributes to the regulation of these functions. However, since the interaction between DBC1 and STAT5A was not found to be GH dependent, the interaction is present even when STAT5A is not activated. Although DBC1 associates with STAT5 regardless of STAT5 activation, the interaction between these two proteins could still affect some of the other functions of STAT5 that are not dependent on GH. This topic should be the focus of future studies on DBC1.

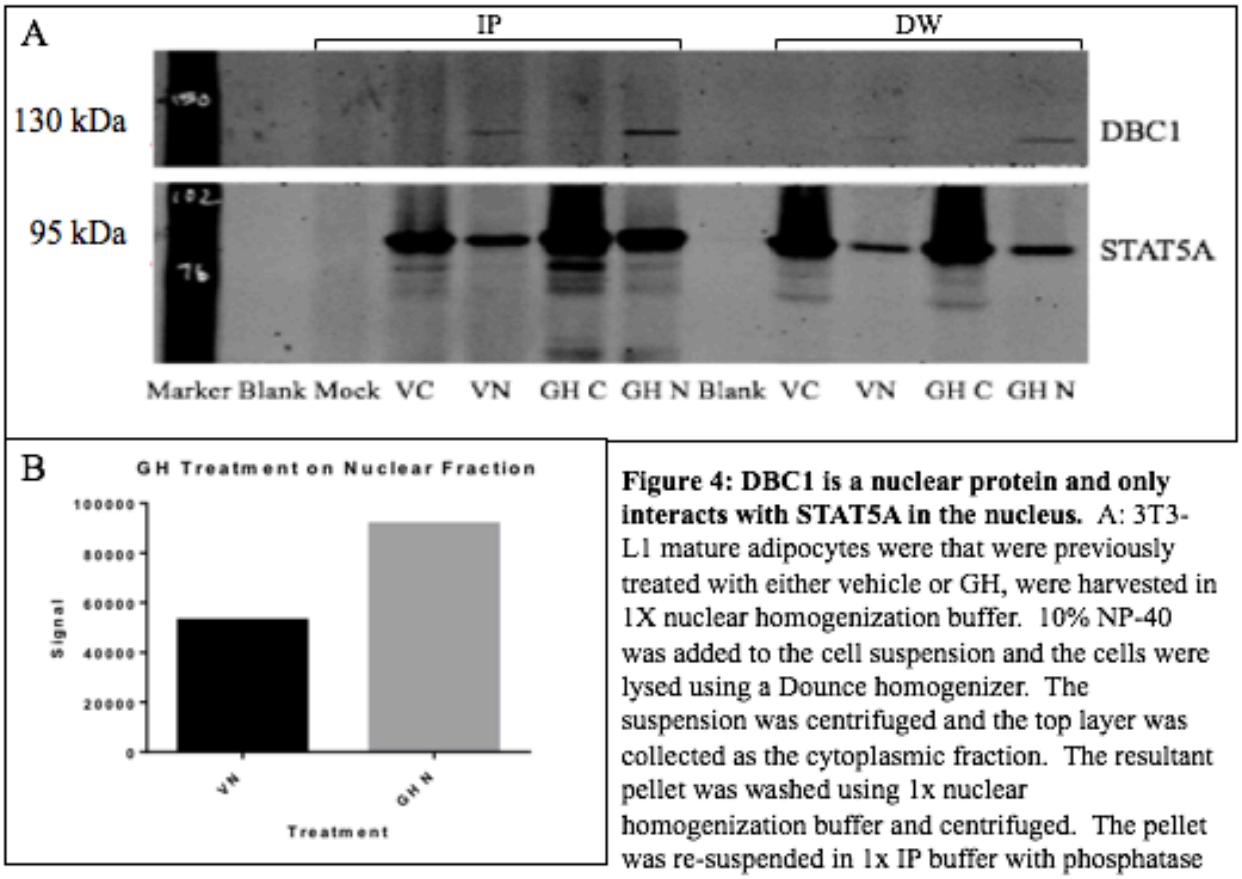

Figure 4: DBC1 is a nuclear protein and only interacts with STAT5A in the nucleus. A: 3T3-L1 mature adipocytes were that were previously treated with either vehicle or GH, were harvested in 1X nuclear homogenization buffer. 10% NP-40 was added to the cell suspension and the cells were lysed using a Dounce homogenizer. The suspension was centrifuged and the top layer was collected as the cytoplasmic fraction. The resultant pellet was washed using 1x nuclear homogenization buffer and centrifuged. The pellet was re-suspended in 1x IP buffer with phosphatase and protease inhibitors and this was collected at the nuclear fraction. A BCA assay was performed, 75 µg of protein was loaded into each well, and gel electrophoresis was performed on a 15% polyacrylamide gel. After the gel ran at 50V overnight, it was taken down and the proteins were transferred to nitrocellulose. The nitrocellulose membrane was blocked with 4% nonfat milk for one hour and then incubated in primary antibodies (1:1000) (Stat5a(L-20) and DBC1 antibody) overnight at 4ºC. The membrane was then washed three times with 1X TBS-T and then probed with secondary antibody (1:10,000) (anti-rabbit secondary antibody) for one hour. The membrane was washed three more times with 1X TBS-T and then visualized using the Odyssey Imaging System. B: Quantification of nuclear STAT5A following GH stimulation using Image Studio and graph generated using Prism software (p-value=0.3243).

This blot shows two biological replicates of vehicle treated cytosolic fractions, vehicle treated nuclear fractions, GH treated cytoplasmic fractions, and GH treated nuclear fractions. As stated previously, DBC1 is a nuclear protein, so the result that DBC1 was only present in the nuclear fractions was as expected (Fig. 4A). The blot was also probed for STAT5A showing a movement of STAT5A into the nucleus in the GH treated samples (Fig. 4A and Fig. 4B). This is due to the fact that GH activates the membrane bound receptor leading to the initiation of the

JAK-STAT pathway and STAT5A tyrosine phosphorylation and activation (Schindler et al. 2007). Once STAT5A is activated, it dimerizes and translocates to the nucleus (Rawlings et al. 2004). The results showing elevated levels of STAT5A in the nuclear fractions treated with GH were as expected. These results suggest that DBC1 does not regulate adipocyte differentiation through its interaction with STAT5A. However, DBC1 is already known to be involved in apoptosis, the lipid storage capacity of adipocytes, and the expression of inflammatory genes (Escande et al. 2015, Kim and Kim 2013, Moreno-Navarrete et al. 2015), so further studies will be necessary to determine how DBC1 functions to do so.

Examination of the potential of adipocyte STAT5 regulation on CD36 expression in liver tissue and HDAC5 expression in iWAT tissue

These next two experiments examine other genes that may be regulated by STAT5 activation or repression. CD36 and HDAC5 are not necessarily STAT5 interacting partners as DBC1 is. The tissues used for these next two experiments were collected from $S5^{AKO}$ mice. $S5^{AKO}$ mice are mice that do not express STAT5A or STAT5B in their adipocytes. We studied tissue from these mice in order to determine how the knockout of STAT5 in adipocytes would alter the expression of genes in other tissues, liver in this case, as well as the expression of genes within adipose tissue itself. CD36 expression in the livers of these mice was tested to determine how the knockout of STAT5 would affect endocrine signaling between the liver and the adipose tissue and how this would affect the CD36 expression in the liver. iWAT tissue was studied to determine how STAT5 knockout in the adipocytes of this tissue would affect the expression of HDAC5 within the tissue and therefore predict STAT5 knockout implications on adipocyte glucose uptake since HDAC5 is a co-repressor of the gene encoding GLUT4.

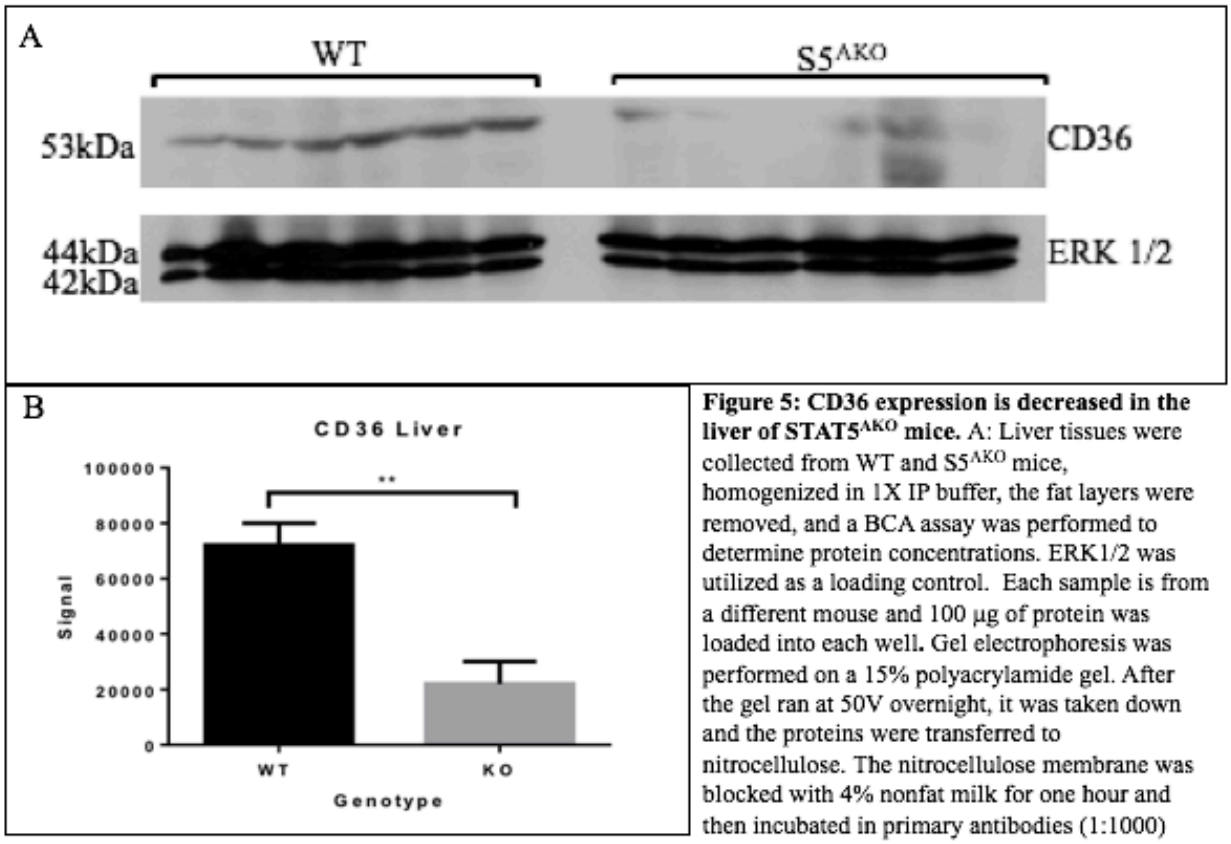

Figure 5: CD36 expression is decreased in the liver of STAT5^{AKO} mice. A: Liver tissues were collected from WT and S5^{AKO} mice, homogenized in 1X IP buffer, the fat layers were removed, and a BCA assay was performed to determine protein concentrations. ERK1/2 was utilized as a loading control. Each sample is from a different mouse and 100 µg of protein was loaded into each well. Gel electrophoresis was performed on a 15% polyacrylamide gel. After the gel ran at 50V overnight, it was taken down and the proteins were transferred to nitrocellulose. The nitrocellulose membrane was blocked with 4% nonfat milk for one hour and then incubated in primary antibodies (1:1000) (CD36 (H-300) and ERK1 (C-16)) overnight at 4ºC. The membrane was then washed three times with 1X TBS-T and then probed with secondary antibodies (1:10,000) (anti-rabbit secondary antibody) for one hour. The membrane was washed three more times with 1X TBS-T and then visualized using film development. B: Quantification of CD36 expression in the liver tissue of WT and S5^{AKO} mice using Image Studio and graph generated using Prism software (p-value: 0.0013).

CD36, also known as fatty acid translocate, is a membrane bound protein responsible for lipid uptake into an adipocyte (Coburn et al. 2000). CD36 has a molecular weight of 53 kDa and ERK1 and ERK2 have molecular weights of 42 kDa and 44 kDa. Expression of CD36 was significantly lower in the STAT5^{AKO} mice livers which suggests that the CD36 gene is regulated differently when STAT5 expression is altered in adipose tissue. ERK1/2 was simply used as a loading control and its expression was constant for all samples.

The liver, adipose tissue, and skeletal muscle are all fuel storage organs that communicate with each other to regulate energy control and prevent metabolic disorders including obesity and T2DM (Shimizu et al. 2015). One of the three main functions regulated by

STAT5 in adipocytes is endocrine action (Ronti et al. 2006). Dysfunctions in adipocyte ability to secrete endocrine hormones leads to metabolic diseases including T2DM because these fuel organs cannot properly communicate with one another (Shimizu et al. 2015). Dysfunction of STAT5 can cause these abnormal metabolic states and since CD36 expression appears to be regulated by STAT5 action, it is likely that CD36 also plays a role in the development of these metabolic states. For this experiment, STAT5 was knocked out in adipocytes, however we tested the liver tissues from these $S5^{AKO}$ mice. Previous studies examining the effect of STAT5 knockout in liver tissue showed that this STAT5 knockout led to greater expression of CD36 in the liver. This higher expression level led to disease states such as fatty liver disease (Baik et al. 2011). The aim of my study was to determine how dysfunction of adipocytes caused by adipocyte specific STAT5 knockout would affect the fuel storage of the liver. Since CD36 expression is down-regulated in the liver of $S5^{AKO}$ mice, this suggests that lipid uptake, which is controlled by CD36, is down-regulated in the liver of $S5^{AKO}$ mice. Bonen et al. support this indication in their study showing that CD36 expression is up-regulated in the adipose tissues of humans who are obese or who have T2DM (Bonen et al. 2006). This suggests that $S5^{AKO}$ mice have greater lipid uptake in adipocytes and lesser lipid uptake in the liver due to the dysfunction of STAT5 in adipocytes. CD36, or fatty acid translocase, allows the cell to physically take in lipids, but it does not necessarily play a role in the capacity of lipids that an adipocyte can hold (Coburn et al. 2000). This increase in lipid uptake in adipocytes can lead to lipid spillage and ectopic fat accumulation (VanHerpen and Schrauwen-Hinderling 2008). $S5^{AKO}$ mice could have a down-regulation of CD36 in the liver due to impaired endocrine function of their adipocytes. Since CD36 is down-regulated in the liver in $S5^{AKO}$ mice, it is unlikely that the ectopic fat caused by lipid spillage by adipocytes is accumulated in the liver. Further studies should test skeletal

muscle since it is another fuel storage organ in communication with adipose tissue to see how this $S5^{AKO}$ affects the CD36 expression of skeletal muscle.

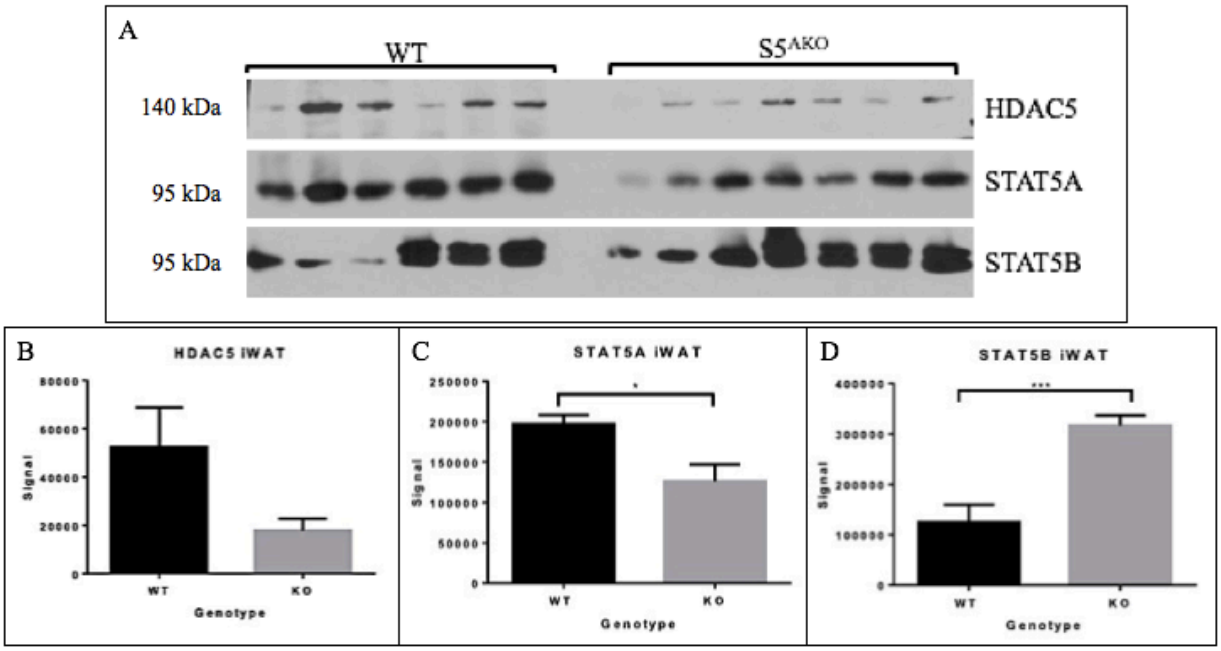

Figure 6: HDAC5 levels are decreased in the adipose tissue of $STAT5^{AKO}$ mice that have increased STAT5 expression in non-adipocytes. A: iWAT tissues were collected from WT and $S5^{AKO}$ mice, homogenized in 1X IP buffer, the fat layers were removed, and a BCA assay was performed to determine protein concentrations. Each sample is from a different mouse and 200 µg of protein was loaded into each well. Gel electrophoresis was performed on a 15% polyacrylamide gel. After the gel ran at 50V overnight, it was taken down and the proteins were transferred to nitrocellulose. The nitrocellulose membrane was blocked with 4% nonfat milk for one hour and then incubated in primary antibodies (1:1000) (HDAC5 (DIJ7V), Stat5a (L-20), and Anti-STAT5B) overnight at 4ºC. The membrane was then washed three times with 1X TBS-T and then probed with secondary antibodies (1:10,000) (anti-rabbit secondary antibody) for one hour. The membrane was washed three more times with 1X TBS-T and then visualized using film development. B: Quantification of HDAC5 expression in iWAT tissue of WT and $S5^{AKO}$ mice (p-value=0.0526). C: Quantification of STAT5A expression in iWAT tissue of WT and $S5^{AKO}$ mice (p-value= 0.0161). D: Quantification STAT5B expression in iWAT tissue of WT and $S5^{AKO}$ mice (p-value: 0.0005). All quantifications were performed using Image Studio and all graphs were generated using Prism software.

HDAC5, histone deacetylase 5, has been implicated as both a co-activator of STAT5 and as a transcriptional co-repressor of GLUT4 (Sparling et al. 2008, Schindler et al. 2007). HDAC5 has a molecular weight of 140 kDa. Adipocyte specific knockout of STAT5 in iWAT tissue led

to the down-regulation of HDAC5 expression in the tissue (Fig. 6A and Fig. 6B). This suggests that the HDAC5 gene is regulated by STAT5 activation. The remaining expression of HDAC5 in the absence of STAT5 in adipocytes could be due to HDAC5 expression in other cell types found in adipose tissue that do not contain the STAT5 knockout. It is also possible that HDAC5 expression is not fully repressed in the absence of STAT5, but is simply up-regulated by STAT5 activation. Since STAT5 is up-regulated during adipogenesis (Harp et al. 2001) and since HDAC5 expression seems to be regulated by STAT5 activation, future studies could examine the effects of knocking out HDAC5 during adipogenesis in the presence of STAT5 to see whether or not STAT5 regulates any of the three main functions of adipocytes by up-regulating the expression of HDAC5.

Expression of STAT5A was lower in the $S5^{AKO}$ tissue than it was in the wild type tissue, but there is still a decently high level of expression (Fig. 6A and Fig. 6C). Expression of STAT5B actually increased in the $S5^{AKO}$ iWAT tissue compared to the wild type iWAT tissue (Fig. 6A and Fig. 6D). Even though STAT5A and STAT5B are both knocked out in the adipocytes of the iWAT tissue, the maintained presence of the two STATs in the $S5^{AKO}$ tissue is due to the presence of other cell types found in adipose tissue. The stromovascular fraction of adipose tissue contains endothelial cells, immune cells, connective tissue cells, and blood cells as well as many other cell types (Richard and Stephens 2011). These other cell types are still capable of expressing STAT5. The increased expression of STAT5B in the stromovascular fraction is suspected to be compensating for the decreased expression of STAT5A in the tissue.

Conclusions and Future Work

DBC1 is already known to play a role in the regulation of inflammation, lipid storage capacity, and apoptosis of adipocytes (Escande et al. 2015, Kim and Kim 2013, Moreno-

Navarrete et al. 2015), however the exact mechanisms behind these regulatory systems were the aim of this experiment. Since DBC1 interacts with STAT5A, it was hypothesized that DBC1 might play a role in how STAT5 regulates the three main functions of adipocytes: insulin sensitivity, lipid storage, and endocrine function (Ronti et al. 2006). However, results showed that DBC1's interaction with STAT5 was not GH dependent and is therefore not responsible for the regulation of the GH mediated effects that STAT5 is known to play in adipocytes. The interaction may play a role in the other functions of STAT5 outside of GH activation. Although not the desired result, trial and error experiments are still vitally important to any type of research. Future experiments should be performed to test the effects of the knockout of DBC1 in adipocytes both *in vitro* and *in vivo* and how its interaction with STAT5 plays a role independent from STAT5 activation by GH.

CD36 expression in the liver is down-regulated by STAT5 knockout in adipocytes. This leads to the understanding that CD36 is a gene that is regulated STAT5 in some manner. External studies have shown that STAT5 knock out in liver tissue leads to higher levels of expression of CD36 in the liver tissue leading to liver disease, such as fatty liver disease (Baik et al. 2011). However, the opposite effect is seen in liver tissues when STAT5 is knocked out in adipocytes. Since CD36 is responsible for a great portion of the lipid uptake into an adipocyte (Coburn et al. 2000), CD36 likely plays a role in the mechanism behind STAT5's regulation of an adipocyte's lipid storage ability. Further studies should be performed both *in vitro* and *in vivo* examining the effects of STAT5 knockout on adipogenesis and CD36 expression affecting the levels of intracellular lipid uptake in adipose tissue and skeletal muscle.

HDAC5 has been shown to be both a co-activator of STAT5 and a co-repressor of GLUT4 transcription (Sparling et al. 2008, Schindler et al. 2007). Since STAT5 is up-regulated

during adipogenesis (Harp et al. 2001), and since GLUT4 is required to prevent hyperglycemia (Sparling et al. 2008), future *in vivo* studies should examine the effects of HDAC5 knockout on the metabolic health of mice, mainly dealing with blood glucose levels. *In vitro* studies should also be performed to examine the effect of the knock out of HDAC5 on adipogenesis. STAT5A and STAT5B are both present in the stromovascular fraction of iWAT tissue. Since increased STAT5B expression in the stromovascular fraction seems to be compensating for the decreased STAT5A expression, further studies should examine the effect of STAT5A adipocyte specific knockout and test whether or not STAT5B compensates for the loss of STAT5A in adipocytes as well.

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