ABSTRACT

Title of dissertation: THE ROLE OF ATAXIA TELANGIECTASIA MUTATED AND THE CATALYTIC SUBUNIT OF DNA-DEPENDENT PROTEIN KINASE DURING ADIPOGENESIS

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Ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}), are DNA damage response (DDR) proteins that have well established roles in the repair of DNA double strand breaks (DSBs). A growing body of scientific evidence suggests that these kinases have important, but not yet well-understood, immune and metabolic functions. ATM and DNA-PK_{cs} can regulate cellular oxidative stress and combat reactive oxygen species (ROS) (Chen et al 2012). Since oxidative stress is a driving factor in the pathogenesis of many obesity related complications, ATM and DNA-PK_{cs} may help respond to and regulate oxidative stress in adipocytes.

Precise oxidative signaling is necessary for adipocyte differentiation and lipid accumulation. Selenium, a nutrient with suspected insulin mimetic properties, can alter cellular oxidative stress (H Gandhi et al 2013). Therefore, it is plausible that selenium may affect adipogenesis and change lipid accumulation patterns in cells.

The primary research questions were as follows: What is the role of ATM and DNA-PK_{cs} in adipogenesis and adipocyte homeostasis? Are the actions of these kinases in adipocytes explained by changes in oxidative stress levels? Do proposed nutrients with

insulin mimetic properties, like selenium, affect adipogenesis via the suspected ATM - DNA-PK_{cs} - ROS pathway?

Time dependent mammalian cell culture based experiments that measured outcome variables like lipid accumulation, senescence activity, protein expression and ROS levels were conducted to assess the action of ATM and DNA-PK_{cs} during adipogenesis and the effect of selenium on the ATM - DNA-PK_{cs} – ROS dependent adipogenic pathway.

This study found that ATM and DNA-PK_{cs} are necessary proteins for maintaining adipocyte integrity as well as having a significant role in the differentiation of preadipocyte to adipocyte. Furthermore, we confirmed that the kinases act via a ROS dependent pathway and that dietary nutrients, such as selenium, can exert additional control over this pathway, therefore highlighting the impact adipocyte metabolism exerts over total metabolic health.

THE ROLE OF ATAXIA TELANGIECTASIA MUTATED AND THE CATALYTIC SUBUNIT OF DNA-DEPENDENT PROTEIN KINASE DURING ADIPOGENESIS

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DEDICATION

To my mother, Karen Rocourt and boyfriend, Stanton Salter, the most important people in my life and my biggest supporters. To my undergraduate research assistants, Chris Maino and Louis Spear; both of whom I consider collaborators and lifelong friends. To Jallah Rouse and Yongmei Qi, who started out as lab mates and became my closest and dearest friends. To my animal companions Beau the beagle, Flossie the cat, and my horse Charlotte for providing unconditional love and pet therapy.

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LIST OF ABBREVIATIONS

A-T	ataxia- telangiectasia
ATM	ataxia- telangiectasia mutated
BMI	body mass index
BSA	bovine serum albumin
C/EBPα	CCAAT/enhancer binding protein α
CM-H_2DCFDA	5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester
DAPI	4,6-diamidino-2-phenylindole
Dex	dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA-PK_{cs}	the catalytic subunit of DNA-dependent protein kinase
DSBs	double-strand breaks
FBS	fetal bovine serum
FSC	forward scatter
GLUT1	glucose transporter 1
GLUT4	glucose transporter 4
GPX1	glutathione peroxidase-1
H_2O_2	hydrogen peroxide
HU	hydroxyurea
IBMX	3-isobutyl-1-methylxanthine
IgE	immunoglobin E
IL-1	interleukin 1
IL-6	interleukin 6
iNOS	inducible nitric acid synthase
KOH	potassium hydroxide
MDI	adipogenic induction media
MEFs	mouse embryonic fibroblasts
MHC	major histocompatibility complex
MSeA	methylseleninic acid
Na_2SeO_3	sodium selenite
NAC	*N*-acetylcysteine
NHANES	U.S. National Health and Nutrition Examination Surveys
NHEJ	non-homologous end-joining pathway
ORO	oil-red-o

***p*ATM Ser-1981**	phospho-ATM at Ser-1981
PBS	phosphate-buffered saline
***p*DNA-PK$_{cs}$ Thr-2647**	phospho-DNA-PK$_{cs}$ at Thr-2647
PI(3)K	phosphoinositide 3-kinase
PIKK	PI(3)K- like protein kinase
PP1	protein phosphatase 1
PPARγ	peroxide peroxisome proliferator-activated receptor gamma
RDA	recommended daily allowance
ROS	reactive oxygen species
RT	room temperature
S.E.M.	standard error of the mean
SA-β-gal	senescence-associated -β-galactosidase
Sec	selenocysteine
SECIS	selenocysteine insertion sequences
SSC	side scatter
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
TG	triglyceride
TNF-α	tumor necrosis factor α
TZD	thiazolidinediones
USF-1	upstream stimulatory factor 1
WT	wild type
γH2A.X	phospho-histone2A.X at Ser-139

INTRODUCTION

Obesity has become a global health concern, significantly affecting populations in the United States and other westernized countries (James et al 2001, Malik et al 2013, Ogden et al 2006, Popkin and Doak 1998, Seidell 2000). Obesity is not only a burden financially, emotionally, and socially, but it also predisposes individuals to a range of health problems, such as type 2 diabetes, metabolic syndrome, cardiovascular disease and hypertension, which are comorbidities frequently associated with obesity (Kelly et al 2008, Kress et al 2005, Must et al 1999, Thompson and Wolf 2001, Wang and Dietz 2002). The molecular mechanisms that explain how obesity is a causative agent in many disease states remain largely unclear. However, it is known that adipose tissue becomes dysfunctional with increasing adiposity (Guilherme et al 2008) and it is evident that the adipocyte unit itself is an indicator of metabolic health. Thus, the key to understanding obesity-associated metabolic diseases lies in the study of adipose tissue and adipocytes (Efrat et al 2013, Moest et al 2013).

We chose to conduct experiments to help elucidate adipose tissue dysfunction by studying the role of the PI(3)K serine/threonine kinases, ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) during adipogenesis. We hope our research helps to highlight the importance of oxidative tone as a likely causative agent in the comorbidities associated with obesity.

The following explains how this dissertation is organized. Chapter 1 consists of a literature review that explains in detail the ATM and DNA-PK kinases, their role in the DNA damage response and provides ample evidence to support our hypothesis of their involvement in adipogenesis. An overview of adipose tissue biology, the differentiation process, the function of mature adipocytes and a close-up look at adipocyte physiology that focuses on lipid droplets & adipocyte remodeling is also included in the Chapter 1. The literature review concludes with an in depth explanation of metabolic dysfunctions and a summary on how the origins of dysfunctions stem from disturbances in adipose tissue dysfunction.

Chapter 2 and 3 are prepared in the style of a manuscript for publication. Chapter 2 is an inquiry into the role of ATM and DNA-PK during adipogenesis. Chapter 3 builds on the critical interpretations and conclusions from Chapter 2 by using selenium to disturb the ROS-ATM-DNA-PK pathway. Chapter 4 advances a summary of the research and discusses the conclusions of the dissertation. The last sections are the appendix followed by the bibliography.

CHAPTER 1: REVIEW OF LITERATURE

The PIKK Family of DNA Damage Response Proteins: ATM and DNA-PK$_{cs}$

The DNA damage response & DNA double strand breaks

Mammalian cells have evolutionary conserved DNA damage response pathways that serve to relay, amplify and translate the DNA damage signal and to coordinate cell cycle arrest and subsequent DNA repair (Zhou and Elledge 2000). The DNA damage response pathway is orchestrated by members of the PI(3)K- like protein kinases (PIKKs) family of proteins, which are recruited to the sites of DNA damage. DNA damage is a causative agent in many disease states, such as cancer (Martin et al 2011). Persistent DNA damage is thought to be an underlying mechanism in aging and age-related diseases (Karanjawala and Lieber 2004, Schumacher et al 2008).

Furthermore, there are many diseases, both inherited and lifestyle attributable, which are associated with DNA damage and/or defective DNA repair (For a review, please see ref. (O'Driscoll 2012)). In most cases, DNA damage is caused by ROS, which can originate from both exogenous and endogenous sources (Sirbu and Cortez 2013). An example of ROS originating from an exogenous source is the ROS generated via ionizing radiation (Leach et al 2001). The byproducts of oxidative phosphorylation are an endogenous example of ROS production (Droge 2002).

DNA double strand breaks

DNA double strand breaks (DSBs), a particularly harmful type of DNA lesion (Lees-Miller et al 1990), can be created after DNA damage, but also can be a consequence of replication failure (Pardo et al 2009). The proper repair of DSBs is critical for genomic stability in order to prevent cancer and delay aging. Following the creation of a DSB, the PIKK family members ATM and DNA-PK_{cs} are activated then initiate the DNA damage response. These proteins recognize the DNA lesions and initiate downstream processes that relay and amplify the damage signal (Shrivastav et al 2009). After appropriate signaling and amplification, effector proteins are activated, which modulate the cell cycle, reconstruct chromatin and control DNA repair (Khanna and Jackson 2001). For detailed reviews on the DNA damage response and DSB repair, please see refs. (Lees-Miller and Meek 2003, Sirbu and Cortez 2013, Vignard et al 2013).

Ataxia- telangiectasia mutated (ATM)

The *Atm* gene is responsible for encoding ATM protein kinase, whose best-known function is as the main activator of the DNA damage response following the formation of DNA DSBs (Shiloh and Ziv 2013). The ATM protein is primarily found in the nucleus and is necessary for a proper DNA damage response (Lavin 2008). Recently, ATM was shown to be activated by oxidative stress without the presence of DNA DSBs (Ditch and Paull 2012, Guo et al 2010a, Guo et al 2010b). ATM is likely to have a role in regulating oxidative stress independent of, and in addition to, its role in DNA DSB repair. The role of ATM has been further expanded after research shows that it has a critical role in immune function (Huang et al 2013, Landoure et al 2013).

Mutations in ATM cause the disease ataxia- telangiectasia (A-T), which is a premature aging syndrome characterized by ataxia and ocular telangiectasia (Huang, et al. 2013). Interestingly, more than 500 mutations are known to cause the disease, with the severity of the disease depending on the location and type of mutation. Approximately 75% of *Atm* mutations are frameshifts or nonsense mutations (Concannon and Gatti 1997, Wright et al 1996), which truncate the entire protein. Other mutations, such as missense mutations or errors in splicing, can result in genomic duplication or deletion.

There is emerging evidence that strongly supports a role of ATM in metabolism outside of its role in the DNA damage response (Shiloh and Ziv 2013, Stagni et al 2013, Stracker et al 2013). Although the DNA damage response function of ATM is carried out in the nucleus, there is a considerable concentration of cytoplasmic ATM that are capable of binding proteins involved in cellular transport (Alexander and Walker 2010, Boehrs et al 2007, Lim et al 1998, Rotman and Shiloh 1999). In addition to their neurological symptoms, A-T patients also have higher incidences of developing type 2 diabetes, insulin resistance (Bar et al 1978), adipocyte lipotoxicity, mitochondrial dysfunction and also have increased systemic oxidative stress (Blevins and Gebhart 1996, Ersoy et al 1991, Lavin and Shiloh 1997, Morrell et al 1986, Schalch et al 1970). Many growth abnormalities and problems with cellular homeostasis in A-T patients cannot be explained by defective DNA damage repair alone. Most A-T patients do not have diabetes, but this may be because they do not live long enough to develop the disease. In one study, 10 out of 17 A-T patients developed type 2 diabetes (Morrell, et al. 1986).

ATM is insulin responsive (Ching et al 2013, Yang and Kastan 2000); and its kinase activity is responsible for the insulin stimulated phosphorylation of Akt/Protein Kinase B (a key insulin signaling protein intermediate) at serine 473 after insulin treatment (Halaby et al 2008). Furthermore, a downstream target of ATM, p53, is involved in several stress signaling pathways that can influence metabolic and glucose homeostasis (Armata et al 2010). For example, one of the well-characterized masters of cellular metabolism the mammalian target of rapamycin (mTOR) is inhibited by p53 activation (For a review of p53 & metabolic regulation please see refs. (Armata, et al. 2010, Berkers et al 2013)).

Glucose transport into cells is often impaired in people with type 2 diabetes and causes reduced glucose clearance, leading to hyperglycemia. ATM plays a role in glucose transport in cultured cells; ATM mutant myoblasts display decreased glucose transport. Glucose transporter 1 (GLUT1)(Andrisse et al 2013) is responsible for the physiologically relevant portion of basal glucose transport, contains a phosphorylation site specific for the ATM kinase. Phosphorylation of Serine 490 of GLUT1 is critical for GLUT1-mediated glucose uptake. Specifically, ATM kinase chemical inhibition diminishes basal glucose transport and that ATM kinase activity is necessary to phosphorylate Serine 490, which when taken together, suggests that ATM is needed for GLUT1 cell surface localization and subsequent glucose uptake. Furthermore, the chemical inhibition of ATM decreases the rate at which it is recycled to the cell surface, suggesting that GLUT1 is in fact an *in vivo* ATM substrate. Interestingly, ROS formation can down regulate glucose transporter expression and promote insulin resistance in mature adipocytes (Kozlovsky et al 1997, Rudich et al 1997), which could be explained by changes in ATM kinase activity.

The GoDarts study in 2011 found that individual variation in the *Atm* gene was shown to alter the glycemic response to the common diabetic drug metformin (GoDarts et al 2011). Specifically, those with the single nucleotide polymorphism rs11212617, at a locus containing the *Atm* gene, had better treatment success with metformin. Furthermore, the group provided mechanistic evidence to suggest the possible mechanistic underpinnings of their observations. Firstly, they chose to study AMP-activated protein kinase (AMPK), because it is the pharmacological target of the insulin sensitizing drug metformin. Although the exact mechanism is unclear, metformin does activate AMPK by inhibiting the mitochondrial respiratory chain (Zhou et al 2001). Inhibiting the mitochondrial respiratory chain increases cellular AMP, which is widely considered to be the mechanism responsible for metformin's insulin sensitizing benefits.

The GoDarts researchers treated rat H4IIE hepatoma cells with the selective ATM kinase inhibitor KU 55933 and found that the activation of AMPK by metformin was significantly reduced. To confirm further their genome wide association study data, they studied the activation of the well-characterized marker of AMPK activation, Serine 79 of Acetyl-CoA carboxylase. Western blot analysis showed that after KU 55933 treatment, phosphorylation of Acetyl-CoA carboxylase was significantly decreased.

Due to the novelty of the GoDarts study, three separate research groups responded to the original article. Woods and her colleagues provided data that showed that when cells were treated with other ATM chemical inhibitors, such as caffeine, that there was no decrease in metformin induced AMPK activation, suggesting that KU 55933 reduces AMPK activity independent of inhibiting ATM kinase (Woods et al 2012). Another group suggests that the GoDarts data can be reinterpreted and that the inhibitor KU 55933 reduces

cellular uptake of metformin by reducing organic cation transporter 1, which is necessary for metformin uptake in cells (Yee et al 2012).

Furthermore, the same researchers argue that the structure of KU 55933 makes it likely an inhibitor of organic cation transporter 1, which offers another plausible explanation of the cell culture data from the GoDarts study. We also can point out alternate hypothesis based on the findings of the genome wide association data. Since ATM is involved in GLUT4 translocation (Halaby, et al. 2008), and metformin increases fatty acid oxidation and GLUT4 phosphorylation (Collier et al 2006), perhaps the association between the particular locus and the varying responses to metformin can be explained by the necessity of the ATM kinase in GLUT4 phosphorylation.

The authors of the original publication responded to their critics by defending their rationale for choosing ATM as the likely gene candidate at that locus (Zhou et al 2012). They further cited the human, mouse and cell studies that indicated ATM is likely involved in insulin signaling and glucose metabolism. They also state the interpretation of their cell culture data cannot be used to assert that ATM is the casual gene with absolute certainty. However, none of the data from the groups responding to the article provides evidence to 1) exclude ATM as the candidate gene or 2) support that other genes at the locus are in fact the causal genes. The GoDarts study and the corresponding letters to the editor emphasize that genome wide association studies are powerful tools, but highlight the necessity of testing all possible gene candidates with equal rigor and without prejudice.

Despite conflicting data relating ATM to a role in metabolic function, we believe that ATM has a regulatory role in lipid accumulation and we conducted experiments to

determine the contribution of ATM to metabolic processes associated with adipogenesis and lipid droplet formation.

DNA-dependent protein kinase (DNA-PK)

Recently, new roles for DNA-PK have been proposed (Chen, et al. 2012, Kong et al 2011, Lin et al 2013, Wong and Sul 2009) that expand beyond its well characterized role in repairing DNA DSBs via the non-homologous end-joining pathway (NHEJ) (Burma et al 2006, Taleei and Nikjoo 2013). We have found DNA-PK is a necessary intermediate in the senescence response following selenium exposure (Rocourt et al 2012, Wu et al 2010) and others have shown that DNA-PK is activated during hypoxia (Bouquet et al 2011), which is a common phenomenon associated with obesity (Trayhurn 2013, L Zhang et al 2011).

The Sul lab has demonstrated a relationship between DNA-PK and insulin signaling (Wong et al 2009). Specifically, they found that after activation of protein phosphatase 1 (PP1) by insulin, PP1 then dephosphorylates DNA-PK_{cs}, which activates DNA-PK kinase function. Activated DNA-PK then phosphorylates upstream stimulatory factor 1 (USF-1) at Serine 262. USF-1 is a transcription factor that controls genes related to lipogenesis, such as fatty acid synthase which converts glucose to fatty acids. The group determined that *de novo* lipogenesis and activation of lipogenic genes are decreased in a DNA-PK deficient severe combined immune deficiency mouse model. Furthermore, the DNA-PK deficient severe combined immune deficient mice also have lower plasma lipids and adiposity than the control mice. Taken together, this model suggests that overfeeding induces insulin release, which then can activate PP1. PP1 can then target DNA-PK and

activate it by phosphorylation. Once activated, DNA-PK can regulate genes for lipolysis and lipogenesis to change the way the body stores and uses fat.

Adipose Tissue and Adipocytes

Differentiation

Adipocytes and their precursors (preadipocytes) are renewed in the body in a tightly controlled fashion. In adults with a healthy body mass index (BMI), the number of total adipocytes remains constant throughout life (Boroumand et al 1980, Roche 1981). In order for a preadipocyte to become a mature adipocyte, exposure to a well-characterized and highly ordered sequence of hormones that promote lipid accumulation is required. Adipose tissue is predominantly composed of mature adipocytes. However, there are other cell types present in adipose tissue that are necessary for its proper function such as preadipocytes, connective tissue cells, and immune cells.

Insulin, glucocorticoids and phosphodiesterase inhibitors activate receptor mediated signaling cascades that are involved in adipogenesis whereby transcriptional regulation changes preadipocytes into adipocytes. After hormonal stimulation of the preadipocyte, transcription factors that regulate transcription of adipocyte specific genes, such as peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα), coordinate cell proliferation, inflammatory responses and regulate insulin sensitivity (Choy and Derynck 2003, Kliewer et al 1995, Siersbaek et al 2010). There is an equally impressive body of studies that identify agents that inhibit adipogenesis such as the cytokines interleukin 1 (IL-1) and TNF-α, cell surface proteins, and various intracellular pathways that involve mitogen activated protein kinases (Sul et al

1998). Thus, the induction and inhibition of adipogenesis from preadipocyte to adipocyte is subject to tight regulation. The replacement of aged and damaged adipocytes is also tightly regulated, and these functions must remain in balance in order to supply energy demand and maintain homeostasis (Iyer et al 2010).

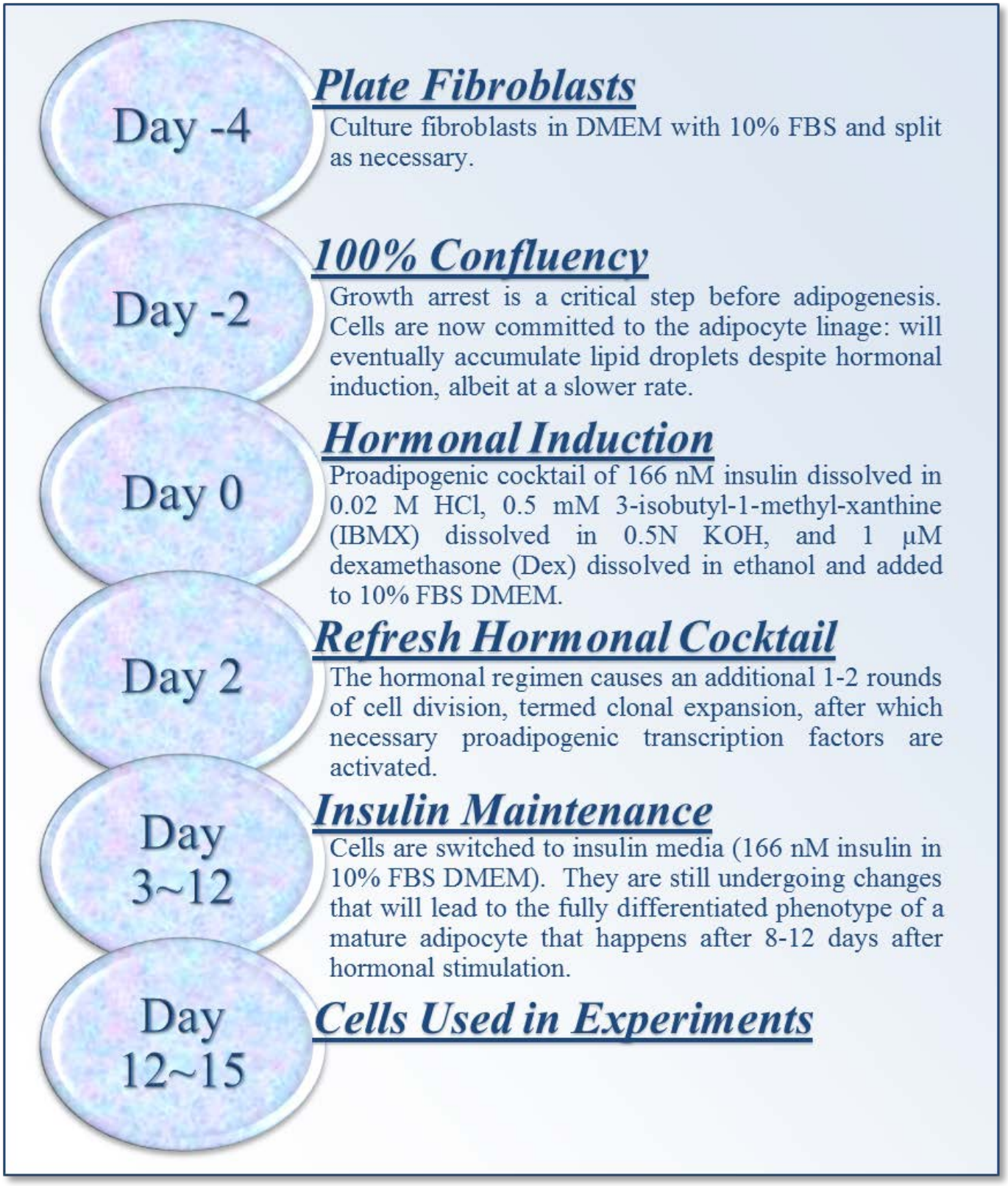

Figure 1: Timeline of Adipocyte Differentiation

One of the most commonly used cellular models to study adipogenesis are murine 3T3-L1 cells. 3T3-L1 cells are considered fibroblasts until 2 days after they have reached 100% confluency. At this point, the cells are exposed to a hormonal cocktail of insulin, dexamethasone (Dex), and isobutylmethylxanthine (IBMX) in media supplemented with fetal bovine serum (FBS) to stimulate differentiation (Kuri-Harcuch and Castro-Munozledo 1984). *In vitro*, the hormonal regimen causes the cells to undergo an additional 1-2 rounds of cell division, termed clonal expansion, after which key transcription factors such as PPARγ and C/EBPα are activated (Brun et al 1996). These two transcription factors then interact with cyclin-dependent kinase inhibitors and co-activators, such as the p160 family proteins and CBP/p300. The proteins then assist with transactivation and promote adipogenesis (Rosen and MacDougald 2006). Often times, the co-activators possess chromatin-remodeling ability, such as histone acetyltransferase activity.

The full understanding of the induction of the transcription factors is unclear; however, after up-regulation of these genes, preadipocytes undergo a final permanent growth arrest and are now committed to the adipocyte lineage. From this point, the cells are referred to adipocytes, although they are still undergoing changes that will lead to the fully differentiated phenotype of a mature adipocyte. This will happen as soon as 10 days after hormonal stimulation. Once differentiated, mature adipocytes are terminally differentiated and thought to be incapable of cell division (Findeisen et al 2011, Hallenborg et al 2012, Rosen and MacDougald 2006, Tang et al 2003).

Adipocyte function

A primary function of adipocytes, with an approximate lifespan of 10 years, is to serve as reservoir of energy in the form of triacylglycerol and to supply non-esterified fatty acids to the body as needed. Adipose tissue plays a role in cushioning and protecting our internal organs as well. Brown adipose tissue is present mainly in infants and hibernating mammals and functions to generate body heat (Cannon and Nedergaard 2004), unlike white adipose tissue, which is non-thermogenic. In humans, it is difficult to detect depots of brown fat past 1-2 years of age (Knittle et al 1979, McDonald and Horwitz 1999). Furthermore, brown adipose tissue has little to do with the development of excess body fat in adults. Therefore, only white adipose tissue is associated with the accumulation of excess body fat, and thus the focus of our research.

In the 1980s, the incorrect assumption was made that adipocytes were merely depots in which to store fat. It is now known adipose tissue is highly complex, immune regulating, metabolically active organ that is necessary to sustain life (Trayhurn and Beattie 2001). We need adipocytes to produce hormones such as resistin, estrogen, leptin, adiponectin, interleukin 6 (IL-6), estradiol, plasminogen activator inhibitor-1 and TNF-α among others (Beltowski 2003, Trayhurn 2001, Trayhurn and Wood 2004).

Ideally, adipocytes are performing their function correctly which is storing (lipogenesis) and releasing fat (lipolysis) as necessary to meet the demands of the individual, while remaining constant in size (Arner et al 2011). The pathways regulating lipolysis and lipogenesis depend heavily on genetic and environmental factors (Brasaemle 2007). Lipoprotein lipase, located on the endothelial surface of adipocytes, is a triglyceride hydrolase. Lipoproteins in circulation give up their free fatty acids to adipocytes after

hydrolyzation by lipoprotein lipase. The free fatty acids (FFAs) are reassembled into triglycerides (TG) in the adipocyte by esterifying the FFAs onto a glycerol molecule. Feeding and fasting conditions determine how fast adipocytes will uptake lipids. When newly recruited adipocytes are in the presence of excess feeding, they grow at a higher rate than adipocytes exposed to normal conditions. Therefore, under high energy conditions, newly formed adipocytes can reach their threshold sooner, possibly contributing to some types of metabolic dysfunction (Wellen and Hotamisligil 2003).

Adipocytes are highly dynamic and constantly shuttle lipids between various adipose tissue depots throughout the body (Casteilla et al 2005). The process of how adipose tissue uptakes lipids and redistributes them throughout the body in times of need is not fully understood, however their function is critical in maintaining energy homeostasis and whole body insulin sensitivity. Unfortunately, this process is difficult to study because adipocytes are highly dynamic, constantly redistributing TG as well as regenerating and recycling their own adipocyte pool (Spalding et al 2008). A major component of the mobilization of FFAs from adipose TG stores are the enzymes adipose tissue triacylglycerol lipase and hormone sensitive lipase that account for most of the hydrolase activity in adipose tissue (Brasaemle 2007). Specifically, these enzymes hydrolyze TGs to produce FFAs and glycerol that are released from the adipocyte to meet the metabolic demands of the individual (Reshef et al 2003).

Adipocytes can fluctuate in size and vary in metabolism over their lifespan (YH Lee et al 2013) depending on a wide variety of factors such as; age, feeding status, growth cycle, cytokines production, gender and nutritional status (Berthiaume et al 2004, Bourlier et al 2008, de Souza et al 2001, Pang et al 2008, Saillan-Barreau et al 2003, Strissel et al

2007, Suga et al 2009, Wilson-Fritch et al 2004). Hormone levels play a large role in the regulation of adipocyte size (Carmean et al 2013, Havel 2004). For example, glucocorticoids such as 11-beta hydroxysteroid dehydrogenase 1 promote fat deposition (MJ Lee et al 2013), while other hormones, like adiponectin can, confer insulin sensitivity and lower FFAs and TGs (Berg et al 2002).

Lipid droplets

Lipid droplets are cellular lipid storage organelles that are the preferred reservoir of TG storage (Walther and Farese 2012). Because lipid droplets are central to energy homeostasis, their function and structure have been elucidated as critical factors in the development of metabolic disease (Konige et al 2013). Lipid droplets confer protection and provide a high degree of organization for TG storage (Paar et al 2012). Lipids that accumulate extracellularly or ectopically are often associated with lipid metabolism dysfunction (Mlinar and Marc 2011, Previs et al 2013). In white adipose tissue, lipid droplets begin small and then coalesce into a fewer larger droplets over time (Z Sun et al 2013). In fact, most mature adipocytes have only a single lipid droplet that occupies around 90 percent of its volume (Fujimoto and Parton 2011). Lipid droplets found in other tissues are usually less than 1 μm in diameter. In tissues affected by metabolic disturbances, such as the liver during hepatic steatosis, the size of a lipid droplet can be up to 10 μm (Reue 2011). To see a representative photo of a differentiated 3T3-L1 adipocyte, refer to Figure 2: Lipid accumulation in 3T3-L1 adipocytes.

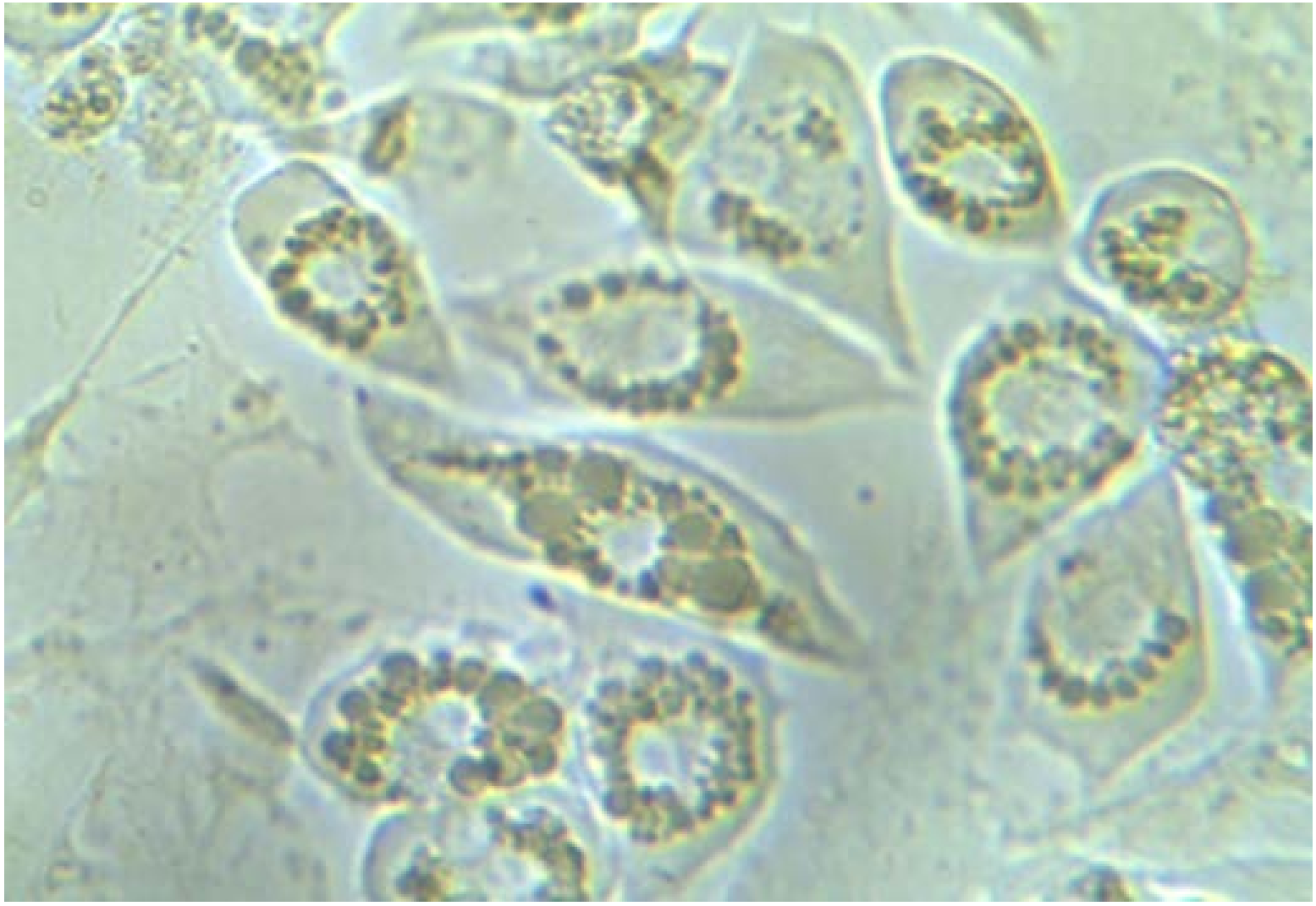

Figure 2: Lipid accumulation in 3T3-L1 adipocytes

Example of 3T3-L1 adipocytes, differentiated as indicated above, viewed under a light microscope at 400x. Round droplets inside cells are triglycerides. Note that not all fibroblast are able to differentiate into adipocytes, as evidenced by their fibroblasts like shape and lack of lipid accumulation.

Individual lipid droplets are covered in proteins that serve metabolic and structural functions (Smith and Ordovas 2012). The proteins provide lipid droplets with their only form of mobility within the cell. The first lipid droplet associated proteins that were identified were perilipins (Greenberg et al 1991), and they serve to help store and release of TG from the lipid droplets (Brasaemle 2007, Granneman et al 2011, Tai and Ordovas 2007, Xu et al 2006). Phosphorylation of perilipins is essential for hormone sensitive lipase translocation to the surface lipid droplet, which is a key factor in stimulating lipolysis (Londos et al 2005). Perilipin under- or over- expression has the potential to promote lipid storage disorders and metabolic disturbances, such as lipodystrophy (Wang et al 2011).

Other proteins associated with lipid droplets are various enzymes involved in triacylglycerol and phospholipid biosynthesis (Brasaemle 2007, Konige, et al. 2013). Such example are: acyl-CoA diacylglycerol acyltransferases, which catalyze the formation of TG from diacylglycerol and acyl CoA; acyl-CoA synthetase enzymes, which are required for fatty acid catabolism & remodeling of membranes; and phosphocholine cytidylyltransferase, which is the rate controlling enzyme in the phosphatidylcholine biosynthesis pathway during de novo lipid synthesis.

Adipose Tissue Plasticity

Preadipocytes as multipotent stem cells

Adipocytes are metabolically and phenotypically distinct from preadipocytes (Sorisky 1999). Mature adipocytes are terminally differentiated, express lipogenic genes, have extensive lipid accumulation and respond to hormonal signals such as insulin (Hauner et al 1987, Shimizu et al 1986, Wiederer and Loffler 1987). Preadipocytes are sensitive to

their environment and have key roles in adipose tissue maintenance, beyond their role in serving as adipocyte precursors (Meijer et al 2011). Preadipocytes are multipotent and have the potential, given the right hormonal signaling, to become osteoblasts, endothelial cells, myoblasts, chondrocytes and several other cell types (Miranville et al 2004, Zuk 2001, Zuk et al 2002). The number of preadipocytes that are able to differentiate successfully into mature adipocytes is dependent on the BMI of the individual and the size of the adipocyte (Sorisky et al 2013). The larger the BMI and size of the adipocyte, the less likely preadipocytes are to differentiate (Kelly, et al. 2008).

Proinflammatory cytokines such as TNF-α, IL-6, transforming growth factor and interferon-γ can inhibit preadipocyte differentiation; however, the precise mechanisms of how they affect adipocyte differentiation remains unclear (Tchkonia et al 2010). Beyond being immature adipocytes, preadipocytes have important immune modulating functions (Cousin et al 1999, Isakson et al 2009, Sorisky, et al. 2013). For example, plasma IL-6, a cytokine associated with aging, is produced by preadipocytes (Mohamed-Ali et al 1997). Furthermore, preadipocytes express known immune cell markers CD11 and CD68, which are associated with an inflammatory response (Trayhurn and Wood 2004, Zeyda and Stulnig 2007).

Preadipocytes can behave similarly to macrophages and have analogous functions, such as making chemokines and engulfing bacteria (Tchkonia, et al. 2010). Preadipocytes from obese individuals do not behave like typical adipocyte precursors when co-cultured with TNF-α, but instead express a proinflammatory macrophage-like phenotype (Isakson, et al. 2009). Due to the macrophage-like state of preadipocytes from obese individuals, and that macrophages accumulate in the adipose tissue of obese individuals (Weisberg et

al 2003), it is probable that many adipose precursor cells are be unable to undergo adipogenesis. This could cause decreased lipid accumulation capacity, thus setting the foundation for lipid storage diseases and promoting adipocyte tissue dysfunction (Moest, et al. 2013).

Adipose tissue remodeling

Adipose tissue remodeling is a hot topic for obesity researchers, but research has generated more questions than answers (Arner, et al. 2011). A better understanding exists for the mechanisms causing adipocyte differentiation than the mechanisms responsible for maintaining adipocytes throughout their lifespan (Erener et al 2012, Hallenborg, et al. 2012, Hou et al 2012, Tchkonia et al 2007). It is estimated that around 10% of all adipocytes in adults are replaced every year, independent of the individual's age and BMI (Spalding, et al. 2008). It has yet to be determined if adipocyte size is related to the age of the adipocyte (Faust et al 1978, Obst et al 1981).

Adipocytes, like other terminally differentiated cells, are comparatively long-lived and more resistant to cell death (Strissel, et al. 2007). However, during obesity there is an increase in adipocyte necrosis, death and senescence which is associated with an increase in macrophage and immune cell infiltration (Lafontan 2013). The necrotic tissue is characterized by the formation of macrophage syncytia and the increased expression of proinflammatory cytokines (Cinti et al 2005). Adipocytes have an upper size limit and cannot continue to increase their size indefinitely under positive energy balance. Large adipocytes that are incapable of storing more fat are fragile and undergo cell death or necrosis (Monteiro et al 2006).

Large adipocytes can be metabolically distinct from small adipocytes (Jo et al 2009, Skurk et al 2007). It is unclear, and somewhat controversial, if size offers any advantage especially given the metabolic differences and functions of various adipose depots (subcutaneous fat vs. visceral fat or white adipose tissue vs. brown adipose tissue). Obese individuals that remain insulin sensitive have smaller adipocytes and less macrophages in their adipose tissue, particularly in their subcutaneous depot, than compared to obese individuals who are insulin resistant controls, (Molgat et al 2011). Thus, the dynamics regulating adipocyte cells involves several organs and body systems (LK Meyer et al 2013).

In the case of overfeeding, it is possible that preadipocytes do not or cannot normally replicate, especially since the cellular dynamics controlling differentiation are adipose depot dependent. For example, visceral and subcutaneous adipose depots have different differentiation responses to the similar environmental stimuli (Tchoukalova et al 2010). If the preadipocyte pool only undergoes partial adipogenesis, it will resulting in a non-functional, immature adipocytes that are likely to increase local and systemic inflammation.

Under fasting conditions, fat can be mobilized for fuel by lipolysis. During lipolysis, the stored TG in the adipocyte is hydrolyzes and it causes the adipocyte to shrink. Conversely, during times of excess, adipose tissue can expand by storing more TG. There are physical limitations to storing TG in adipocytes. When adipocytes are forced to replicate due to extreme conditions, they do so with many errors, giving rise to malfunctioning adipocytes or promote ectopic lipid accumulation (Girousse and Langin 2012), which perpetuate the cycle of inflammation and insulin resistance (Yang et al 2012).

Hypertrophy and hyperplasia

The expansion of adipose tissue can be due to hypertrophy, an increase in the adipocyte size, hyperplasia, an increase in adipocyte number, or both (Jo, et al. 2009). Factors affecting hyperplasia and/or hypertrophy are the individual's genes, age, gender, environment, total body fat percentage, nutritional status (total calories vs. macronutrient breakdown) and the adipose depot origin (subcutaneous, visceral etc.) among others. It is likely that hypertrophic and hyperplastic cells exist together in dysfunctional adipose tissue, and both create conditions that favor the propagation of the other. If this is the case, hypertrophic and hyperplastic adipocytes contribute to the toxic cellular environment that is present in many diseases associated with obesity.

Hyperplasia occurs when adipocytes are unable to take up any more TG, inducing the creation of new adipocytes from adipose cell precursors in the mesenchymal stem cell pool. It is thought hypertrophic adipocytes are inefficient for storing or releasing energy, which creates an environment that promotes hyperplasia. Therefore, hypertrophy affects not only the enlarged cells themselves, but create a toxic environment and promote dysfunction in neighboring cells (Sun et al 2012).

During obesity, the rate of lipolysis is proportional to the adipocyte's size, with larger adipocytes having increased rates of lipolysis (Wueest et al 2009). The size of the adipocyte may correlated with the extent of insulin resistance and other pathologies associated with obesity. Hypertrophic adipocytes are thought to secrete proinflammatory factors, which could explain why hypertrophic adipocytes are associated with a risk of type 2 diabetes (Yang, et al. 2012). Neither hypertrophy nor hyperplasia are ideal (Hanamoto et al 2013, Jo, et al. 2009), and further research is needed to elucidate how adipocytes

maintain equilibrium and to determine if adipose expansion causes or contributes to adipocyte dysfunction. However, there are obvious difficulties with gathering this data because real time monitoring of individual adipocytes for a sufficient amount of time *in vivo* is not feasible at this point (Day 1999).

Metabolic Dysfunctions

Obesity

Obesity can be simply defined as a BMI greater than 30 kilograms/meters squared. Because of the complex and highly regulated signaling pathways within the body that control metabolism, obesity is highly resistant to treatment (Ahima 2006, Fonseca-Alaniz et al 2007, Galic et al 2010, Mohamed-Ali et al 1998). Usually obesity results from an overabundance of white adipose tissue (WAT). The overabundance of WAT can stem either from an increased number of adipocytes storing fat or from larger adipocytes that continue to store more fat (Spalding, et al. 2008). Obese individuals usually have an increased risk of chronic disease, however not all of the risk can be attributed to an increase in WAT (Ahima and Lazar 2013). Obesity is a causative agent in various metabolic syndromes, in part due to the increased oxidative stress burden (Berkemeyer 2010). Oxidative stress is known to promote inflammation and vice versa (Bondia-Pons et al 2012, Fernandez-Sanchez et al 2011), but it was unknown until recently that fat accumulation is correlated with systemic oxidative stress in humans (Furukawa et al 2004). Regardless of the origins underlying the inflammatory state, infiltration by macrophages and other immune cells further burden adipose tissue promoting insulin resistance (Calabro and Yeh 2007, Siklova-Vitkova et al 2012, Zeyda and Stulnig 2007, Zhang and Huang 2012).

During obesity, adipocytes can become dysfunctional. These hallmarks of adipocyte dysfunction are increased lipolysis, abnormal cytokine secretion, increased insulin resistance and impairments in triglyceride storage (Guilherme, et al. 2008, Hotamisligil et al 1995, Weisberg, et al. 2003). Furthermore, dysfunctional adipose tissue will attract macrophages and promote an inflammatory state (O'Rourke et al 2012, Trayhurn and Wood 2004). Obese individuals with high levels of FFAs may undergo extreme lipogenesis in attempts to store the FFAs (McGarry and Dobbins 1999). However, this will disturb homeostasis, encourage lipodystrophy and cause adipocyte dysfunction (Arner, et al. 2011).

Dysfunctions in lipogenesis and lipolysis can promote various lipodystrophies (Vigouroux et al 2011) and greatly increase the risk of lipotoxicity. Lipotoxicity causes excess fat accumulation in non-adipose tissue such as skeletal muscles, liver, heart, pancreatic beta-cells and kidneys (McGarry and Dobbins 1999, Unger 1995). Obesity and the related comorbidities are associated with lipotoxicity, and overfeeding increases levels of FFAs which dramatically increases lipogenesis in attempts to lower plasma FFAs by shuttling them into adipocytes (McGarry and Dobbins 1999). In dysfunctional adipose tissue lipotoxicity can; increase ROS causing beta-cell deterioration (Lenzen 2008), disturb mitochondrial homeostasis (Schrauwen and Hesselink 2004, Supale et al 2012), promote nonalcoholic steatohepatitis (Cusi 2012) and impair insulin sensitivity (Vigouroux, et al. 2011) all driving mechanisms behind the comorbidities associated with obesity.

Type 2 diabetes

Type 2 diabetes is characterized by insulin resistance and typically develops over a period of many years (Stumvoll et al 2005). Insulin resistance is the inability of insulin sensitive cells to utilize glucose correctly (Ye 2013), usually due to the defects in the insulin-signaling pathway. During the initial stages of insulin resistance, the pancreatic beta-cells will continuously produce insulin in response to hyperglycemia. However, the beta-cells will eventually reach a critical point at which they can no longer sustain insulin production. This is believed to be one of the most important factors that precipitate the onset of type 2 diabetes (Eberle et al 2013).

The formal diagnosis of type 2 diabetes (Alberti and Zimmet 1998) occurs when fasting blood glucose levels are greater than 125 mg/dL, but the exact event or process that tips the scale from insulin resistance to diabetes is harder to determine and predict. There are many factors that affect this process in individuals (Stumvoll, et al. 2005), and that is why we are attempting to elucidate the processes that lead to the progression of insulin resistance and type 2 diabetes by studying how the key proteins, ATM and DNA-PK, regulate oxidative tone during adipogenesis.

Cellular senescence

Normal somatic cells undergo senescence after they reach their proliferative capacity (Goldstein 1990), which is tissue specific. Senescence is the loss of proliferative potential, not permanent cell cycle arrest because cells can undergo cell cycle arrest without being senescent (Blagosklonny 2011). Senescent cells are still metabolically active and can carry out various cellular processes (Goldstein 1990). Senescence due to irreversible growth-

arrest is termed replicative senescence, during which the cells appear flat. Senescence promotes aging, but is also a barrier of tumorigenesis, stifling cancer at an early stage (Patil et al 2005). At a pH of 4.5- 6.0, senescence-associated β-galactosidase activity can be detected in senescent cells. The reason for increased senescence-associated β-galactosidase activity in senescent cells is due to the β-galactosidase originating from the lysosomes. During senescence, there is an increase in the size and number of lysosomes. β-galactosidase activity is an outcome of senescence, but the activity of the enzyme is not required for the induction of senescence (Lee et al 2006).

In the field of DNA damage repair and cancer, the cellular senescent phenotype is well studied (Campisi 2011); however, we are interested in the effects of senescence in adipose tissue. Principally, we are interested in the mechanisms that control preadipocyte senescence, which we believe to be a factor contributing to dysfunctional adipose tissue. Emerging lines of evidence show that differentiated adipocytes in fat tissue display senescent-like characteristics, challenging the basic concepts of cellular senescence (Tchkonia, et al. 2010). Under oxidative stress, adipocytes exhibit a senescent/proinflammatory phenotype characterized by increased production of ROS, more DNA damage, shortened telomeres and impaired glucose uptake (Monickaraj et al 2013). Interestingly, removal of senescent cells delays age related pathologies in various tissues in mice (Baker et al 2011). Thus, prevention, elimination or treatment of senescent cells in adipose tissue is a possible therapeutic intervention for the treatment of the comorbidities of obesity.

The age related changes in adipose tissue of obese individuals, whether they are young or old, may have severe systemic consequences and lead to premature onset of chronic disease and death. Senescent cells secrete factors that can negatively alter their microenvironment (Coppe et al 2010), which is termed the bystander effect. The bystander effects also encompasses the increased oxidative stress and concentration of proinflammatory cytokines (Campisi and d'Adda di Fagagna 2007, Nelson et al 2012) in the vicinity of senescent cells. It has been hypothesized that DNA damage response proteins facilitate this process to warn surrounding cells of their potentially harmful condition (Rodier et al 2009).

Mechanisms of Metabolic Dysfunction

Oxidative stress

Since oxidative stress and inflammation are central to obesity, it is important to understand adipocytes in terms of their contributions to and role in ROS metabolism. ROS is a collective term for chemical species (e.g. superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO\bullet$)) that are formed after incomplete reduction of oxygen. ROS are potentially toxic because they contain oxygen, which makes them highly chemically reactive. The imbalance of antioxidant events and production of ROS generates oxidative stress, which can lead to tissue damage (Bergamini et al 2004). For example, oxidative stress can cause structural and conformational damage to proteins, thereby generating new antigens that provoke immune responses and result in inflammation (Adler et al 1999, Cabiscol et al 2000).

Oxidative damage to proteins can affect the function of the entire organism (i.e. by damaging enzymes, receptors, transport proteins, cytoskeleton components and disrupt DNA repair pathway activity). In particular, insulin exposure to ROS increases the concentration of free carbonyl groups, which encourages the formation of peroxidation products on insulin's amino acid side chains. The resulting changes can cause the insulin protein to aggregate, cross-link, and fragment, thus affecting immunoreactivity (Olivares-Corichi et al 2005), and perhaps giving biological evidence of insulin resistance *in vivo*.

ROS are required signaling molecules that are necessary in most basic biological processes. However, they are harmful and causative agents in many diseases when their balance is disturbed. ROS molecular recognition is at the level of amino acids, or the atomic level, unlike other types of specific signaling, in which a ligand binds non-covalently to its receptor via conformational changes. To date, there is much controversy regarding the specificity of ROS signaling (Adler, et al. 1999). ROS signaling occurs through chemical signaling that leads to covalent protein modifications, which presumably would lead to indiscriminate and non-specific ROS signaling. However, ROS redox mechanisms have high specificity, but it is not clear how this is achieved *in vivo* (D'Autréaux and Toledano 2007, Møller and Sweetlove 2010).

ROS are crucial regulators in most systemic processes in the body. In many diseased states, such as obesity, ROS and oxidative stress are increased (Gutowski and Kowalczyk 2013). The genetic, proteomic, cellular and systemic damage from oxidative stress during obesity could be responsible for inflammation and many of the pathologies associated with the obese state. Chronic low-level inflammation is a hallmark of most diseases related to obesity, such as atherosclerosis and type 2 diabetes (Duncan et al 2003, Wellen and

Hotamisligil 2005). Obese people also have increased biomarkers of oxidative stress due to over nutrition and inflammation (Block et al 2002, Dandona 2002, Dandona and Aljada 2002). Further, obese people have more plasma ROS than people of a normal weight because their excessive fat may promote an oxidizing extracellular environment (Gletsu-Miller et al 2009).

When adipose tissue is subject to increased oxidative stress and inflammation, it impairs adipocyte maturation from preadipocyte precursors (Findeisen, et al. 2011). This can cause defective adipokine (cytokines released from adipocytes) and insulin signaling (Stentz et al 2004). Defective signaling can cause an infiltration of inflammatory cells, increased cell death, and the loss of antioxidant protection (Fernandez-Sanchez, et al. 2011, Mlinar and Marc 2011, Parola and Marra 2011). Therefore, oxidative stress is perhaps the most common pathology that all of the various comorbidities of obesity have in common (Kral et al 2010, Lepor et al 2013, Malik, et al. 2013, Must, et al. 1999).

Immune response

When a pathogen is able to infect our bodies, constantly circulating leukocytes will detect the infection and cross the blood barrier to the site of the infection. This then starts the inflammatory process, which is a consequence of the body's immune response. Inflammation is characterized by redness, heat, swelling and pain and is a normal response in the process of healing. Varying degrees of the inflammatory response are present at all times in many disease states, particularly those that are associated with obesity, such as type 2 diabetes (Donath et al 2013, Ye 2013). Thus, inflammation is a double-edged sword,

in that it is necessary for healing from various infections but also can exacerbate many disease states.

The attraction of leukocytes to tissue is regulated by chemokines, a type of cytokine. Chemokines are secreted by damaged tissues, which cause a local inflammation response which directs circulating immune cells to the damaged tissue (Luster 1998). Circulating leukocytes enter infected tissue via extravasation. Extravasation is the process where leukocytes adhere to and pass between endothelial cells lining walls of blood vessels (Carmeliet et al 2001). The endothelial cells express specific leukocyte cell adhesion molecules that direct leukocytes to the site of the infection. Some cell adhesion molecules are expressed constitutively while others are only expressed after cytokine stimulation. Therefore, the expression of cell adhesion molecules on both endothelial cells and leukocytes is important for the induction of the inflammation response.

The biological activity of cytokines in promoting inflammation in response to an infection is necessary to promote healing. We believe this same process may exacerbate and induce many of the comorbidities of obesity. Below, is an explanation of how cytokines affect disease resistance, in terms of B and T cell activation, and insights on how this helpful process may be harmful in perpetuating the comorbidities of obesity.

B cells and T cells both recognize antigens, but B cells can directly recognize antigens via their membrane immunoglobins, while T cell antigen recognition requires the interaction of its T cell receptor with the antigen/ major histocompatibility complex (MHC). In addition, MHC's are only found on antigen presenting cells, while B cells can interact with any cell that express the correct surface markers. Once B and T cells are activated, they can exert numerous biological effects, many of which are done through the

secretion of cytokines. For example, activated T-helper cells can secrete interleukins 2, 4 or 5 that can cause B cell proliferation and induce class switching of B cells to IgE. On the other hand, activated T-helper cells can also secrete interferon gamma, which blocks the action of interleukin 4 in the class switching of B cells to IgE.

Cytokines are low-molecular-weight proteins or glycoproteins that play an important role in cell-to-cell communication. Cytokines have a short half-life in plasma or the blood stream and are principally produced by T-helper cells, dendritic cells and macrophages. Cytokines can regulate the development of immune effector cells and some cytokines possess the ability to have direct immune effector functions themselves. The susceptibility of cells to cytokines is dependent on specific membrane receptors. Most cytokines have very high affinity for their receptors, so biological effects can result from picomolar concentrations of cytokines (Nathan and Sporn 1991).

Once cytokines are released, they can activate and start signal transduction pathways that can affect a large network of interacting cells (Starr et al 1997). Cytokines are required for many responses, such as the development of the cellular and humoral response, induction of the inflammatory response, regulation of hematopoiesis, control of proliferation and differentiation and wound healing. The functions of cytokines are exerted in an antigen-nonspecific manner. Whichever cells have appropriate surface receptors are susceptible to cytokine signaling. Thus, the specificity of cytokines is primarily controlled by expression of cytokine receptors on individual cells.

The term cytokine usually refers to proteins secreted from immune cells. However, preadipocytes, can function similarly to immune cells and secrete cytokines (Wang et al 2004). Cytokines secreted from adipose tissue are technically termed adipokines (Lau et

al 2005, Trayhurn and Wood 2004, Trayhurn and Wood 2005), although there is inconsistency among the literature regarding nomenclature and the term cytokine is commonly used. Unfortunately, a hallmark of many diseases is chronic inflammation, which is usually the accumulation and activation of macrophages. This accumulation can stimulate fibroblast production, resulting in fibrosis and scar tissue that interferes with normal tissue function. An applicable example of how this happens during metabolic dysfunction would be the fibrotic conditions that affect adipose tissue and the kidney, liver and heart during metabolic dysfunction (K Sun et al 2013). There is even a life-threatening disease related to chronic macrophage activation called macrophage-activation syndrome. The disease is commonly observed in children with chronic immune dysfunction (such as those with juvenile idiopathic arthritis or systemic lupus erythematosus). This disease is often under- or mis- diagnosed and the only available therapies are immune suppressing drugs, which are known to be a difficult long-term treatment option.

Linking Metabolic Derangements to Adipocytes

Inflammation

The obese state is usually synonymous with increased oxidative stress(Furukawa, et al. 2004, Karbownik-Lewinska et al 2012), chronic inflammation (Cildir et al 2013) and a distorted immune response (Cancello and Clement 2006, Donath, et al. 2013). Adipose tissue of obese people has more macrophages than adipose tissue from normal individuals. This fact was some of the earliest evidence that demonstrated a mechanistic link between increased inflammation and the obese state (Kress, et al. 2005). An expression analysis of macrophage and non-macrophage cell populations in adipose tissue using macrophage

markers, such as CD68 was performed, and revealed that obese people have more macrophages in their adipose tissue. In addition, macrophages of obese individuals participate directly in adipose tissue inflammation by increasing the expression of IL-6, TNF-α and inducible nitric oxide synthase (iNOS) (Bourlier et al 2012).

TNF-α and IL-6 are proinflammatory cytokines of innate immunity and serve many necessary purposes such as regulating cell death, promoting vasculature, activating neutrophils and help influencing adaptive immunity by promoting proliferation and antibody secretion (Seidell 2000). In terms of insulin resistance and type 2 diabetes, the secretion and plasma concentrations of IL-6 and TNF-α are higher than in healthy individuals and their dysregulation causes problems during many diseased states (Abraham et al 2007). For instance, TNF-α can effect changes that directly impact insulin resistance, such as inhibiting phosphorylation of the insulin receptor and preventing its translocation from the cytoplasm to the membrane and thus promoting hyperglycemia (Popkin and Doak 1998). In addition, IL-6 inhibits insulin receptor signal transduction and insulin action in hepatocytes (Must, et al. 1999). Taken together, the increased concentrations of IL-6 and TNF-α in type 2 diabetics might be explained by dysfunctional preadipocytes acting as immune cells. In acting as immune cells, preadipocytes may secrete high levels of cytokines and causing a feed-forward loop and promoting the disease state typical of obesity and its comorbidities (See Figure 3: Cytokines).

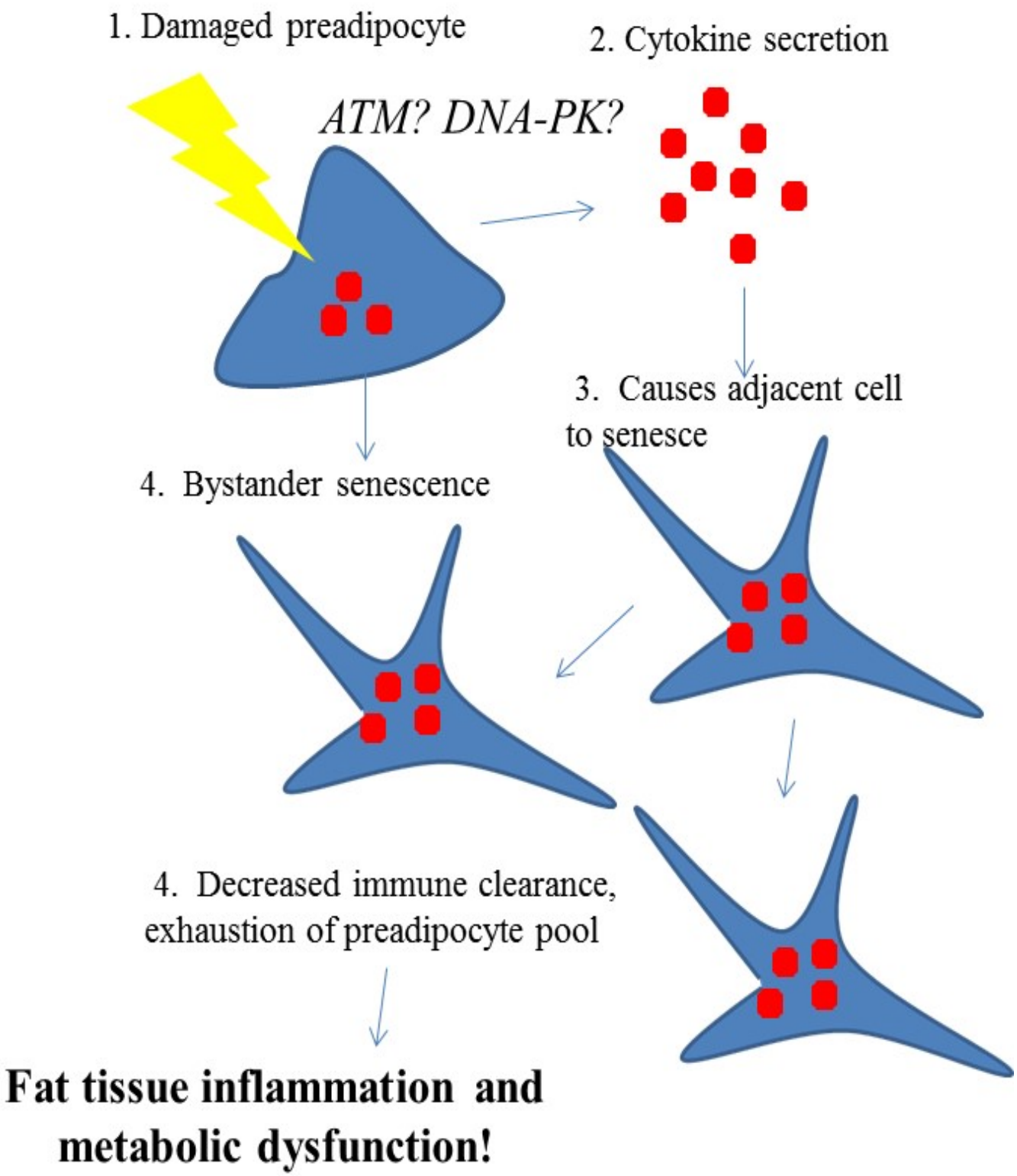

Figure 3: Cytokines

The toxic effects of cytokines may damage preadipocytes beyond repair, causing them to lose their ability to differentiate, even if normal extracellular homeostasis exists. If ability to differentiate is lost, and differentiation is blocked, overall metabolism is negatively affected because the renewal pool of adipocytes comprised, which leaves adipocytes vulnerable to dysfunction.

Insulin resistance

Children, and other mammals during early development, have the unique ability to increase adipocyte number. Adults typically do not make adipocytes unless they to replace ones that have malfunctioned or died (Chumlea et al 1981, Kirkland et al 1990, Strawford et al 2004). Therefore, individuals that were obese as children may be more efficient in storing more fat, either from their diet or from metabolic processes. This is because they have more adipocytes, and thus storage capacity, in adulthood than their peers who were not obese during childhood (Bonnet et al 1970, Spalding, et al. 2008). In adults, there is decreased ability for the body to increase total adipocyte number, and this usually only happens under extreme obesity conditions (Kovsan et al 2011, McDonald and Horwitz 1999). Unfortunately, this inability to make new adipocytes can inflict severe stress on existing adipocytes, causing them to expand to an unnaturally large size, becoming unresponsive, and eventually becoming insulin resistant (West 2009).

The insulin sensitivity of obese individuals varies (Arner, et al. 2011). The location of the excess the adipose tissue is thought to play a key role in determining the insulin sensitivity. An obese individual with a gynoid body shape may retain their insulin sensitivity more than an obese individual with an android body shape with the same body fat percentage (Dolinkova et al 2008, Epel et al 2000, Kaplan 1989). However, more recent studies debate the validity of this presumption. It has been shown that an increase in body fat, not dependent on fat depot distribution, is the strongest correlation for an increase in the risk of developing obesity related diseases (Gregg et al 2005), not body shape or location of fat stores.

There is research that shows the subcutaneous adipose tissue of insulin resistant individuals is composed of smaller adipocytes than adipose tissue from insulin sensitive individuals. Interestingly, these small adipocytes from the insulin resistant individuals had lower lipid storage capacity, which is a hallmark of adipose tissue dysfunction, compared to the adipocytes of insulin sensitive individuals (McLaughlin et al 2010). Yet, drugs used to treat insulin resistance in type 2 diabetics, such as a thiazolidinediones (TZDs) (Day 1999), which are PPARγ agonists (Spiegelman 1998), spur the maturation of new adipocytes, creating increased lipid storage capacity that can help alleviate hyperglycemia and promote insulin sensitivity. TZDs increase the number of small adipocytes and promote insulin sensitivity in these cells, therefore not all small adipocytes are unable to take up lipids properly (Spiegelman 1998).

Some data support that the insulin responsiveness of large and small adipocytes from the same depot does not vary (Wueest, et al. 2009), suggesting that adipocyte size is not related to insulin resistance. Conversely, there are other data that show larger, or hypertrophic adipocytes, are the predominate type of adipocytes found in individuals with insulin resistance (Yang, et al. 2012). Also adipocyte hypertrophy is associated with proinflammatory factors and cellular necrosis (Jo, et al. 2009), both which are known factors that help drive insulin resistance. Furthermore, obese insulin resistant individuals have larger adipocytes and more macrophages in their subcutaneous fat than do obese insulin sensitive individuals (Molgat, et al. 2011). The molecular mechanisms that perpetuate this phenotype are largely misunderstood, but understanding this insulin resistant state will provide clinicians with valuable treatment options in dealing and managing type 2 diabetes (Arner, et al. 2011).

CHAPTER 2: THE KINASE ACTIVITY OF ATM AND DNA-PK_{CS} IS CRITICAL DURING ADIPOGENESIS

Abstract

Adipogenesis, the process of adipocyte differentiation, and lipid droplet formation are key processes regulating adipocyte integrity and preventing dysfunction. It has been shown that both processes are regulated by oxidative stress. The DNA damage repair proteins ATM and DNA-PK_{cs} can be activated in response to oxidative stress in normal human fibroblasts. Therefore, we hypothesize ATM and DNA-PK_{cs} play a critical role in maintaining adipocyte integrity. In this study, the standard adipocyte differentiation process of murine 3T3-L1 preadipocytes and mouse embryonic fibroblasts were manipulated. We used chemical inhibitors of ATM and DNA-PK_{cs}, antioxidants, and prooxidants. After treatments, we studied the cellular morphology, lipid accumulation, oxidative damage, and the expression of lipid droplet associated proteins. We found that ATM and DNA-PK are necessary for lipid accumulation and they exert their effects via a ROS dependent pathway. Based on our results, ATM and DNA-PK could emerge as molecular targets for intervention of obesity and related comorbidities.

Introduction

Rationale

Although the initial studies of ATM and DNA-PK focused on their role in DNA repair, emerging evidence indicates roles beyond their function in the DNA damage

response. ATM is activated by oxidative stress (Guo, et al. 2010a, Guo, et al. 2010b) and we have shown that DNA-PK is necessary to relay the selenium-induced ROS response in human fibroblasts (Rocourt et al 2013). ATM and DNA-PK_{cs} are involved in cellular senescence (Rocourt, et al. 2013, Wu, et al. 2010) and the secreted components of senescent cells are thought to disrupt tissue function and structure (Campisi and d'Adda di Fagagna 2007). As the complications of obesity can in part be explained by the structure and function of adipocytes, our central hypothesis is that ATM and DNA-PK_{cs} are critical for maintaining preadipocyte and adipocyte integrity.

Significance

Although attempts have been made to understand obesity related diseases at the molecular level, obesity, and the related complications, are predicted to continue to increase (Malik, et al. 2013). The mechanism by which being overweight or obese predispose individuals to comorbidities is not clear (Allison et al 1999, Guh et al 2009, Paeratakul et al 2002). To further complicate matters, current screening methods for identifying those at risk for obesity associated comorbidities and metabolic dysfunctions are primarily anthropometric measurements such as BMI and waist circumference (Abate 2012). These fail to capture a percentage of individuals that are risk for developing metabolic diseases because they do not meet the criteria or cut offs (Waugh et al 2013).

Not only is there a need for better screening for obesity-related comorbidities, but there is also a need to better understand the obesity-mortality paradox, in which ~ 10% people who are overweight or mildly obese are metabolically healthy while ~ 8-24% of people with a normal BMI are metabolically unhealthy (Ahima and Lazar 2013, Phillips

2013). We and others (Guilherme, et al. 2008, Trayhurn 2013, Vigouroux, et al. 2011) believe that unifying factors among individuals with metabolic dysfunctions are disturbances in adipose tissue functioning.

Hypothesis

We believe that oxidative stress is central to the comorbidities of obesity. It is our hypothesis that the DNA damage response proteins, ATM and DNA-PK$_{cs}$, serve a critical role in regulating ROS throughout the lifecycle of adipose tissue. It is our view that dysfunctional adipocytes are responsible for the comorbidities of obesity; with the central driving force being ROS and oxidative damage triggering a senescence-associated secretory phenotype (Kirkland et al 2002, Rodier, et al. 2009, Tchkonia, et al. 2010) in preadipocytes, thus creating toxic adipose tissue that causes systemic effects.

To test our hypothesis, we used murine 3T3-L1 fibroblasts and murine embryonic fibroblasts and employed the use of specific kinase inhibitors to determine the requirement of ATM and DNA-PK during adipogenesis. After critical analysis of the data, we performed experiments that attempted to explain the molecular underpinnings of how ATM and DNA-PK contribute to adipocyte homeostasis.

Methods

Cell lines and maintenance

SV40-transformed wild-type, DNA-PK$_{cs}^{-/-}$, Ku70$^{-/-}$ and Ku80$^{-/-}$ Mouse Embryonic Fibroblasts (MEFs) from E13.5 embryos were obtained from UT Southwestern Medical Center. MEFs were grown as previously reported (Rocourt, et al. 2013, S Zhang et al

2011). Murine 3T3-L1 preadipocytes (CL173) were purchased from American Type Culture Collection (Manassas, VA) (See Figure 2: Lipid accumulation in 3T3-L1 adipocytes). Preadipocytes were cultured in Dulbecco's minimum essential medium (DMEM) (GibcoBRL, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (GibcoBRL) and 1% penicillin/streptomycin (GibcoBRL) and were maintained in a 37° C humidified atmosphere in either 20% oxygen or 3% oxygen.

Adipocyte differentiation

Adipogenesis was induced by using induction media; 10% FBS DMEM was supplemented with the hormonal proadipogenic cocktail of 166 nM insulin dissolved in 0.02 M HCl, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) dissolved in 0.5N potassium hydroxide (KOH) and 1 µM dexamethasone (Dex) dissolved in ethanol (Hemati et al 1997) to cells that were two days past 100% confluency (designated Day 0). After two days, the induction media was refreshed for 1 day and then the cells were maintained in insulin media (166 nM insulin in 10% FBS DMEM) until used for experiments, typically on day 12~15 (See Figure 1: Timeline of Adipocyte Differentiation).

Lipid accumulation

To detect lipid droplets, the coverslips were incubated with HCS LipidTOX™ Green neutral lipid stain (1:100 in BSA, Invitrogen Molecular Probes, Carlsbad, CA) for 1 h at room temperature. Then the coverslips were washed with 1x PBS and mounted onto slides with a drop of Prolong® Gold mounting media containing 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen Molecular Probes), which serves to adhere the coverslip to the slide, stain the nuclei and prevent fading of fluorescence.

Treatments

Cells were treated with, without, or in combination with the following: the ATM kinase inhibitor KU 55933 (10 µM in DMSO, Tocris, Ellisville, MO), the DNA-PK kinase inhibitor NU 7026 (10 µM in DMSO, Tocris), the antioxidants N- acetylcysteine (NAC) (10 mM in phosphate buffered saline (PBS), Sigma- Aldrich) and 2, 2, 6, 6-tetramethyl-piperidine-1-oxyl (TEMPO) (1 mM in PBS, Sigma- Aldrich), the prooxidant hydrogen peroxide (H_2O_2) (10 µM in double distilled water, Fischer Scientific, Waltham, MA) and Dimethyl sulfoxide (DMSO) (Vehicle, Sigma- Aldrich,). All chemicals were added to the cell culture and incubated at 37° C for the times specified by the experiment. Please refer to

Appendix for more detailed information.

Co-culture

For co-culture experiments, undifferentiated 3T3-L1 fibroblasts were grown on porous wells that nestle into standard 6-well plates. The fibroblasts were treated with ATM and DNA-PK inhibitors to induce senescence. Upon senescence, the wells with the treated cells were added to individual wells of a 6-well dish that contained 3T3-L1 cells, grown on coverslips that had been hormonally induced to undergo adipogenesis. Thus, by co-culturing, the differentiated 3T3-L1 cells were exposed to the cellular environment and culture medium of the senescent preadipocytes. The differentiated cells were then subjected to immunofluorescence microscopy.

Oil-Red-O staining

Oil-Red-O (ORO) (Sigma-Aldrich) staining was conducted, as previously described (Ramirez-Zacarias et al 1992, Ross et al 1999, Ross et al 2000, van Goor et al 1986) with little modification, in order to determine lipid accumulation _ENREF_7 Dye preparation: 0.5 g of ORO was dissolved in 250 mL isopropanol to form a stock solution. 30 minutes before staining, 20 mL of deionized water was added to 30 mL of the stock solution to make the working solution, and then put on a stir plate at 50° C until adipocyte staining was completed.

Cell staining: Adherent cells were gently rinsed with phosphate-buffered saline (PBS) then fixed in a 4% paraformaldehyde solution for 1 h, washed with 1x PBS then refixed with 4% paraformaldehyde solution for 2-3 days. After fixation, the cells were rinsed with 1x PBS followed by 60% isopropyl alcohol, which was added and allowed to evaporate. The cells were then stained with the ORO working solution for 10 min. Plates were rinsed 3x with tap water and allowed to dry. Photographs were taken, and cells were subsequently analyzed as described below.

Quantification of stained cells: Stained cells were counted under a light microscope (40x- 400x magnification) and given a score based on the percent lipid accumulation when compared to the differentiated control, which we designated to be 100%.

Senescence

To determine senescence, we detected the expression of senescence associated-β-galactosidase (SA-β-gal) by using a Senescence Detection Kit (MBL Co. Ltd., Woburn,

MA) according to the manufacturer's instructions and our previous publication (Rocourt, et al. 2013).

Coverslip preparation

Immunofluorescence analysis was performed as described previously with slight modifications (Nioi et al 2007, Rocourt, et al. 2013) _ENREF_9. MEFs and 3T3-L1 cells were seeded on coverslips in 6-well plates at 70% confluency then differentiated and treated as described above. At the end of the experiment, coverslips were rinsed in PBS, fixed in 4 % paraformaldehyde solution in PBS for 30 min at room temperature and then washed twice with 1x PBS.

Antibody detection

Cells were prepared as described above with minor modifications as follows; after 15 min fixation with a paraformaldehyde solution, the cells were permeabilized with ice-cold methanol for 10 min at -20° C. Next, the cells were permeabilized again for 10 min in 0.3% Triton X-100 and then blocked in 10% normal goat serum / 0.3 M glycine in 1x PBS for 1 h to block non-specific protein-protein interactions. Individual coverslips were hybridized with either of the following antibodies for 1 hour (h) at room temperature (RT): monoclonal anti-γH2A.X at Serine 139 (γH2A.X) (1: 250 in BSA, Abcam, Boston, MA) and perilipin D418 (1:200 in BSA, Cell Signaling, Danvers, MA).

After incubation with antibodies, the coverslips were washed in 1x PBS, and incubated with the appropriate secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit for perilipin and Alexa Fluor 594-conjugated goat anti-mouse γH2A.X both from Invitrogen Molecular Probes) in BSA (1:200 dilution) for 1 h at RT in the dark. Cells

were then washed 3x in 1x PBS and the coverslips were mounted onto slides that contained a drop of DAPI and were visualized using immunofluorescent microscopy.

Quantification of antibody data

The immunostaining was visualized using the 63x oil-immersion lens on a Zeiss Axio Observer Z1m (Zeiss, Thornwood, NY). A z-stack of 10 photos was taken of the appropriate channels (DAPI, GFP and DsRed). Images were obtained using the same parameters of brightness, contrast and exposure time (DAPI 5 ms, LipidTOX 125 ms, γH2A.X 40 ms, and perilipin 75 ms). Images were then processed using the deconvolution function of the software AxioVision Release 4.7.2.0. Lipid droplet data were quantified by comparing the saturation, size and distribution of lipid droplets (LipidTOX) as well as counting foci as described previously (Rocourt, et al. 2013) for perilipin and γH2A.X data.

Granularity

Flow cytometry was conducted to determine the degree of lipid accumulation as previously described (Lee et al 2004). In preparation for flow cytometry, the samples were trypsinized for 3 min, then serum-containing media was added to stop digestion and the suspension was centrifuged (1000 rpm, 2 min at RT) to form a pellet. The supernatant was discarded, the cells resuspended in ice-cold 1x PBS and were immediately analyzed using a BD FACSCanto II flow cytometer. Increasing side scatter (SSC) and forward scatter (FSC) were used to indicate lipid accumulation and cell size, respectively. The data was analyzed using FlowJo version 7.6.5 (Tree Star Inc., Ashland, OR).

Statistics

All the experiments were independently performed and repeated three times. We defined the control as differentiated 3T3-L1 or MEF cells, induced to undergo adipogenesis as described above unless stated otherwise in the text. The data were analyzed using GraphPad Prism software version 5.04 (GraphPad Software Inc., La Jolla, CA). Two tailed, one- sample t-tests were applied to determine statistical significance (*, $p<0.05$) between treatments.

Results

ATM and DNA-PK kinase activity are necessary for lipid accumulation

Lipid accumulation in 3T3-L1 cells was significantly decreased after treatment with the ATM and DNA-PK competitive protein kinase inhibitors, NU 7026 and KU 55933 (See Figure 4: ATM and DNA-PK kinase activity are necessary for lipid accumulation). The maximal effect of kinase inhibition on lipid droplet formation occurred when inhibition was induced prior to hormonal induction of adipogenesis (See Figure 5: ATM and DNA-PK kinase inhibition has maximal effect on lipid accumulation when added before inducing adipogenesis).

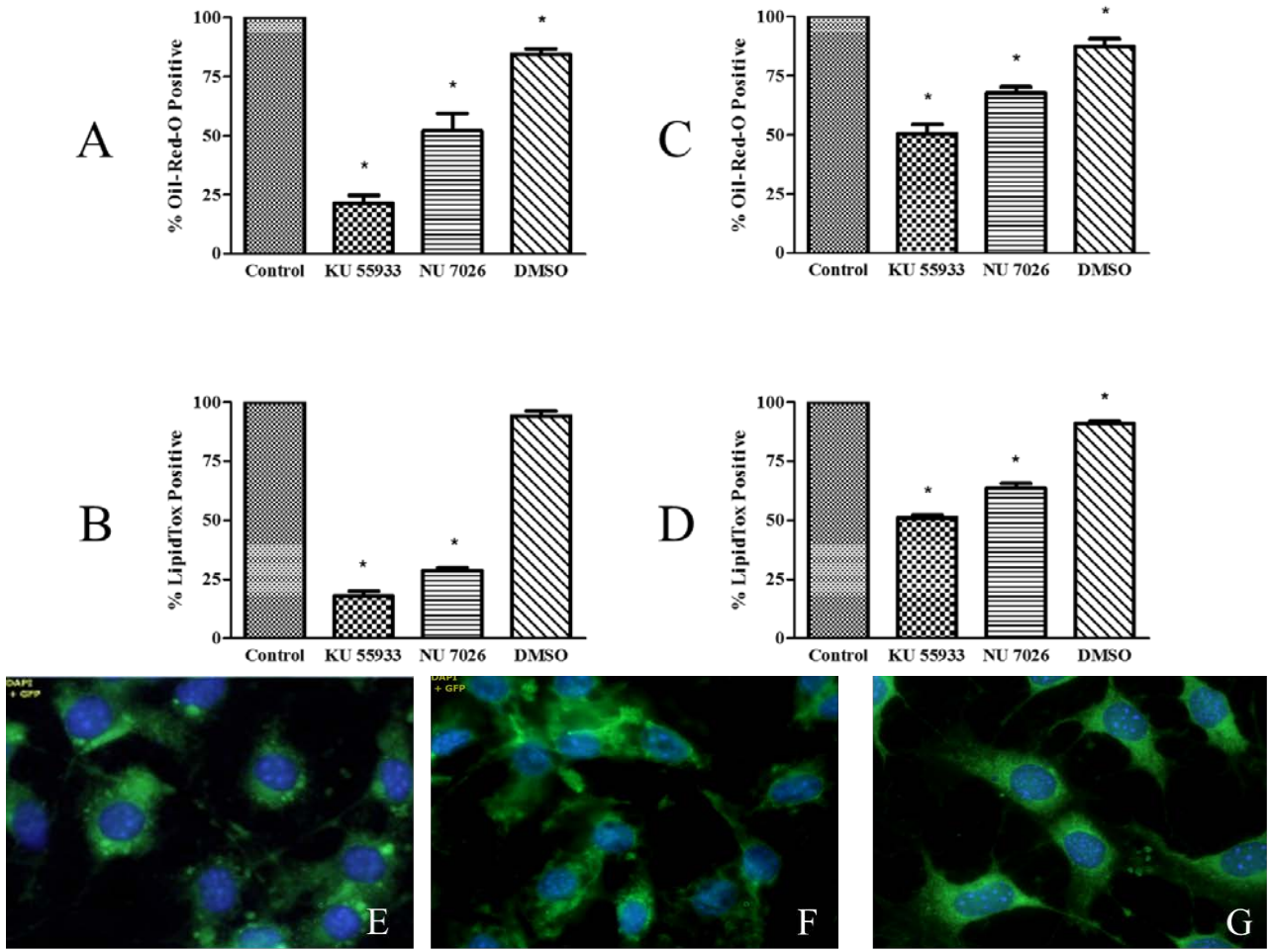

Figure 4: ATM and DNA-PK kinase activity are necessary for lipid accumulation

3T3-L1 fibroblasts were differentiated and treated with the ATM inhibitor, KU 55933, (10 µM in DMSO; 48 h treatment started day 0 of adipogenesis) or the DNA-PK inhibitor, NU 7026, (10 µM in DMSO; 48 h treatment started day 0 of adipogenesis) and cultured in 20% oxygen (A & B) or 3% oxygen (C & D). Lipid accumulation was determined by Oil-Red-O staining (A & C), analyzed using a light microscope (400x), or LipidTOX (1:200 in BSA, 1 h RT) staining (B & D), analyzed using an immunofluorescent microscope (63x oil-immersion). Each treatment was given a score based on the percent Oil-Red-O or LipidTOX saturation when compared to the differentiated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells). (E-G) Representative oil-immersion photographs (DAPI, blue color, nuclear stain; GFP, green color, lipid droplet stain) of differentiated 3T3-L1 fibroblasts (E) treated with 10 µM KU 55933 (F) or 10 µM NU 7026 (G) and cultured in 20% oxygen.

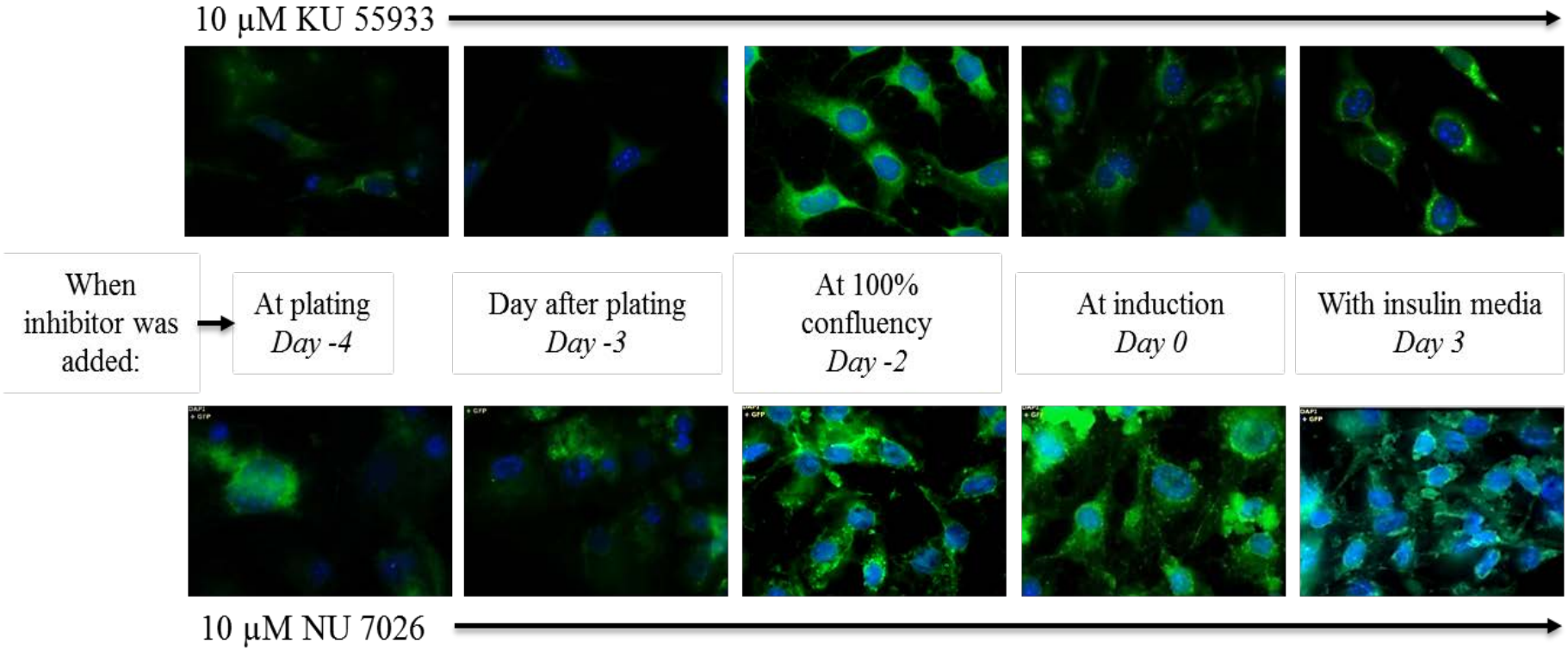

Figure 5: ATM and DNA-PK kinase inhibition has maximal effect on lipid accumulation when added before inducing adipogenesis

3T3-L1 cells were hormonally induced to undergo differentiation (166 nM Insulin, 0.5 mM IBMX, and 1 µM dexamethasone in 10% FBS DMEM) treated with the ATM kinase inhibitor KU 55933 (10 µM dissolved in DMSO) or the DNA-PK kinase inhibitor NU 7026 (10 µM dissolved in DMSO) at the indicated time points then hybridized with LipidTOX (1:200 in BSA, 1 h RT) to visualize lipid droplets. Cells were observed with a fluorescence microscope to observe lipid staining (GFP, green color) and nuclear staining (DAPI, blue color).

ATM and DNA-PKcs are critical for lipid accumulation in mouse embryonic fibroblasts

Because of the reliability and accuracy of using chemical inhibition to study kinase activity, we employed mouse embryonic fibroblasts (MEFs) that have *Atm* and *PRKDC* (which encodes the DNA-PK_{cs}) knocked out. We chose these cells lines to study the role of the ATM and DNA-PK_{cs} proteins in adipogenesis, without the use of ATP competitive protein kinase inhibitors, which could potentially have off target effects. MEFs without the ATM protein accumulated significantly less lipids than did the wild type counterpart (See Figure 6: Knockout of the ATM gene decreases lipid accumulation in MEFs).

MEFs with DNA-PK_{cs} knocked out revealed minuscule lipid accumulation following hormonal induction of adipogenesis (See Figure 7: DNA-$PK_{cs}^{-/-}$ MEFs show little lipid accumulation despite hormonal induction of adipogenesis). MEFs without DNA-PK_{cs} had a distinct fibroblast-like phenotype. Furthermore, differentiated DNA-$PK_{cs}^{-/-}$ MEFs were much larger in size and had much smaller lipid droplets than the wild type control (See Figure 8: DNA-$PK_{cs}^{-/-}$ MEFs are larger and have smaller lipid droplets than wild type MEFs).

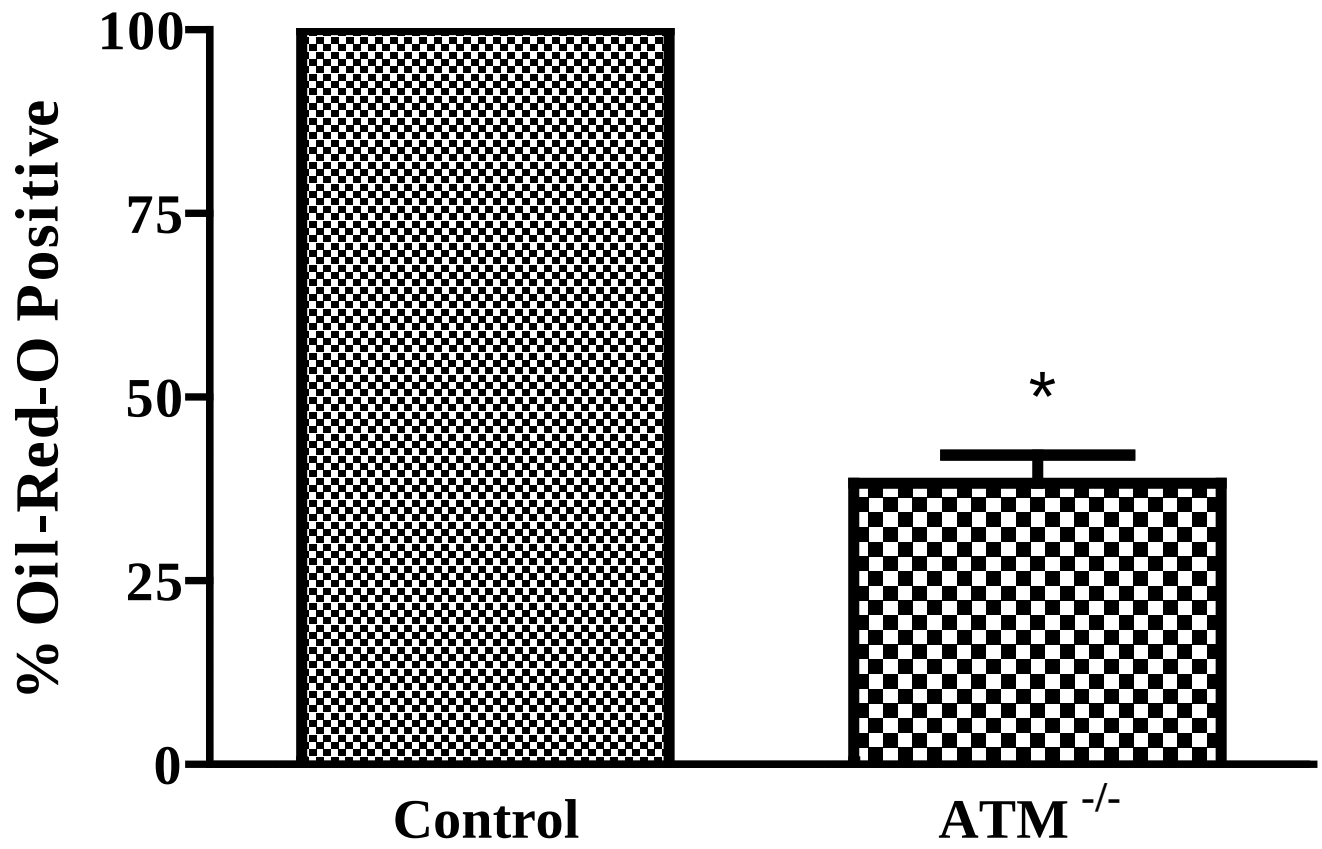

Figure 6: Knockout of the ATM gene decreases lipid accumulation in MEFs

Normal mouse embryonic fibroblasts (MEFs), and those without the ATM gene, were subject to hormonal stimulation to undergo adipogenesis (166 nM Insulin, 0.5 mM IBMX and 1 µM dexamethasone in 10% FBS DMEM). Lipid accumulation was determined by analyzing Oil-Red-O staining using a light microscope (400x). Each treatment was given a score based on the percent Oil-Red-O saturation when compared to differentiated wild type MEFs, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to wild type MEFs).

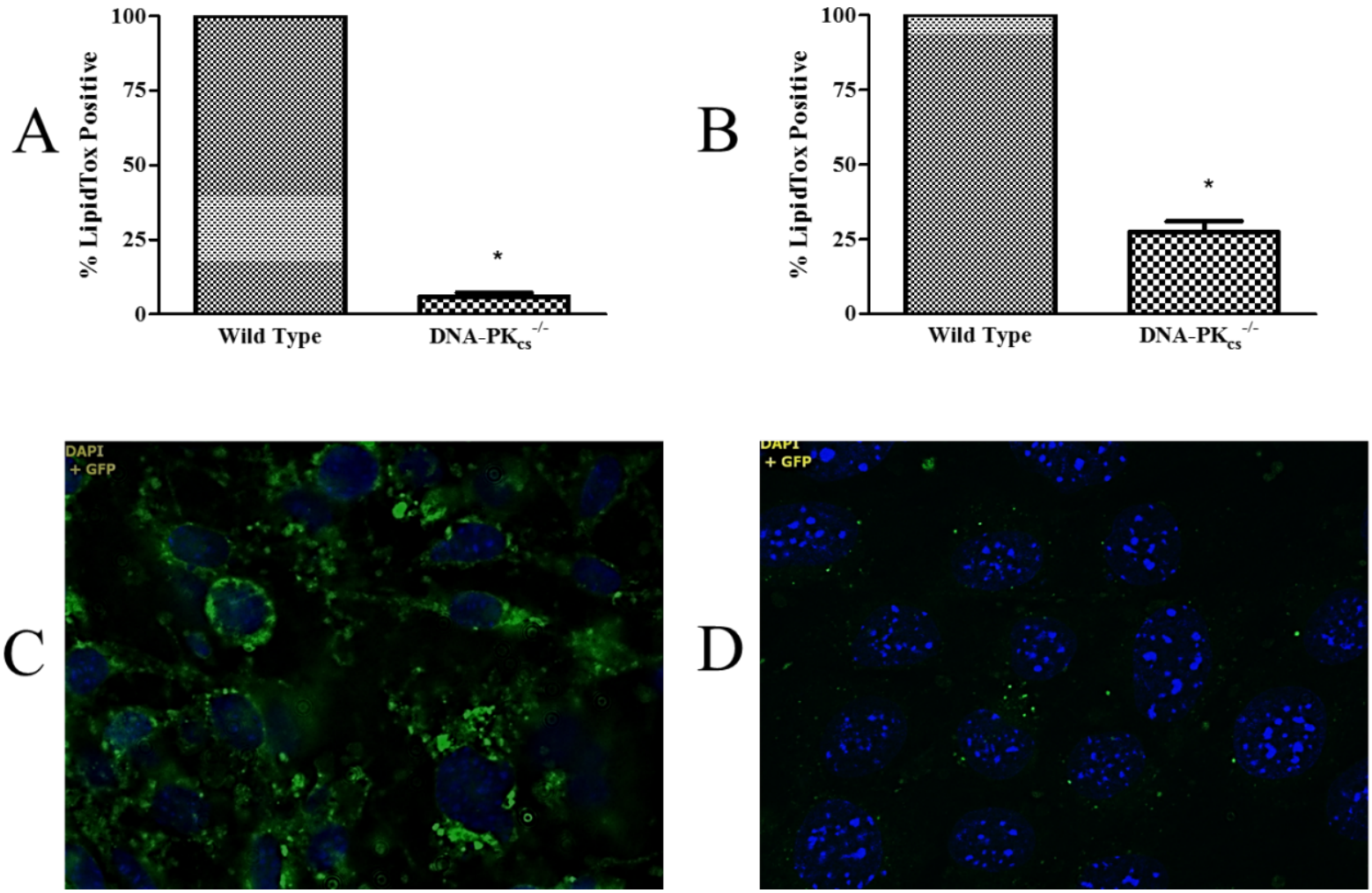

Figure 7: DNA-PK$_{cs}^{-/-}$ MEFs show little lipid accumulation despite hormonal induction of adipogenesis

Wild type and DNA-PK$_{cs}^{-/-}$ mouse embryonic fibroblasts (MEFs) were subject to hormonal stimulation to undergo adipogenesis (166 nM Insulin, 0.5 mM IBMX, and 1 µM dexamethasone in 10% FBS DMEM) and cultured in 20% oxygen (A) and 3% oxygen (B). Lipid accumulation was determined by LipidTOX staining (1:200 in BSA, 1 h RT) visualized using an immunofluorescent microscope (63x oil-immersion). Scores were based on the percent LipidTOX saturation of differentiated wild type MEFs, which we designated as 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated wild type MEFs). Representative oil- immersion photographs (DAPI, blue color; nucleus GFP, green color; lipid droplet stain) of differentiated wild type (C) and DNA-PK$_{cs}^{-/-}$ (D) MEFs.

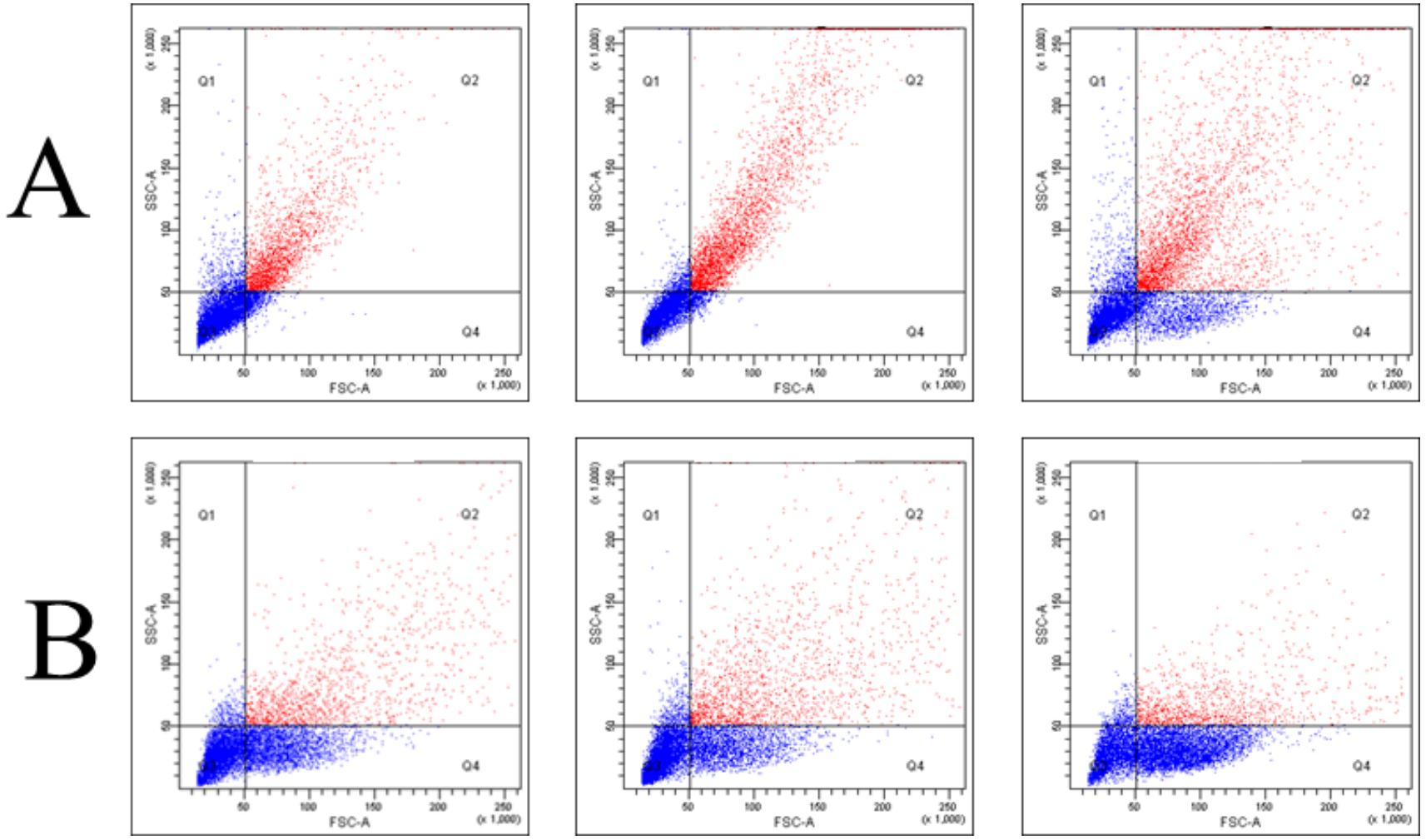

Figure 8: DNA-PK$_{cs}^{-/-}$ MEFs are larger and have smaller lipid droplets than wild type MEFs

Differentiated (A) wild type and (B) DNA-PK$_{cs}^{-/-}$ mouse embryonic fibroblasts (MEFs) cells hormonally induced to undergo adipogenesis (166 nM Insulin, 0.5 mM IBMX, and 1 µM dexamethasone in 10% FBS DMEM). Upon differentiation, cells were analyzed by observing the side scatter (y-axis) to forward scatter (x-axis) ratio, with higher values indicating increasing lipid accumulation and increasing cell size, respectively.

DNA-PK acts independently of the NHEJ pathway during adipogenesis

We also employed MEFs that were homozygous negative for Ku80 and Ku70, necessary proteins for the non-homologous end-joining pathway (NHEJ). The Ku80 and Ku70 proteins make up a heterodimer that together with DNA-PK$_{cs}$, make up the holoenzyme DNA-PK (Lees-Miller, et al. 1990). This heterodimer is the key kinase in the NHEJ pathway that repairs DNA DSBs (Lees-Miller and Meek 2003). In our experiment, the silencing of Ku70 and Ku80 did not significantly affect lipid accumulation in differentiated MEFs. This finding suggests that DNA-PK$_{cs}$ affects lipid droplet formation independent of its role and function in the NHEJ pathway (See Figure 9: DNA-PK's role in adipocyte differentiation is independent from its function in the non-homologous end-joining pathway).

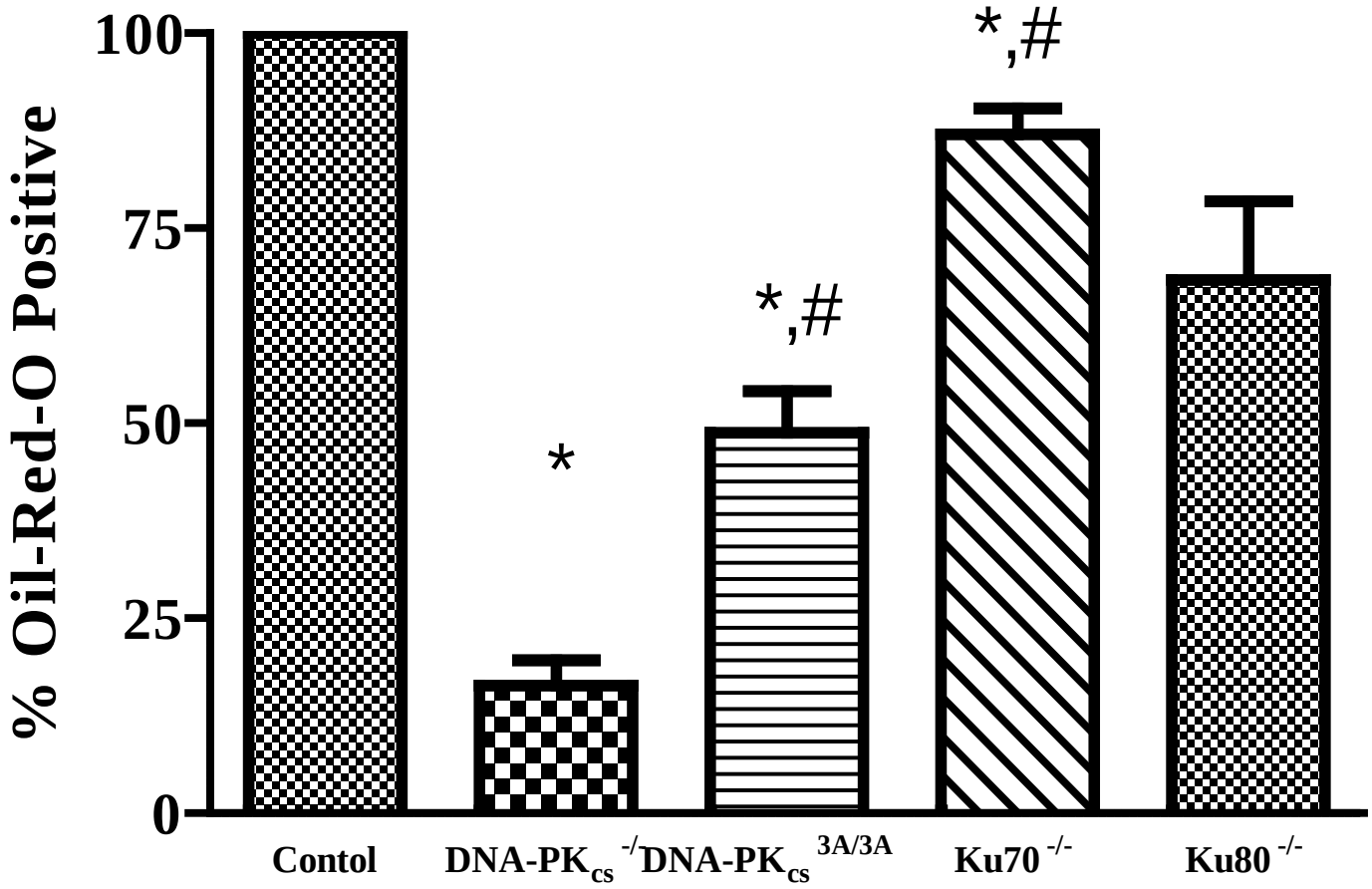

Figure 9: DNA-PK's role in adipocyte differentiation is independent from its function in the non-homologous end-joining pathway

Wild type mouse embryonic fibroblasts (MEFs) and MEFs with the following mutations; $Ku70^{-/-}$, $Ku80^{-/-}$ and $DNA\text{-}PK_{cs}^{-/-}$, and $DNA\text{-}PK_{cs}^{3A/3A}$ (alanine mutation at the catalytically active site which results in significantly decreased kinase activity) were subjected to hormonal stimulation to undergo adipogenesis (166 nM Insulin, 0.5 mM IBMX, and 1 µM dexamethasone in 10% FBS DMEM). Lipid accumulation was determined by analyzing Oil-Red-O staining using a light microscope (400x). Each treatment was given a score based on the percent Oil-Red-O saturation when compared to differentiated wild type MEFs, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated wild type MEFs, # denotes significance ($p<0.05$) as compared to differentiated $DNA\text{-}PK_{cs}^{-/-}$ MEFs

ATM and DNA-PK inhibition decrease perilipin concentration

Because we saw decreased lipid droplet formation when we treated 3T3-L1 with ATM and DNA-PK kinase inhibitors, we wanted to investigate which factors were responsible. Therefore, we chose to investigate the expression of the lipid droplet protein, perilipin 1. Perilipins coat lipid droplets and serves as a scaffolding protein. Furthermore, chromosomal variations at the perilipin locus are associated with obesity risk (Tai and Ordovas 2007) and some perilipin family members direct lipase to the lipid droplet in order to facilitate lipolysis (Wang, et al. 2011, Wang et al 2003). For these reasons, we hypothesize that ATM and DNA-PK inhibition could be decreasing perilipin localization to lipid droplets, which might explain why we observed decreased lipid accumulation.

In ATM and DNA-PK chemically inhibited 3T3-L1 adipocytes, perilipin concentrations were significantly decreased when compared to normally differentiated cells (See Figure 10: ATM and DNA-PK inhibition decreases perilipin concentration). Interestingly, we observed that the vehicle used to dissolve the chemical inhibitors, DMSO, which is known to have anti-inflammatory or antioxidant properties, slightly increased basal perilipin concentrations and their association with lipid droplets.

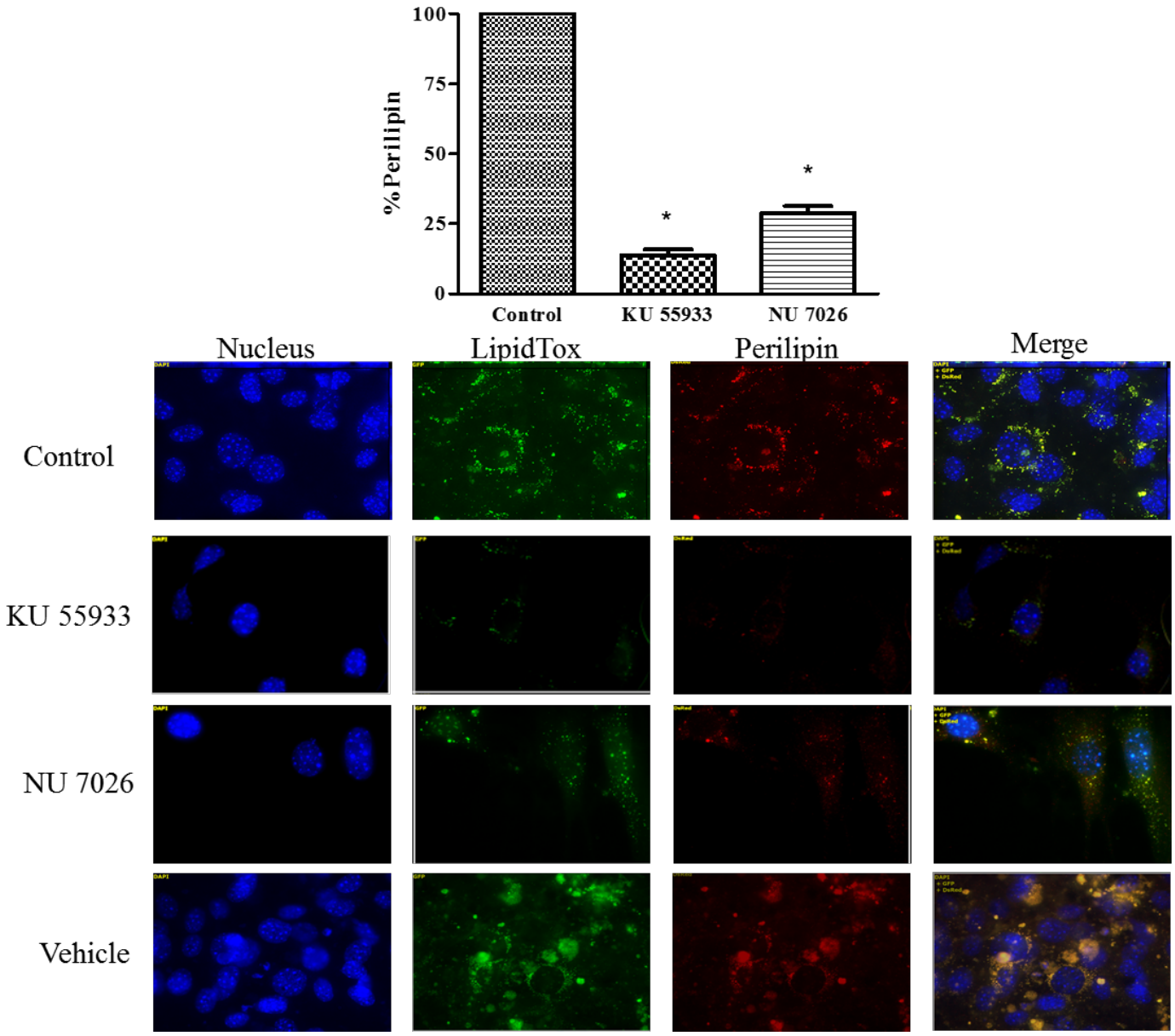

Figure 10: ATM and DNA-PK inhibition decreases perilipin concentration

3T3-L1 fibroblasts were differentiated and treated with the ATM inhibitor, KU 55933, (10 µM in DMSO; 48 h treatment started day 0 of adipogenesis) or the DNA-PK inhibitor, NU 7026, (10 µM in DMSO; 48 h treatment started day 0 of adipogenesis) and cultured in 20% oxygen. Differentiated cells were probed for perilipin (1: 200 in BSA, 1 h RT; 2° Ab Alexa Fluor 488-conjugated goat anti-rabbit 1: 200 in BSA, 1 h RT) and LipidTOX (1: 100 in BSA, 1 h RT) then visualized using an immunofluorescent microscope (63x oil-immersion). Each treatment was given a score based on the total perilipin concentration when compared to the differentiated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells). Photographs are representative of data in bar graph and the vehicle, DMSO, used to dissolve the inhibitors. DAPI, blue color, nuclear stain; GFP, green color, lipid droplet stain; DsRed, red color, perilipin antibody; Merge, yellow color, co-localization of perilipin and lipid droplet.

ROS promotes lipid accumulation in 3T3-L1 and MEF models of adipogenesis

Because we have previously shown that ATM and DNA-PK are activated in response to oxidative stress in human normal fibroblasts (Wu, et al. 2010), we hypothesized that the decreased adipogenesis after KU 55933 and NU 7026 treatment was due to changes in oxidative stress levels. Furthermore, literature has indicated that oxidative stress is required for differentiation (Higuchi et al 2013) but that chronic increased oxidative stress can inhibit adipogenesis (Findeisen, et al. 2011) and promotes adipogenesis. Whatever the mechanism, it is likely that oxidative stress is a critical regulator of adipogenesis.

In order to test our hypothesis that ATM and DNA-PK act via a ROS dependent pathway during adipogenesis, we first established the effect of oxidative stress in our models. Briefly, we treated differentiating 3T3-L1 with the antioxidants NAC and TEMPO as well as the ROS generating prooxidant H_2O_2 and cultured them in 3% and 20% oxygen and measured the lipid accumulation (See Figure 11: The effect of oxidative stress on lipid accumulation in 3T3-L1 cells). Our results suggest that increased ROS levels results in increased lipid droplet accumulation and that antioxidant treatment slightly diminishes lipid droplet formation.

We also performed similar experiments in our wild type MEFs to establish the effect that oxidative stress played in their ability to accumulate lipids (See Figure 12: The effect of oxidative stress on lipid accumulation in MEFs). We found that differentiated wild type MEFs, when pretreated with H_2O_2 and NAC, had similar lipid accumulation patterns to the 3T3-L1-cell line. H_2O_2 promoted increased lipid droplet formation while NAC slightly decreased or had no effect on lipid accumulation.

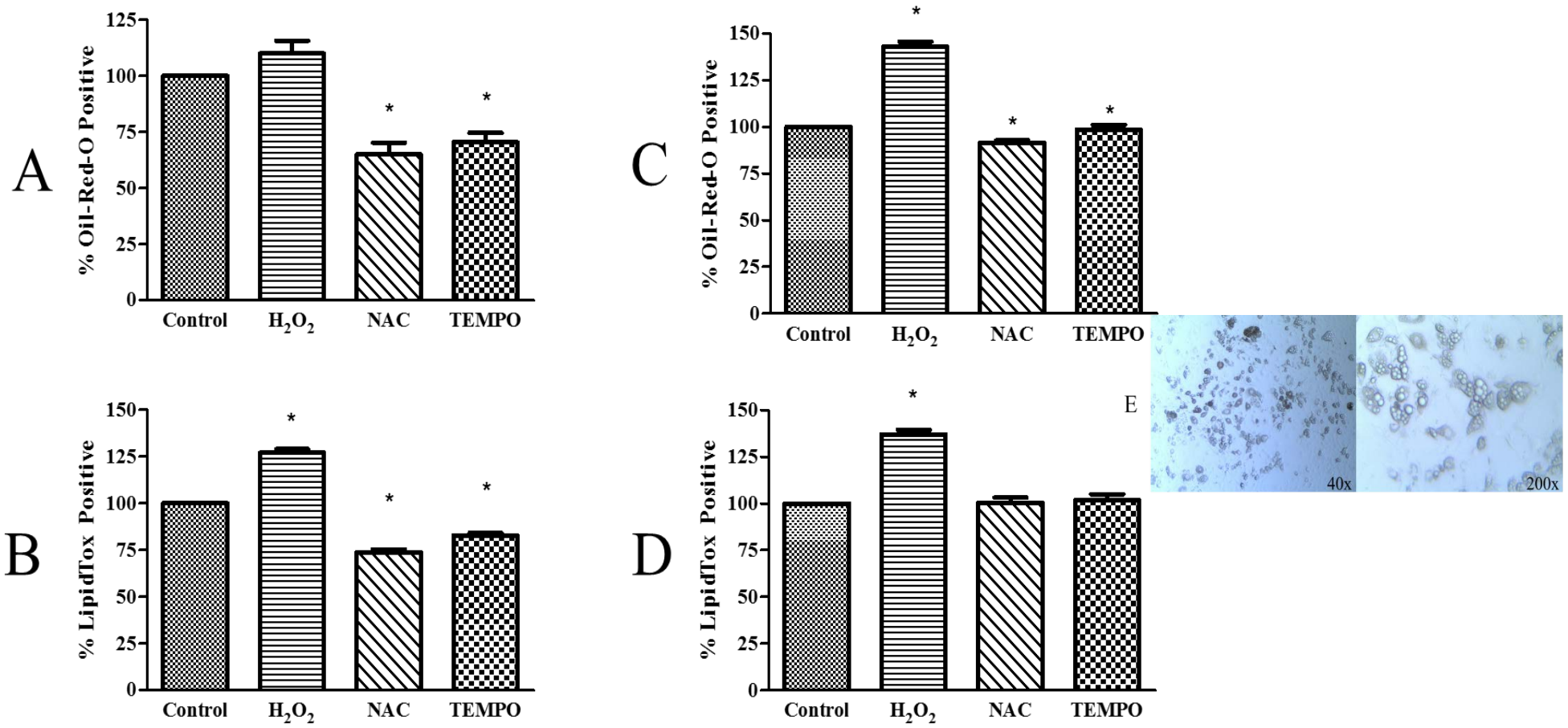

Figure 11: The effect of oxidative stress on lipid accumulation in 3T3-L1 cells

3T3-L1 fibroblasts differentiated and treated with the prooxidant H_2O_2 (10 µM in dd H_2O, 48 h treatment started day 0 of adipogenesis) or the antioxidants NAC (10 mM in PBS, 48 h treatment started day 0 of adipogenesis) and TEMPO (1 mM in PBS, 48 h treatment started day 0 of adipogenesis) and cultured in 20% oxygen (A & B) or 3% oxygen (C & D). Lipid accumulation was determined by analyzing Oil-Red-O staining (A & C) using a light microscope (400x) or by LipidTOX staining (B & D) using an oil-immersion immunofluorescent microscope (63x). Each treatment was given a score based on the percent Oil-Red-O or LipidTOX saturation when compared to the untreated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells). (E) Light microscope photograph of differentiated 3T3-L1 adipocytes illustrating the proadipogenic additive effect of H_2O_2 treatment (10 µM in dd H_2O, 48 h treatment started day 0 of adipogenesis) and culturing cells in 3% oxygen.

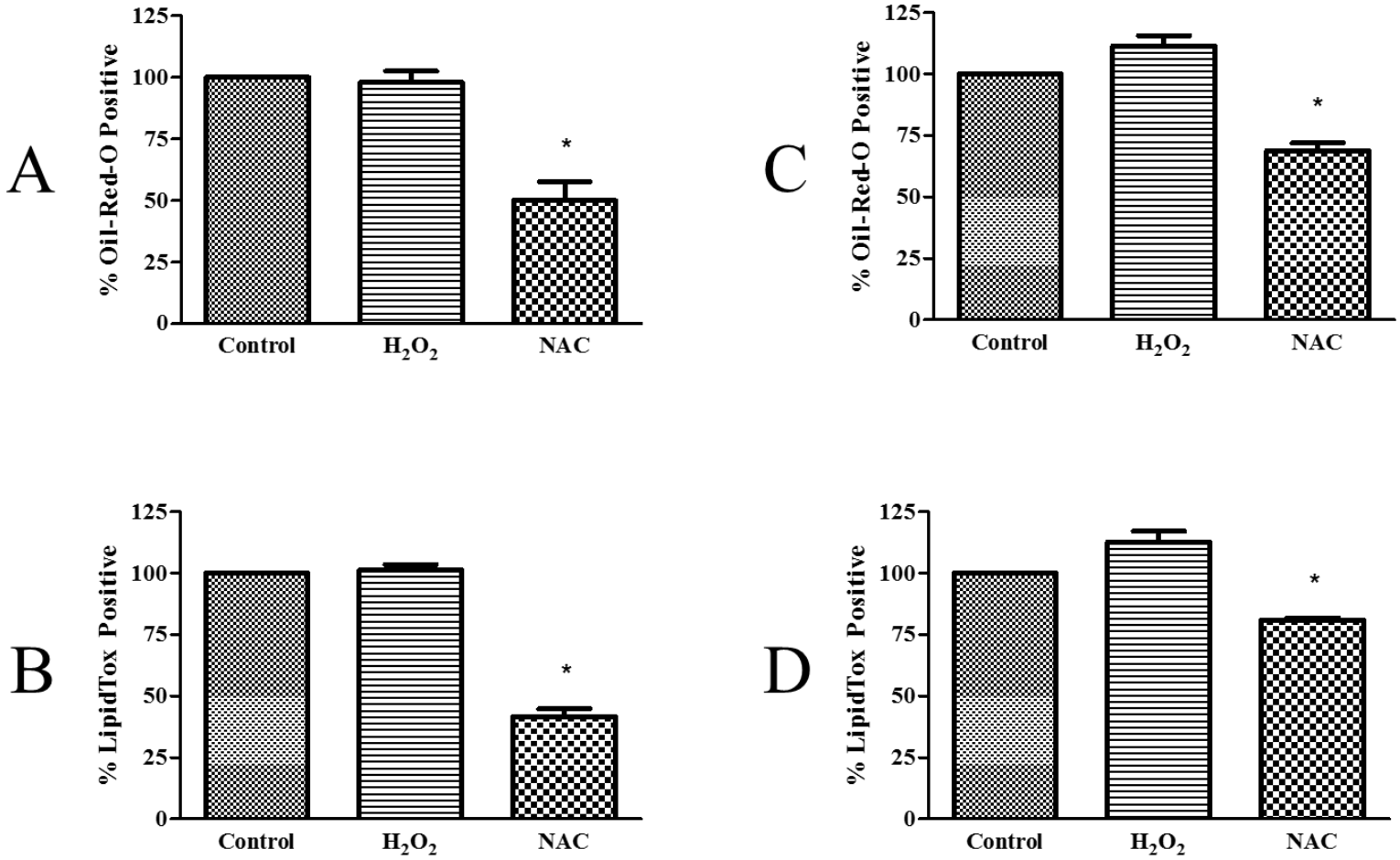

Figure 12: The effect of oxidative stress on lipid accumulation in MEFs

Wild type mouse embryonic fibroblasts were subject to hormonal stimulation to undergo adipogenesis (166 nM Insulin, 0.5 mM IBMX, and 1 µM dexamethasone in 10% FBS DMEM) and treated with the prooxidant H_2O_2 (10 µM in dd H_2O, 48 h treatment started day 0 of adipogenesis) or the antioxidant NAC (10 mM in PBS, 48 h treatment started day 0 of adipogenesis) and cultured in 20% oxygen (A & B) or 3% oxygen (C & D). Lipid accumulation was determined by analyzing Oil-Red-O staining (A & C) using a light microscope (400x) or by LipidTOX staining (B & D) using an oil-immersion immunofluorescent microscope (63x). Each treatment was given a score based on the percent Oil-Red-O or LipidTOX saturation when compared to the untreated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated MEFs).

ATM and DNA-PK orchestrate adipogenesis and lipid accumulation by regulating oxidative tone

In order link oxidative stress, lipid droplet accumulation and the role of ATM and DNA-PK kinases, we pretreated with H_2O_2 or NAC followed by KU 55933 and NU 7026 treatment during adipogenesis. Both antioxidant and prooxidant pretreatment significantly altered the capacity of the fibroblasts to differentiate and accumulate lipids (See Figure 13: ATM and DNA-PK influence lipid droplet formation via a ROS dependent pathway). Although antioxidant pretreatment had the largest effect, both treatments increased the lipid droplet formation and restored adipocyte morphology, despite the ATM or DNA-PK chemical inhibition.

This effect was greatest in cells that had ATM chemically inhibited, suggesting that ATM is a more critical regulator, or is upstream of DNA-PK, in the regulation of ROS before and during adipogenesis. Taken together, ATM and DNA-PK_{cs} respond to ROS and oxidative stress and preserve a normal pattern of lipid retention in 3T3-L1 adipocytes.

Pretreating DNA-$PK_{cs}^{-/-}$ MEFs with NAC and H_2O_2 revealed a similar, but less significant, trend to the 3T3-L1 fibroblasts. Specifically, both antioxidant and prooxidant treatment increased the lipid accumulation in DNA-$PK_{cs}^{-/-}$ MEFs (See Figure 14: Both antioxidant and prooxidant treatment causes DNA-$PK_{cs}^{-/-}$ MEFs to increase lipid accumulation).

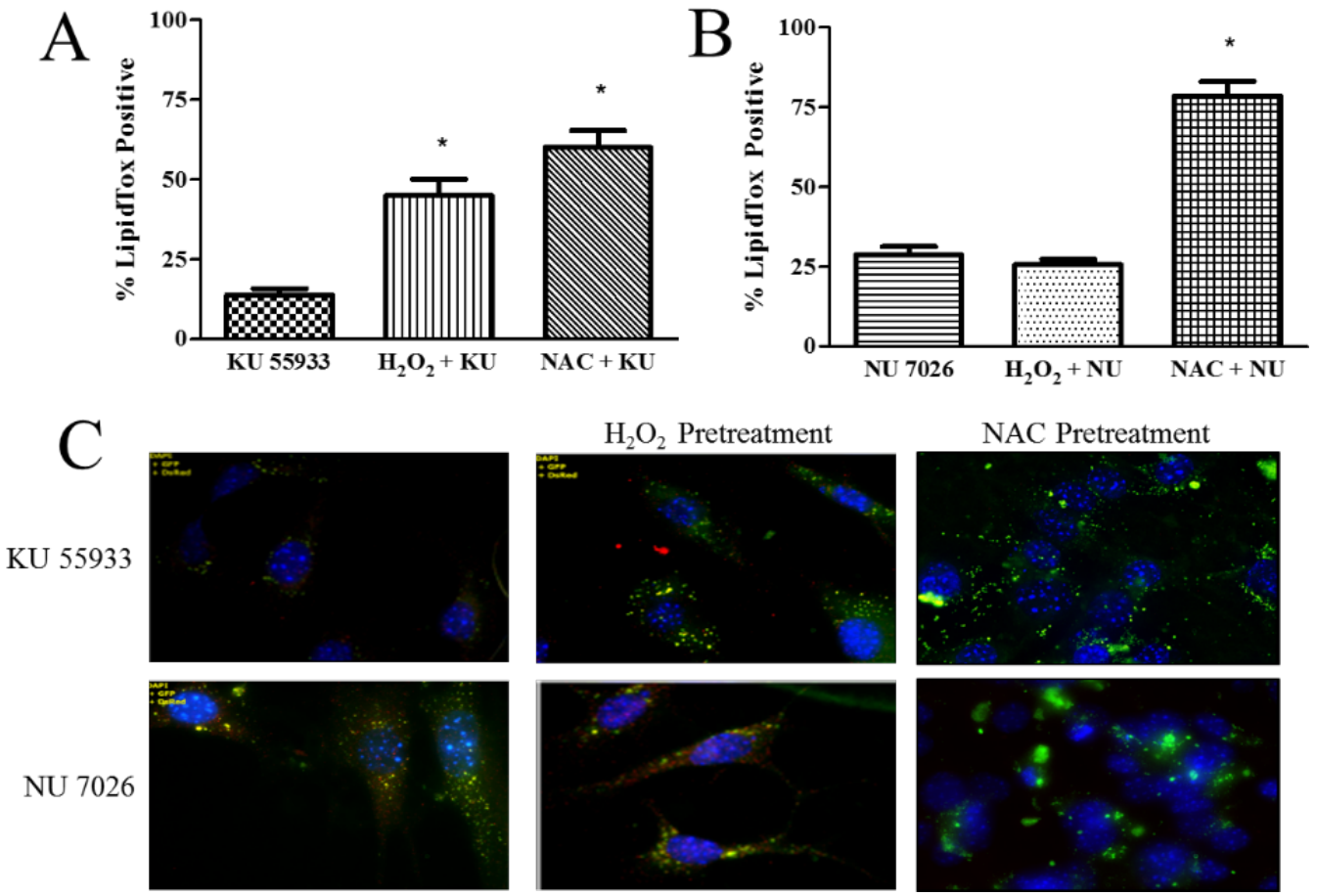

Figure 13: ATM and DNA-PK influence lipid droplet formation via a ROS dependent pathway

3T3-L1 fibroblasts were pretreated with the prooxidant H_2O_2 (10 µM in dd H_2O, 48 h treatment started day -2 of adipogenesis) or the antioxidant NAC (10 mM in PBS, 48 h treatment started day -2 of adipogenesis) followed immediately by hormonal stimulation to undergo adipogenesis (166 nM Insulin, 0.5 mM IBMX, and 1 µM dexamethasone in 10% FBS DMEM) and (A) treatment with the ATM inhibitor, KU 55933, (10 µM in DMSO, 48 h treatment started day 0 of adipogenesis) or (B) the DNA-PK inhibitor, NU 7026, (10 µM in DMSO, started day 0 of adipogenesis) and cultured in 20% oxygen. Lipid accumulation was determined by LipidTOX staining using an oil-immersion immunofluorescent microscope (63x). Each treatment was given a score based on the percent Oil-Red-O or LipidTOX saturation when compared to the untreated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to inhibitor only treated 3T3-L1 cells). (C) The top row of pictures are representative photographs of bar graph A and the bottom row of pictures is representative of bar graph B; where the blue color is DAPI, a nuclear stain and the green color is LipidTOX, a lipid stain.

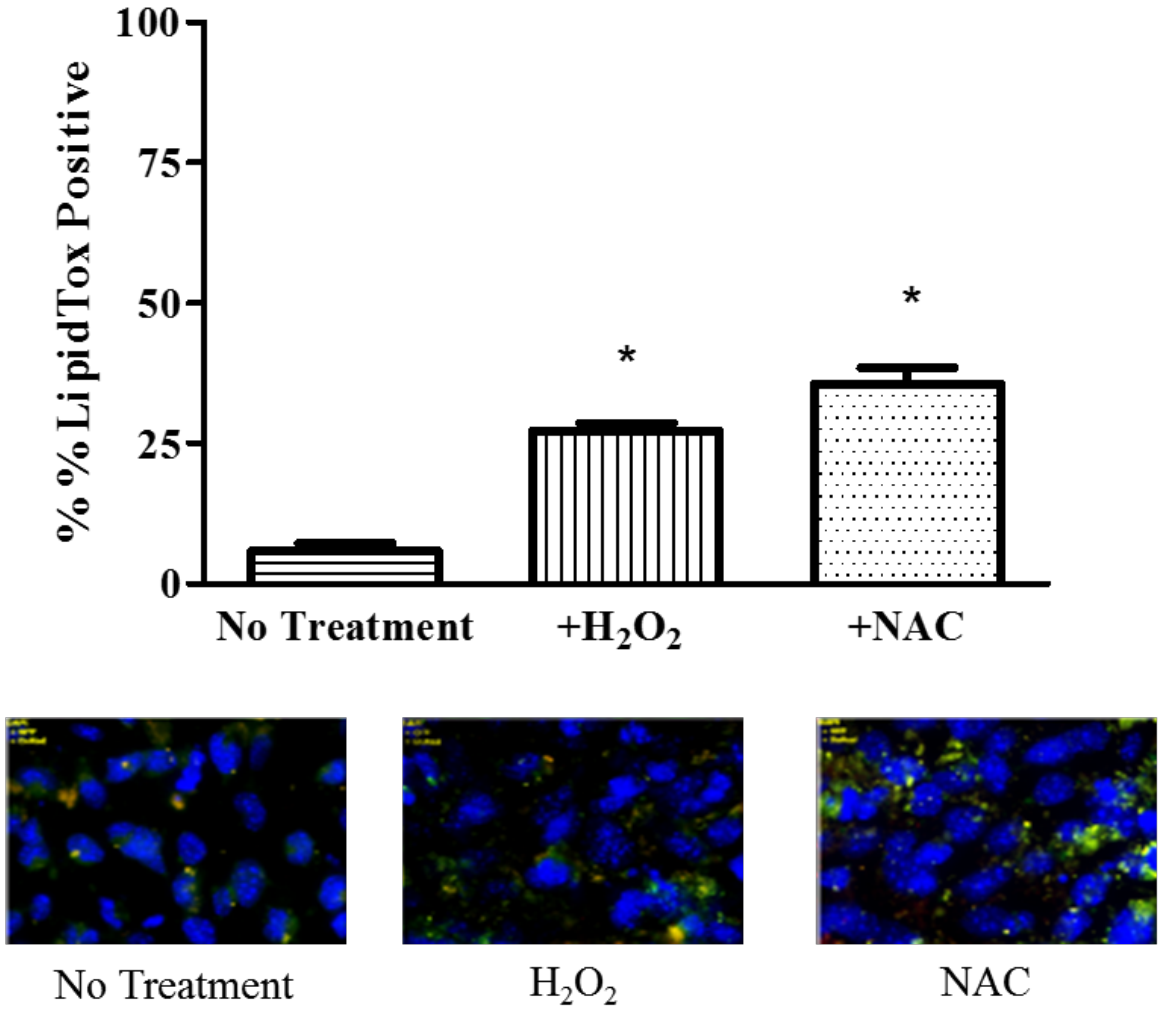

Figure 14: Both antioxidant and prooxidant treatment causes DNA-PK$_{cs}^{-/-}$ MEFs to increase lipid accumulation

DNA-PK$_{cs}^{-/-}$ MEFs were treated with the prooxidant H_2O_2 (10 µM in dd H_2O, 48 h treatment started day -2 of adipogenesis) or the antioxidant NAC (10 mM in PBS, 48 h treatment started day -2 of adipogenesis) followed by hormonal stimulation to undergo adipogenesis (166 nM Insulin, 0.5 mM IBMX, and 1 µM dexamethasone in 10% FBS DMEM) and then cultured in 20% oxygen. Lipid accumulation was determined by LipidTOX staining using an oil-immersion immunofluorescent microscope (63x). Each treatment was given a score based on the percent LipidTOX saturation when compared to the untreated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated DNA-PK$_{cs}^{-/-}$ MEFs). The photographs represent the treatments seen in the bar graph; where the blue color is DAPI, a nuclear stain and the green color is LipidTOX, a lipid stain.

Requirement of DNA damage in differentiation

There are various cellular rearrangements required for adipogenesis such as mitotic clonal expansion (Lee et al 2009), permanent growth arrest (Ailhaud et al 1990), rearrangement of nuclear architecture (Xiong et al 2013), cytoskeleton changes (Liu et al 2005, Selvarajan et al 2001), up-regulation of genes (Cristancho and Lazar 2011), etc.). It is our hypothesis that ATM and DNA-PK kinases could affect some of the processes regulating cellular rearrangement during adipogenesis via an oxidative stress related pathway.

Since it was found that ATM and DNA-PK could influence adipogenesis via a ROS dependent pathway, we were interested in determining which pathways these kinases regulate. We set out to test if γH2A.X is a mediator or intermediate in the ATM and DNA-PK pathway during adipogenesis. We chose to study γH2A.X, a marker of DNA damage, because it is phosphorylated by ATM and DNA-PK in response to DNA damage (An et al 2010, B Meyer et al 2013) or oxidative stress (Wrann et al 2013).

Because our previous experiments demonstrated that ATM and DNA-PK are critical for adipocyte differentiation, most likely through an ATM- DNA-PK- ROS dependent pathway, γH2A.X expression was the most logical mechanistic candidate to carry out and relay/amplify the oxidative stress signaling. ATM and DNA-PK inhibition is altered by prooxidants and antioxidants and γH2A.X phosphorylation is known to occur during oxidative stress (Zhao et al 2008) and during to hypoxia (Trayhurn 2013). Both are common in adipose tissue of obese individuals. Taken together, these facts make it reasonable to suspect that γH2A.X has an important role in adipogenesis.

Differentiated 3T3-L1 fibroblasts were treated with the ATM inhibitor KU 55933 or the DNA-PK inhibitor NU 7026, as well as antioxidants, and cultured in both 3% and 20% oxygen incubator environments. Lipid accumulation and γH2A.X signals were detected using immunofluorescence microscopy. The results suggest that γH2A.X phosphorylation is a required step in adipogenesis and that cells in the 20% oxygen environment were more prone to oxidative DNA damage and have decreased lipid accumulation (See Figure 15: DNA damage in adipocytes)

In addition, cells treated with the ATM inhibitor, KU 55933, had more γH2A.X foci than those treated with the DNA-PK inhibitor, NU 7026. From literature, it is known that γH2A.X and DNA-PK activity display unusual and unexpected activity during adipogenesis (Meulle et al 2008). However, the question lies in why there is an increased presence of γH2A.X in differentiated cells treated with an antioxidant or those treated with the inhibitors.

Figure 15: DNA damage in adipocytes

3T3-L1 fibroblasts were differentiated and treated with the ATM inhibitor, KU 55933, (10 µM in DMSO; 48 h treatment started day 0 of adipogenesis) or the DNA-PK inhibitor, NU 7026, (10 µM in DMSO; 48 h treatment started day 0 of adipogenesis) and cultured in 20% oxygen (A) or 3% oxygen (B). Differentiated cells were then hybrized with a γH2A.X antibody (1: 250 in BSA, 1 h RT; 2° Ab Alexa Fluor 488-conjugated goat anti-mouse 1: 200 in BSA, 1 h RT), which is a marker for DNA damage, and LipidTOX (1: 100 in BSA, 1 h RT) then visualized (DAPI, blue color, nuclear stain; GFP, green color, lipid droplet stain; DsRed, red color, γH2A.X) using the 63x oil-immersion lens of an immunofluorescent microscope.

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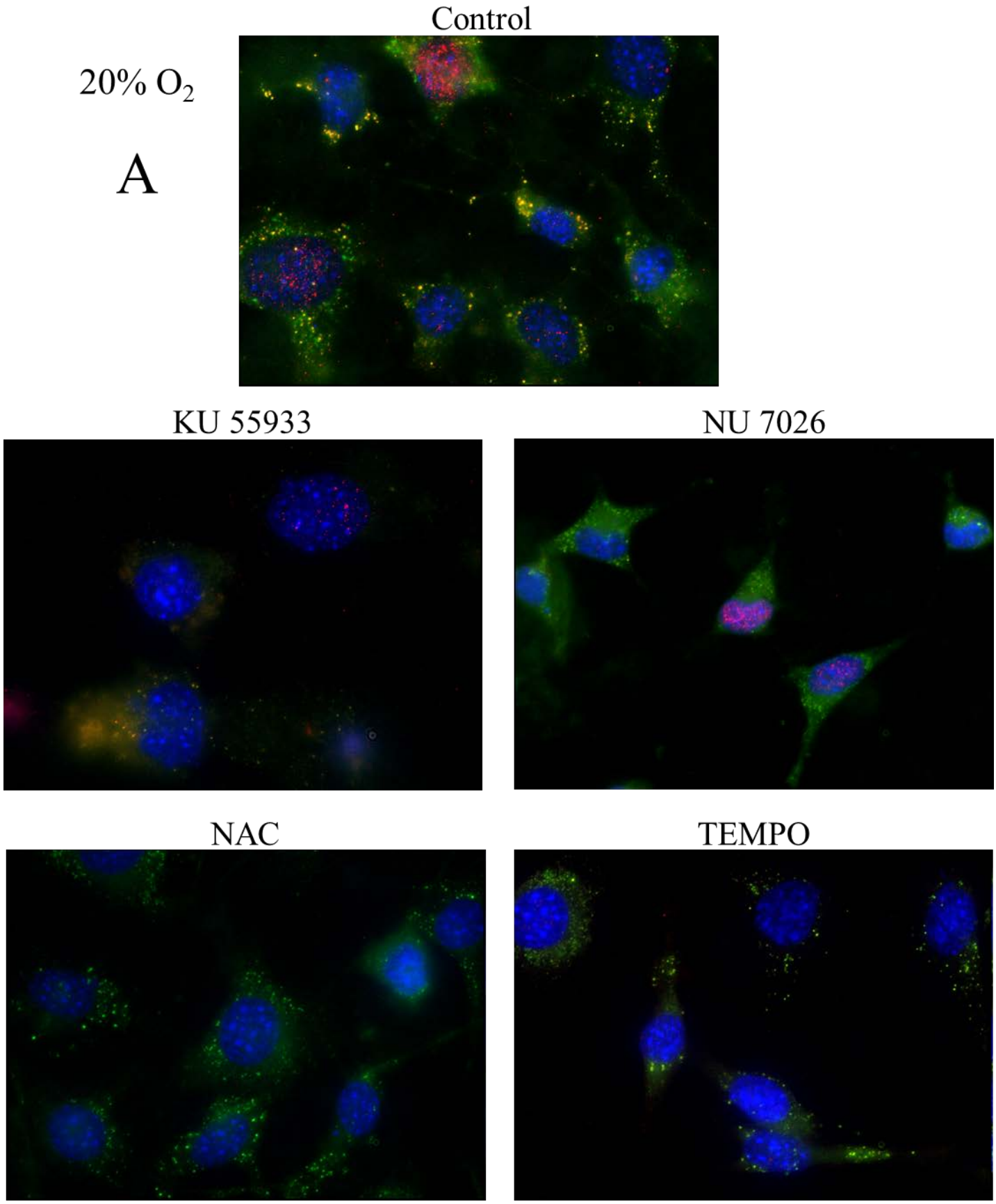
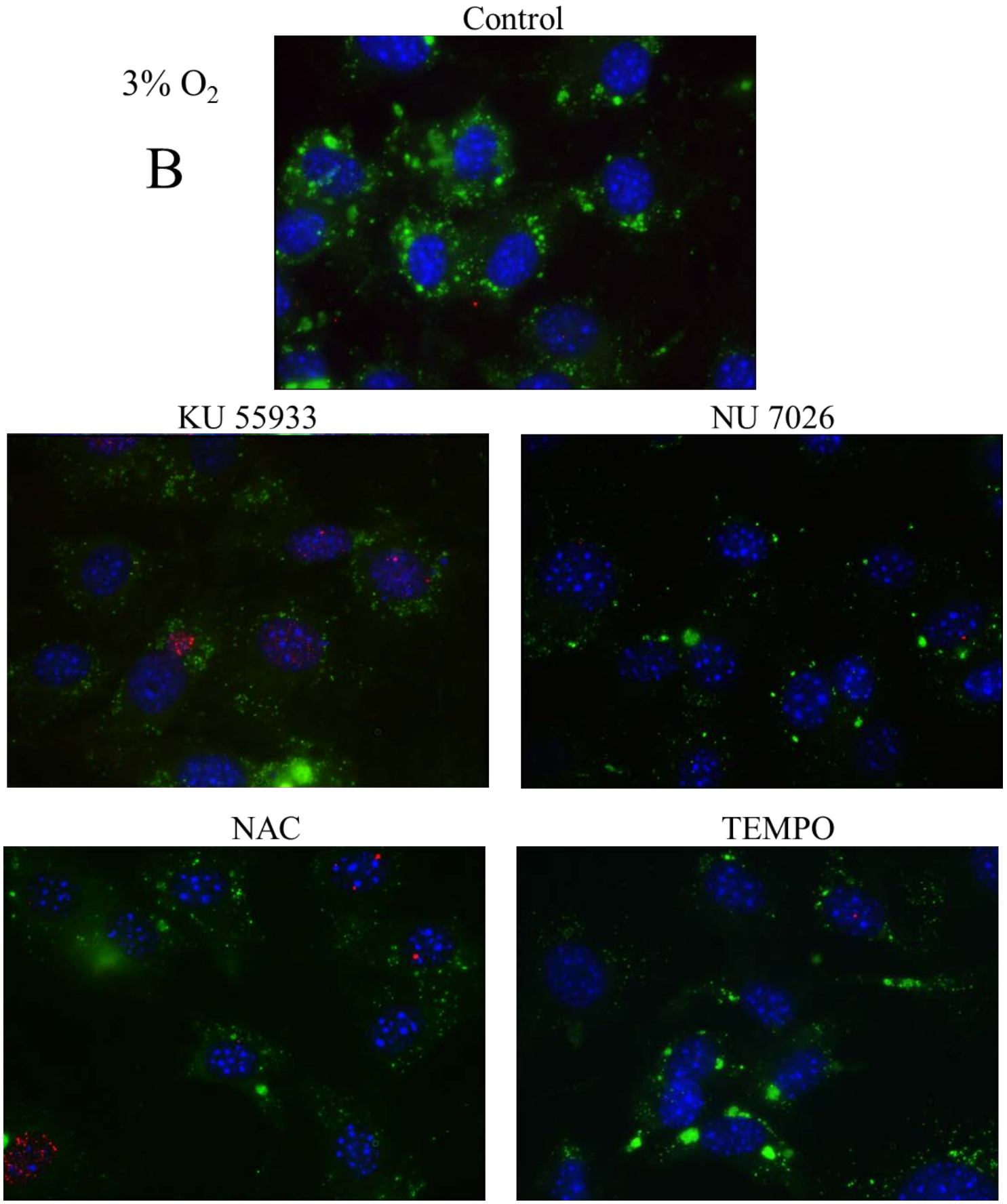

Control
20% O_2
A
KU 55933
NU 7026
NAC
TEMPO


Control
3% O_2
B
KU 55933
NU 7026
NAC
TEMPO

Preadipocyte senescence is toxic to adipose tissue

Increased oxidative stress may cause preadipocytes to senescence (Tchkonia, et al. 2010). Furthermore, senescent preadipocytes may create a toxic environment that contributes to and exacerbates adipocyte dysfunction, paving the way for insulin resistance and type 2 diabetes (Guilherme, et al. 2008). It is our hypothesis that ATM and DNA-PK kinases have a role in the propagation of senescent preadipocytes (Please refer to Figure 27: Conceptual Model for more details).

In order to test this hypothesis, we treated undifferentiated 3T3-L1 cells with the ATM kinase inhibitor KU 55933 or DNA-PK kinase inhibitor NU 7026. We observed a senescent-like phenotype in the cells (See Figure 16: ATM and DNA-PK kinase inhibition causes a senescent-like phenotype). Not only was the phenotype of the preadipocytes affected, but an increased presence of SA-β-gal was more detected in cells treated with DNA-PK kinase inhibitor, NU 7026 and ATM kinase inhibitor, KU 55933, and the general PIKK inhibitors wortmannin and caffeine, than cells with no treatment at all (See Figure 17: ATM and DNA-PK inhibition cause preadipocyte senescence).

We experimented with a co-culturing preadipocytes and adipocytes to see if the extracellular environment of one could illicit cellular changes in the other. Preadipocytes have functions beyond serving as a reservoir for adipogenesis. During adipose tissue dysfunction preadipocytes can behave like macrophages and participate directly in inflammatory processes (Cousin, et al. 1999).

Differentiated 3T3-L1 adipocytes were co-cultured with preadipocytes treated with the ATM kinase inhibitor, KU 55933, or the DNA-PK kinase inhibitor, NU 7026, to assess the effect the senescent phenotype of the preadipocyte would have on differentiated

adipocytes. The differentiated adipocytes and the senescent preadipocytes were co-cultured for 72 hours, then measured for lipid accumulation. The most apparent phenotype of the differentiated 3T3-L1 cell was a diminished scheme of lipid accumulation. Additionally, the cells took on different shapes from their normal adipocyte counterparts (See Figure 18: Senescent preadipocytes alter phenotype of adipocytes in co-culture model).

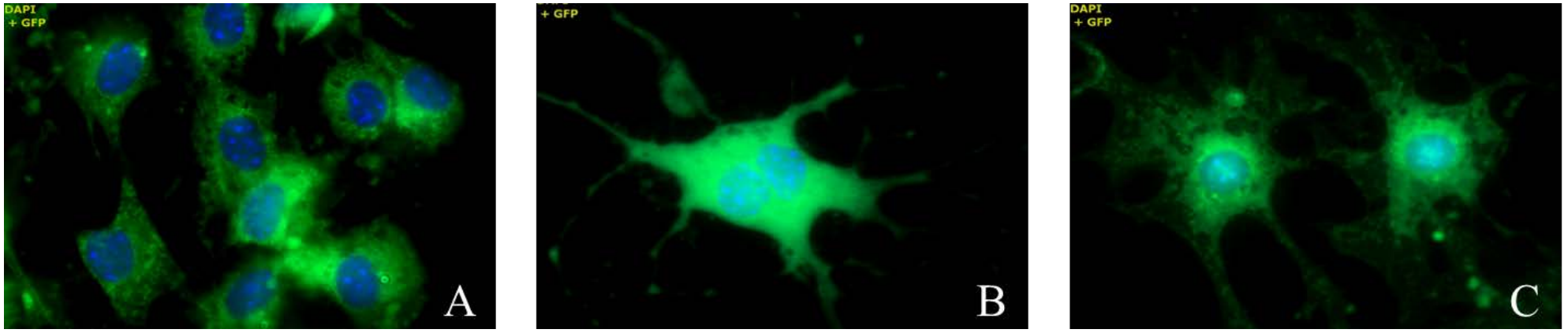


Figure 16: ATM and DNA-PK kinase inhibition causes a senescent-like phenotype

Undifferentiated 3T3-L1 cells (preadipocytes) (A) were treated with the ATM kinase inhibitor KU 55933 (10 µM dissolved in DMSO, 6 days) (B) or the DNA-PK kinase inhibitor NU 7026 (10 µM dissolved in DMSO, 10 days) (C) then harvested for analysis. Cells were observed with a fluorescence microscope to observe LipidTOX staining (GFP, green color) and nuclear staining (DAPI, blue color).

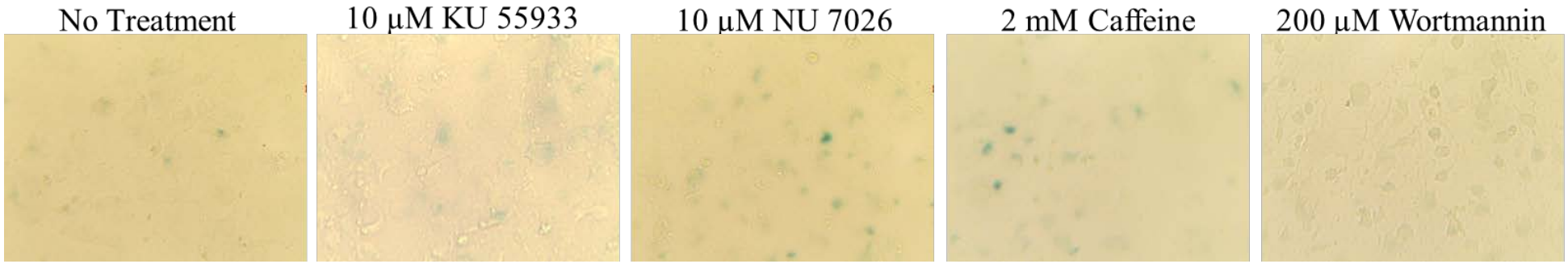


Figure 17: ATM and DNA-PK inhibition cause preadipocyte senescence

Undifferentiated 3T3-L1 cells (preadipocytes) were treated with the ATM kinase inhibitor KU 55933 (10 µM dissolved in DMSO, 48 h), the DNA-PK kinase inhibitor NU 7026 (10 µM dissolved in DMSO, 48 h) and the general PIKK inhibitors caffeine (2 mM in dd H_2O) and wortmannin (200 µM in DMSO) then allowed to recover for 10 days. Cells were harvested and tested for senescence by detecting the presence of senescence associated-β-galactosidase present (blue color) visualized using a light microscope at 200x.

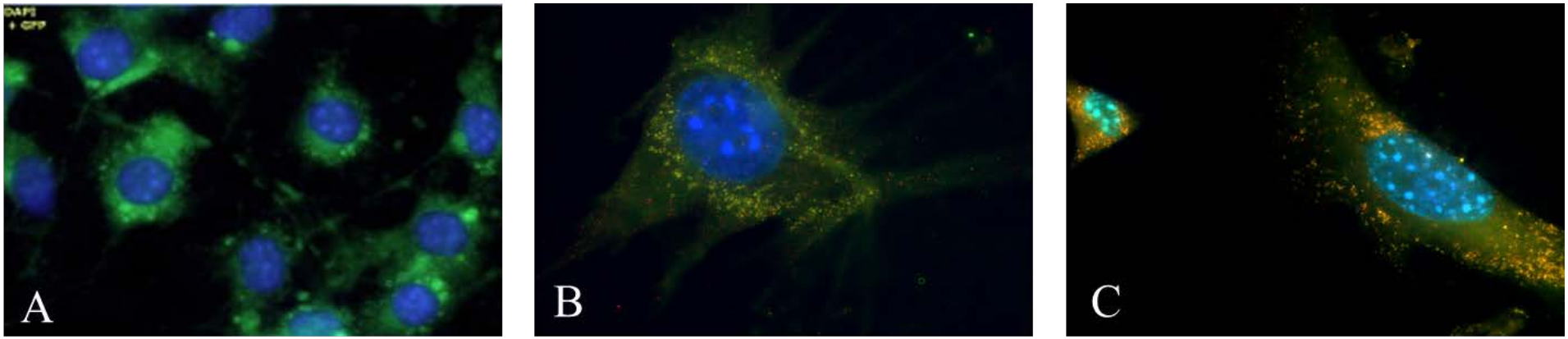


Figure 18: Senescent preadipocytes alter phenotype of adipocytes in co-culture model

Undifferentiated 3T3-L1 fibroblasts were grown on porous wells and made to senescence with the ATM inhibitor, KU 55933, (10 µM in DMSO; continuous) or the DNA-PK inhibitor, NU 7026, (10 µM in DMSO; continuous) and cultured in 20% oxygen (not shown). The well containing cells treated with KU 55933 was transferred to a culture of differentiated 3T3-L1 cells (B) and the well containing cells treated with NU 7026 was transferred to a different culture of differentiated 3T3-L1 cells (C). The differentiated cells and senescent preadipocytes were co-cultured together for 72 h, then the adipocytes were harvested and stained with LipidTOX (1: 100 in BSA, 1 h RT, green color) and mounted on slides containing DAPI (1 drop, nuclear stain, blue color) then visualized using an immunofluorescent microscope (63x oil-immersion) and compared to differentiated 3T3-L1 adipocytes that were grown under normal conditions (A).

Discussion

Our results support the hypothesis that ROS, oxidative stress, and senescence can play a central role in metabolic dysfunction (Nelson, et al. 2012) and that the kinases ATM and DNA-PK are key orchestrators of this response. Furthermore, this study helps elucidate possible mechanisms whereby preadipocytes and adipocytes cause dysfunctions in adipose tissue during obesity.

We believe our results help explain why, during obesity, it is possible that preadipocytes do not or cannot normally replicate and only undergo partial adipogenesis, giving rise to non-functional, immature adipocytes. This could increase local and systemic inflammation, resulting in infiltration by macrophages (O'Rourke, et al. 2012, Trayhurn and Wood 2004).

The increase in H2A.X phosphorylation in differentiated adipocytes can be explained by a recent study that indicates high levels of phosphorylation of H2A occurs after mitotic arrest in normal cells (Tu et al 2013). Preadipocytes have to undergo mitotic arrest before committing to the adipocyte linage, so perhaps H2A phosphorylation by ATM and/or DNA-PK in response to oxidative stress is a key event in mitotic clonal expansion. If that were the case, it would also help explain why preadipocytes that have ATM and DNA-PK inhibited fail to undergo full differentiation. Further studies are needed to confirm these hypotheses.

Recently, the role of dysfunctional beta-cells has been tied closely with the diabetic state (Bensellam et al 2012, Gilbert and Liu 2012). Beta- cells primarily self-replicate and do not draw heavily on stem-cell progenitors. Thus, cell cycle machinery and checkpoints

are critical to ensuring proper replication and function of beta-cells (Tavana and Zhu 2011). The DNA damage proteins ATM and DNA-PK are key regulators of the cell cycle and are involved in checkpoint activation (Lisby et al 2004, Zhou and Elledge 2000), so they may play a large role in protecting beta-cells.

Although our study was not done in beta-cells, we show that disrupted DNA damage signaling in adipocytes causes incomplete differentiation and decreased lipid accumulation, both factors which contribute to type 2 diabetes and metabolic dysfunctions (Alves et al 2010, Guilherme, et al. 2008, Ravussin and Smith 2002, Scherer 2006). Furthermore, phosphorylation of histone 2A, a common marker of DNA damage, is greatest in differentiated adipocytes (See Figure 15: DNA damage in adipocytes), suggesting that the DNA damage response may play a necessary role during adipogenesis (Meulle, et al. 2008).

CHAPTER 3: THE EFFECT OF SELENIUM SPECIES ON ADIPOGENESIS IS ORCHESTRATED BY OXIDATIVE STRESS AND INVOLVES DNA DAMAGE RESPONSE PROTEINS

Abstract

We have shown that the DNA damage response proteins ATM and DNA- PK_{cs}, members of the PI(3)K family of serine/threonine kinases, are activated in response to oxidative stress in normal human fibroblasts. This protein family is implicated in insulin-stimulated glucose uptake. We manipulated the differentiation of murine 3T3-L1 preadipocytes and mouse embryonic fibroblasts employing chemical inhibitors of ATM and DNA- PK_{cs} exposed to pharmacological levels of insulin. We also tracked the phosphorylation patterns of ATM and DNA- PK_{cs} after selenium treatment to examine DNA damage responses in adipocytes. ATM and DNA- PK_{cs} were both activated in response to selenium treatments in differentiated adipocytes. Taken together, selenium's involvement in adipogenesis is dependent on oxidative stress pathways that may be mediated by DNA damage response proteins.

Introduction

Obesity is a worldwide concern, especially in the westernized world. Molecular studies done to elucidate the pathways that lead to and maintain obesity have yet to provide concrete evidence to support any treatment regarding the rise in obesity. One of obvious

targets for obesity prevention and treatment is adipocyte differentiation; the process by which precursor cells become committed specialized cells that store triglycerides. Obesity is not only a burden financially, emotionally and socially; it predisposes individuals to a range of health problems, such as diabetes and metabolic syndrome. The loss of glucose homeostasis is the hallmark of diabetes mellitus, a metabolic disease caused by impaired tissue responsiveness to insulin or by decreased pancreatic insulin secretion.

Selenium can act as an insulin mimetic (Ezaki 1990, Heart and Sung 2001, Heart and Sung 2003, Hwang et al 2007). Most diabetics have elevated serum selenium. (Bleys et al 2007a, Bleys et al 2007b, Ezaki 1990). However, selenium pretreatment protects hepatocytes against damage from drug-induced diabetes in rats (Can et al 2005). The only irrefutable evidence in the literature about selenium's relationship with diabetes is that high selenium status is positively associated with diabetes, with the exception of pregnant women (Farhat et al 1993, Farhat et al 1995, Tan et al 2001). Selenium supplements are not recommended for the prevention of diabetes.

Murine 3T3-L1 preadipocyte cells are one of the most commonly used models to study obesity, metabolic disease and diabetes. Confluent 3T3-L1 preadipocytes differentiate to adipocytes in the presence of insulin, dexamethasone and isobutylmethylxanthine. We hypothesized that selenium treatment will alter adipogenesis in 3T3-L1 preadipocytes, possibly via the ATM-DNA-PK-ROS pathway characterized in Chapter 2.

Methods

Cell lines and maintenance

Cells were grown. Murine 3T3-L1 preadipocytes (CL173) were purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's minimum essential medium (DMEM) (GibcoBRL, Carlsbad, CA) as previously reported (Rocourt, et al. 2013, Zhang, et al. 2011). The media was supplemented with 10% fetal bovine serum (FBS) (GibcoBRL) and 1% penicillin/streptomycin (GibcoBRL) and were maintained in a 37° C humidified atmosphere in either 20% oxygen.

Adipocyte differentiation

Adipogenesis was induced by using media (Hemati, et al. 1997) supplemented with 10% FBS DMEM. The media was then supplemented with the hormonal proadipogenic cocktail of 166 nM insulin dissolved in 0.02 M HCl, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) dissolved in 0.5N potassium hydroxide (KOH) and 1 µM dexamethasone (Dex) dissolved in ethanol to cells that were two days past 100% confluency (designated Day 0). After two days, the MDI media was refreshed for 1 day and then the cells were maintained in insulin media (166 nM insulin in 10% FBS DMEM) until used for experiments, typically on day 12~15 (See Figure 1: Timeline of Adipocyte Differentiation).

Treatments

For differentiation and immunofluorescence assays, 3T3-L1 cells were treated with, without or in combination with the following chemicals; 2 µM sodium selenite (Na_2SeO_3) in PBS (Sigma-Aldrich, St. Louis, MO), 2 µM methylseleninic acid, 95% (MSeA) in PBS (Sigma), 10 µM NU 7026 in DMSO (Tocris, Ellisville, MO), 10 µM KU 55933 in DMSO

(Tocris), 500 μM paraquat in double distilled water (Sigma-Aldrich), 1 mM hydroxyurea (HU) in double distilled water (MP Biomedicals, Santa Ana, CA). All chemicals were added to cell culture for the indicated times and at the indicated concentrations and incubated at 37 °C. Please refer to the appendix for more detailed information.

Oil-Red-O staining

Oil-Red-O (ORO) (Sigma-Aldrich, St. Louis, Missouri) staining was conducted, as previously described (Ramirez-Zacarias, et al. 1992, Ross, et al. 1999, Ross, et al. 2000, van Goor, et al. 1986) with little modification, in order to determine lipid accumulation. Dye preparation: 0.5 g of ORO was dissolved in 250 mL isopropanol to form a stock solution. 30 minutes before staining, 20 mL of deionized water was added to 30 mL of the stock solution to make the working solution, and then put on stir plate at 50° C until adipocyte staining. Cell staining: Adherent cells were gently rinsed with phosphate-buffered saline (PBS) then fixed in a 4% paraformaldehyde solution for 1 h, washed with 1x PBS then refixed with 4% paraformaldehyde solution for 2-3 days. After fixation, the cells were rinsed with 1x PBS followed by 60% isopropyl alcohol, which was added and allowed to evaporate. The cells were then stained with the ORO working solution for 10 min. Plates were rinsed 3x with tap water and allowed to dry. Photographs were taken, and cells were subsequently analyzed as described below. Quantification of stained cells: Stained cells were counted under a light microscope (40x- 400x magnification) and given a score based on the percent lipid accumulation when compared to the differentiated control, which we designated to be 100%.

Coverslip preparation

Immunofluorescence analysis was performed as described previously with only minor modification (Nioi, et al. 2007, Rocourt, et al. 2013)_ENREF_9. 3T3-L1 cells were seeded on coverslips in 6-well plates at 70% confluency then differentiated and treated as described above. At the end of the experiment, coverslips were rinsed in PBS, fixed in 4% paraformaldehyde solution in PBS for 30 min at room temperature and then washed twice with 1x PBS.

Antibody detection

Cells were prepared as described above with minor modifications as follows; after 15 min fixation with a paraformaldehyde solution, the cells were permeabilized with ice-cold methanol for 10 min at -20° C. Next, the cells were permeabilized again for 10 min in 0.3% Triton X-100 and then blocked in 10% normal goat serum / 0.3 M glycine in 1x PBS for 1 h to block non-specific protein-protein interactions. Individual coverslips were hybridized with either of the following antibodies for 1 h at room temperature (RT): phospho-DNA-PK_{cs} Thr-2647 (lot 903801) (1: 250 in BSA, Abcam, and Boston, MA) and phospho-ATM Ser-1981 (lot 20772) (1:250 in BSA, Rockland, Gilbertsville, PA). After incubation with antibodies, the coverslips were washed in 1x PBS, and incubated with the appropriate secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit for phospho-DNA-PK_{cs} Thr-2647 and Alexa Fluor 594-conjugated goat anti-mouse for phospho-ATM Ser-1981 both from Invitrogen Molecular Probes, Carlsbad, CA) in BSA (1:200 dilution) for 1 h at RT in the dark. Cells were then washed 3x in 1x PBS and the

coverslips were mounted onto slides that contained a drop of DAPI and were visualized using immunofluorescent microscopy.

Quantification of antibody data

Immunostaining was visualized using the 63x oil-immersion lens on a Zeiss Axio Observer Z1m (Zeiss, Thornwood, NY) and analysis was performed as described (Rocourt, et al. 2013, Wu, et al. 2010). A z-stack of 10 photos was taken of three random areas on each cover slip using the appropriate channels (DAPI, GFP and DsRed). Images were obtained using the same parameters of brightness, contrast and exposure time and were processed using the deconvolution function of the software AxioVision Release 4.7.2.0.

Statistics

All the experiments were independently performed and repeated three times. We defined the control as differentiated 3T3-L1 or MEF cells, induced to undergo adipogenesis as described above unless stated otherwise in the text. The data were analyzed using GraphPad Prism software version 5.04 (GraphPad Software Inc., La Jolla, California). Two tailed, one- sample t-tests were applied to determine statistical significance (*, $p<0.05$) between treatments.

Results

Selenium speciation differentially effects lipid accumulation

In order to investigate the suspected insulin-mimetic effect of selenium (Heart and Sung 2003, Xu et al 2011) on adipocyte differentiation and lipid accumulation, cells were treated with 2 µM methylseleninic acid (MSeA) or 2 µM sodium selenite (Na_2SeO_3) during

the differentiation process (See Figure 1: Timeline of Adipocyte Differentiation for timeline and day designation).

Interestingly, the amount of lipid accumulation and adipocyte differentiation were found to be dependent on the selenium species used. We found that Na_2SeO_3 treatment decreased lipid accumulation when added at the initiation of hormone induction (See Figure 19: Sodium selenite treatment decreases lipid accumulation when added at the start of adipogenesis) and that MSeA treatment slightly increased 3T3-L1 differentiation when added at the start of adipogenesis, as well as when added after the initial hormone exposure period (See Figure 20: Methylseleninic acid promotes lipid accumulation).

Furthermore, both species of selenium elicited maximal effects on lipid accumulation when they were added after the standard hormonal induction period. This response indicates that the insulin-mimetic effect of selenium is observed only following the commitment to adipocyte lineage. Therefore, the effects of selenium treatment during adipocyte differentiation are likely explained by properties of selenium that are not related to its role in insulin signaling or glucose metabolism.

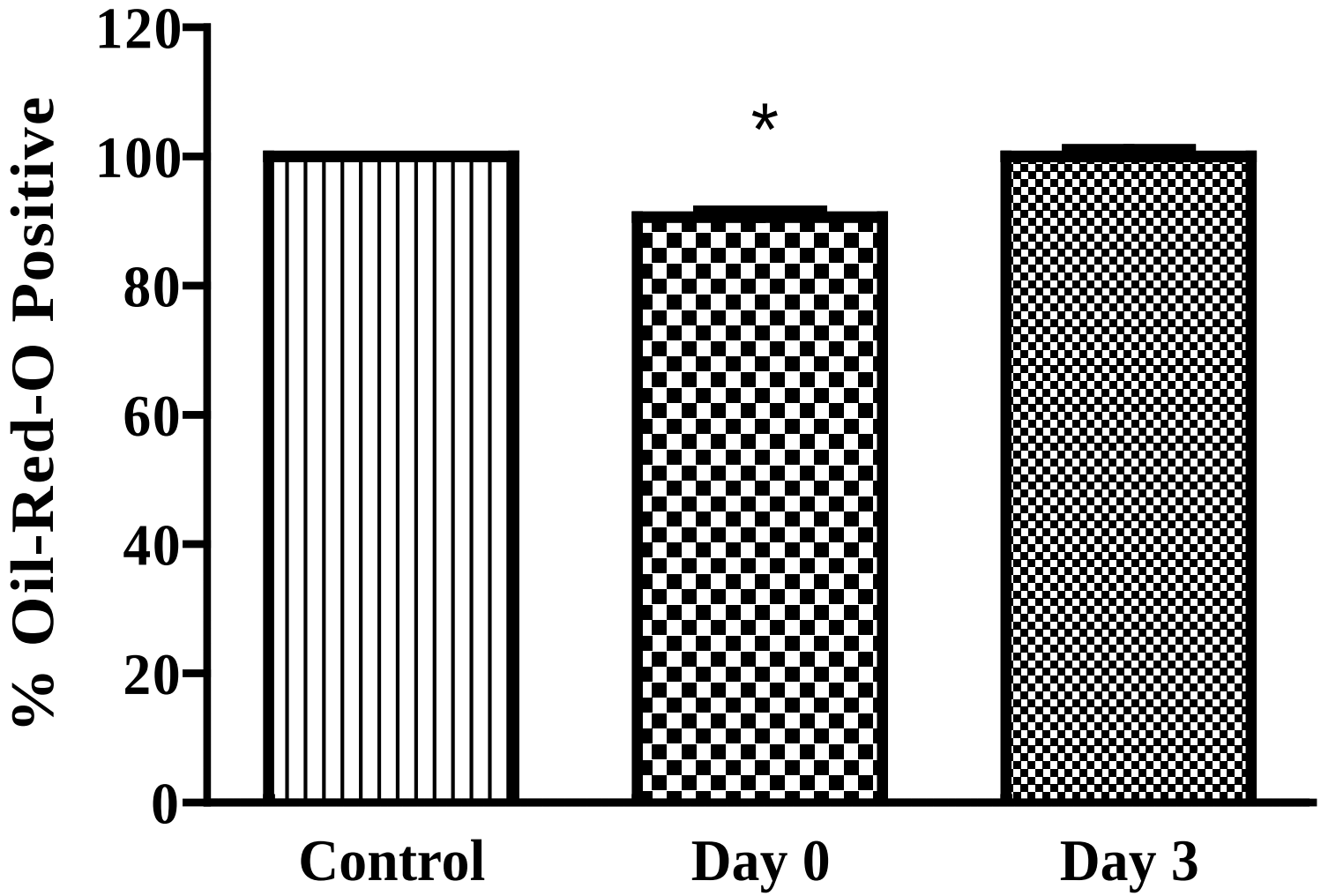


Figure 19: Sodium selenite treatment decreases lipid accumulation when added at the start of adipogenesis

3T3-L1 fibroblasts were differentiated and treated with Na_2SeO_3, (2 μM in PBS, continuous treatment beginning either on day 0 or 3 of adipogenesis) and cultured in 20% oxygen. Lipid accumulation was determined by Oil-Red-O staining and analyzed using a light microscope (400x). Each treatment was given a score based on the percent Oil-Red-O saturation when compared to the differentiated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells).

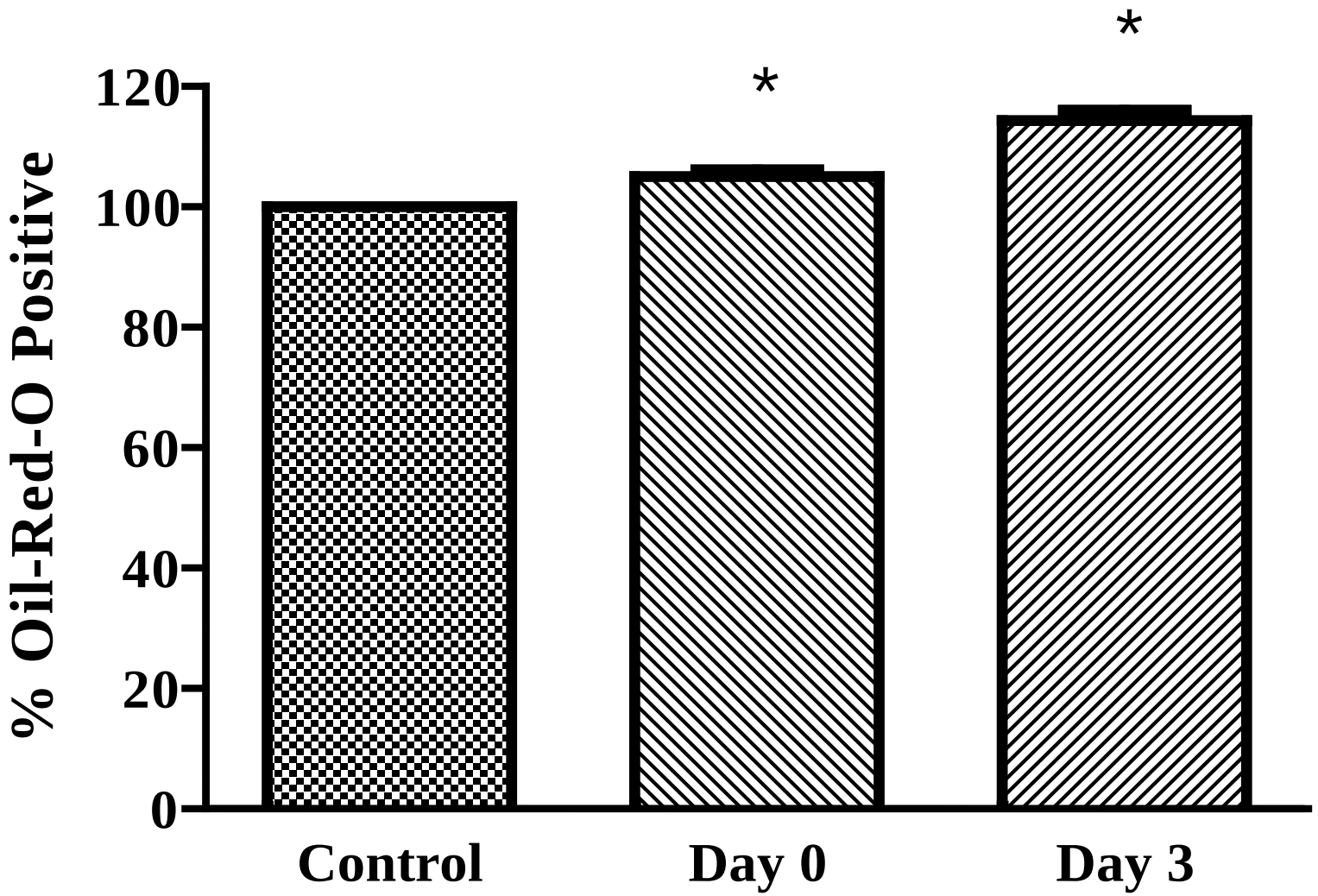


Figure 20: Methylseleninic acid promotes lipid accumulation throughout adipogenesis

3T3-L1 fibroblasts were differentiated and treated with methylseleninic acid, (2 µM in PBS, continuous treatment beginning either on day 0 or 3 of adipogenesis) and cultured in 20% oxygen. Lipid accumulation was determined by Oil-Red-O staining and analyzed using a light microscope (400x). Each treatment was given a score based on the percent Oil-Red-O saturation when compared to the differentiated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells).

The role of DNA damage kinases in a selenium treated adipocyte model

Because of the unique observations that lipid accumulation is dependent on the DNA damage kinases ATM and DNA-PK, and that selenium species affects lipid accumulation during adipogenesis, we aimed to determine whether this response to selenium species occurs via the ATM or DNA-PK pathways. To address this question, the kinase activity of ATM and DNA-PK in 3T3-L1 cells were chemically inhibited using the commercially available inhibitors KU 55933 for ATM inhibition and NU 7026 for DNA-PK inhibition. The cells were then challenged with sodium selenite and lipid accumulation measured by ORO staining.

Sodium selenite treatment significantly reduced lipid accumulation in cells treated with the ATM inhibitor (See Figure 21: Sodium selenite further decreases lipid accumulation in ATM inhibited cells). Sodium selenite reduced lipid accumulation in cells that have had DNA-PK inhibited, although analysis revealed the observation was not statistically significant (See

Figure 22: Sodium selenite slightly reduces lipid accumulation in DNA-PK inhibited cells). Taken together, these results suggest that the effect of sodium selenite on lipid accumulation occurs through an ATM-dependent pathway, with ATM located upstream of DNA-PK in mediating the cellular response to sodium selenite.

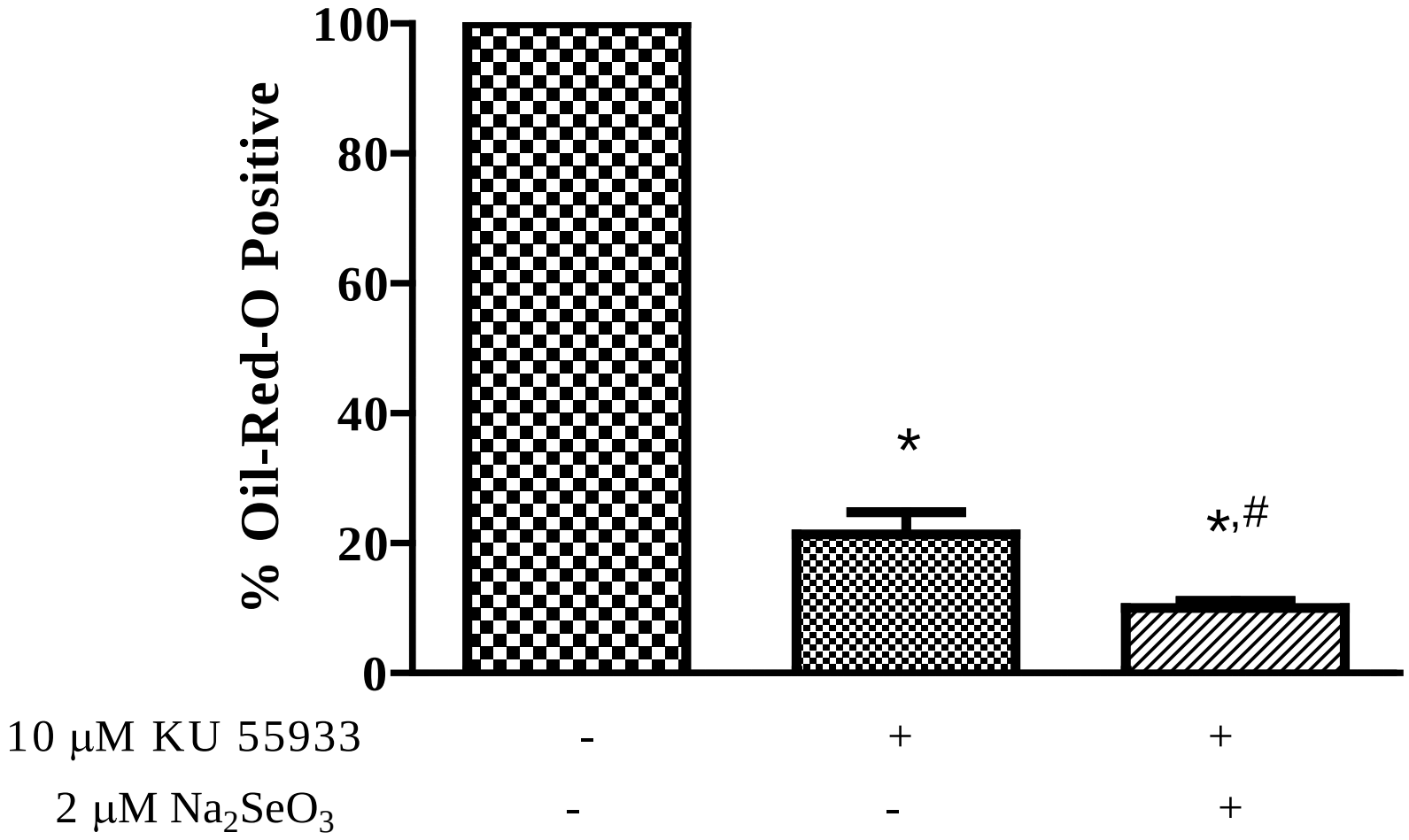


Figure 21: Sodium selenite further decreases lipid accumulation in ATM inhibited cells

Differentiated 3T3-L1 fibroblasts were treated with the ATM inhibitor, KU 55933, (10 μM in DMSO; 48 h treatment started day 0 of adipogenesis) and Na_2SeO_3 (2 μM in PBS; 48 h treatment started day 0 of adipogenesis) and cultured in 20% oxygen. Lipid accumulation was determined by Oil-Red-O staining and analyzed using a light microscope (400x). Each treatment was given a score based on the percent Oil-Red-O saturation when compared to the differentiated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells, # denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells with ATM chemically inhibited).

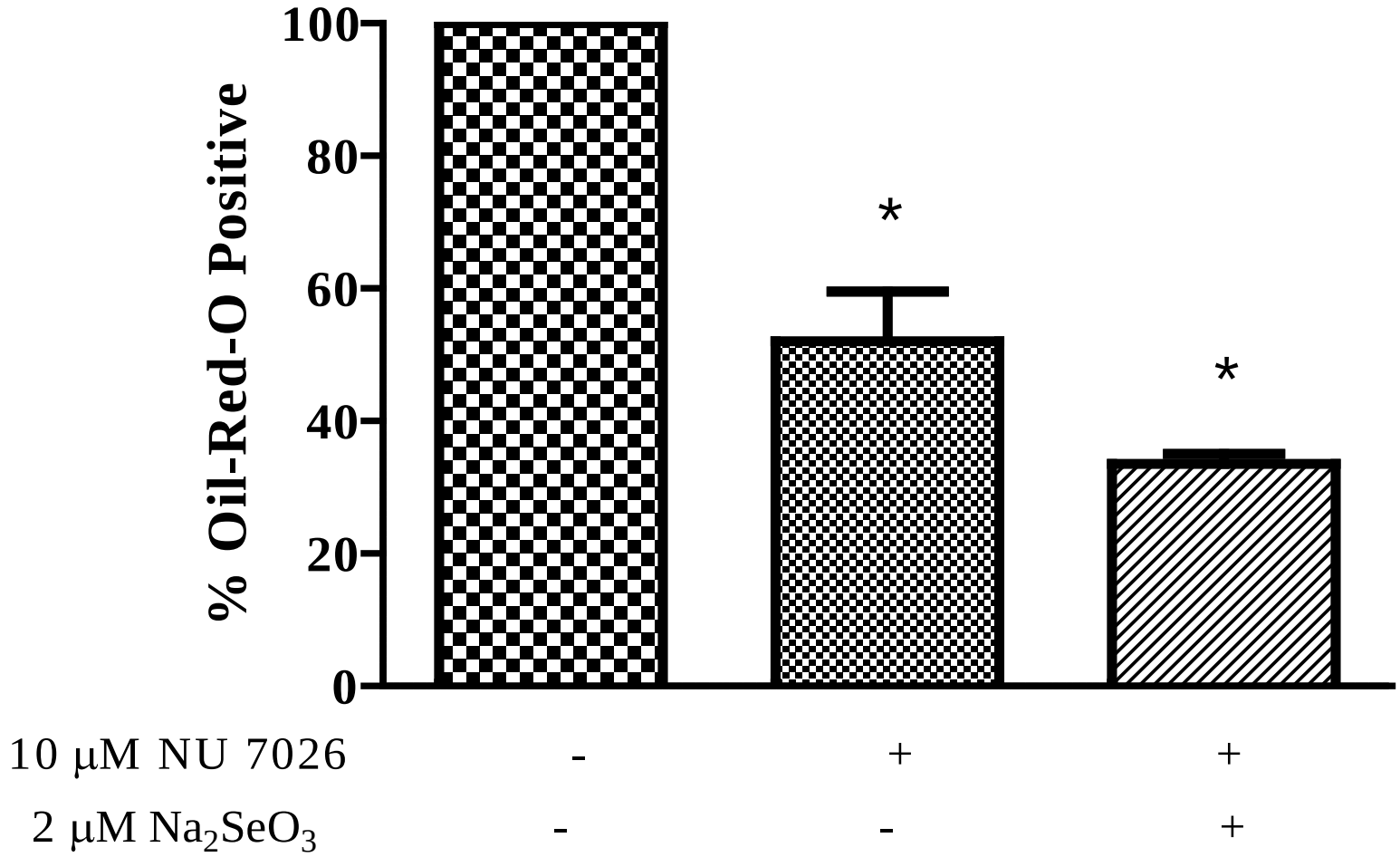


Figure 22: Sodium selenite slightly reduces lipid accumulation in DNA-PK inhibited cells

Differentiated 3T3-L1 fibroblasts were treated with the DNA-PK inhibitor, NU 7026, (10 μM in DMSO; 48 h treatment started day 0 of adipogenesis) and Na_2SeO_3 (2 μM in PBS; 48 h treatment started day 0 of adipogenesis) and cultured in 20% oxygen. Lipid accumulation was determined by Oil-Red-O staining and analyzed using a light microscope (400x). Each treatment was given a score based on the percent Oil-Red-O saturation when compared to the differentiated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells).

Selenium species restores lipid accumulation in cells challenged with known DNA stressors

Cells treated with paraquat (500 µM, 48 h) during differentiation failed to survive. We suspect that the cause of death of these cells (apoptosis, necrosis, autophagy etc.) was most likely due to the high oxidative stress burden. When cells were co-treated with sodium selenite, there were no effects on lipid accumulation and cell survival. However, when cells were exposed to methylseleninic acid, we were able to prevent cell death and restore lipid accumulation (See Figure 23: Methylseleninic acid, but not sodium selenite, restores lipid accumulation in paraquat treated cells).

The treatment of cells with HU (1 mM, 48 h) treatment decreased lipid accumulation, which in *in vivo* models, is a unifying symptom for lipid dystrophies. HU treated adipocytes co-treated with either sodium selenite or methylseleninic acid promoted an increase in lipid accumulation (See Figure 24: Selenium reverses the effect of hydroxyurea on adipogenesis), confirming the role of selenium in restoring lipid accumulation in cells challenged with DNA stressors.

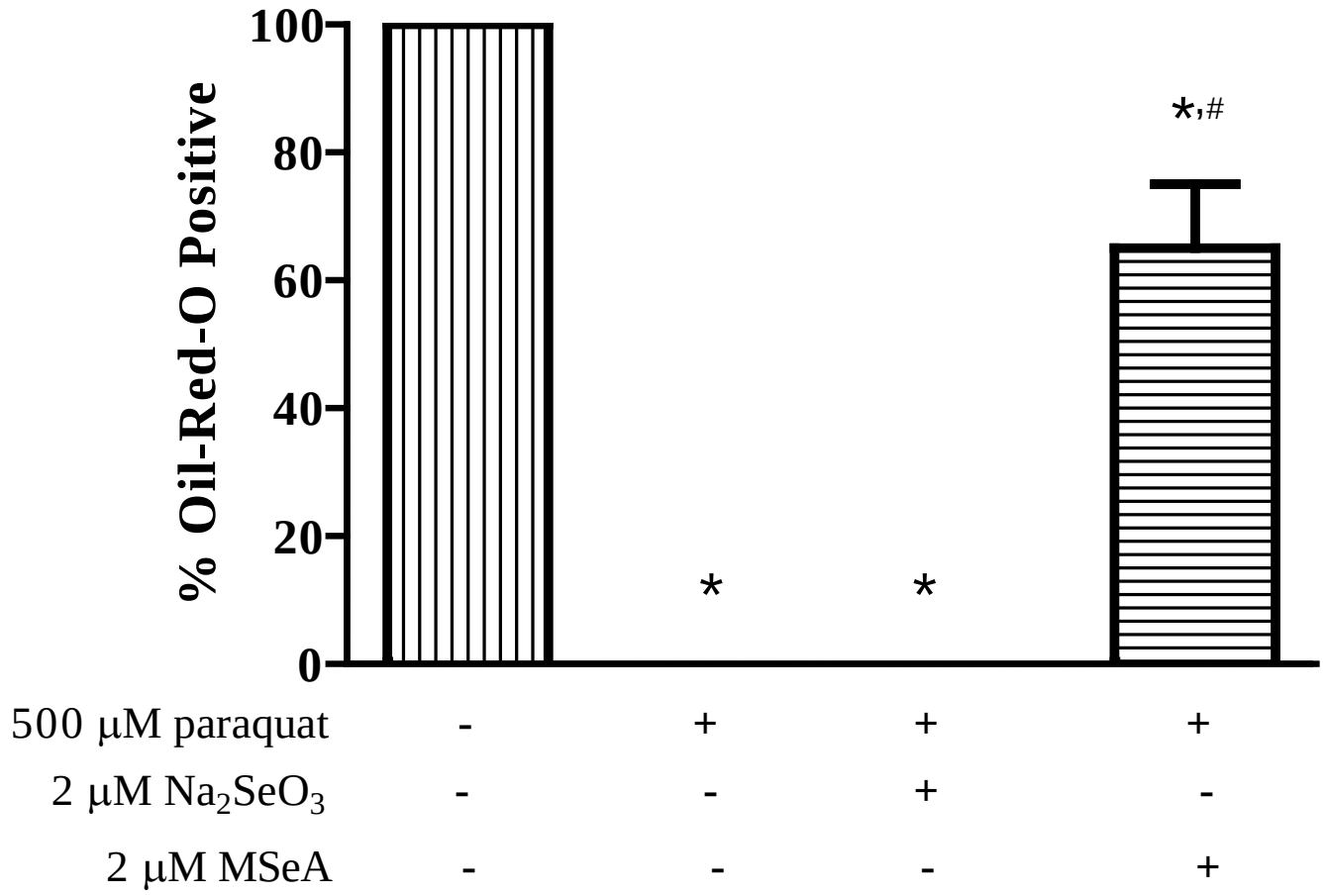


Figure 23: Methylseleninic acid, but not sodium selenite, restores lipid accumulation in paraquat treated cells

Differentiated 3T3-L1 fibroblasts were treated for 48 h starting at day 0 of adipogenesis with the ROS generator paraquat (500 μM in dd H_2O), Na_2SeO_3 (2 μM in PBS), MSeA (2 μM in PBS) and cultured in 20% oxygen. Lipid accumulation was determined by Oil-Red-O staining and analyzed using a light microscope (400x). Each treatment was given a score based on the percent Oil-Red-O saturation when compared to the differentiated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells, # denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells treated with paraquat only).

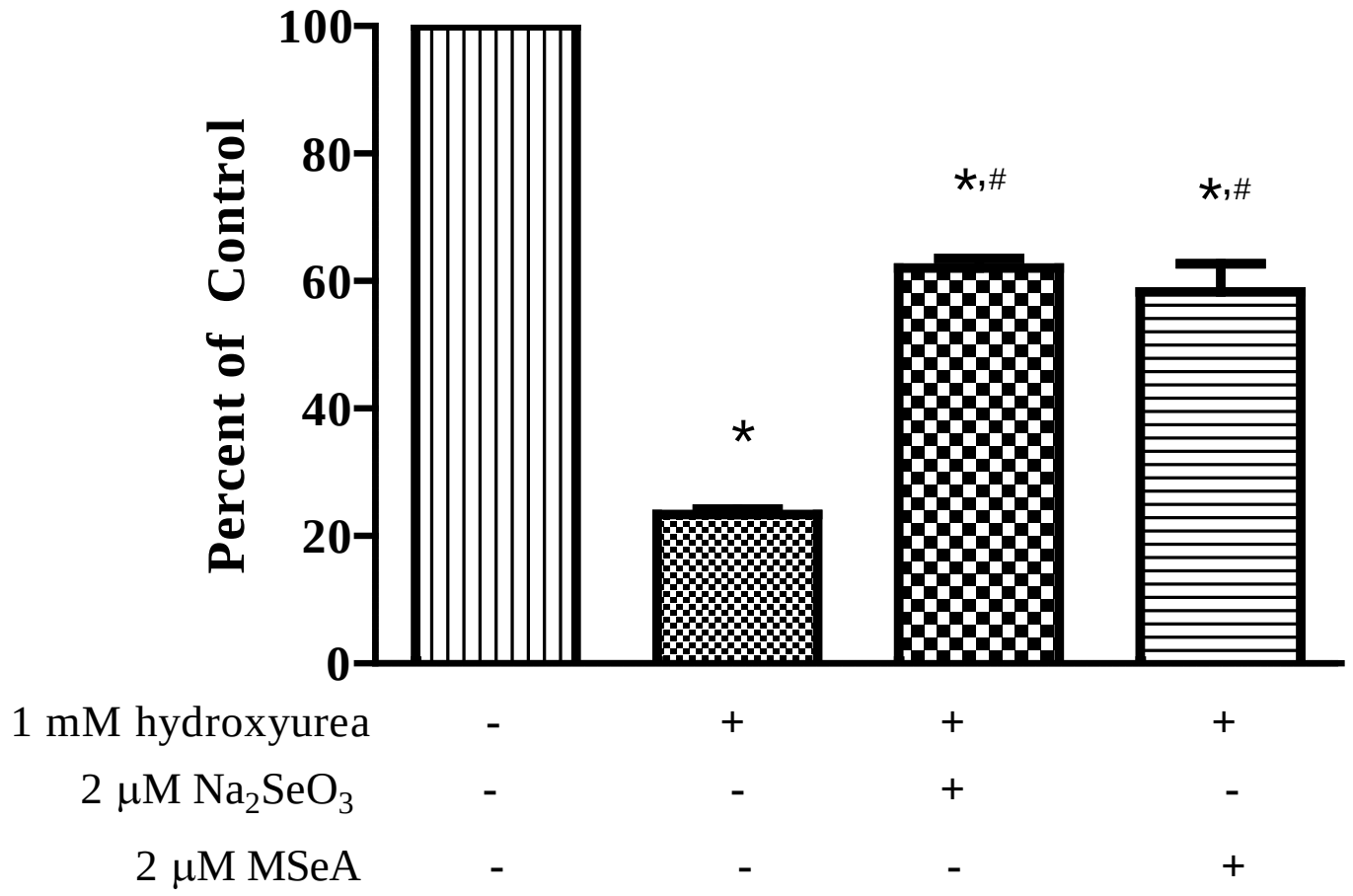


Figure 24: Selenium reverses the effect of hydroxyurea on adipogenesis

Differentiated 3T3-L1 fibroblasts were treated for 48 h starting at day 0 of adipogenesis with the replication stress inducer hydroxyurea (HU) (1 mM in dd H_2O), Na_2SeO_3 (2 μM in PBS), MSeA (2 μM in PBS) and cultured in 20% oxygen. Lipid accumulation was determined by Oil-Red-O staining and analyzed using a light microscope (400x). Each treatment was given a score based on the percent Oil-Red-O saturation when compared to the differentiated control, which we designated to be 100%. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells, # denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells treated with hydroxyurea only).

The role of the selenium induced DNA damage response during adipogenesis

In order to determine the activation of the ATM kinase during adipogenesis, ATM kinase activation was assessed. We measured the anti-*p*ATM Ser-1981 antibody, and DNA-PK activation, as indicated by the anti- *p*DNA-PK_{cs} Thr-2647 antibody. The latter phosphorylation site was selected because it is known to be downstream of ATM in the DNA damage response (Chen et al 2007). Phosphorylation at Thr-2647 is required for cellular resistance to radiation (Soubeyrand et al 2003) and promotes the repair of DNA double strand breaks by the NHEJ pathway (Povirk et al 2007).

The acid and salt forms of selenium activated *p*ATM Ser-1981 (See Figure 25: Selenium treatment is a potent activator of the ATM kinase during adipogenesis) but not *p*DNA-PK_{cs} Thr-2647 (See Figure 26: Selenium induced phosphorylation of DNA-PK_{cs} at Thr-2647 is downstream of ATM kinase). However, sodium selenite treatment results in statistically significant increased phosphorylation of ATM at Ser-1981 than methylseleninic acid treatment. These results were expected based on the metabolism and metabolites of both forms of selenium.

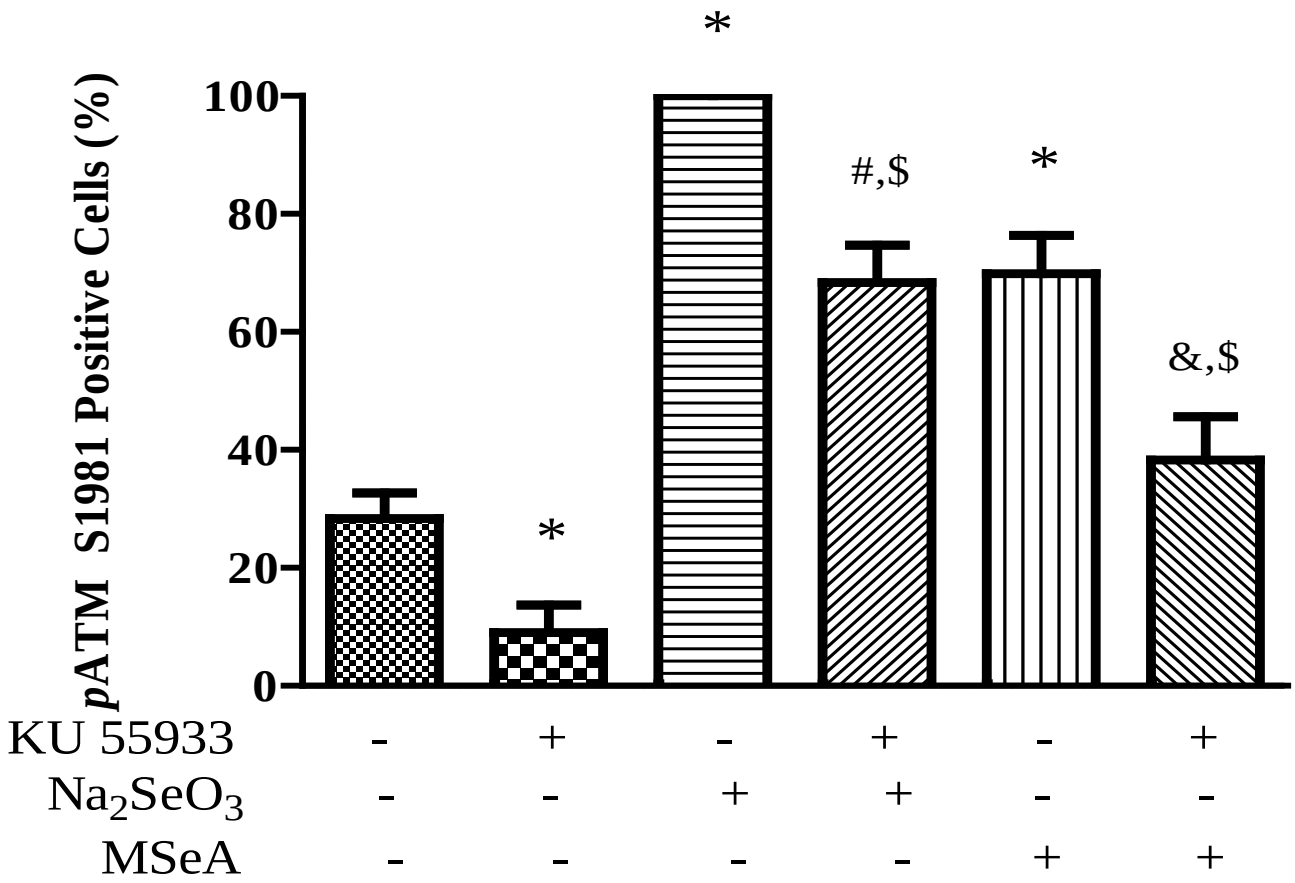


Figure 25: Selenium treatment is a potent activator of the ATM kinase during adipogenesis

Cells were treated with, without or in combination with KU 55933 (10 µM), Na_2SeO_3 (2 µM) or MSeA (2 µM) at the time of induction with MDI media. Cells were maintained in insulin media for five days, then fixed, probed for total ATM and pATM Ser-1981 and then visualized using AxioVision software. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells, # denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells treated with Na_2SeO_3 only, & denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells treated with MSeA, and $ denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells treated with KU 55933 only.

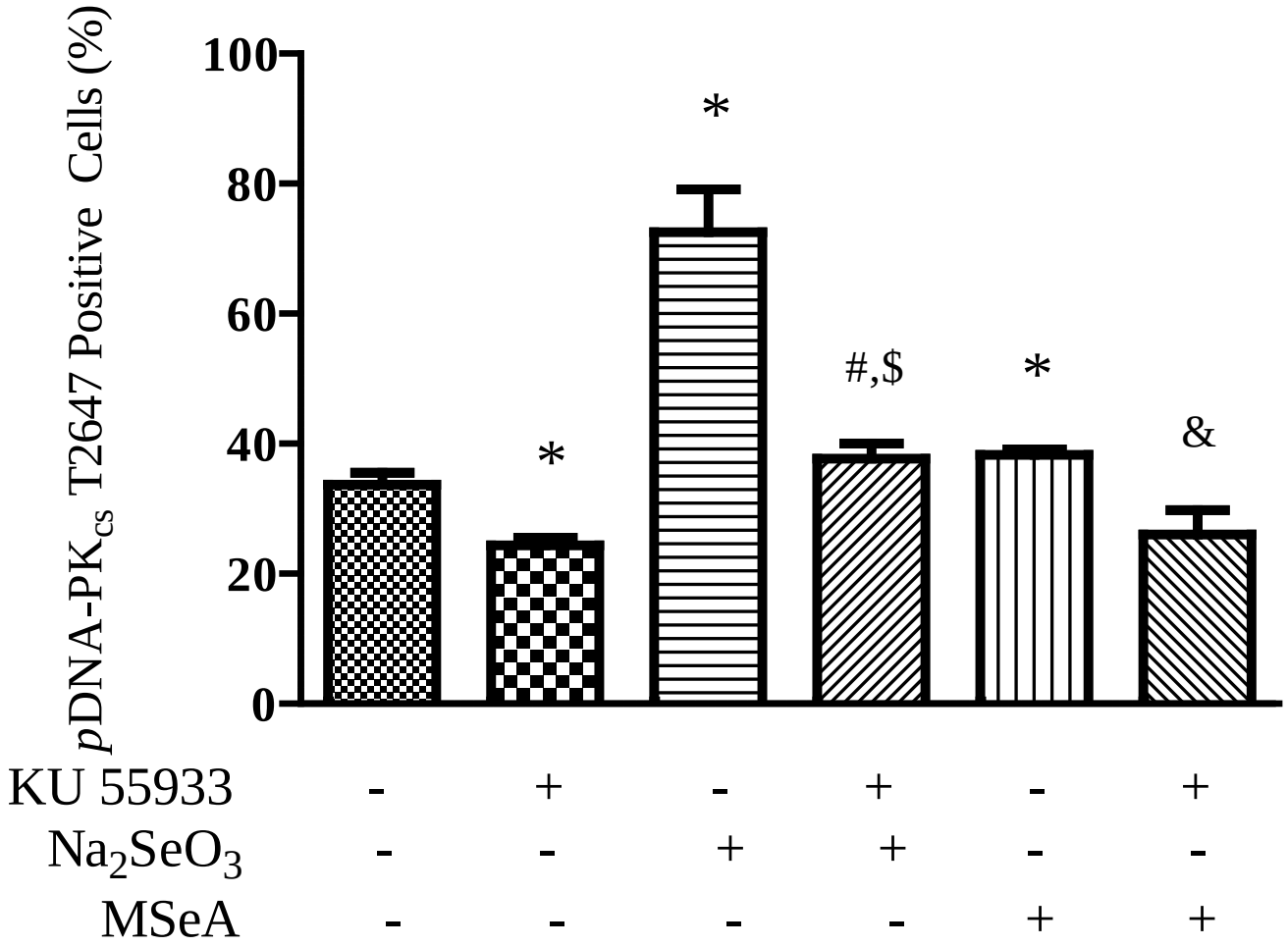


Figure 26: Selenium induced phosphorylation of DNA-PK$_{cs}$ at Thr-2647 is downstream of ATM kinase

Cells were treated with, without or in combination with KU 55933 (10 µM), Na_2SeO_3 (2 µM) or MSeA (2 µM) at the time of induction with MDI media. Cells were maintained in insulin media for five days, then fixed, probed for total DNA-PK$_{cs}$ and *p*DNA-PK$_{cs}$ Thr-2647 and then visualized using AxioVision software. Values are means ± S.E.M. (n= 3; * denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells, # denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells treated with Na_2SeO_3 only, & denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells treated with MSeA, and $ denotes significance ($p<0.05$) as compared to differentiated 3T3-L1 cells treated with KU 55933 only.

Discussion

Selenium has both insulin (Ezaki 1990) and non-insulin (Heart and Sung 2003) like actions in adipocytes. These help to explain this process on a molecular level and could account for the protective effect of selenium in diabetic animals. Selenium has altered function and status in disease states; particularly diabetes (Ammar et al 2004, Ayaz et al 2002, Ayaz et al 2004, Ayaz and Turan 2006, Battell et al 1998, Bleys, et al. 2007b, Brajovic et al 1987, Can, et al. 2005, Douillet et al 1996, He et al 2009, Kilinc et al 2008, Laclaustra et al 2009, Marcason 2008, Mueller et al 2009, Sheng et al 2004, Soska and Kunert 2004, Stranges et al 2007, Vinceti et al 2009, Yadav et al 1991), cardiovascular disease (Agbor et al 2007, Laclaustra, et al. 2009, Rajpathak et al 2005, Soska and Kunert 2004, Toyran et al 2005, Turan et al 2005) and obesity (Douillet et al 1998, Gjorup et al 1988, Kanarek and Aprille 1986, Maquoi et al 2002, McClung et al 2004).

The results of the study agree with others in the literature who have found that selenium is an insulin mimetic (Ezaki 1990). However, we go further and show that selenium speciation is a critical factor in the insulin promoting effect of selenium. Both forms of selenium preferentially targeted sites on ATM as opposed to DNA-PK_{cs}. In addition, sodium selenite was more potent than the acid form of selenium in stimulating phosphorylation of ATM at Ser-1981 and DNA-PK_{cs} at Thr-2647. We hypothesized that, because sodium selenite is more effective in generating ROS than the acid form, more sites were activated.

Paraquat, a herbicide, is an inducer of ROS and is a known toxicant (Ali et al 1996, Ross et al 1979). The damage from paraquat toxicity is processed via the selenium requiring enzyme glutathione peroxidase 1 (GPX1) (Cheng et al 1998). Mice that have

homozygous null mutations for GPX1 have a higher susceptibility to paraquat (de Haan et al 1998). We hypothesized that selenium may have beneficial effects on lipid accumulation in 3T3-L1 cells that were exposed to DNA stressors, such as paraquat or hydroxyurea (HU), because of the protective role of selenium in antioxidant defenses that guard against genome instability.

HU inhibits DNA synthesis and cellular proliferation but does not inhibit protein or RNA synthesis (Sinclair 1965, Young and Hodas 1964). Specifically, HU is a ribonucleotide reductase inhibitor (Elford 1968) that has antineoplastic properties, making it a popular chemotherapy drug (Nocentini 1996, Shao et al 2006). HU is a widely used reagent in studies investigating DNA damage response due to its ability to stall DNA replication by depleting cellular deoxyribonucleotide pools (Reichard 1988). This depletion activates the DNA damage response (Lisby, et al. 2004, Zhou and Elledge 2000). Interestingly, HU can induce both gene expression and synthesis of proinflammatory cytokines, such as TNF-α and IL-6 (Navarra et al 1997). Both cytokines are known to be increased during obesity related complications (Trayhurn and Wood 2004).

We were able to reverse the effects of HU on adipocyte differentiation. We found that when we treated preadipocytes with HU on induction day there was a significant decrease in differentiation. When we co-treated the cells with both forms of selenium we rescued the cells and a larger percentage of preadipocytes became mature adipocytes.

HU is a popular treatment for sickle cell disease because it inhibits the polymerization of sickle hemoglobin by increasing the synthesis of fetal hemoglobin and can prolong the lifespan of the individual (Charache 1997, Steinberg et al 2003). HU treatment increases the expression of the selenium containing GPX1 enzyme in a p53 dependent manner (Cho

et al 2010). Increased GPX1 could possibly explain the effectiveness of HU in sickle cell patients. This suggests that HU may have a direct interaction with selenium, supporting our data that shows an interaction between selenium and HU.

We showed that there is a significant increase in lipid accumulation in HU treated cells following selenium treatment. This data, and the similar findings from the paraquat treated cells, suggest that selenium co-treatment could reverse or decrease lipodystrophies and cachexia that commonly occur because of medications used to treat chronic diseases such as sickle cell, cancer, AIDS and other wasting diseases. These results could also support the fact that supranutritional levels of selenium could reverse HU's intended effects and render it useless for chemotherapy patients.

In the future, we hope to describe the mechanism of selenium and DNA damage response in the differentiation of adipocytes and elucidate how these things affect the bigger picture of metabolic disease, diabetes and obesity.

CHAPTER 4: SUMMARY AND CONCLUSIONS

Summation of Objectives

The first objective was to understand if ATM and DNA-PK kinase inhibition have an effect on adipocyte integrity during adipogenesis and throughout the lifespan of the adipocyte. This objective was met by confirming the role of ATM and DNA-PK kinase activity during adipogenesis and by demonstrating that their role was directly dependent on intracellular ROS levels.

The second objective was to understand how ATM and DNA-PK maintain adipocyte and preadipocyte integrity by preventing some of the associated senescent properties of damaged cells. We have found that inhibition of ATM and DNA-PK kinase activity causes a senescent-like phenotype in preadipocytes.

We believe the senescent preadipocyte is the foundation for the impaired adipogenesis and unusual adipocyte phenotype we see after stimulation of adipogenesis in cells that have ATM and DNA-PK inhibited. We propose that ATM and DNA-PK inhibition in preadipocytes causes cellular senescence and prevents the cells from undergoing complete adipogenesis, even after the standard pro-adipogenic hormonal treatment (See Figure 1: Timeline of Adipocyte Differentiation).

It is possible that by inhibiting ATM and DNA-PK in preadipocytes, we have induced senescence and consequently created a model for studying adipocyte dysfunction. Our study can help comprehend how adipocyte dysfunction is triggered and maintained, and therefore, we can speculate on potential therapeutic targets to reverse or mitigate the

consequences of adipocyte dysfunction, which would help address and treat the comorbidities associated with obesity.

Furthermore, we propose a ROS-driven hypothesis of how ATM and DNA-PK inhibition contribute to dysfunctional adipocytes. After inhibiting enzymes that respond to and regulate ROS, what we believe we have done is induced a preadipocyte environment with high/discordant oxidative stress levels. Our studies suggest that if increased ROS in dysfunctional adipocytes can be regulated, the consequences of adipose tissue dysfunction can be prevented or treated.

The third objective was to understand how nutrients could affect adipogenesis, particularly by using selenium to manipulate the ROS-ATM-DNA-PK dependent adipogenic pathway. Interestingly, it was found that selenium speciation was a key factor in altering lipid accumulation, most likely due to differences in redox potentials of selenium metabolites. Therefore, adding more complexity to weight maintenance theories that rely on strict calorimetery rules.

Significance

Obesity is a worldwide health epidemic that contributes to and exacerbates many disease states (See Metabolic Dysfunctions). Current treatment strategies for treating obesity and its complications have failed. Understanding normal adipose tissue metabolism, physiology and differentiation could be a foundation for obesity research and a cornerstone to develop therapeutic interventions. We believe that there is a critical gap in our knowledge of how dysfunctional adipocytes are propagated (See Mechanisms of Metabolic Dysfunction). By understanding adipocyte dysfunction, treatment of obesity-

induced disease states might be possible. The work summarized in this dissertation is presented in the hope to be able to help guide current therapeutic strategies and identify molecular targets for the treatments of the comorbidities of obesity.

Often times in obese individuals, particularly those with metabolic diseases, there is increased oxidative DNA damage (Al-Aubaidy and Jelinek 2011) which may lead to genomic instability (Ahima 2009), thus perpetuating the chronic disease cycle. This inflammatory state might directly affect beta-cell homeostasis and disrupt self-replication, causing hyper- and hypoplasia of beta-cells thereby driving the type 2 diabetic phenotype. In our research, we believe dysfunctional preadipocytes, perhaps caused by these cytokines, activate the innate immune system creating the subclinical inflammatory response indicative of type 2 diabetes, that go on to induce insulin resistance and hyperglycemia (See Figure 27: Conceptual Model).

Our research further elucidates the management of oxidative stress as a key factor to maintain adipocyte tissue homeostasis in order to prevent metabolic dysfunctions. Further research is required to elucidate the mechanisms by which adipocytes maintain homeostasis within the body and whether adipose disequilibrium causes or contributes to dysfunction, senescence, tissue remodeling or a combination of these within the adipocyte.

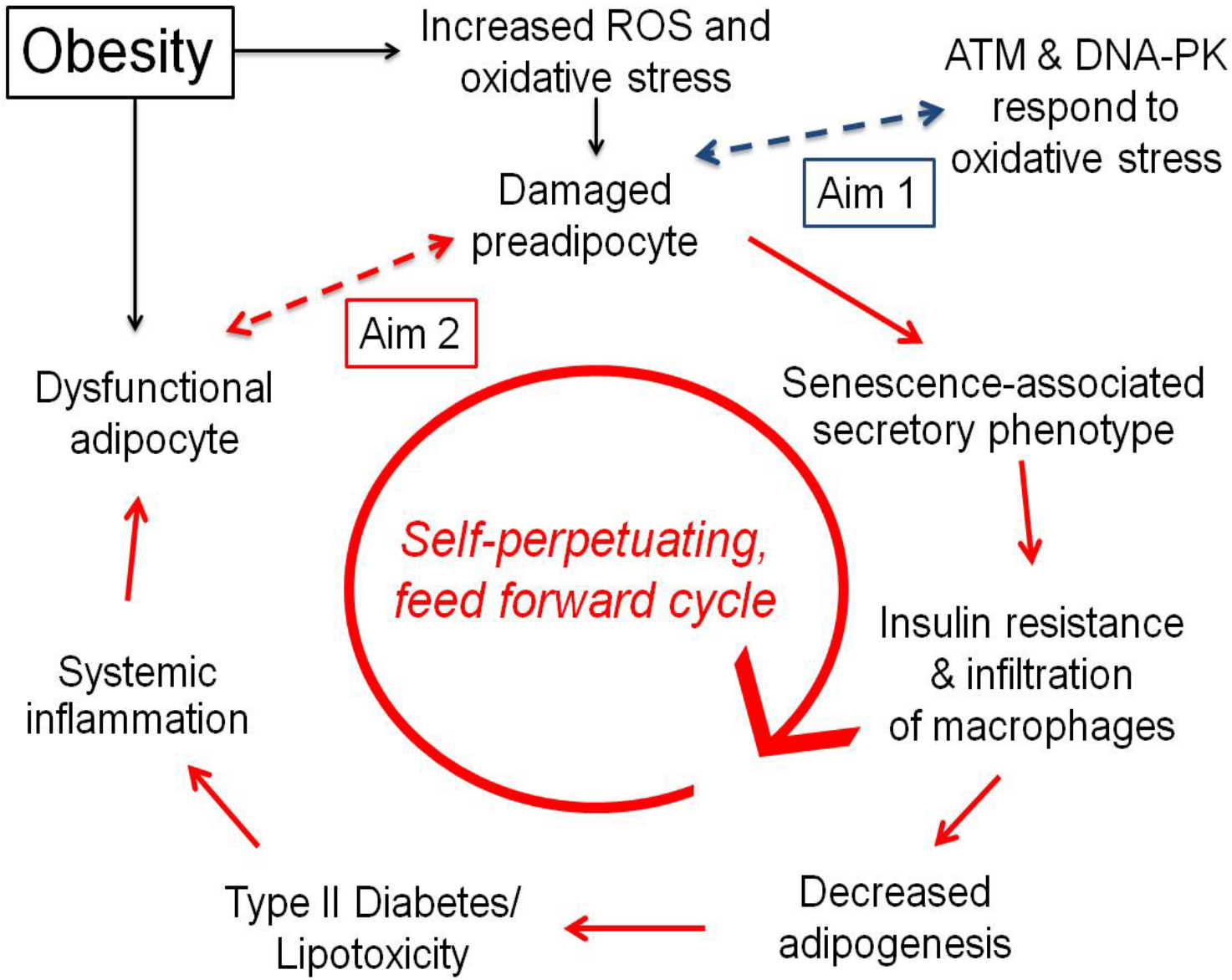


Figure 27: Conceptual Model

The ROS- ATM- DNA-PK pathway as a missing link in unifying the systemic effects of dysfunctional adipocyte.

Adipocyte dysfunction includes increased lipolysis, impairment of triglyceride storage and increased cytokine production (Coppack 2001). All of these are linked to insulin resistance (de Heredia et al 2012). It is clear that there is an excess production of IL-6 and TNFα in type 2 diabetes. However, the origins, order of events, and molecular mechanisms linking these cytokines to type 2 diabetes are not well understood or defined. IL-6 and TNFα stimulate the production of acute phase proteins (James, et al. 2001, Ogden, et al. 2006) that then cause inflammation. Since inflammation is a common facet that most metabolic diseases related to obesity share (Candore et al 2010, Emanuela et al 2012, Fuentes et al 2013, Kitade et al 2012, Sun, et al. 2012, Ye 2013), understanding how cytokines promote immune system imbalance is critical in the prevention and treatment of the comorbidities of obesity.

We have attempted to elucidate how obesity can promote conditions that favor chronic disease on the molecular level by studying DNA damage response proteins in adipocytes. The work summarized here represents the initial investigation of the roles of ATM and DNA-PK_{cs} in the formation and maintenance of adipocytes. In particular, we show that maintenance of oxidative tone via the ATM and DNA-PK pathway is necessary to promote lipid accumulation in adipocytes. Moreover, our work helps to explain the importance of balancing oxidative stress in order to maintain normal adipose tissue function and thus stifle the onset or progression of some of the comorbidities of obesity (See Linking Metabolic Derangements to Adipocytes). These findings portray a novel approach to addressing the comorbidities of obesity and attempt to provide insight that could improve the quality of life for overweight and obese individuals and to help understand the evolution of chronic diseases.

APPENDIX

Cell Culture Reagents

- 1% penicillin/streptomycin (GibcoBRL, Carlsbad, CA, Cat# 15140148)
- IBMX (Sigma-Aldrich, St. Louis, MO, Cat# I-7018)
- Dex (Sigma-Aldrich, St. Louis, MO, Cat# D-4902)
- DMEM (GibcoBRL, Carlsbad, CA, Cat# 11965-084)
- FBS (GibcoBRL, Carlsbad, CA, Cat# 10099-141)
- Insulin (Bovine; Sigma-Aldrich St. Louis, MO, Cat# I-5500)
- Pen/Strep/Glutamine (100x P/S/G; GibcoBRL, Carlsbad, CA, Cat#10378-016)

Cell Culture Chemicals and Treatments

- Caffeine (2 mM, Sigma-Aldrich, St. Louis, MO, Cat# 485635)
- DMSO (Vehicle, Sigma- Aldrich, St. Louis, MO, Cat# 472301)
- H_2O_2 (10 µM, Sigma- Aldrich, St. Louis, MO, Cat# 216763)
- HU (1mM, MP Biomedicals, Santa Ana, CA, Cat# 0210202301)
- KU 55933 (10 µM, Tocris, Ellisville, MO, Cat# 3544)
- MSeA (2 µM, Sigma-Aldrich, St. Louis, MO Cat# 541281)
- Na_2SeO_3 (2 µM, Sigma-Aldrich, St. Louis, MO Cat# S9133)
- NAC (10 mM, Sigma- Aldrich, St. Louis, MO, Cat# A-7250)
- NU 7026 (10 µM, Tocris, Ellisville, MO, Cat# 2828)
- Paraquat (500 µM, Sigma-Aldrich, St. Louis, MO Cat# 36541)
- TEMPO (1 mM, Sigma-Aldrich, St. Louis, MO, Cat# 214000)
- Wortmannin (200 µM, Sigma-Aldrich, St. Louis, MO, Cat# 95455)

Experimental Reagents

- Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1: 200 in BSA, Invitrogen, Carlsbad, CA, Cat# A11008)
- Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (1: 200 in BSA, Invitrogen, Carlsbad, CA, Cat# A11005)
- CM-H_2DCFDA (10 µM in PBS, Invitrogen, Carlsbad, California, Cat# C6827)
- HCS LipidTOX™ (Green neutral lipid stain 1: 100 in BSA, Invitrogen Molecular Probes Inc., Carlsbad, CA, Cat# H34475)
- ORO (0.5 g dissolved in 250 mL isopropanol, Sigma-Aldrich, St. Louis, MO, Cat# O0625-100G)
- *p*ATM Ser-1981, phospho-ATM at Ser-1981
- *p*DNA-PK_{cs} Thr-2647 Antibody (lot # 903801) (1:200 in BSA, Abcam, Boston, MA, Cat# ab61045)
- Perilipin D418 Antibody (1: 200 in BSA, Cell Signaling Technology, Danvers, MA, Cat# 3470)
- Prolong® Gold mounting media containing 4,6-diamidino-2-phenylindole (Prolong® Gold mounting media containing DAPI or DAPI) (1 drop, Invitrogen, Carlsbad, CA, Cat# P36930)
- Senescence-associated β-galactosidase kit (SA- β-gal) (Biovision, Milpitas, CA, Cat# k320-620)
- γH2A.X at Serine 139 Antibody (γH2A.X) (lot 41665603, Abcam, Boston, MA, Cat# ab2893)

Table 1: Cell culture reagents

Chemical	Company	Catalog Number	Treatment Concentration	Treatment Duration
Alexa Fluor® Anti-Mouse	Invitrogen	A11005	1:200	1 h, RT
Alexa Fluor® Anti-Rabbit	Invitrogen	A11008	1:200	1 h, RT
Caffeine	Sigma	485635	2 mM	48 h
DAPI Prolong® Gold	Invitrogen	P36930	1 drop	
Dexamethasone	Sigma	D-4902	1 µM	48 h
IBMX	Sigma	I-7018	0.5 mM	48 h
Insulin	Sigma	I-5500	166 nm (1 µg/mL)	48 h as induction media, then constant when used for adipocyte maintenance
KU 55933	Tocris	3544	10 µM	48 h
LipidTOX	Invitrogen	H34475	1:100	1 h, RT
NAC	Sigma	A-7250	10 mM	48 h
NU 7026	Tocris	2828	10 µM	48 h
***p*DNA-PK$_{cs}$ Thr-2647**	Abcam	ab61045	1:200	Overnight 4° C
Perilipin D418	Cell Signaling	3470	1:200	1 h, RT
TEMPO	Sigma	214000	1 mM	48 h
Wortmannin	Sigma	95455	200 µM	48 h
γH2A.X at Serine 139	Abcam	ab2893	1:250	1 h, RT

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