p107 is crucial to the switch in adipose lineage commitment: a missing link in the PRDM16 pathway

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Abstract

This study shows that p107 is at the crux in determining adipose lineage commitment. It was completely absent in BAT and only expressed in the stem cell compartment of WAT depots. Moreover, contrary to PRDM16, a major brown adipocyte determination factor, p107 was significantly decreased in subcutaneous compared to intra-abdominal WAT reservoirs. In vivo activation of brown adipocyte formation within subcutaneous WAT by β3-adrenergic agonist treatment, further significantly decreased levels of p107. Importantly, the absence or the depletion of p107 results in the formation of brown adipocytes in p107 genetically deleted primary mesodermal stem cells and p107 knockdown mesenchymal stem cell lines, respectively. The establishment of brown adipocytes was dependant on the knockdown of p107 during growth or a very early time point during differentiation, suggesting a function in brown adipose lineage commitment rather than the differentiation pathway.

Consistent with the hypothesis that PRDM16 is a brown adipocyte determination factor, PRDM16 over expressing cell lines had significantly reduced levels of p107 concomitant with expression of brown adipocyte markers after differentiation. The control of p107 function by PRDM16 was confirmed by expression assays using a 900 bp p107 promoter fused to the luciferase gene. Notably, the importance of p107 to the PRDM16 brown determination pathway is highlighted by MSC cell lines overexpressing PRDM16 transduced with overexpressing p107 retrovirus. In this case, though the cells undergo adipocyte differentiation, they are blocked from brown adipocyte formation with sustained p107 expression.

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Abbreviations

AD	adipose depot
ANOVA	analysis of variance
ATP	adenosine triphosphate
BAT	brown adipose tissue
BMP7	bone morphogenic protein 7
BMPR1A	bone morphogenic protein receptor 1A
BMR	basal metabolic rate
bp	base pairs
BrdU	5-bromodeoxyuridine
c/ebp	CCAAT/enhancer binding protein
$cPGI_2$	carbaprostacyclin
DMEM	Dulbecco's modified eagle medium
DMEM/F-12 (1:1)	serum free DMEM
EBD	E2F binding domain
EPI	epididymal
FACS	fluorescent activated cell sorting
FADH2	flavin adenine dinucleotide, reduced
FBS	fetal bovine serum
H&E	Hematoxylin and eosin
HDAC	histone deacetylases
hMADS	human mesenchymal adipose derived stem cells
IG	inguinal
IL-6	interleukin-6
INT	interscapular
KD	knockdown
KO	knockout
MEF	murine embryonic fibroblasts
mg	milligram
MSC	mesenchymal stem cells
MSC-Ctrl	mesenchymal stem cells transduced with pMSCV control vector
MSC-scr	mesenchymal stem cells transduced with scrambled p107 RNAi
NADH	nicotinamide adenine dinucleotide, reduced
PERI	retroperitoneal
PET/CT	positron emission tomography - computed tomography
PGC-1α	ppary coactivator 1 alpha
PGE_2	prostaglandin E2
pMSCV	murine stem cell virus plasmid
PPARγ	peroxisome proliferator activated receptor gamma
PPRE	PPARγ responsive elements
pRB	retinoblastoma protein
PRDM16	PRD1-BF1-RIZ1 homologous domain containing 16

qChIP	quantitative chromatin immunoprecipitation
qPCR	quantitative polymerase chain reaction
R998Q	point mutation at arginine 998 for glutamine
RB	retinoblastoma gene
RNAi	ribonucleic acid interference
RNase	ribonuclease
rpm	rotations per minute
rWAT	retroperitoneal WAT
sh	short hair pin
SV	stromal vascular
T_3	triiodothyronine
TAGs	triacylglycerides
TNF-α	tumor necrosis factor alpha
UCP-1	uncoupling protein 1
WAT	white adipose tissue
WT	wild type
β3	CL316,243
µl	microlitre

Contents

Literature Review

1. Obesity

Obesity has reached epidemic proportions, affecting people independently of age, gender and race. There are at least 2.8 million people dying a year due to complications of obesity (OECD, 2010). The World Health Organization states that obesity has more than doubled since 1980. In 2008, approximately 1.5 billion adults were overweight and of these over 500 million were obese, indicating that more than 1 in 10 adults of the world's population are overweight (WHO, 2008). Notably, by 2010, nearly 43 million children under the age of five were overweight, a statistic with dire consequences as childhood obesity is highly correlated with the onset of diabetes later in adulthood (WHO, 2003). With today's sedentary lifestyle and "cafeteria" diets obesity rates are on the rise. It is predicted that by 2020, 75% of Americans will be overweight and 55% of Canadians, let alone the increases occurring worldwide (OECD, 2010). Alarmingly, 65% of the world's population live in countries where being overweight and obese kills more people than being underweight (WHO, 2008).

In obesity, there is a long term imbalance of energy intake which outweighs energy expenditure due to high-caloric, high-fat and high-carbohydrate ingestion leading to hyperplasia and hypertrophy of WAT (Rosen and Spiegelman, 2006). Obesity is the leading cause for the metabolic syndrome and leads to insulin resistance, hypertension, coronary heart disease, some cancers and eventually type II diabetes (Calle and Kaaks, 2004; Donahue et al., 1987; Fujioka et al., 1987; Hjartåker et al., 2008; Kopelman, 2000). In order to lose weight, energy balance must be shifted to energy expenditure through diet

and exercise. The degree to which this is applicable to humans is dependent on genetic and environmental factors (Dudley et al., 2011; Na et al., 2011; Qi et al., 2011; Tanofsky-Kraff et al., 2009; Tounian, 2011). Therefore, we must devise effective counter strategies to combat the obesity pandemic and its many complications tailored to all individuals in society.

1.2 White Adipose Tissue

White adipose tissue (WAT) an important reservoir for energy storage in the form of triacylglycerides (TAGs) and is an endocrine organ that is crucial for whole body homeostasis (Rosen and Spiegelman, 2006). WAT is made up of white adipocytes that morphologically have a unilocular appearance with a single lipid droplet containing TAGs (Cinti, 2005). One function of white adipocytes is to hydrolyze TAGs into fatty acids, which can then be oxidized in mitochondria in other tissues to generate energy in the form of ATP during times of energy need (Lafontan, 2008). White adipocytes have a great capability to expand with over nutrition and it is when these cells are in excess and hypertrophic that WAT becomes dysfunctional. High-fat diets that cause obesity are related to elevated TAG concentrations in adipose tissue and increased lipolysis (Lottenberg et al., 2012).

WAT is able to communicate with the brain and peripheral organs through the secretion of hormones such as leptin, interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and other cytokines released by adipose tissue known as adipokines (Trayhurn and Bing, 2006). Increased WAT results in the dysfunctional secretion of inflammatory

markers including IL-6 and TNF-α and exacerbation of adipokine signalling by decreases in anti-inflammatory adipokines (Lafontan, 2008; Trayhurn and Bing, 2006). Overall, this causes chronic low-grade inflammation which leads to insulin resistance, dyslipidemia, type II diabetes and indirectly to cardiovascular disease (Rosen and Spiegelman, 2006). The increase in these risk factors leads to a shorter life expectancy (Peeters et al., 2003; Walls et al., 2012; Zheng and Yang, 2012). Therefore, finding new and innovative ways to combat this disease is important in order to help give people healthier and longer lives.

WAT is subdivided into visceral (intra-abdominal) and subcutaneous (between fascia and muscle) depots (Cinti, 2000). In humans the visceral depot is located in the abdomen and the subcutaneous depots are located beneath the skin and away from internal organs. Visceral adipose tissue is located closer to vital organs and when in excess is strongly correlated to type II diabetes, insulin resistance, hypertension, and coronary heart disease (Achike et al., 2011; Brietzke, 2010; Donahue et al., 1987; Hermsdorff and Monteiro, 2004; Hjartåker et al., 2008). However, subcutaneous depots help to prevent against these diseases by storing lipids away from vital organs and the heart (McLaughlin et al., 2011; Wajchenberg, 2000).

1.3 Brown Adipose Tissue and UCP-1

Another type of adipocyte is the multi-lipid filled, multilocular appearing, brown adipocyte that makes up brown adipose tissue (BAT). Brown adipocytes are filled with mitochondria which gives them a brownish color, hence its name. It is important in

regulating body temperature under cold conditions (van Marken Lichtenbelt and Schrauwen, 2011).

Normally in the mitochondria, there is oxidation of substrates such as glucose or fatty acids. These substrates generate reducing equivalents, NADH and $FADH_2$, which supply the electrons that pass along the respiratory chain through the innermembrane of the mitochondria. This processes forms a proton gradient in the intermembrane space and the gradient is used to drive the production of ATP through ATP Synthase (Figure 1) (Fernie et al., 2004).

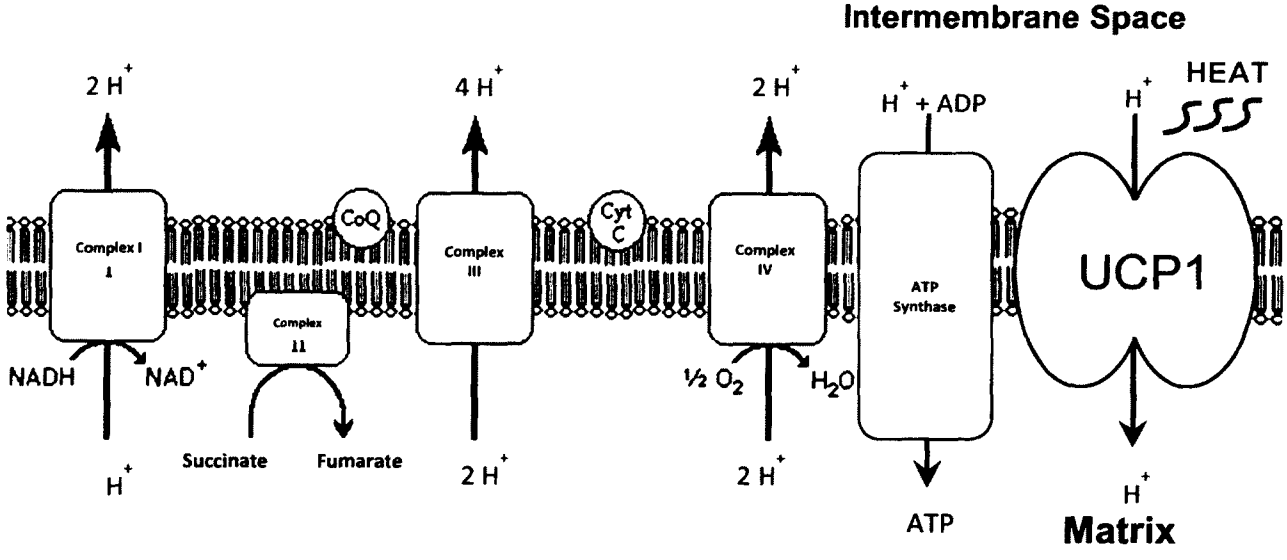


Figure 1. Electron transport chain

See text for detail and referencing.

However, brown adipocyte mitochondria contain the protein uncoupling protein-1 (UCP-1) (Cinti, 2006). When activated UCP-1, acts as a pore, providing an alternate pathway for protons to leak back into the matrix of the mitochondria. The result is the release of energy as heat at the expense of ATP production (Figure 1) (Argyropoulos and

Harper, 2002; Cannon and Nedergaard, 2004). This necessitates the increased utilization of glucose and fatty acids to maintain the proton gradient to produce sufficient ATP. Since more glucose is being used and fatty acid break down is up-regulated, energy expenditure is therefore increased independently of exercise (Lidell and Enerback, 2010; Tseng et al., 2010). The heat produced is termed non-shivering thermogenesis and it is important because it is an adaptation for warm blooded mammals to generate heat in times of cold exposure (Bartness, 2005; van Marken Lichtenbelt and Schrauwen, 2011).

Thermogenesis, the process of heat production in mammals, can be subdivided into two broad categories; obligatory and facultative or adaptive. Obligatory thermogenesis is essential for the life of all cells of the body and includes processes that support normal and consistent body temperatures. It includes necessary energy expenditure involved in ingesting, digesting, and processing food (Himms-Hagen, 1989). The largest component of obligatory thermogenesis is provided by the basal metabolic rate (BMR) (Acheson et al., 1984; Argyropoulos and Harper, 2002). BMR is the minimal amount of energy that needs to be used for the functioning of all vital organs in the body. A defining factor between the two categories is that obligatory thermogenesis occurs continuously in all organs of the body and facultative thermogenesis can be rapidly switched on or off and occurs mainly in two tissues: skeletal muscle and BAT (van Marken Lichtenbelt and Schrauwen, 2011). When in a cold environment mammals have the capacity to increase their heat production in skeletal muscle and BAT. Shivering thermogenesis takes place in muscle, and non-shivering thermogenesis occurs in BAT (Argyropoulos and Harper, 2002; Block, 1994; Himms-Hagen, 1989). Activation and

transcription of UCP-1 is the hallmark of non-shivering thermogenesis. Other genes important for thermogenesis (pro-thermogenic) that are expressed preferentially in brown adipocytes include, Cidea which controls UCP-1 activation, PPARγ coactivator-1 alpha (PGC1-α) involved in mitochondrial biogenesis, Elovl3 a fatty acid elongation factor and Cox8b a protein in the electron transport chain (Petrovic et al., 2010).

UCP-1 activity is tightly controlled by the sympathetic nervous system (SNS) and the β3 adrenergic receptor (Bachman et al., 2002; Hansen and Kristiansen, 2006). For UCP-1 to function it must be activated by an additional stimulus such as cold exposure. Low temperatures are sensed by the skin and the message is relayed back to the central nervous system. From there the message is transmitted along preganglionic neurons to postganglionic neurons which densely innervate BAT. Postganglionic neurons release catecholamines, in this case norepinephrine, which is able to activate the adrenergic receptors on target tissues such as the β3-adrenergic receptor found on the plasma membrane of BAT (Cannon and Nedergaard, 2004). Once the receptor is triggered it causes a signalling cascade to activate UCP-1 and to increase its transcription.

Another more controversial method of achieving an increase in UCP-1 activity is through overeating that is thought to cause diet induced thermogenesis (Bachman et al., 2002; Cannon and Nedergaard, 2004; Feldmann et al., 2009; Lladó et al., 1991; Pérez-Echarri et al., 2007; Rothwell and Stock, 1979). It has been shown that UCP-1 levels increase during times of over feeding in order to try and maintain energy homeostasis. Lladó et al. showed that high fat fed mice had increased brown adipose tissue to try to help compensate for the excess calories (Lladó et al., 1991). Thus they hypothesized that

the obese state itself induces brown adipose tissue activation and recruitment, but that it disappears with a prolonged state of obesity. This was shown by an elegant study by Fieldmann et al., when mice were placed at thermoneutrality that is reared at 30 °C, when the need for non-shivering thermogenesis and thus BAT activation is no longer necessary. In this scenario, mice without BAT quickly became obese (Feldmann et al., 2009). However, contradicting diet induced thermogenesis is the correlation of leaner human individuals having more BAT (Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Vijgen et al., 2011).

BAT was first discovered over 400 years ago by Konrad Gesner (Gesner, 1551). He described BAT as being 'neither fat nor flesh (muscle)', an amazingly accurate description for its time. A large quantity of BAT is found in small mammals in the interscapular region and in new born humans in the axillary, cervical, perirenal and periadrenal regions (Hu et al., 2012; Lean et al., 1986). In humans it was thought to disappear soon after birth. However, in more recent years, through the use of advanced positron emission tomography - computed tomography (PET/CT) technology, BAT has been located in the supraclavicular and spinal regions of adults (Cypess et al., 2009; Saito et al., 2009). PET/CT scans measuring fluorescently tagged glucose (18-fluorodeoxyglucose) uptake in cancer patients noted an increased uptake at the supraclavicular, spinal and suprarenal regions that were not tumors but attributed to BAT (Cypess et al., 2009; Saito et al., 2009). Virtanen et al. in 2009, were the first to show that the areas of cold-induced 18-fluorodeoxyglucose uptake in healthy adult subjects did

in fact have the histologic features of brown adipose tissue and expressed mRNA and proteins that distinguish it from white adipose tissue (Virtanen et al., 2009).

1.4 Brown Adipose Tissue – Potential to Increase Energy Expenditure

Metabolically active BAT has an immense capacity for blood triglyceride clearance and glucose disposal (Bartelt et al., 2011; Cannon and Nedergaard, 2004; Jakus et al., 2008). Only 50 grams of activated BAT is sufficient to utilize 20% of an individual's daily energy needs, revealing the weight loss potential of BAT that could be used to help combat obesity and its many risk factors (Rothwell and Stock, 1983). Genetically altered rodents that have increased amounts of brown adipose tissue are leaner and more resistant to obesity and its complications (Bartelt et al., 2011; Cousin et al., 1992; Dali-Youcef et al., 2007; Fitzgibbons et al., 2011; Scimè et al., 2005; Seale et al., 2007; Sellayah et al., 2011; Tseng et al., 2008). Alternatively, rodents with genetically deleted UCP-1 are susceptible to becoming obese (Feldmann et al., 2009; Hamann et al., 1995, 1996; Lowell et al., 1993)

In human, the amount of active BAT found in the supraclavicular region of some subjects, 63 grams, would be sufficient to metabolize an amount of energy equivalent to about four kg of WAT during the course of one year (Frühbeck et al., 2009; Virtanen et al., 2009). One group noted that BAT can be activated in humans, as 96% (23/24) of their subjects that underwent PET/CT scanning showed BAT activation under cold conditions (van Marken Lichtenbelt et al., 2009). More importantly, Ouellet et al. have shown that BAT present in humans contributes to energy expenditure (Ouellet et al., 2012).

Moreover, human studies also reveal that there is a strong correlation between leaner individuals having more BAT compared to obese individuals with little or no BAT (van Marken Lichtenbelt et al., 2009; Vijgen et al., 2011). Realizing that humans have active BAT brought its potential as a source of energy expenditure to the forefront of research as an innovative method to combat obesity.

1.5 Beige Adipocytes

Interestingly, it has been found that white and brown adipocytes are not restricted to their respective tissue depot reservoirs (Cinti, 2000; Cousin et al., 1992; Ishibashi and Seale, 2010; Kajimura et al., 2010; Petrovic et al., 2010; Xue et al., 2007). Brown adipocytes that are found in WAT are known as adaptive or recruitable fat cells, brite (brown in white) or beige adipocytes (Enerbäck, 2009; Ishibashi and Seale, 2010). It is not clear if the brown adipocytes found in white adipose depots may actually be a third adipocyte cell type. However, a very recent study has shown that these beige cells might arise from a unique precursor cell. It has been found that a subset of SV cells isolated by fluorescent activated cell sorting (FACS) for the molecular marker, PDGFRα, are able to differentiate into beige adipocytes (Lee et al., 2012b; Wu et al., 2012). Once differentiated these cells express a unique gene expression pattern different from both white and brown adipocytes (Wu et al., 2012). Regardless, beige adipocytes express UCP-1 and other pro-thermogenic markers. They emerge in white adipose depots in response to various environmental stresses, such as cold exposure or treatment with β3-adrenergic agonist (Barbatelli et al., 2010; Cinti et al., 2002; Ma et al., 2012). Similarly,

thyroid hormone, triiodothyronine, and ephedrine can up-regulate the production of beige adipocytes (Dulloo et al., 1991; Lee et al., 2012a). When mice are placed in a cold environment their subcutaneous, inguinal, depots show increased expression of UCP-1 and other pro-thermogenic factors compared to little in the visceral, epididymal, depots (Barbatelli et al., 2010; Cannon and Nedergaard, 2004; Himms-Hagen et al., 2000; Ishibashi and Seale, 2010; Madsen et al., 2010; Seale et al., 2011). Understanding and utilizing these beige adipocytes in humans could help increase weight loss.

2. Adipocyte differentiation

For stem cells to turn into adipocytes, it requires that they first commit to the adipocyte lineage followed by terminal differentiation that is accomplished by the increase and decrease of specific proteins and transcription factors (Figure 2). Mesodermal stem cells restricted to forming tissues of the mesoderm are able to commit and differentiate into adipocytes. Postnatal, mesodermal stem cells known as mesenchymal stem cells (MSCs) are readily available in adipose tissue and have the ability to be differentiated into various mesodermal cell types including but not limited to adipocytes, such as myofibers, osteoblasts and chondrocytes (Abumaree et al., 2012; Elabd et al., 2007, 2009; Liu et al., 2009; Rodriguez et al., 2005). Recently, research has suggested that MSCs are in fact pericytes which can be found in the vasculature surrounding endothelial cells of the highly vascularized adipose depots (Caplan and Correa, 2011; Crisan et al., 2008). In tissue culture, the most commonly used MSC line is C3H10T1/2, which have proven very useful in studying commitment and differentiation

of adipocytes (Bowers et al., 2006; Tang et al., 2004; Tang and Lane, 2012; Tseng et al., 2008).

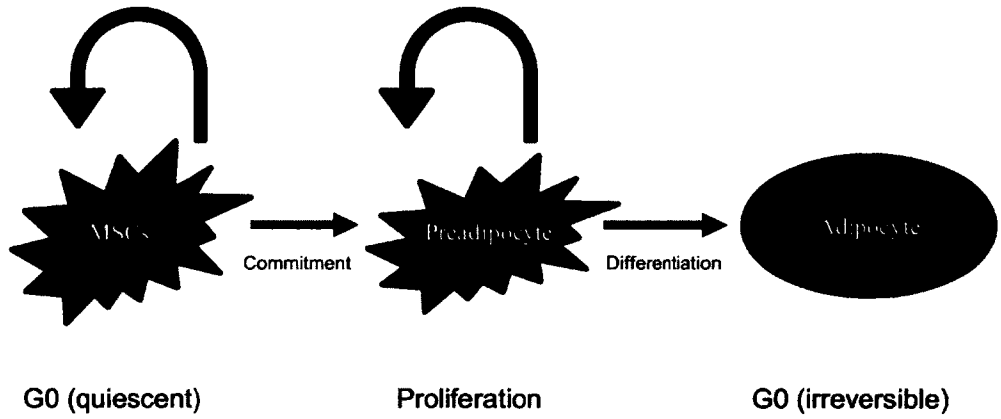


Figure 2. Stem cell commitment and differentiation

Mesenchymal stem cells (MSCs) found in adipose tissue are in a quiescent state until given a signal to become activated. They are able to self-renew their population as well as become committed as a white or brown cycling adipocyte progenitor (preadipocyte) and to finally differentiate into their specific adipocyte lineage.

There are over 100 transcription factors that are expressed in adipocytes and certain ones play a role in either the commitment or differentiation step (Rosen and MacDougald, 2006). Including this study there is very little known regarding the commitment step to the white or brown adipocyte lineage. However, in either brown or white adipocyte differentiation there are two key regulators, which include peroxisome proliferator activated receptor γ (PPARγ) and the CCAAT/enhancer binding protein (C/EBP) family of transcription factors (Rosen and MacDougald, 2006). When these factors activate gene transcription they initiate a cascade of events leading to the development of a terminally differentiated adipocytes.

PPARγ is considered to be a master regulator of adipocyte differentiation (Koppen and Kalkhoven, 2010; Rosen and Spiegelman, 2006). It is a member of the nuclear hormone receptor transcription factor superfamily and is both necessary and sufficient for adipogenesis (Barak et al., 1999; Kubota et al., 1999; Rosen et al., 1999). Additionally, no other factor has been identified that promotes adipogenesis in the absence of PPARγ. It is a ligand dependent transcription factor and once dimerized with retinoid X receptors (RXR) it targets genes by binding to PPARγ Responsive Elements (PPREs) located on gene promoters (Koppen and Kalkhoven, 2010). Additionally, PPARγ directly controls the expression of many genes involved in the key functions of adipocytes such as lipid transport, lipid metabolism, insulin signalling and adipokine production (Frohnert et al., 1999; Graves et al., 1992; Rangwala and Lazar, 2004; Schoonjans et al., 1996).

2.1 Beige adipocyte differentiation

It is not clear how beige cells originate (Scimè, 2012). One hypothesis suggests that they could be formed through transdifferentiation of mature white into beige adipocytes (Barbatelli et al., 2010; Cannon and Nedergaard, 2004; Cinti, 2002, 2011; Himms-Hagen et al., 2000; Perwitz et al., 2010; Walden et al., 2011). Transdifferentiation is the conversion of one differentiated cell type to another (Tosh and Slack, 2002). The Cinti group has suggested that β3-adrenergic agonist CL316,243 transdifferentiates white adipocytes into beige adipocytes directly. This is due to the observation that the number of proliferating cells which include stem cells and their progenitors are unchanged with the addition of CL316,243 (Barbatelli et al., 2010).

However, a constant stem cell population may be a reflection of the cells differentiating at the same rate as they are proliferating. Pisani et al. in agreement with this theory showed human mesenchymal adipose derived stem cells (hMADS) first differentiated into white adipocytes before subsequent transdifferentiation into brown adipocytes (Pisani et al., 2011).

The other possible method of beige cell formation is thought to be through the de novo commitment and differentiation of MSCs and/or their progenitors located in the WAT depots. Recently, Zpf423, a cell surface marker, has identified an MSC population found in the vasculature of WAT that has the ability to differentiate into a white or brown adipocyte (Gupta et al., 2010, 2012; Tran et al., 2012). This would suggest that both beige and white adipocytes found in WAT arise from a common progenitor, thus giving credence to de novo adipogenesis. Furthermore, in mesodermal stem cells the expression of bone morphogenic protein 7 (BMP7), PRDM16, and Orexin, or the depletion of pRB and p107 have been implicated in the formation of beige adipocytes (Hansen et al., 2004b; Dali-Youcef et al., 2007; Schulz and Tseng, 2009; Scimè et al., 2005; Seale et al., 2007, 2008; Sellayah et al., 2011; Tseng et al., 2008).

2.2 PPARγ co-activator-1α

A key player in brown adipogenesis is PGC-1α. It is expressed in much higher levels in brown adipocytes compared to white adipocytes and is induced by cold exposure in vivo, or by β3-adrenergic agonists in vivo and in vitro (Puigserver et al., 1998; Puigserver, 2005). PGC-1α is a master regulator of mitochondria biogenesis, stimulates

the expression of electron transport chain genes, and together with PPARγ activates the expression of UCP-1 in mice and in human adipose tissue (Puigserver et al., 1998; Tiraby et al., 2003). Furthermore, overexpression of PGC-1α in vivo has been shown to cause the expression of multilocular cells expressing UCP-1 in WAT, indicative of beige adipocytes (Tiraby et al., 2003; Uldry et al., 2006).

2.3 PRDM16

Although, brown and white adipose tissues express many of the same proteins, a global transcription factor expression profile discovered that PRD1-BF1-RIZ1 homologous domain containing 16 (PRDM16) was one of three differentially expressed transcription factors. PRDM16 has been shown to have a 15 fold up-regulation in BAT versus WAT (Seale et al., 2007). Expression of PRDM16 is sufficient to drive the differentiation of stem cells and their uncommitted progenitors, from WAT or BAT, as well as myoblasts into the brown adipocyte cell fate (Kajimura et al., 2009, 2010; Seale et al., 2008). Overexpression of PRDM16 in both WAT or BAT pre-adipocytes results in an increase in mitochondrial biogenesis, increased cellular respiration, and expression of brown adipocyte specific pro-thermogenic genes including UCP-1, PGC-1α, Cidea and Elovl3 (Seale et al., 2007, 2011).

PRDM16 is thought to play an important role in beige adipocyte formation in subcutaneous depots. Notably, it has recently been shown that PRDM16 levels are higher in more metabolically beneficial subcutaneous WAT depots compared to visceral depots (Kajimura et al., 2009; Seale et al., 2011). Moreover, the specific transgenic expression

of PRDM16 in adipose tissue showed increased formation of beige adipocytes in subcutaneous WAT depots under β-adrenergic stimulation (Seale et al., 2007, 2008, 2011).

Nevertheless, how PRDM16 functions in a pathway to bring about the brown adipocyte fate is unclear. It is known that the PRDM16 protein contains two zinc-finger domains that bind DNA. However, a recent study showed that when those binding sites are mutated, PRDM16 retains the ability to activate brown adipocyte specific genes (Seale et al., 2007). It is thought to bind in a complex with transcription factors such as PPARγ and c/ebpβ (Kajimura et al., 2010; Seale et al., 2008). Kajimura et al., have suggested the PRDM16 binds c/ebpβ to initiate the commitment step of MSCs to the brown adipocyte lineage (Kajimura et al., 2009, 2010). This PRDM16-c/ebpβ complex can then activate PGC-1α and PPARγ, which in turn activate the pro-thermogenic program (Figure 3).

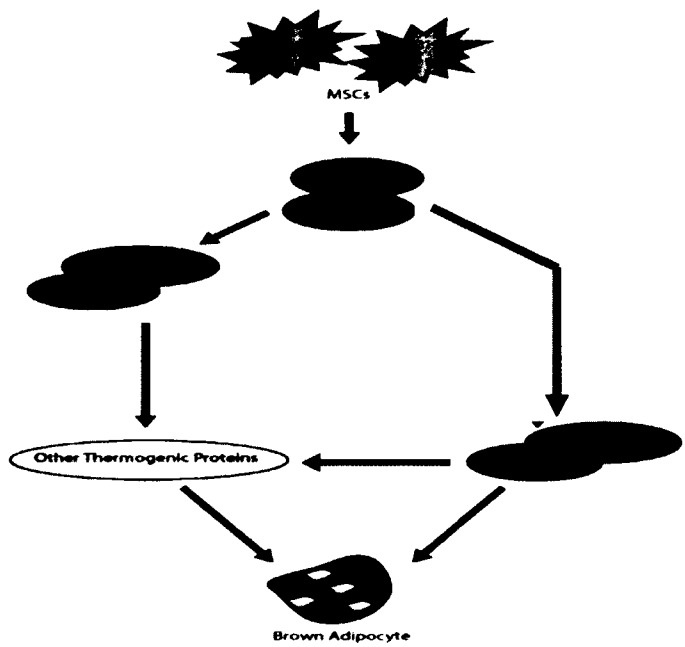


Figure 3. PRDM16 brown adipocyte forming pathway
Once bound to c/ebpβ, PRDM16 can activate the brown adipocyte formation pathway by increasing the transcription of both PPARγ and PGC-1α. These proteins then in turn activate other pro-thermogenic proteins in order to form a fully differentiated brown adipocyte.

3. Retinoblastoma Family

The retinoblastoma susceptibility protein (RB) gene family contains 3 members; pRB, p107 and p130. The RB family members are critical as evident by their homologs being conserved across species such as humans, reptiles, mice, chickens, flies and some plants (Wirt and Sage, 2010). pRB, the first of its family members to be discovered is known to function as a tumor suppressor (Lee et al., 1987; Manning and Dyson, 2011). Inactivating mutations in the pRB gene or deregulation of the protein's pathway lead to a variety of human cancers (Burkhart and Sage, 2008). Its ability to suppress tumors is due to its role in regulating cell cycle exit (Bester et al., 2011; Burkhart and Sage, 2008; Jacks et al., 1992).

p107 and p130 are structurally similar to pRB and have some functional overlap (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Wirt and Sage, 2010). As with pRB, they contain similar structural regions at the C-terminus, and within their "pocket" domains. Although, the RB family is not able to bind DNA directly, they act as transcriptional co-repressors binding to members of the E2F family of transcription factors (Chong et al., 2009; Landsberg et al., 2003). Upon hyperphosphorylation the RB family become inactivated such that binding to E2F proteins is abrogated and gene transcription is derepressed. Together the RB family members with E2Fs bind histone deacetylases (HDAC), to repress gene transcription via chromatin remodeling (Brehm et

al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). Although the RB family has some functional overlap in regulating the cell cycle (Classon and Dyson, 2001), it is thought that the individual members are functionally different and important in different cell types (Classon et al., 2000; Jiang et al., 2000; Scimè et al., 2010; Thomas et al., 1998; Vanderluit et al., 2007).

The RB family members are expressed at different levels throughout the cell cycle (Figure 4) (Classon and Dyson, 2001). p130 is found with high expression levels in quiescent (G_0) cells and as the cell cycle progresses its levels drop off (Classon and Dyson, 2001; Mayol et al., 1995). pRB levels tend to stay at a relatively constant level throughout the cell cycle and p107 levels gradually increase up to S-phase (Beijersbergen et al., 1995). Unique only to p107 is that its levels are generally low in quiescent and differentiated cells. Furthermore, it controls cell proliferation by regulating exit from G1 through the repression of genes (Classon and Dyson, 2001; Zhu et al., 1993).

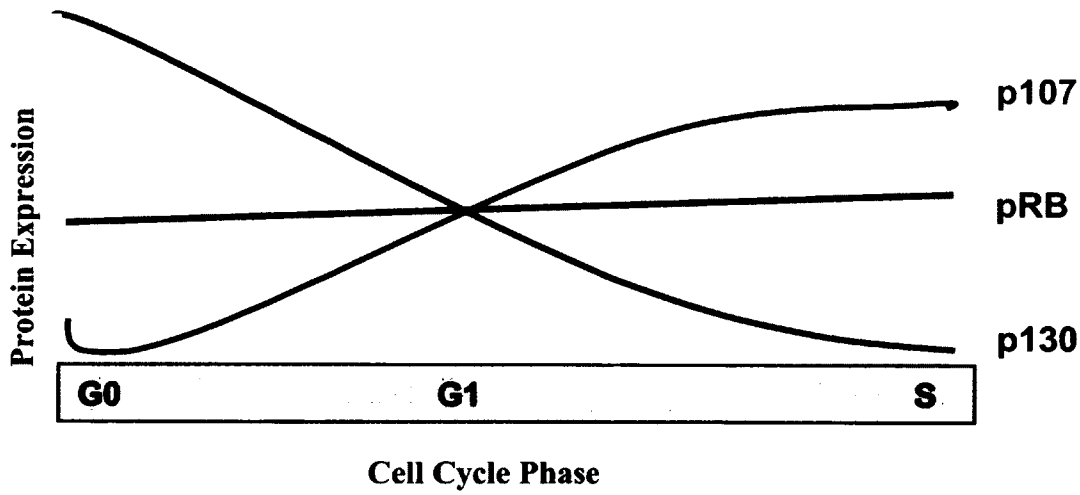


Figure 4. Expression patterns of pRB, p107 and p130 during cell cycle

See text for description and referencing.

3.1 p107 and Adipogenesis

The Rb family members pRB and p107 play an important role in adipogenesis (Dali-Youcef et al., 2007; Hansen et al., 2004a, 2004b; Scimè et al., 2005). The genetic deletion of RB in mesodermal stem cells and in terminally differentiated white adipocytes results in the formation of brown adipocytes (Dali-Youcef et al., 2007; Hansen et al., 2004a, 2004b; Scimè et al., 2005). p107 has been implicated in the regulation of numerous cell types including neurons, muscle and adipose tissue during development (Scimè et al., 2005, 2010; Vanderluit et al., 2007). However, its specific role in the different cell types is much less well defined. While studying the phenotypic effects of p107 specific genetic deletion (p107 KO), LeCouter et al. noted that the mice had postnatal growth deficiency and myeloid hyperplasia in the spleen and liver (LeCouter et al., 1998). Scimè et al. explored the changes in the adipose tissue and metabolism. It was determined that p107 KO mice were leaner, with significantly smaller adipose depots (Scimè et al., 2005), they were resistant to weight gain on a high fat diet and were more insulin sensitive (Scimè et al., 2010). More intriguing still was the presence of UCP-1 in their inguinal subcutaneous white adipose depot (Scimè et al., 2005). This research was the first to hint at the importance of p107 in brown/beige adipocyte formation.

Study Hypothesis and Objectives

We hypothesize that p107 is an integral player in the adipocyte lineage commitment step of mesodermal/mesenchymal stem cells and that it has an important role in the PRDM16 brown adipocyte forming pathway.

Objectives

1. To further understand and characterise p107 in various adipose depots.
2. To determine if p107 knockdown is necessary for brown/beige adipocyte commitment and/or differentiation.
3. To determine if there is a link between p107 and PRDM16 in the brown adipocyte forming pathway.

Methods

Mice and dissections

All animal experiments were performed according to procedures approved by the institutional Animal Care Committee of York University. All experimental procedures were performed in adult (8 to 16 week old) mice of the Balb/c genetic background, including those with a genetic deletion of p107 (p107 KO) (LeCouter et al., 1998). Mice were maintained on a standard rodent chow diet with 12-hour light and dark cycles. Adipose depots for analysis were dissected as follows: Inguinal WAT, bilateral superficial subcutaneous WAT depots between the skin and muscle fascia just anterior and posterior to the lower segment of the hind limbs; interscapular WAT, posterior bilateral superficial WAT depots between the skin and muscle fascia flanking either side of the BAT depot; testicular or ovarian WAT, prominent bilateral intra-abdominal visceral depots in male mice attached to the epididymides or female mice attached to the ovaries; peritoneal WAT, bilateral depots in the abdominal cavity behind the peritoneum on the dorsal side of the kidney; BAT, bilobed tissue between the scapulae.

Morphological analysis

Hematoxylin and eosin (H&E) staining of tissue sections was performed by the Mount Sinai Hospital Molecular and Cellular Biology Core Facility (Toronto) according to standard procedures.

β3-adrenergic agonist treatment

The β3-adrenergic agonist, CL316,243 (Sigma-Aldrich), was injected at 1mg/kg into the intraperitoneum of Balb/c mice for 7 days.

Cell types used

Murine embryonic fibroblasts (MEFs) representing an enriched source of primary mesodermal stem cells were isolated from embryos of a heterozygous p107 KO mating pair at 14.5 days post coitum. After embryos were isolated and the placenta removed, ectodermal and endodermal cells located within heads, tails, and internal organs were discarded and some tissue saved for genotyping. The remaining mesodermal tissue was minced, resuspended in DMEM with 10% FBS and 1% penicillin/streptomycin, plated and allowed to grow.

For isolation of the stromal vascular (SV) fraction, specific adipose depots were dissected as above, minced and digested with 1mg/ml collagenase I (Sigma) in 10% fetal bovine serum (FBS) at 37°C for 45 minutes. To recover the cells after digestion, the cell digested slurry was passed through a 100 μm cell strainer and cells were pelleted by centrifugation at 250g for 5 minutes.

C3H10T1/2 cells are a mesenchymal stem cell line obtained from the American Type Culture Collection (ATCC).

Cell culture and adipocyte differentiation

All cells were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. MEFs or C3H10T1/2 cells were differentiated to adipocytes after reaching confluency for 48 hours (time 0) according to the standard protocols employing the serum-based or serum-free regimens. For the serum-based differentiation method, at time 0, DMEM with 10% FBS and 1% penicillin/streptomycin was supplemented with 0.5 mM isobutylmethylxanthine, 125 nM indomethacin, 1uM dexamethasone, 850 nM insulin, 1nM T_3, and 1 uM rosiglitazone for 2 days. Afterwards at time day 2, cells were switched to maintenance medium containing 10% FBS, 1% penicillin/streptomycin, 850 nM insulin, 1 nM T_3, and 1 uM rosiglitazone for 5 days. The serum-free regimen used only DMEM/F-12 (1:1) media instead of DMEM with 10% FBS. For the differentiation time course, cells were grown and differentiated for the stated times before lysis.

Virus production and Cell line derivation

For p107 RNAi and control virus production, 293FT cells were passaged to 30% confluency and were transfected using PolyJet (FroggaBio) with p107 RNAi (TRCN0000218550; Sigma-Aldrich) sequence GTACCGGATCTTTGCCAATGCTATAATGCTCGAGCATTATAGCATTGGCAAAGATTTTTTTG or control scrambled p107 RNAi (Sigma-Aldrich) lentiviral plasmids, with packaging vectors according to manufacturer's protocol. 18 hours post transfection cells were washed and at 48 and 72 hours media containing virus was collected.

For PRDM16, pMSCV and p107 virus production, Phoenix cells were passaged to 30% confluency and were transfected with either pMSCV-PRDM16, pMSCV or pBrit-p107 retroviral plasmids respectively using PolyJet transfection reagent (FroggaBio) according to manufacturer's protocol. Cells were washed 18 hours post transfection and at 48 and 72 hours media containing virus was collected.

For transducing cells, 10 mls of the various collected viral media was added to C3H10T1/2 cells with 8 μg/ml of polybrene. After 24 hours cells were lysed or washed and re-fed for cell line derivation.

Cell lines were derived by selecting with 2 mg/ml puromycin to produce MSC-shp107, MSC-scr, MSC-PRDM16, MSC-Ctrl, MSC-p107.

Cell and Tissue Protein Isolation

Cells in culture were lysed in RIPA buffer (0.5% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl pH 7.5) containing protease inhibitor cocktail (Roche) for 10 minutes on ice. The lysate was clarified at 13,000 g for 10 minutes at 4°C with the pellet and any lipid layer discarded.

For protein extraction from adipose tissue, 500 mg of isolated tissue was homogenized using the cell disruptor Retsch MM400 with two beads in 600 μl RIPA buffer containing protease inhibitors. The lysate was then clarified for 10 minutes at 13,000 g at 4°C and the lipid layer and pellet were discarded.

Western blot analysis

For Western blot analysis protein lysates were loaded onto polyacrylamide gels, ranging from 7.5%-15% acrylamide, and Western blotted according to standard protocol. Samples to be loaded were first boiled for 3 minutes in sample buffer. 15 μg of sample was loaded into 7.5% SDS polyacrylamide gel and 50 μg of sample was loaded into 15% gels. The proteins were separated by electrophoresis for 2 hours at 30 milliamps per gel at R.T. Proteins were transferred on a 0.45 PVDF membrane at 4 °C for 60 at 100V, using a wet transfer method. After blocking for 60 minutes at room temperature in 5% non-fat milk in tris buffer saline (TBS) containing 0.1% tween, the membranes were probed with antibodies diluted in 5% non-fat milk in TBS. Incubation with primary antibodies was done overnight at 4 °C and under light agitation. Membranes were then probed for 60 minutes at R.T, under light agitation, with the appropriate secondary antibodies diluted in 5% non-fat milk in TBS. Antibodies used were rabbit polyclonal anti-UCP-1 (Calbiochem), rabbit polyclonal C18 anti-p107 (Santa Cruz), rabbit polyclonal PRDM16 (a kind gift of Dr. Patrick Seale, University of Pennsylvania), monoclonal antibodies M-2 anti-Flag and anti-Tubulin (Sigma-Aldrich) and secondary antibodies goat anti-mouse or anti-rabbit (Santa Cruz).

Cell and Tissue RNA Isolation

For RNA isolation, cells were lysed in RNA lysis buffer (FroggaBio). RNA was then purified using the Total RNA mini kit according to manufacturer's instructions (FroggaBio).

For RNA isolation from adipose tissue, the fat pad was first weighed and 1 ml of TRIzol (Invitrogen) was added per 50-100mg of tissue. The sample was then homogenized using a cell disruptor, transferred to a 15 ml Oak Ridge tube and centrifuged for 5 minutes at 10,000 rpm at 4°C. The lipid layer was removed and 0.2 ml of chloroform per 1 ml of TRIzol was added. The sample was shaken vigorously for 30 seconds then centrifuged for 15 minutes at 10,000 rpm at 4°C. The bottom layer was discarded and the top aqueous layer was transferred to a fresh 50 ml conical tube. 0.5 ml of isopropanol was added to the tube and centrifuged at 4°C for 15 minutes at 5,000 rpm. The RNA pellet was washed in 75% ethanol and resuspended in 100µl of RNase free H_20.

qPCR analysis

1 mg of RNA was reverse transcribed into cDNA employing the GeneAmp Kit (Applied Biosystems). 25 ng of cDNA was employed for PCR analysis. The quantitative real time polymerase chain reaction, (qPCR), assays were performed on the ABI 7500 Fast cycler using SYBR green PCR Master mix (Invitrogen) with the primer sets listed in Table 1. Relative expression of cDNAs was determined after normalization with β-actin using the $^{\Delta\Delta}$Ct method. Student's t-test was used for comparison and to obtain statistics.

Table 1. Primer sets

Primers	Forward Primer (5' – 3')	Reverse Primer (3' – 5')
p107	ttc cag aga cat ggt gaa ac	tta ttg gag aca ttg gca tc
PRDM16	cag cac ggt gaa gcc att c	gcg tgc atc cgc ttg tg
UCP-1	act gcc ac acct cca gtc att	ctt tgc ctc act cag gat tgg

Cidea	tgc tct tct gta tcg ccc agt	gcc gtg tta agg aat ctg ctg
Cox8b	gaa cca tga agc caa cga ct	gcg aag ttc aca gtg gtt cc
Elovl3	tcc gcg ttc tca tgt agg tct	gga cct gat gca acc cta tga
PGC-1α	ccc tgc att gtt aag acc	tgc tgc tgt tcc tgt ttt c
PPARγ	cag gct tgc tga acg tga ag	gga gca cct tgg cga aca
Adiponectin	gca ctg gca agt tct act caa	gta ggt gaa gag aac ggc ctt gt
aP2	aca ccg cgc ttt ctt caa ctg	cca tct agg gtt atg atg tct tca
β-actin	gct ctg gct cct agc acc at	cca ccg atc cac aca gcg tac

Luciferase assay

C3H10T1/2 cells at 50% confluency were transfected with various amounts of pMSCV-PRDM16, pMSCV-R998Q, pCMV-E2F1 along with the promoter luciferase construct to be tested and Renilla luciferase plasmid as an internal control. The constructs tested consisted of 900 bp proximal to the transcriptional start site of the p107 promoter fused to the luciferase gene or this same promoter containing either one of both mutations of two E2F binding domains (kind gifts of Dr. Julien Sage, Stanford University). Transfections were performed with Polyjet (Froggabio) according to the manufacturer's protocol. pMSCV and pcDNA3 were used as filler controls. Twenty four hours post transfection, luciferase activity was determined using the Dual-Luciferase assay kit (Promega) according to the manufacturer's protocol.

Transplantation

1 X 10^7 p107 KO MEFs or control wild type (WT) MEFs were resuspended in 200 µl sterile PBS, and transplanted subcutaneously into the sternum region of WT Balb/c mouse hosts. The MEFs were of the same strain as the host to eliminate the possibility of immune rejection. After transplantation, brown adipocyte production was stimulated by daily treatment with β3-adrenergic agonist, CL316,243 (Sigma) at 1 mg/kg injected into the intraperitoneum for 7 days. 8 weeks post transplantation the newly formed adipose tissue pads derived from the transplanted cells were excised and processed for histological analysis using hemotoxylin and eosin on paraffin sections or RNA isolation for qPCR analysis.

Competition Experiments

MSC-PRDM16 or MSC-p107 cells were transduced by passaging at 50% confluency directly into media containing p107 or PRDM16 virus respectively or into fresh media, all containing 8 µg/ml polybrene. After 24 hours cells were washed and re-fed. Once confluent, 72 hours post-transduction, cells were differentiated using the serum protocol stated above. Cells were lysed after 7 days of differentiation and analyzed using qPCR.

Statistical Analysis

Statistical analyses were performed by Microsoft excel and online One-Way ANOVA calculators using a Tukey post-hoc test. Student's paired t-test was used unless otherwise stated. Results were considered to be statistically significant when $p < 0.05$.

Results

p107 levels in adipose depots are inversely proportional to PRDM16

In order to begin understanding the role of p107 in adipocyte lineage commitment and differentiation its protein expression levels were characterized in various adipose depots. To accomplish this, the adipose depots were separated into the stromal vascular (SV) fraction which contains stem cells and their committed progenitors from the terminally differentiated adipocyte fraction (AD) (Figure 5A). Protein expression by Western blot analysis showed that p107 was expressed only in the SV fraction and not in the mature and terminally differentiated adipocytes (Figure 5B). Further Western blot analysis of individual adipose depots revealed that p107 was also not expressed in BAT (Figure 5C). Graphical representation of the Western data demonstrated that p107 was significantly ($p < 0.05$) expressed as much as 3-fold more in the visceral compared to the subcutaneous WAT depots and was negligible in the BAT depot (Figure 5D). This expression for p107 is inversely proportional to the brown adipocyte determination factor PRDM16 that is hypothesized to maintain a lean phenotype (Seale et al., 2011). A potential that these proteins might be involved in the same pathway is highlighted by the relationship between p107 and PRDM16 within adipose tissue depots. Isolation of inguinal fat pad depots, analysed by qPCR, revealed that the higher the weight of the fat pad the higher the ratio between p107/PRDM16 (R^2=0.74) (Figure 5E). This data shows that p107 has a role in the SV fraction, where MSCs and their committed progenitors are found, where its absence is potentially necessary for brown adipocyte formation. In addition, that p107 might interact with PRDM16 in a brown adipocyte forming pathway.

Figure 5

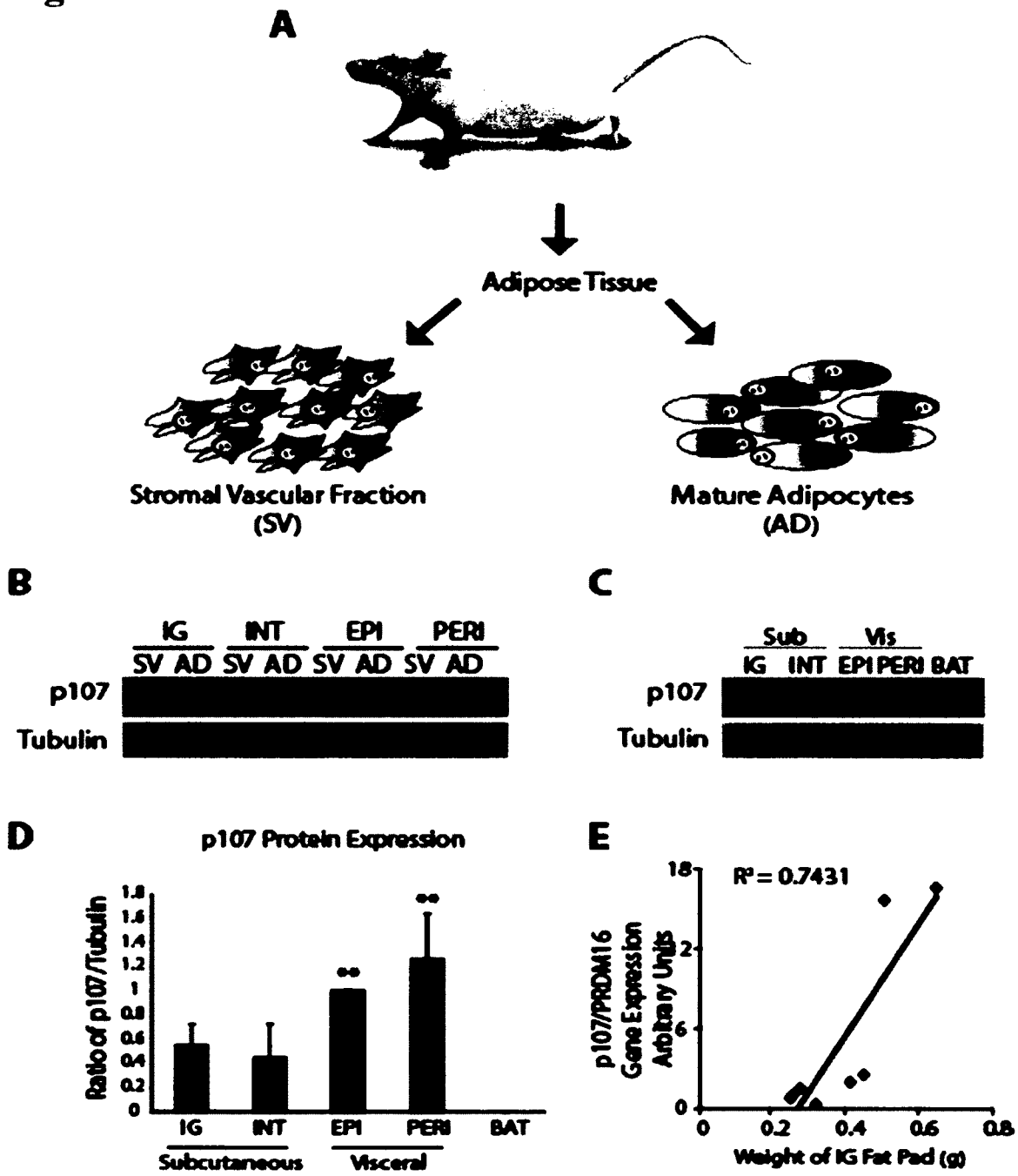


Figure 5. p107 expression pattern in adipose depots

(A) Diagram representing isolation and fractionation of adipose tissue into stromal vascular (SV) and mature adipocyte (AD) fractions from Balb/c mice. (B) Representative Western blot for p107 and Tubulin of the stromal vascular fraction (SV) and adipocyte

fraction (AD) of various adipose depots from adult Balb/c mice. (C) Representative Western blot for p107 and Tubulin of subcutaneous (Sub), visceral (Vis) and BAT depots of Balb/c mice. (D) Graphical representation of p107 protein expression from Western blots of various adipose depots (n=6, ** $p < 0.01$, One-way Anova and Tukey Post-hoc test were used). Inguinal (IG), interscapular (INT), epididymal (EPI), intraperitoneal (PERI) and brown adipose tissue (BAT). (E) Graphical representation of the gene expression ratio for p107 and PRDM16 by qPCR of the inguinal adipose depot from Balb/c mice compared to the weight in grams (g) of the depot.

β3-adrenergic agonist significantly decreases p107 levels in subcutaneous depots in vivo

As β-adrenergic agonists induce brown fat formation in WAT, we tested what effect the β3-adrenergic agonist, CL316,243, would have on p107 expression in vivo within the inguinal depot. As expected, UCP-1 levels were increased after 7 days of treatment (Figure 6A). On the other hand, a representative Western blot for p107 showed that its protein levels were significantly reduced in the presence of the β3-adrenergic agonist (Figure 6B). Further graphical analysis of the Western blot data demonstrated that p107 levels significantly ($p < 0.01$) decreased by as much as 4-fold in the inguinal depots compared to when saline was used (Figure 6C). This suggests that brown adipocyte formation in WAT that result from the differentiation of stem cells or their committed progenitors might require that p107 levels be reduced.

Figure 6

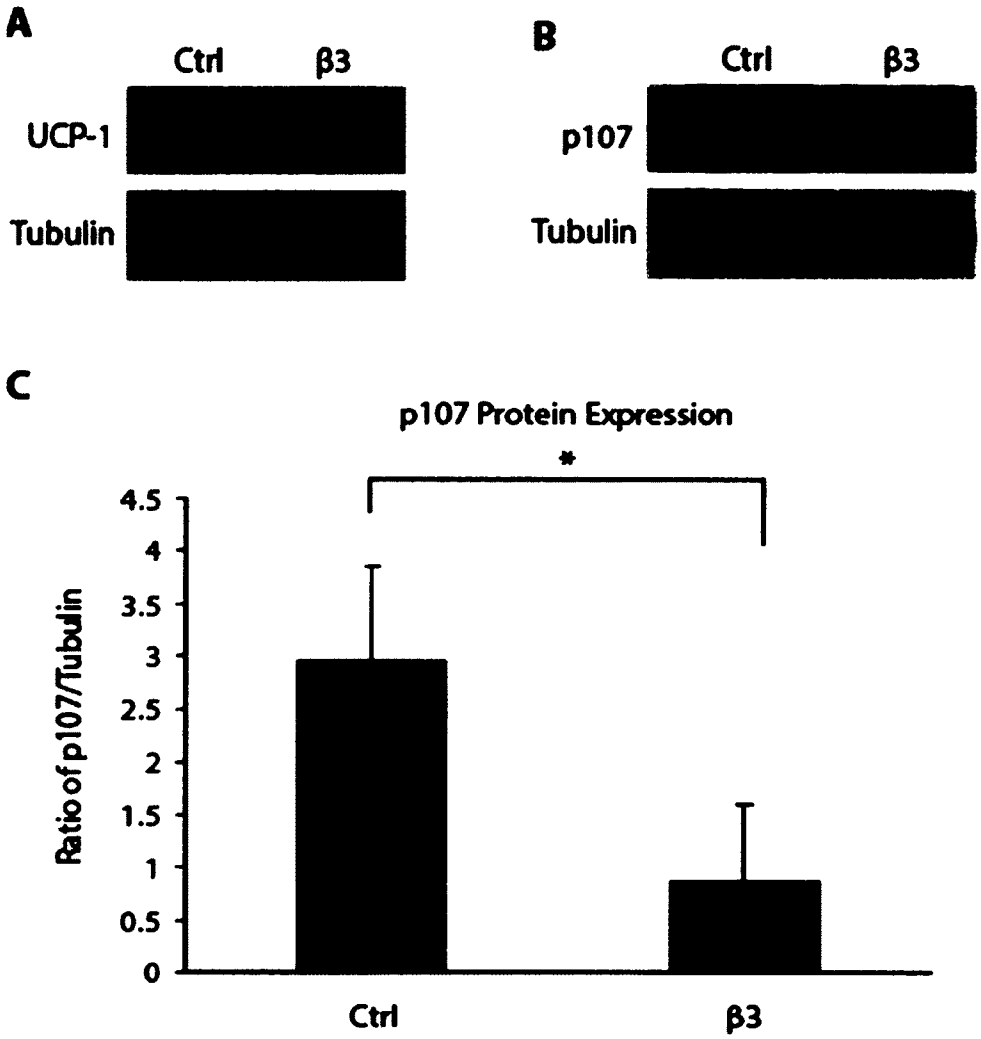


Figure 6. β3-adrenergic agonist decreases p107 levels in subcutaneous adipose depots in vivo

(A) Representative Western blot for UCP-1 and Tubulin from the inguinal adipose depot of adult Balb/c mice treated with β3-adrenergic agonist, CL316,243 (β3) or Saline (Ctrl). (B) Representative Western blot of p107 expression in the inguinal adipose depots of adult Balb/c mice treated with CL316,243 (β3) or Saline (Ctrl) with Tubulin as the loading control. (C) Graphical representation of p107 protein expression from Western blots of inguinal adipose depots for CL316,243 (β3) treated Balb/c mice compared to the Saline control (Ctrl) (n=6, * $p < 0.05$)

The absence of p107 in mesodermal stem cells promote brown adipocyte formation

The role of p107 was assessed in murine embryonic fibroblasts (MEFs), representing a rich source of primary mesodermal stem cells. MEFs, of p107 genetically deleted (p107 KO) and wild type (WT) littermate controls were isolated from 14.5 day embryos of a heterozygous mating pair (Figure 7A). After discarding ectodermal and endodermal tissue, the mesodermal cells were plated and genotyped. The MEFs were assessed for their potential to differentiate into adipose tissue. Oil red O that stains lipid showed essentially an equal amount of accumulation of adipocytes in both the WT and p107 KO MEFs 7 days post adipocyte differentiation (Figure 7B). qPCR confirmed similar expression levels of general adipogenic markers, aP2, Adiponectin and PPARγ in both the WT and p107 KO MEFs (Figure 7C). This is very important because it indicates that the p107 deletion does not affect the ability of the cells to differentiate into adipocytes.

Figure 7

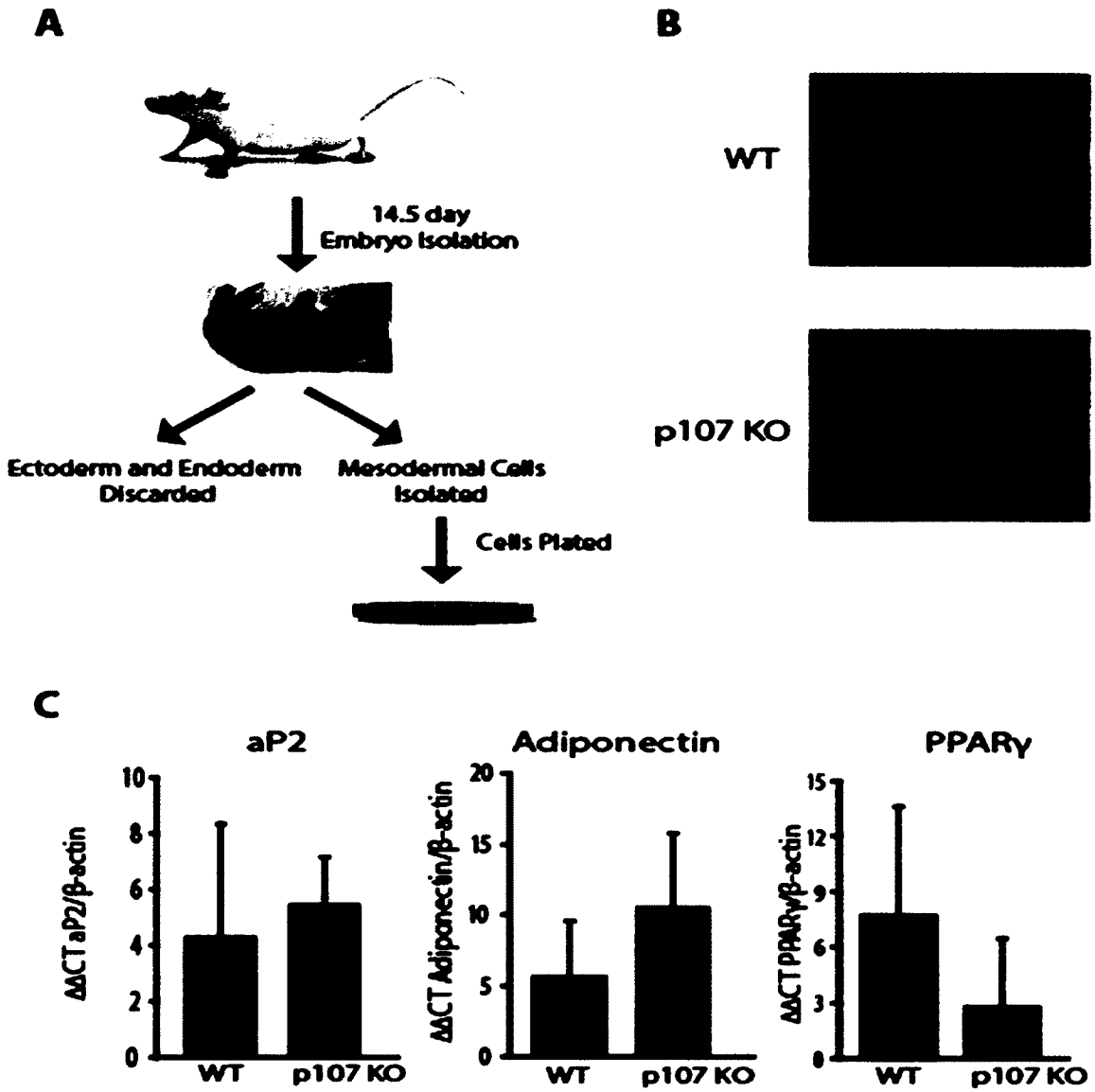


Figure 7. p107 KO MEFs readily differentiate into adipocytes

(A) Diagram representing the isolation of p107 genetically deleted (p107 KO) primary murine embryonic fibroblasts (MEFs) from 14.5 day old embryos originating from heterozygous mating pairs. (B) Oil red O staining of wild type (WT) control and p107 KO MEFs 7 days post adipocyte differentiation. (C) Gene expression analysis by qPCR of general adipogenic factors, aP2, Adiponectin and PPARγ, in WT and p107 KO MEFs 7 days post adipocyte differentiation (n=3).

Next, adipose terminal differentiation was assessed for the expression of the brown adipocyte marker UCP-1 at both the RNA and protein levels. The p107 KO MEFs had significantly ($p < 0.01$) higher levels of UCP-1 gene expression after 7 days of adipocyte differentiation (Figure 8A). Furthermore, protein expression pattern by Western blotting confirmed that UCP-1 was expressed beginning at an earlier time point, compared to the WT controls that had little UCP-1 expression up to 7 days post-differentiation (Figure 8B). The presence of brown adipocytes was corroborated by significantly increased expression of other pro-thermogenic markers such as PGC-1α, Cox8b, Elovl3 and Cidea by qPCR at 7 days post differentiation in the p107 KO MEFs (Figure 8C).

Figure 8

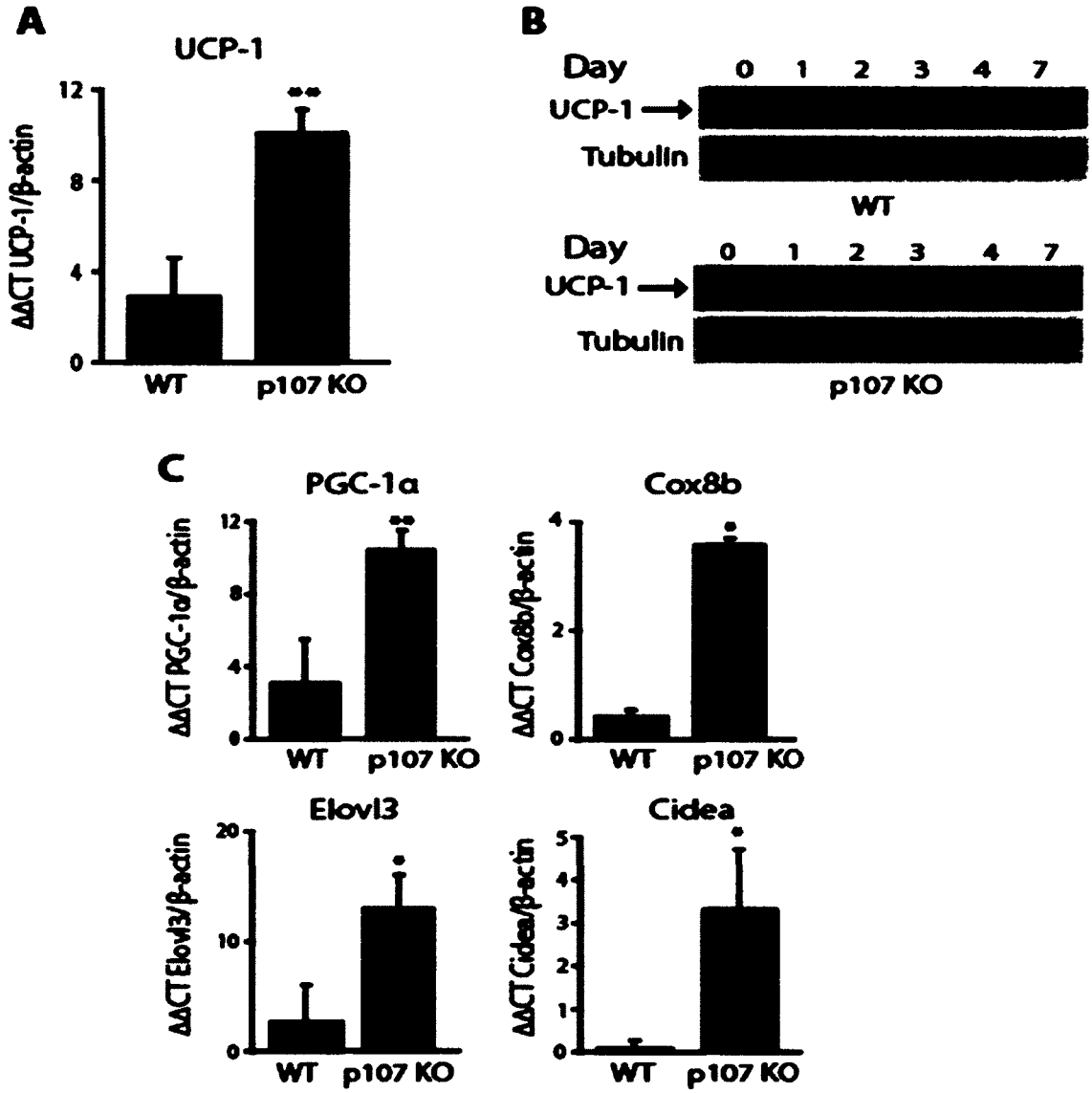


Figure 8. p107 KO MEFs readily differentiate into brown adipocytes

(A) Gene expression analysis by qPCR of UCP-1 in wild type (WT) control and p107 genetically deleted (p107 KO) MEFs 7 days post adipocyte differentiation (n=3, ** $p < 0.01$). (B) Representative Western blots for UCP-1 and Tubulin in WT and p107 KO MEFs during a time course of adipocyte differentiation over 7 days. (C) Gene expression analysis by qPCR of general pro-thermogenic factors, PGC-1α, Cox8b, Elovl3 and Cidea in WT and p107 KO MEFs 7 days post adipocyte differentiation (n=3, * $p < 0.05$, ** $p < 0.01$)

The in vivo potential of brown adipocyte formation for p107 KO MEFs was also tested. In this regard, WT or p107 KO MEFs were transplanted into Balb/c mice subcutaneously below the sternum region. This area with negligible endogenous adipose stores, has the potential to form a fat pad with transplanted adipocyte precursors within 6-8 weeks (Seale et al., 2007; Tseng et al., 2008; Tran and Kahn, 2010). After transplantation, the mice were injected with β3-adrenergic agonist CL316,243 daily for 7 days in order to induce brown adipocyte formation. After 6 weeks the mice were sacrificed and the newly formed fat pad excised and processed for analysis. Morphological analysis with hemotoxylin and eosin (H&E) staining revealed that the mice transplanted with p107 KO MEFs contained many patches of multilocular appearing cells, a hallmark of brown adipocytes (Cinti, 2005) (Figure 9A). Contrarily, the WT control transplanted MEFs showed only the presence of unilocular cells characteristic of white adipocytes. Further analysis of the newly formed tissue by qPCR revealed that the p107 KO transplanted MEFs had significantly ($p < 0.05$) more UCP-1 expression when compared to the WT transplanted or saline transplant controls (Figure 9B). Together, this data emphasizes the requirement of p107 reduction in primary mesodermal stem cells in vivo for brown adipocyte formation to occur.

Figure 9

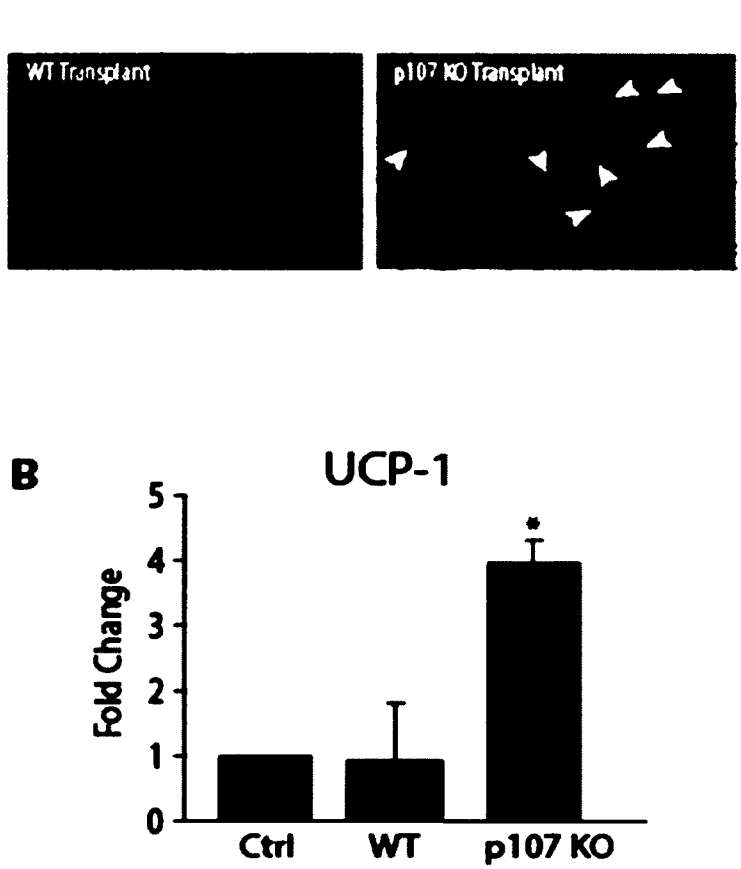


Figure 9. p107 KO transplanted MEFs form brown adipocytes in vivo

(A) Representative hemotoxylin and eosin (H&E) staining of newly formed tissue sections from host Balb/c mice after 6 weeks of incubation with subcutaneously transplanted donor wild type (WT) control or p107 KO Balb/c MEFs. Arrow heads denote the multilocular appearance of cells indicative of the presence of brown adipocytes. (B) Gene expression analysis by qPCR of UCP-1 in newly formed tissue from host Balb/c mice arising from mock saline (Ctrl), and wild type (WT) or p107 KO Balb/c MEF transplants after 6 weeks of incubation (n=3, * $p < 0.05$, One-way ANOVA and Tukey Post-hoc test used).

p107 knockdown is required for brown adipocyte formation

In order to discriminate against a possible developmental affect due to p107 chronic loss in MEFs, the MSC line C3H10T1/2 was examined for p107 knockdown (KD). Lentivirus was made that expresses p107 short hairpin (sh) RNA or a scrambled shRNA control to produce cell lines designated MSC-shp107 and MSC-scr, respectively (Figure 10A). Western blot as well as gene expression analysis showed that p107 was significantly ($p > 0.05$) depleted by 80% in 3 different shp107 cell lines (Figure 10B and 10C).

Figure 10

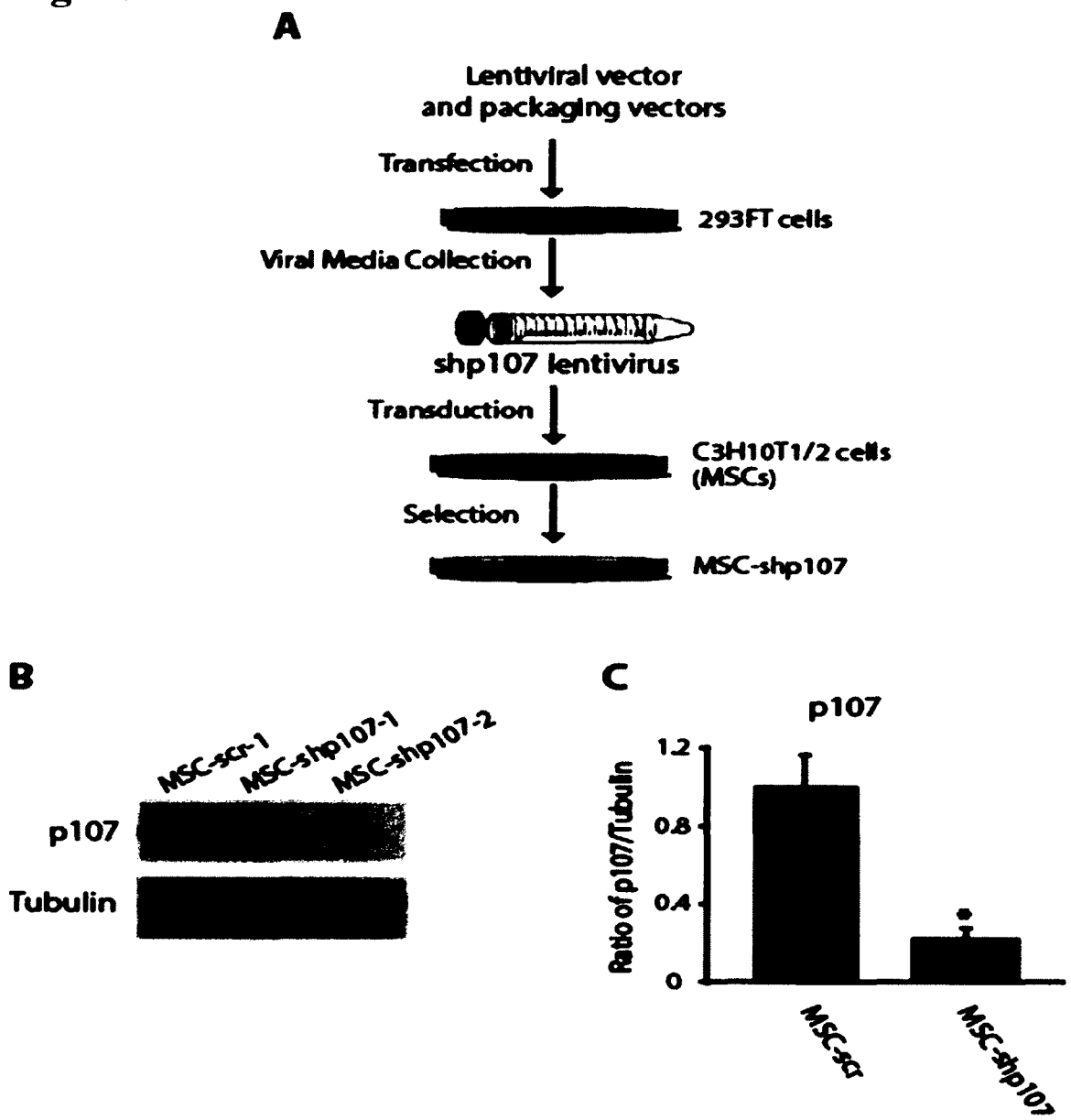


Figure 10. Derivation of the p107 KD mesenchymal stem cell line, MSC-shp107

(A) Diagram representing the process used to derive the p107 knockdown (p107 KD) and control cell lines, MSC-shp107 and MSC-scr, respectively. (B) Representative Western Blot for p107 during growth of mesenchymal stem cell (MSC) lines MSC-scr-1, MSC-shp107-1 and MSC-shp107-2. (C) Graphical representation of p107 RNA expression during growth in different MSC-scr and MSC-shp107 cell lines (n=3, * $p < 0.05$)

The MSC-shp107 and MSC-scr cell lines were similar in lipid accumulation after 7 days of adipocyte differentiation revealed by Oil red O staining (Figure 11A). The gene expression of general adipogenic markers aP2, Adiponectin and PPARγ, were also similar at this time point (Figures 11B). This indicates as with the MEFs, that p107 KD in C3H10T1/2 cells does not influence the cells' ability to differentiate into adipocytes.

Figure 11

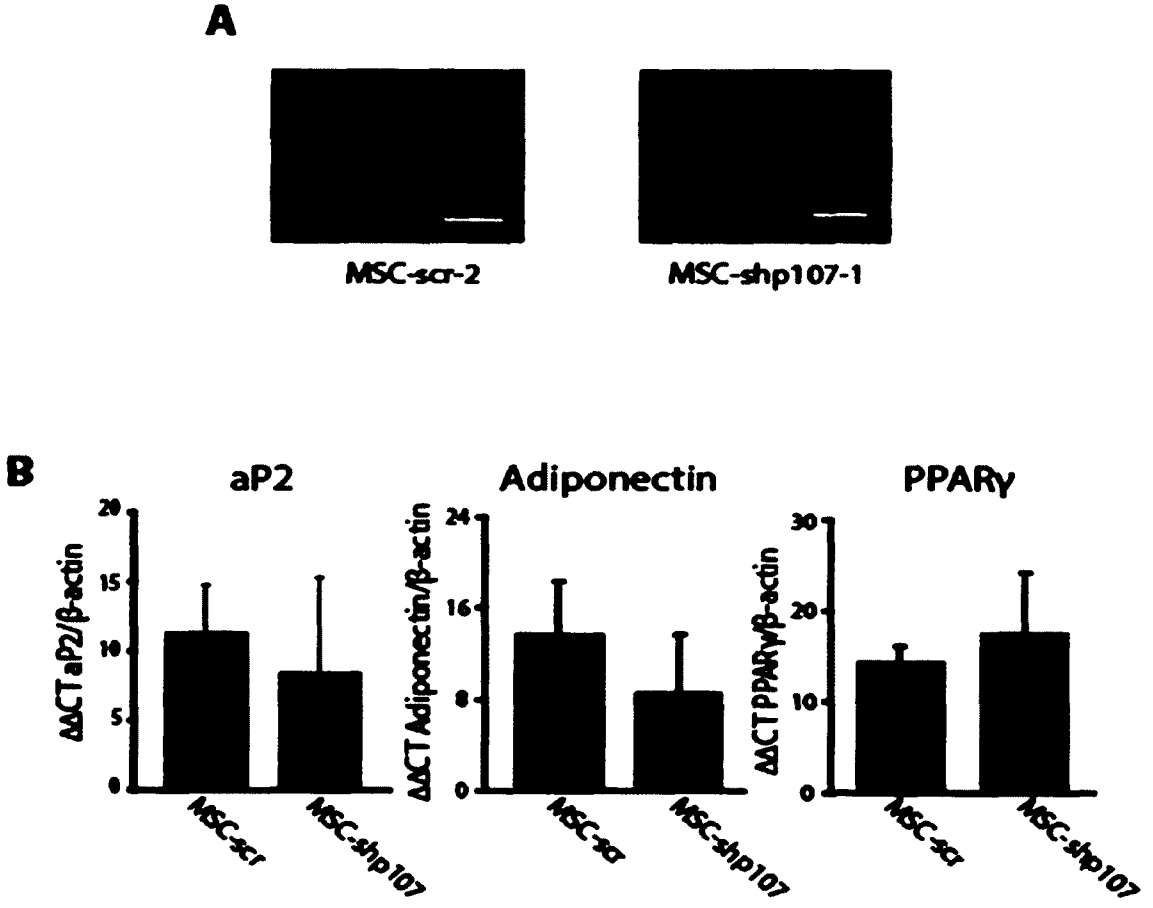


Figure 11. p107 knockdown does not affect adipose differentiation

(A) Oil red O staining of MSC-scr-2 control and MSC-shp107-1 cell lines, scale bar represents 25 μm. (B) Gene expression analysis by qPCR of general adipogenic factors aP2, Adiponectin and PPARγ of 3 different MSC-scr control or MSC-shp107 cell lines, 7 days post adipocyte differentiation.

To test for brown adipocyte formation, the expression of UCP-1 was examined during a time course of adipocyte differentiation. Astoundingly, the UCP-1 gene expression by qPCR, in the MSC-shp107 cell lines was significantly ($p < 0.01$) increased, up to a 100-fold compared to MSC-scr cells (Figure 12A). Furthermore, protein analysis by Western blot during a time course of differentiation showed that UCP-1 expression commenced by day 2 and continued to increase up to 7 days in the MSC-shp107 cell lines (Figure 12B). This is in contrast to the MSC-scr control cell lines that had virtually no UCP-1 protein expressed (Figure 12B). The formation of brown adipocytes was further confirmed by the significantly elevated gene expression of the pro-thermogenic markers, PGC-1α, Cidea and Elovl3 in MSC-shp107 compared to MSC-scr control cell lines (Figure 12C). Together, this data suggests that p107 down-regulation in differentiating stem cells is required for the induction of a brown adipocyte phenotype.

Figure 12

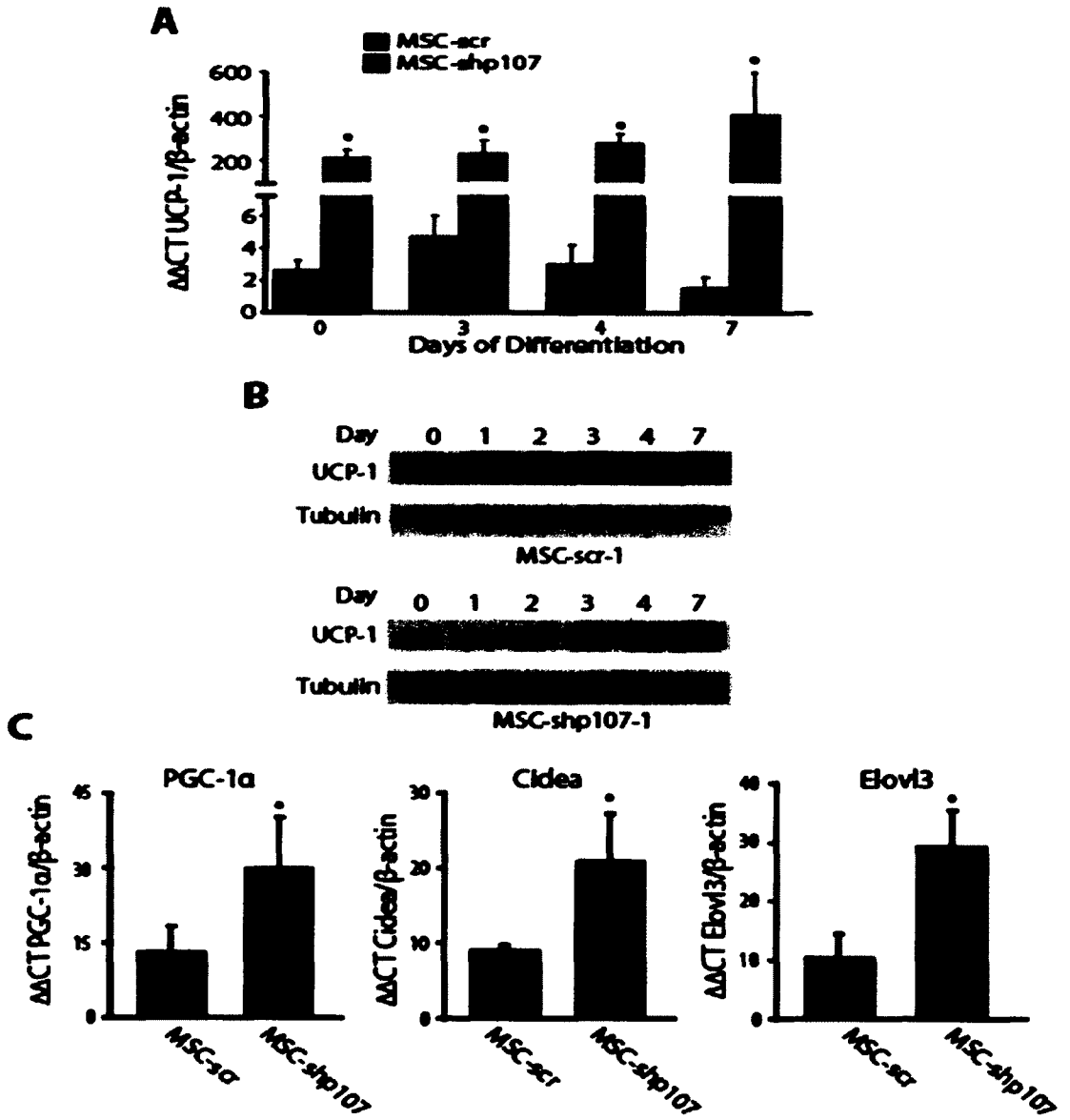


Figure 12. p107 knockdown is required for brown adipocyte differentiation.

(A) Gene expression analysis by qPCR for UCP-1 in MSC-scr control and MSC-shp107 cell lines during 7 days of adipocyte differentiation in arbitrary units (AU) (n=3, ** $p < 0.01$). (B) Representative Western blots for UCP-1 and Tubulin during a time course of adipocyte differentiation in MSC-scr-1 and MSC-shp107-1 cell lines. (C) Gene expression analysis by qPCR of pro-thermogenic factors, PGC-1α, Cidea and Elovl3 in MSC-scr control and MSC-shp107 cell lines 7 days post adipocyte differentiation (n=3, * $p < 0.05$).

p107 knockdown is required for the commitment of the brown adipocyte lineage

As MSCs must first become committed to an adipocyte lineage before differentiation, the role for p107 in these two pathways was evaluated. This was accomplished by controlling the timing of p107 depletion. Normally, p107 levels in the MSC-shp107 are completely reduced by day 2 of differentiation compared to continued expression in the control MSC-scr cell line (Figure 13A). However, reduced p107 levels during differentiation at day 2 by using serum free media, did not increase UCP-1 protein levels 7 days post differentiation in the MSC-scr control cells (Figure 13B). The result indicates that for brown adipocyte formation p107 expression must be down regulated during the lineage commitment phase or at least a very early time point during the differentiation stage.

Figure 13

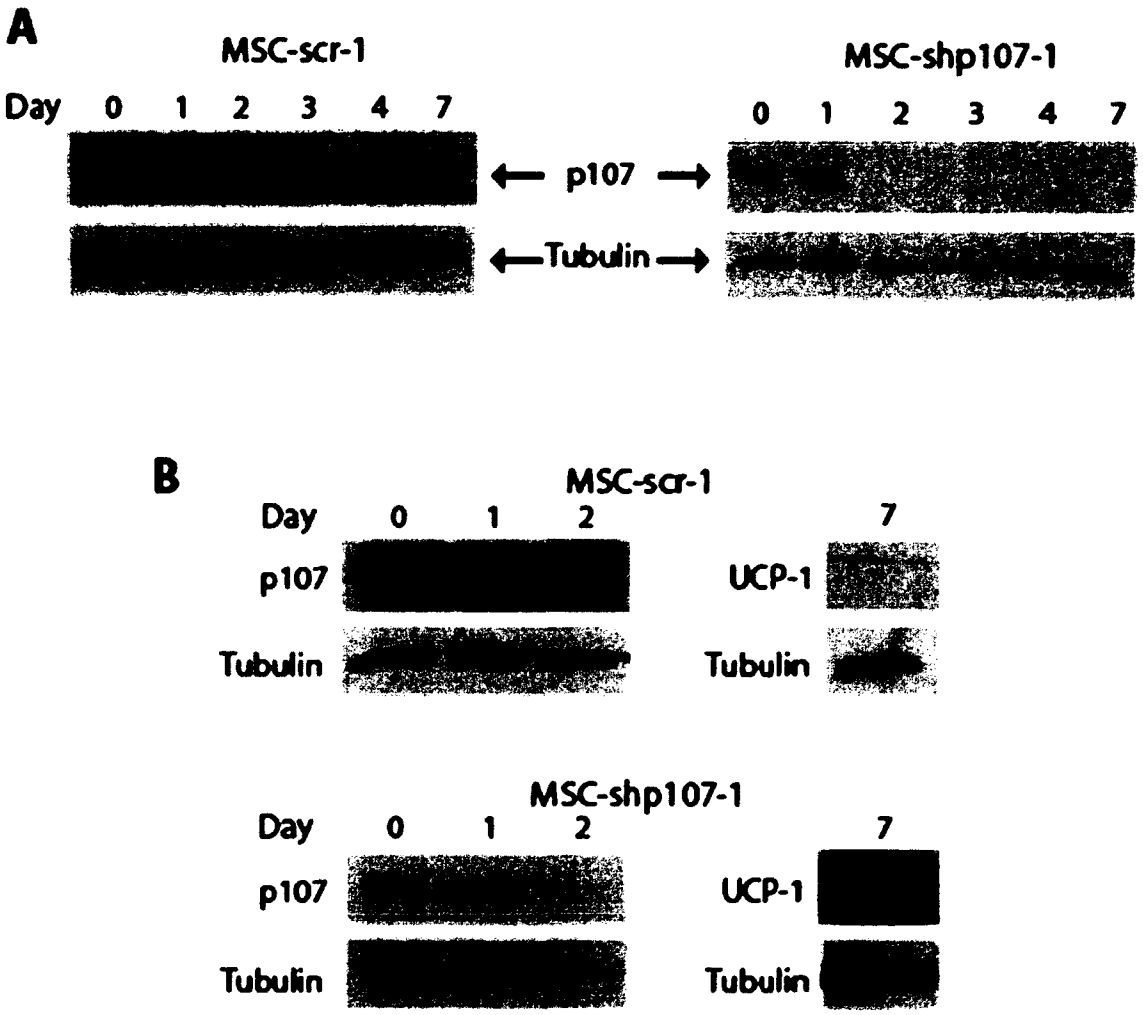


Figure 13. p107 knockdown in MSCs is required for brown adipocyte lineage commitment and not differentiation

(A) Representative Western blots for p107 and Tubulin of the MSC-scr-1 control and the MSC-shp107-1 cell lines during a time course of adipocyte differentiation in days using standard differentiation protocol. (B) Representative Western blots for p107, UCP-1 and Tubulin during a time course of adipocyte differentiation using serum-free media in the MSC-scr-1 control and the MSC-shp107-1 cell lines.

PRDM16 down regulates p107 gene expression levels

Realizing that PRDM16 is a master regulator of brown adipocyte lineage commitment and has an inverse relationship with p107, overexpressing PRDM16 cell lines were made to deduce a potential effect on p107 function. Retroviruses were made that overexpress Flag-tagged PRDM16 or the control empty vector to produce cell lines designated MSC-PRDM16 and MSC-Ctrl, respectively (Figure 14A). Successful exogenously expressed PRDM16 cell lines were confirmed by Western blot analysis with antibodies to PRDM16 and the Flag epitope (Figure 14B). Exogenous PRDM16 expression was also confirmed by the significant ($p < 0.01$) increase in gene expression analyzed by qPCR (Figure 14C). As expected, after a time course of differentiation, the MSC-PRDM16 cells readily differentiated into brown adipocytes by strongly expressing UCP-1 compared to the MSC-Ctrl cell lines (Figure 14D).

Figure 14

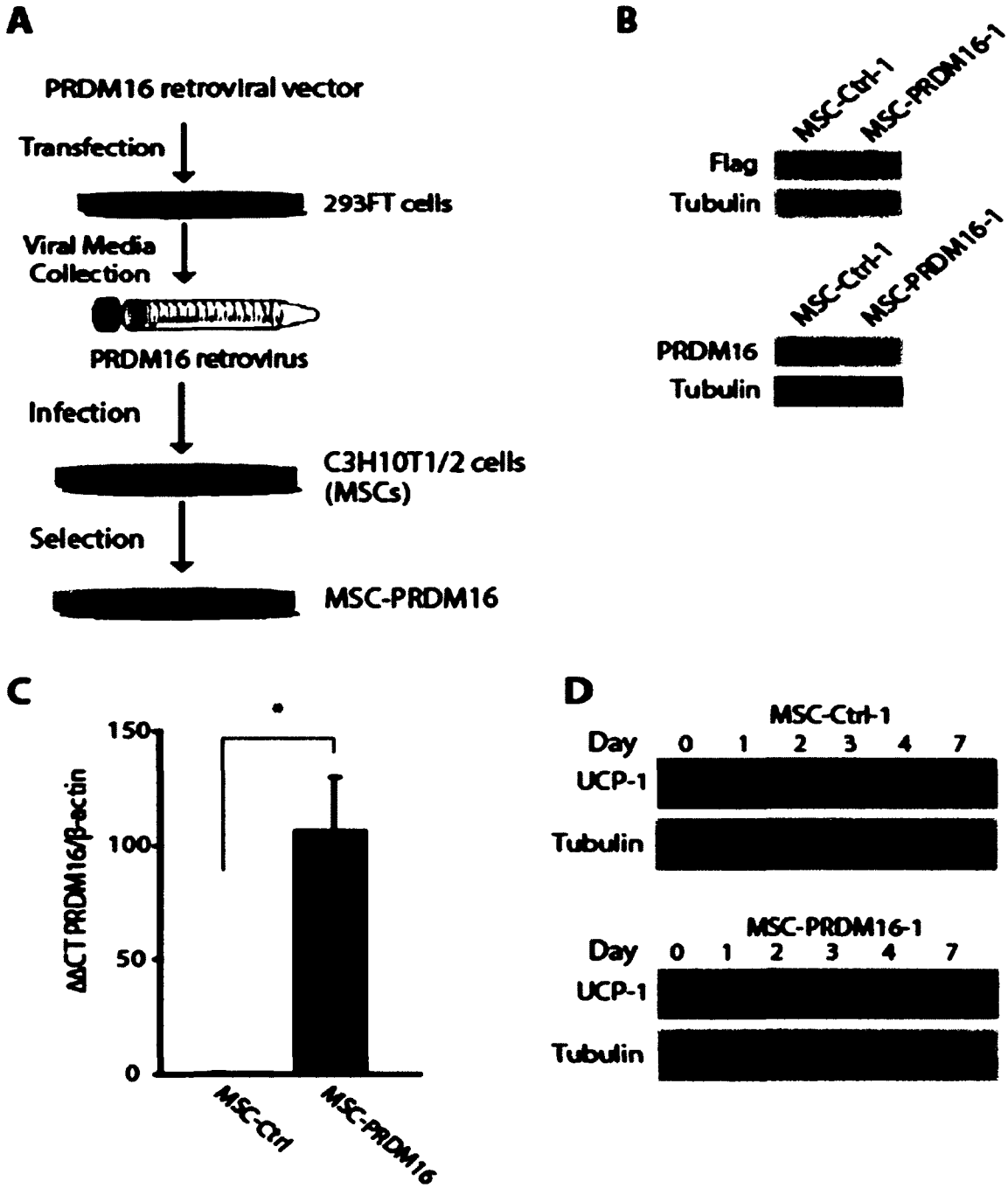

A
PRDM16 retroviral vector
Transfection
293FT cells
Viral Media Collection
PRDM16 retrovirus
Infection
C3H10T1/2 cells (MSCs)
Selection
MSC-PRDM16
B
MSC-Ctrl-1
MSC-PRDM16-1
Flag
Tubulin
MSC-Ctrl-1
MSC-PRDM16-1
PRDM16
Tubulin
C
ΔΔCT PRDM16/β-actin
150
100
50
0
*
MSC-Ctrl
MSC-PRDM16
D
MSC-Ctrl-1
Day 0 1 2 3 4 7
UCP-1
Tubulin
MSC-PRDM16-1
Day 0 1 2 3 4 7
UCP-1
Tubulin

Figure 14. Derivation of the PRDM16 overexpressing MSC line – MSC-PRDM16

(A) Diagram representing the process used to derive the PRDM16 flag tagged overexpressing MSC lines. (B) Representative Western blots for PRDM16, Flag and Tubulin during growth in the MSC-Ctrl-1 and the MSCV-PRDM16-1 cell lines. (C) Gene expression analysis by qPCR of PRDM16 levels in 3 different MSC-Ctrl and MSC-PRDM16 cell lines (n=3, * $p < 0.05$). (D) Representative Western blots for UCP-1 and Tubulin during a time course of adipocyte differentiation in days in the MSC-Ctrl-1 and the MSC-PRDM16-1 cell lines.

Notably, Western blot analysis showed a decrease in the expression of p107 (Figure 15A). This was corroborated by a significant ($p < 0.05$) decrease of up to 8-fold in p107 gene expression in the PRDM16 cell lines during growth (Figure 15B). Contrarily, Western blot and qPCR analysis showed no changes in PRDM16 protein and gene expression with p107 KD, in the MSC-shp107 cell lines, in growth or during a time course of differentiation up to 7 days (Figure 15C and 15D). These results suggest that PRDM16 down regulates p107 gene expression levels, whereas p107 has no control over PRDM16 in determining the brown adipocyte lineage.

Figure 15

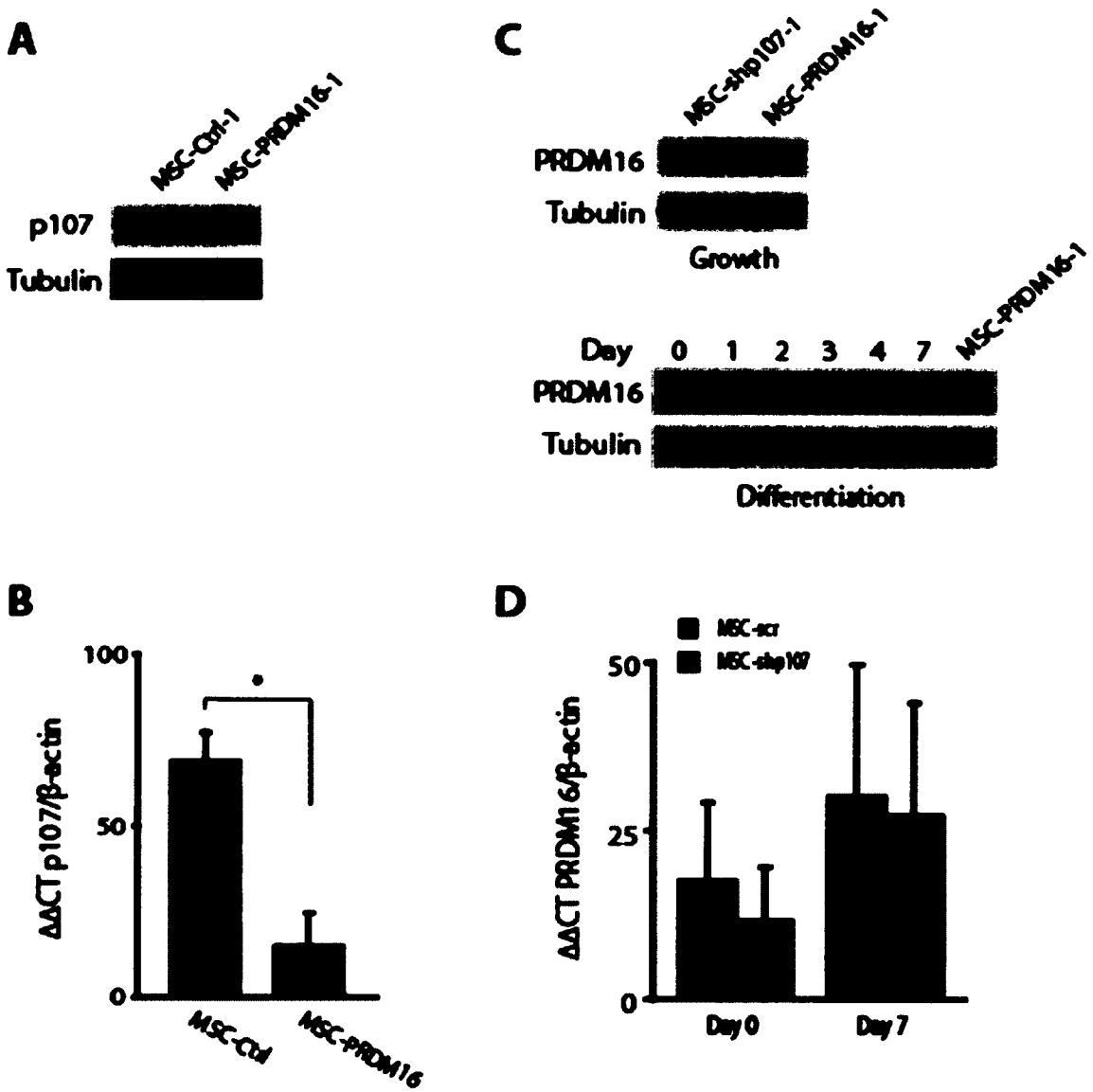


Figure 15. PRDM16 overexpression lowers p107 levels

(A) Representative Western blots for p107 and Tubulin in the MSC-Ctrl-1 and the MSC-PRDM16-1 cell lines at confluency. (B) Gene expression analysis by qPCR for p107 in different MSC-Ctrl and the MSC-PRDM16 cell lines at confluency. (n=3, * $p < 0.05$). (C) Representative Western blots for PRDM16 and Tubulin in MSC-shp107-1 and MSC-PRDM16-1 cell lines during growth and during a time course of adipocyte differentiation. (D) Gene expression analysis by qPCR for PRDM16 in 3 different MSC-scr or MSC-shp107 cell lines at 0 and 7 days post adipocyte differentiation (n=3).

PRDM16 down regulates p107 transcription

In order to evaluate the PRDM16 transcriptional control over p107, luciferase assays were used against a p107 promoter, 900 bp proximal to the transcriptional start site, containing two E2F binding domains (EBD) a kind gift of Dr. Julien Sage, Stanford University (Burkhart et al., 2010) (Figure 16A). With increasing amounts of E2F1, a known activator of p107 transcription, luciferase activity was significantly ($p < 0.01$) increased (Figure 16B). However, if increasing amounts of PRDM16 was added, there was a significant ($p < 0.01$) decrease by as much as 2.5 fold in luciferase activity, indicating a repression of the promoter (Figure 16C).

Figure 16

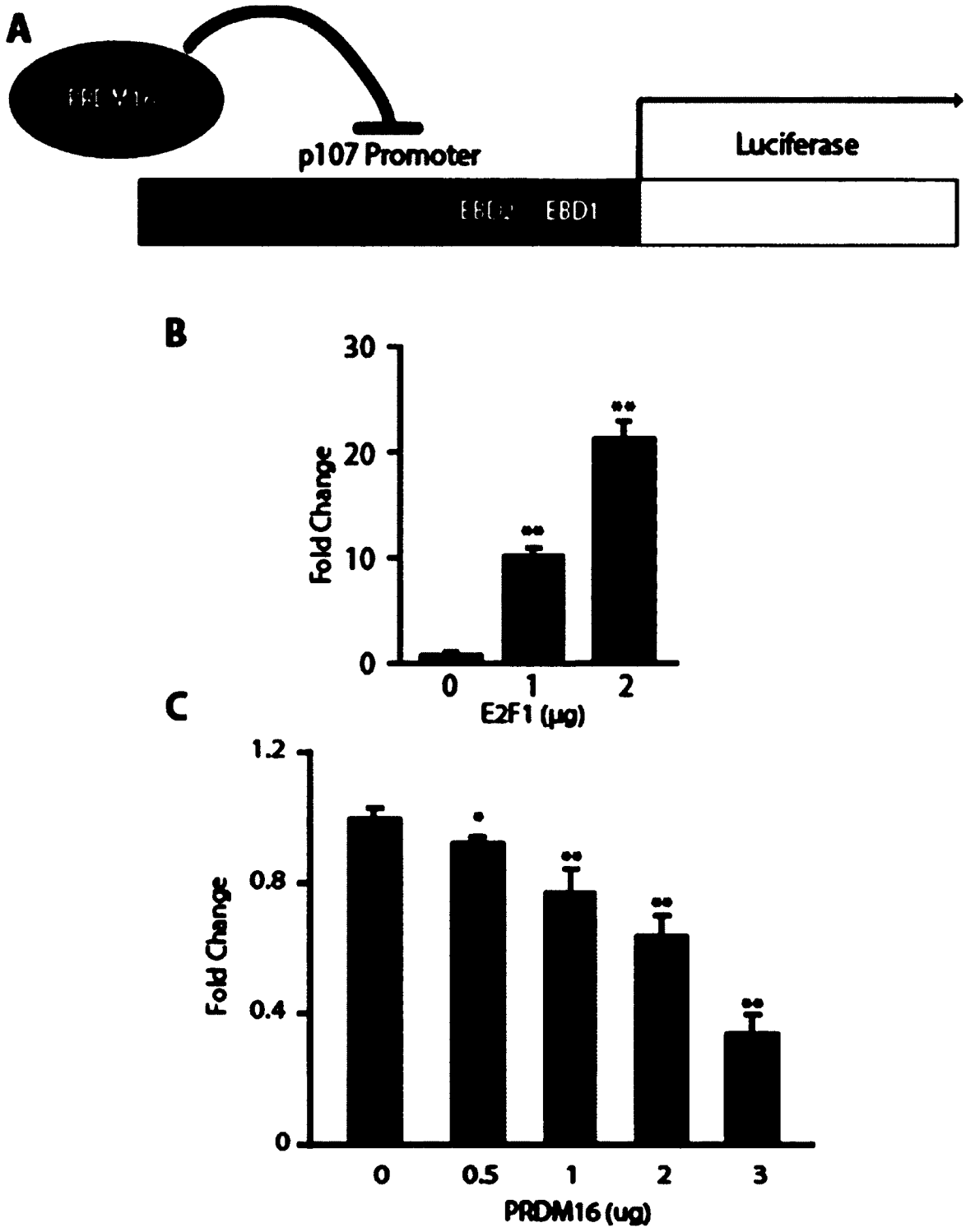

A
p107 Promoter
Luciferase
EBD1
B
Fold Change
30
20
10
0
0
1
2
E2F1 (µg)
C
Fold Change
1.2
0.8
0.4
0
0
0.5
1
2
3
PRDM16 (ug)

Figure 16. PRDM16 down regulates the p107 promoter activity

(A) A diagram of the p107 promoter, 900 base pairs (bp) proximal to the transcriptional start site, fused to the luciferase gene containing 2 E2F binding domains (EBD1 and EBD2). (B) Fold change in luciferase activity using the 900 bp p107 promoter construct transfected with increasing amounts of E2F1 plasmid in C3H10T1/2 cells (n=3, ** $p < 0.01$, One-way ANOVA and Tukey Post-hoc test were used). (C) Fold change in luciferase activity of the p107 promoter construct with increasing amounts of PRDM16 plasmid in C3H10T1/2 (n=7, ** $p < 0.01$, One-way ANOVA and Tukey Post-hoc test were used).

PRDM16 controls the p107 promoter without binding directly to DNA

To delve deeper into understanding how PRDM16 influences the p107 promoter, a mutant version of PRDM16 was used. PRDM16 contains a zinc-finger domain required to bind to DNA. However, despite a mutation within this domain that abrogates DNA binding, the thermogenic program in cells is sufficiently stimulated (Seale et al., 2007). Hence, in order to test if PRDM16 repression of the p107 promoter required its DNA binding activity, a PRDM16 DNA binding deficient zinc finger domain mutant containing a point mutation at arginine 998 for glutamine (PRDM16-R998Q) was tested in luciferase assays. It was found that with increasing amounts of PRDM16-R998Q, there was a sustained significant (* $p < 0.05$, ** $p < 0.01$) decrease in luciferase activity (Figure 17A). This suggests that PRDM16 does not bind directly to the p107 promoter but might bind in a complex, with an unknown protein (protein X) (Figure 17B).

Figure 17

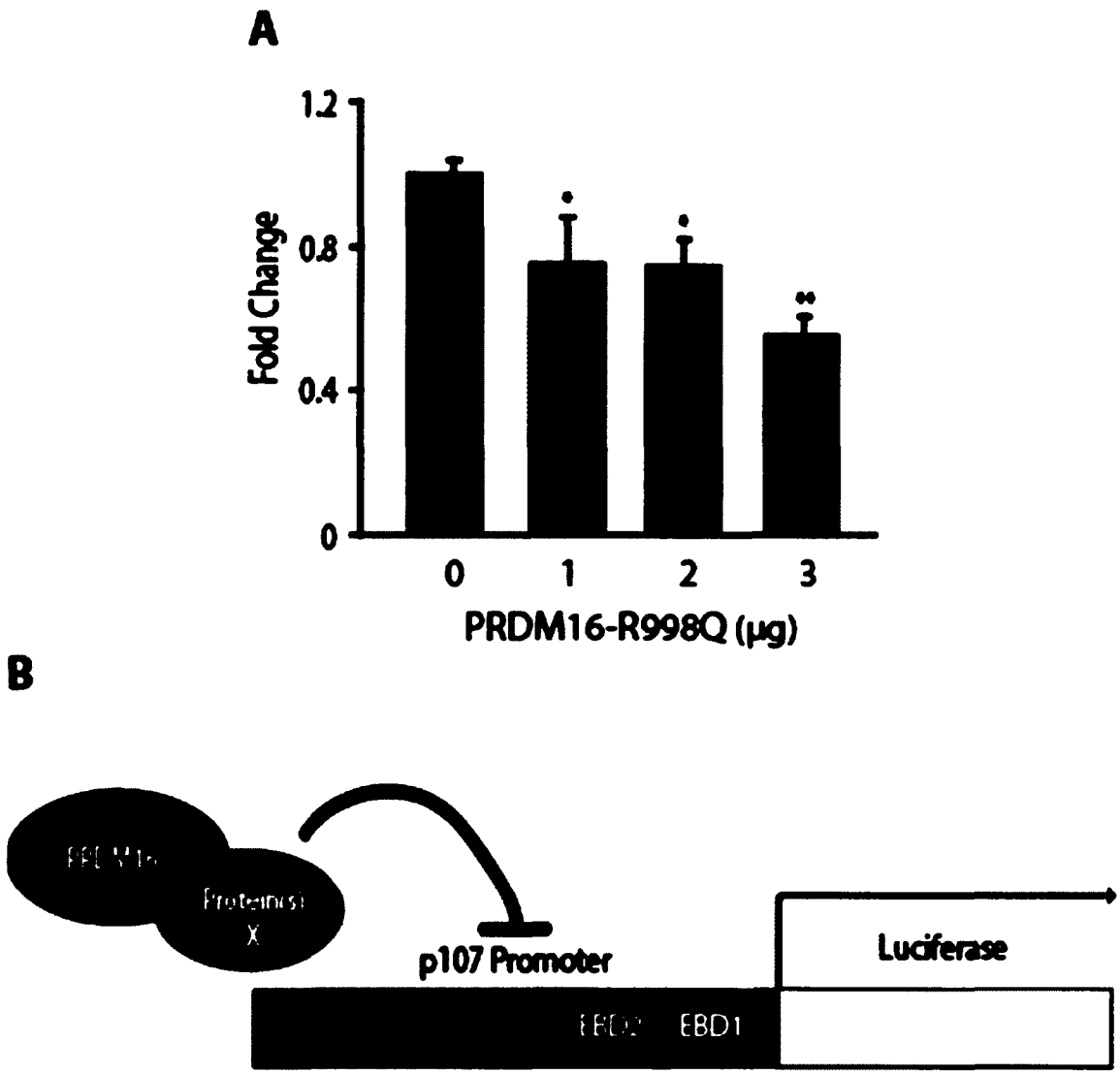


Figure 17. PRDM16 regulates p107 promoter activity without directly binding to DNA

(A) Luciferase assay in C3H10T1/2 cells for the 900 bp p107 promoter construct transfected with increasing amounts of PRDM16-R998Q plasmid (n=6, * $p < 0.05$, ** $p < 0.01$, One-way ANOVA and Tukey Post-hoc were test used). (B) Diagram depicting PRDM16 binding in a DNA independent manner in a complex with an unknown protein(s) X in order to down regulate p107 transcription.

PRDM16 down regulates p107 independently of E2Fs

In order to determine if PRDM16 influences the p107 promoter in a complex that down regulates E2F activity, the EBDs were assessed (Figure 16A). In this case, PRDM16 was tested against the p107 promoter mutated at either one or both of the EBDs. Results showed that when either site was mutated, EBD1 or EBD2, increasing levels of PRDM16 was able to significantly ($p < 0.01$) decrease luciferase activity, and therefore p107 transcription (Figures 18A). Moreover, when both EBD sites were mutated, EBD1/2, increasing amounts of PRDM16 continued to reduce luciferase activity (Figure 18B). Therefore, these results suggest that PRDM16 represses the p107 promoter independently of E2F activity.

Figure 18

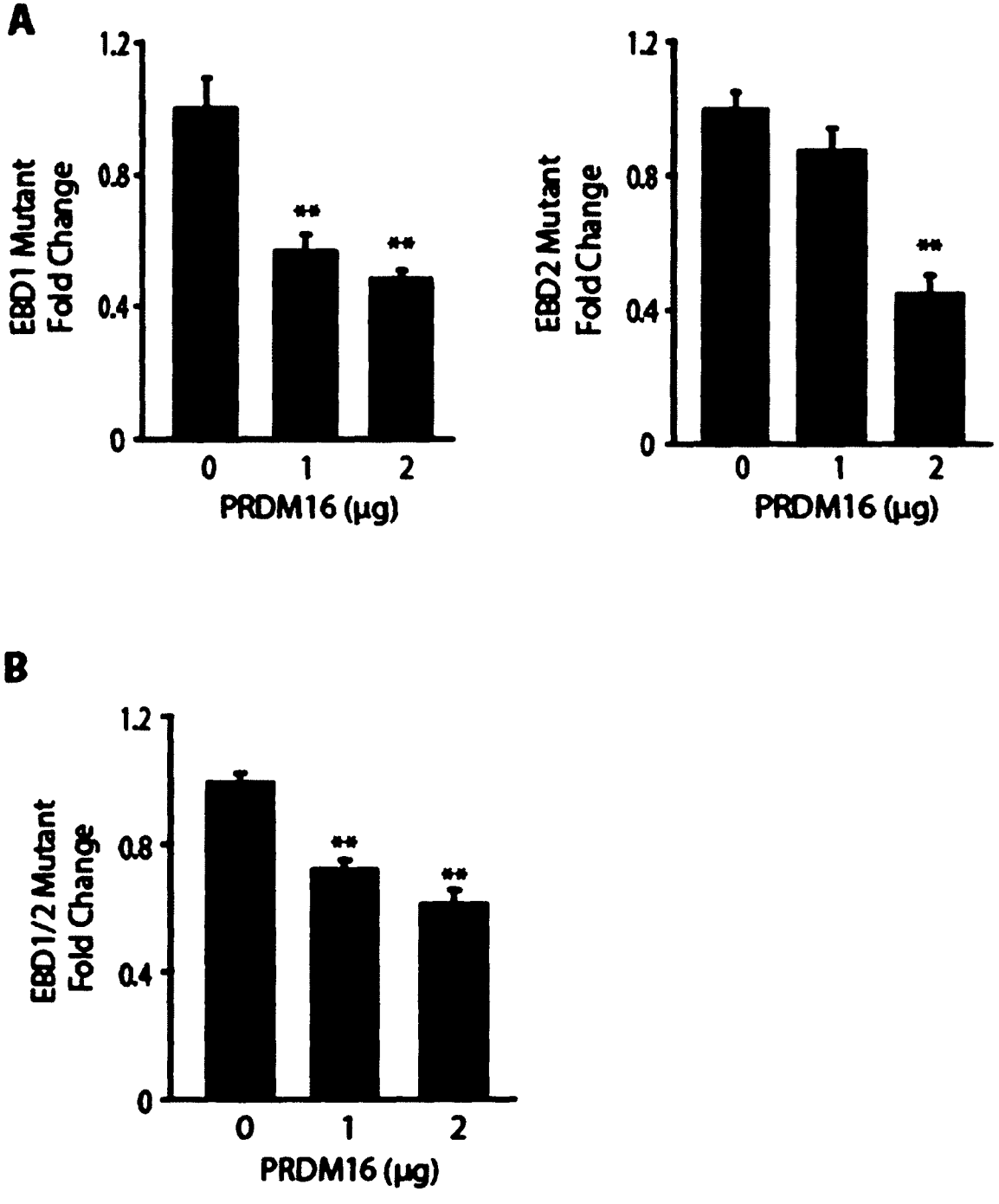

A
EBD1 Mutant Fold Change
1.2
0.8
0.4
0
**
**
0
1
2
PRDM16 (µg)
EBD2 Mutant Fold Change
1.2
0.8
0.4
0
**
0
1
2
PRDM16 (µg)
B
EBD1/2 Mutant Fold Change
1.2
0.8
0.4
0
**
**
0
1
2
PRDM16 (µg)

Figure 18. PRDM16 controls p107 transcription independently of E2Fs

(A) Luciferase assay in C3H10T1/2 cells for the 900 bp p107 promoter construct mutated at the E2F Binding Domain 1 (EBD1) or the E2F binding domain 2 (EBD2) sites with increasing amounts of PRDM16 (n=4, ** $p < 0.01$, One-way ANOVA and Tukey Post-hoc test were used). (C) Luciferase assay in C3H10T1/2 cells for the 900 bp p107 promoter construct mutated at both EBD1 and EBD2 sites (EBD1/2), with increasing amounts of PRDM16 (n=4, ** $p < 0.01$, One-way ANOVA and Tukey Post-hoc test were used).

p107 overexpression attenuates the role of PRDM16 in the brown adipocyte differentiation pathway

Together, from this data, it can be hypothesized that p107 is a downstream target that is inhibited by PRDM16 in the brown adipocyte pathway. Hence, in order to establish the importance of p107 in this pathway competition assays were used. First, MSC cell lines overexpressing PRDM16 (MSC-PRDM16) were transduced with p107 overexpressing retrovirus before differentiation to test if brown adipocyte formation could be blocked. Gene expression by qPCR revealed continued p107 overexpression ($p < 0.01$) 7 days post adipocyte differentiation in transduced MSC-PRDM16 cell lines (Figure 19A). The cells were also assessed for their potential to differentiate into adipocytes (Figure 19B). Oil red O staining showed essentially equal amounts of adipocyte accumulation with and without p107 transduction in MSC-PRDM16 cells 7 days post adipocyte differentiation (Figure 19B). Moreover, qPCR confirmed that adipocyte differentiation was unaffected with similar expression levels of general adipogenic markers, aP2, Adiponectin and PPARγ in the presence and absence of p107 (Figure 19C). This is very important because it indicates that forced p107 expression does not affect the cells' ability to differentiate into adipocytes.

Figure 19

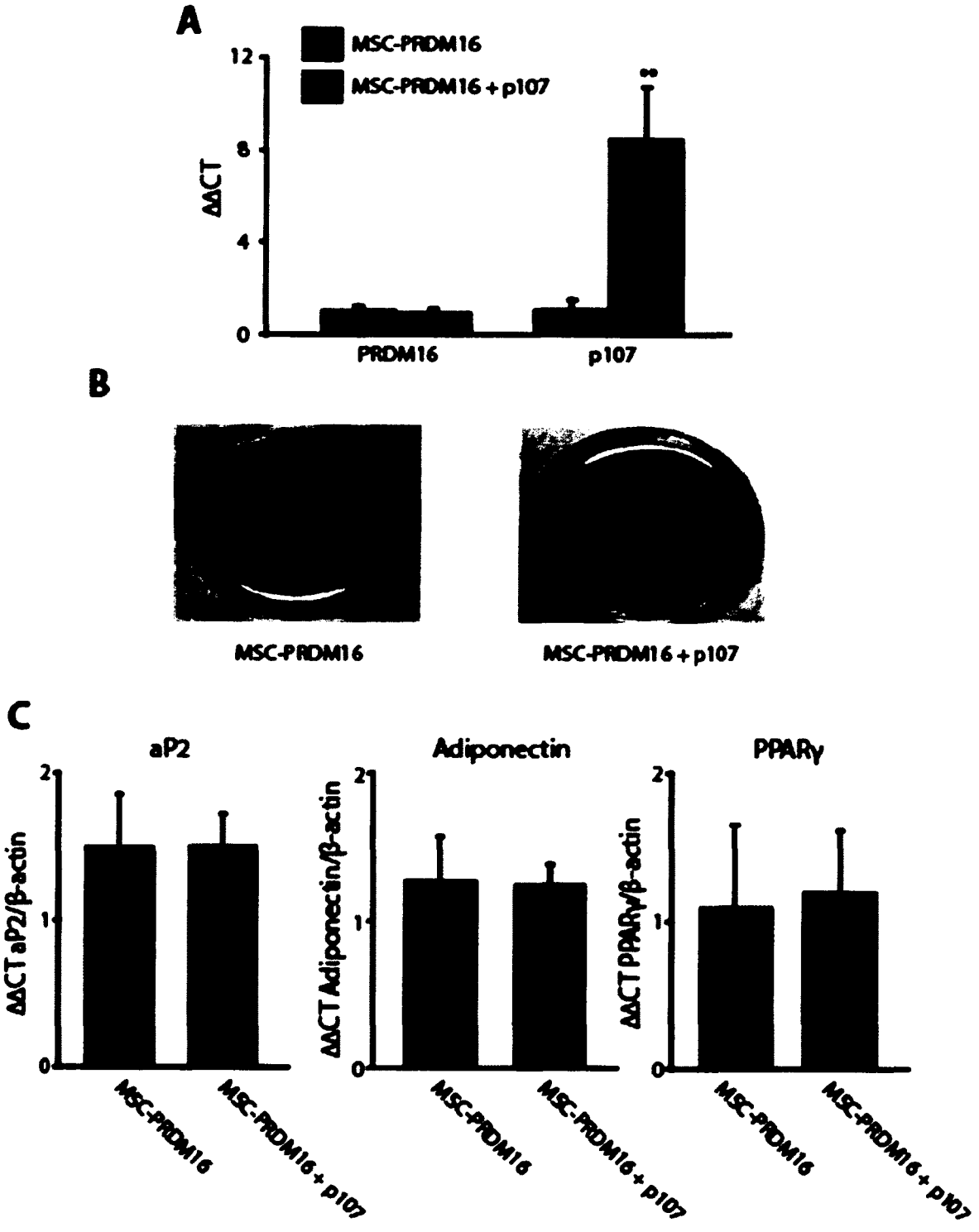

A
MSC-PRDM16
MSC-PRDM16 + p107
ΔΔCT
12
8
4
0
PRDM16
p107
B
MSC-PRDM16
MSC-PRDM16 + p107
C
aP2
Adiponectin
PPARγ
ΔΔCT aP2/β-actin
ΔΔCT Adiponectin/β-actin
ΔΔCT PPARγ/β-actin
2
1
0
MSC-PRDM16
MSC-PRDM16 + p107

Figure 19. Adipocyte differentiation is unaffected in PRDM16 cell lines overexpressing p107

(A) Gene expression analysis by qPCR of p107 and PRDM16 7 days post adipocyte differentiation in MSC-PRDM16 cell lines non-transduced or transduced with p107 retrovirus (n=3, * $p < 0.01$). (B) Representative Oil red O staining of MSC-PRDM16 cell lines non-transduced or transduced with p107 retrovirus 7 days post adipocyte differentiation. (C) Gene expression analysis by qPCR for general adipogenic factors, aP2, Adiponectin and PPARγ, in MSC-PRDM16 cell lines non-transduced or transduced with p107 retrovirus, 7 days post adipocyte differentiation (n=3).

Next, the potential for p107 to block brown adipocyte formation was tested. The MSC-PRDM16 that continued to overexpress p107 had significantly ($p < 0.05$) lower levels of UCP-1 gene expression after 7 days of adipocyte differentiation compared to cells that were not transduced with p107 (Figure 20A). The paucity of brown adipocyte formation was further exemplified by significantly ($p < 0.05$) decreased gene expression of other pro-thermogenic markers Elovl3 and Cidea in the presence of overexpressed p107 (Figure 20B). However, PGC-1α gene expression was not influenced by the persistent expression of p107 levels (Figure 20B). These results suggest that p107 may be important in down-regulating the transcription of pro-thermogenic genes.

Figure 20

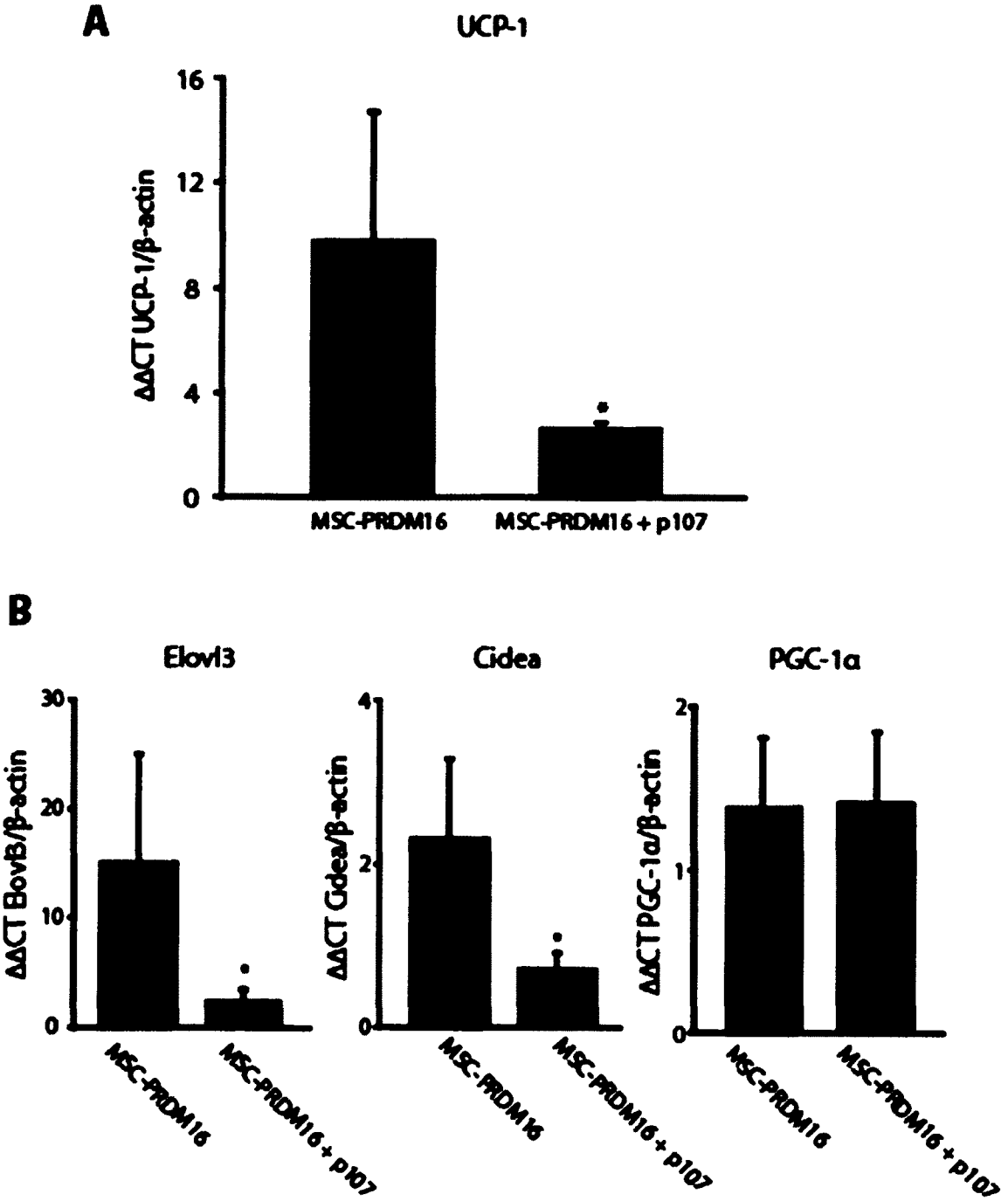

A
UCP-1
16
12
8
4
0
ΔΔCT UCP-1/β-actin
MSC-PRDM16
MSC-PRDM16 + p107
B
Elovl3
30
20
10
0
ΔΔCT Bovb/β-actin
Cidea
4
2
0
ΔΔCT Cidea/β-actin
PGC-1α
2
1
0
ΔΔCT PGC-1α/β-actin
MSC-PRDM16
MSC-PRDM16+p107

Figure 20. The brown thermogenic program activated by PRDM16 is attenuated by the overexpression of p107

(A) Gene expression analysis by qPCR for UCP-1 in MSC-PRDM16 cell lines non-transduced or transduced with p107 retrovirus 7 days post adipocyte differentiation (n=3, * $p < 0.05$). (B) Gene expression analysis by qPCR for pro-thermogenic factors, Elovl3, Cidea and PGC-1α in MSC-PRDM16 cell lines non-transduced or transduced with p107 retrovirus 7 days post adipocyte differentiation (n=3, * $p < 0.05$).

Second, the importance of p107 in the PRDM16 brown adipocyte forming pathway was further corroborated by evaluating overexpressing p107 cell lines, MSC-p107, transduced with PRDM16 retrovirus. qPCR revealed that PRDM16 was significantly (p <0.01) overexpressed after transduction of PRDM16 retrovirus followed by 7 days of differentiation (Figure 21A). Moreover, the cells transduced with PRDM16 were not affected in adipocyte accumulation revealed by Oil red O staining 7 days post adipocyte differentiation (Figure 21B). qPCR confirmed that there were similar expression levels of general adipogenic markers, aP2, Adiponectin and PPARγ, in the absence and presence of PRDM16, 7 days post differentiation (Figure 21C). Together, this demonstrates that overexpression of PRDM16 does not influence the MSC-p107 cells' ability to differentiate into adipocytes.

Figure 21

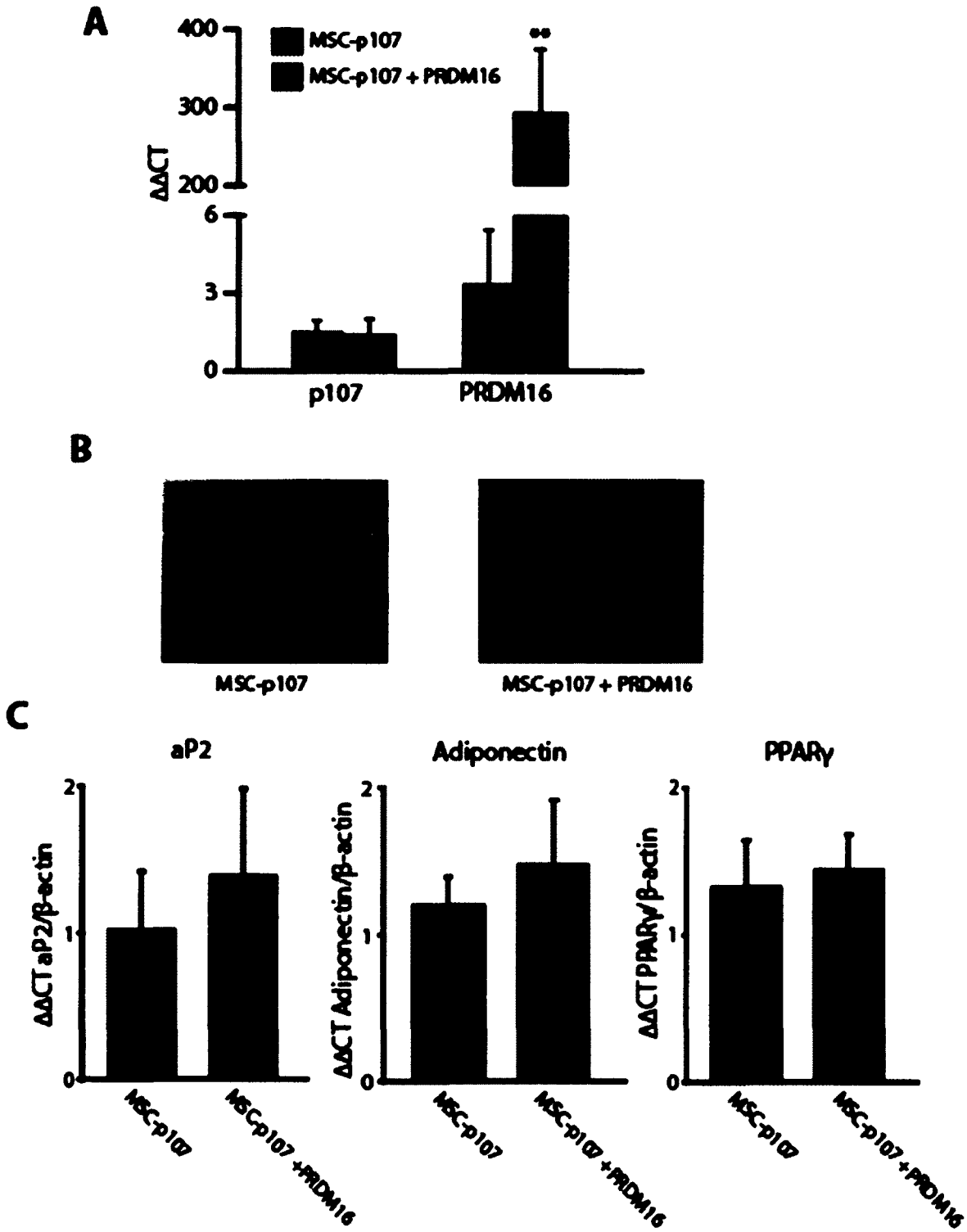

A
400
300
200
6
3
0
ΔΔCT
MSC-p107
MSC-p107 + PRDM16
**
p107
PRDM16
B
MSC-p107
MSC-p107 + PRDM16
C
aP2
Adiponectin
PPARγ
2
1
0
ΔΔCT aP2/β-actin
ΔΔCT Adiponectin/β-actin
ΔΔCT PPARγ/β-actin
MSC-p107
MSC-p107 +PRDM16

Figure 21. Adipocyte differentiation is unaffected in p107 cell lines overexpressing PRDM16

(A) Gene expression analysis by qPCR of p107 and PRDM16 7 days post adipocyte differentiation in MSC-p107 cell lines non-transduced or transduced with PRDM16 retrovirus (n=3, ** $p < 0.01$). (B) Representative Oil red O staining of the MSC-p107 cells non-transduced or transduced with PRDM16 retrovirus, 7 days post adipocyte differentiation. (C) Gene expression analysis by qPCR for general adipogenic factors, aP2, Adiponectin and PPARγ, in MSC-p107 cell lines non-transduced or transduced with PRDM16 retrovirus 7 days post adipocyte differentiation (n=3).

Subsequently, brown adipose terminal differentiation was analyzed for the expression of UCP-1 at the RNA level. The MSC-p107 cells despite the continued presence of PRDM16 did not increase UCP-1 gene expression after 7 days of adipocyte differentiation (Figure 22A). Furthermore, the pro-thermogenic markers Elovl3 and Cidea also did not increase with increasing amounts of PRDM16 (Figure 22B). However, concurrent with the previous data, overexpression of PRDM16 significantly ($p < 0.05$) increased the expression of PGC-1α regardless of the overexpression of p107 (Compare Figures 20B and 22B). This data suggests that p107 has the ability to block part of PRDM16 thermogenic determining function in the brown adipocyte forming pathway.

Figure 22

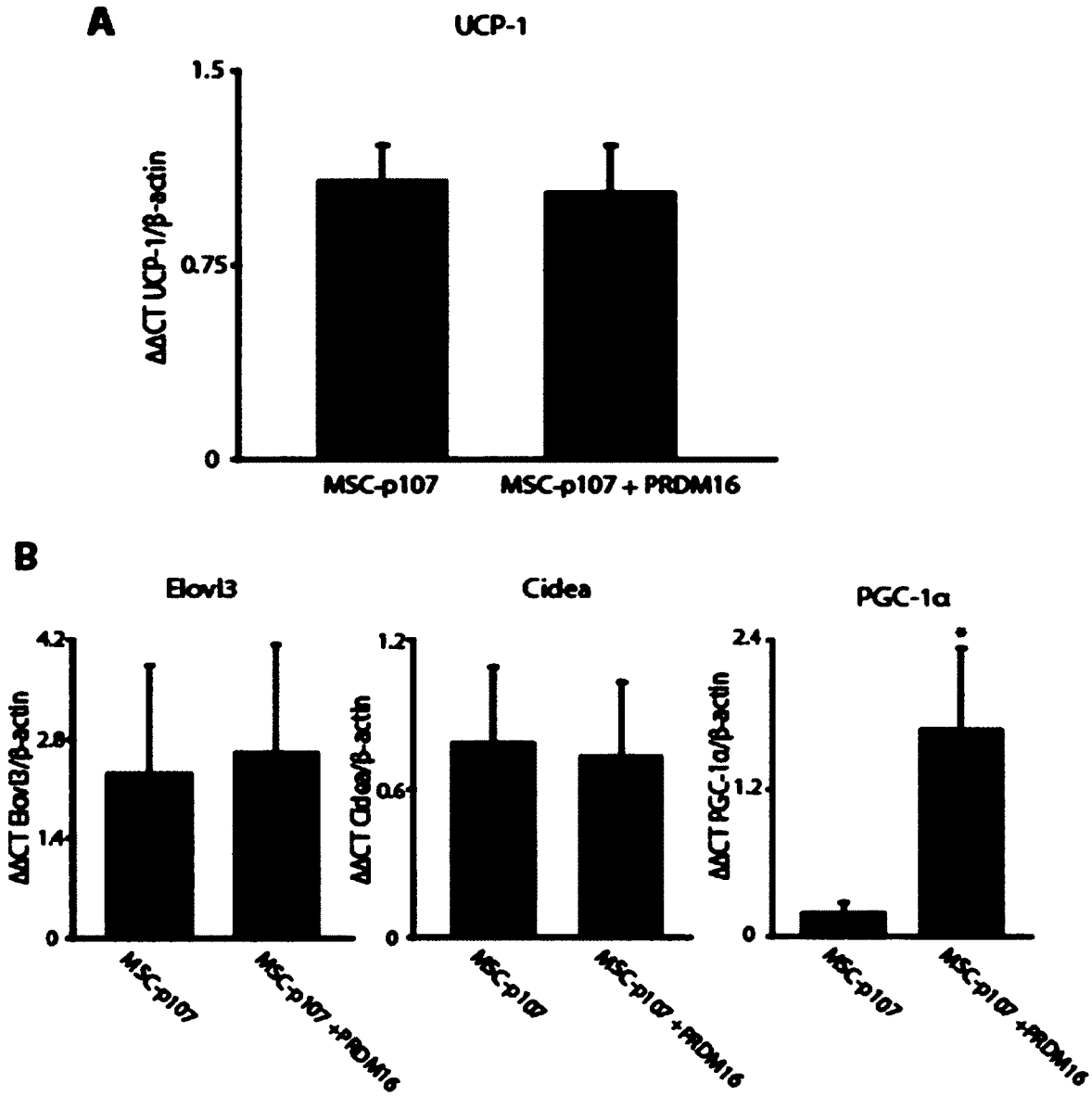


Figure 22. The PRDM16 brown adipogenic program is blocked in overexpressing p107 cell lines

(A) Gene expression analysis by qPCR for UCP-1 in MSC-p107 cell lines non-transduced or transduced with PRDM16 retrovirus, 7 days post adipocyte differentiation (n=3). (B) Gene expression analysis by qPCR for pro-thermogenic factors, Elovl3, Cidea and PGC-1α, in MSC-p107 cell lines in non-transduced or transduced with PRDM16 retrovirus 7 days post adipocyte differentiation (n=3, * $p < 0.05$).

It is apparent through many lines of evidence in this study that the down regulation of p107 is both necessary and required for the complete formation of brown adipocytes. This research is the first to show the molecular importance and connection of p107 in the PRDM16 brown adipocyte forming pathway at the level of the stem cell. Using the knowledge learned from this study, manipulating p107 represents a strong approach that might help combat obesity in humans.

Figure 23

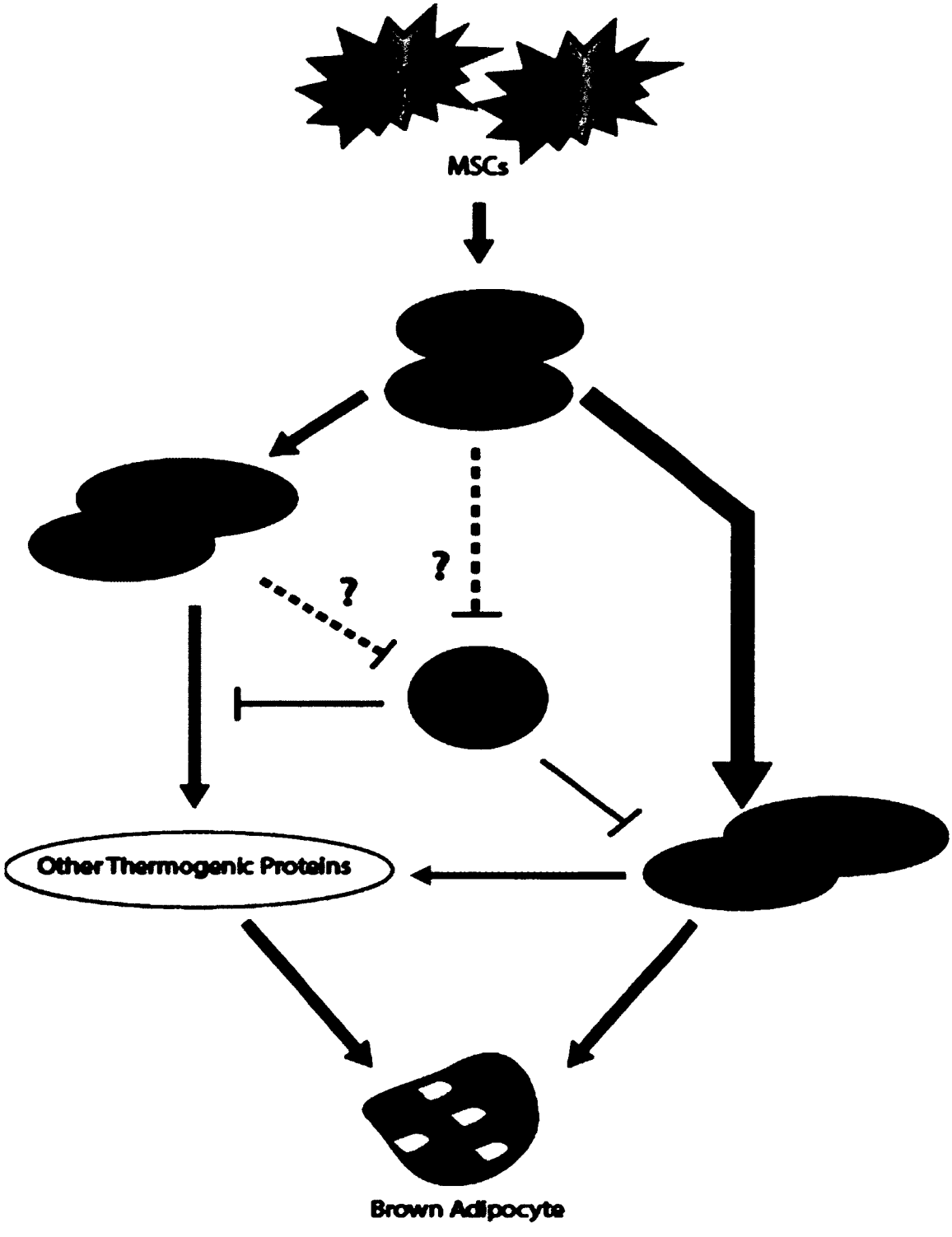


Figure 23. The role of p107 in the PRDM16 brown adipogenic lineage commitment pathway

Mesenchymal/mesodermal stem cells (MSCs) have the potential to differentiate into various mesodermal cell types including brown adipocytes. PRDM16 bound to c/ebpβ has the ability to commit MSCs to the brown adipocyte lineage. It does this by activating both PGC-1α and PPARγ. The PRDM16-c/ebpβ complex can activate PPARγ which can then exert its effects by turning on pro-thermogenic proteins. However, over expression of p107 has the ability to block this process. The PRDM16-c/ebpβ complex activates PGC-1α. Although, p107 blocks PGC-1α activation, over expression of PRDM16 can by-pass this blockade and activate PGC-1α directly, indicated by the thicker arrow. It is unknown which PRDM16 complex inhibits p107, indicated by the hashed lines.

Discussion

The importance of p107 as a stem cell fate deciding factor has come to the forefront of research in recent years. This study explores the cellular and molecular mechanism for how p107 functions during brown/beige adipocyte formation. The down regulation of p107 in myogenic stem cells controls the lineage switch to an oxidative fiber type (Scimè et al., 2010). Additionally, Vanderluit et al. have shown that a reduction in p107 levels controls the commitment of neural progenitors to a neuronal fate (Vanderluit et al., 2007). To date, no other group has shown the importance of p107 during stem cell commitment to brown adipocytes.

In this study, it was shown with many lines of evidence that p107 down regulation is essential in determining the commitment of stem cells to the brown adipocyte lineage. First, upon investigation of the various fat depots in mice, it was noted that p107 expression is only present in the SV fraction, which is where the stem cells and their committed progenitors are located. This indicates that p107 functions at the level of the stem cell and not the terminally differentiated adipocyte. Moreover, the subcutaneous adipose depots that are more readily able to form beige adipocytes have lower levels of p107 compared to visceral depots (Figure 5C and 5D). Indeed, there is an inverse relationship between PRDM16, a master regulator of brown adipocyte formation, and p107 levels in the inguinal adipose depot as a function of the weight of the pad (Figure 5E). This is important as it is thought that increasing levels of PRDM16 in the inguinal depots promotes weight loss through the formation of beige adipocytes (Seale et al., 2011). Second, upon injection of the β3-adrenergic agonist, CL316,243, there is a

significant decrease in p107 expression in the inguinal adipose depot and an increase in UCP-1 expression, concomitant with beige adipocyte formation (Figure 6). Third, p107 KO MEFs, contrary to their WT controls, have the ability to differentiate into brown adipocytes both in vitro and in vivo, confirmed by the expression of UCP-1 and other pro-thermogenic markers (Figure 8 and 9). Fourth, knockdown of p107 in the MSC cell line, C3H10T1/2, facilitates brown adipocyte differentiation (Figure 12). p107 KD is required before cells start to differentiate into brown adipocytes, demonstrating its role during commitment and not differentiation (Figure 13). Fifth, this research shows that p107 is a downstream target of PRDM16 in the brown adipocyte formation pathway (Figures 15 and 16C). Indeed, overexpression of PRDM16 in MSCs down regulates p107 expression (Figure 15A). This is confirmed by luciferase assays demonstrating that PRMD16 represses p107 promoter activity. Finally, PRDM16 cell lines are impeded from initiating the brown adipocyte differentiation program with the continued maintenance of p107 expression (Figures 19 and 20). Moreover, PRDM16 cannot rescue the brown adipocyte program in p107 overexpressing cell lines (Figures 21 and 22). Together, these data show compelling evidence that p107 is a stem cell fate deciding factor for adipocytes downstream of PRDM16 in the brown adipocyte formation pathway.

Current studies suggest MSCs that differentiate into white or brown adipocytes are in fact perivascular cells (pericytes) (Caplan and Correa, 2011; Crisan et al., 2008; Rodeheffer et al., 2008; Tang et al., 2008; Zannettino et al., 2008). Recently, it has been shown that preadipocytes with the cell surface marker, Zfp423, can give rise to white or brown adipocytes (Gupta et al., 2010, 2012). These cells arise from pericytes found in the

endothelium of the vasculature of both WAT and BAT (Gupta et al., 2012; Tran et al., 2012). In WAT or BAT pads they are normally committed to the specific lineage of their depot. Hence, it is not surprising that there is no expression of p107 in the stem cell compartment of BAT that would consist of brown pre-adipocytes (Figure 5C and 5D). Also, there is less expression of p107 in subcutaneous depots where beige adipocytes are formed (Figure 5C). The importance of p107 in the committed preadipocyte would be confirmed by a closer inspection of its expression in the Zfp423 population in WAT and BAT.

The method by which beige adipocytes form in vivo is still a topic for debate. Although, this study supports data for de novo commitment of stem cells into beige cells, some researchers argue that transdifferentiation of white adipocytes occurs (Barbatelli et al., 2010; Cinti, 2002, 2009, 2011; Cannon and Nedergaard, 2004; Himms-Hagen et al., 2000; Perwitz et al., 2010; Walden et al., 2011). Transdifferentiation describes how a terminally differentiated cell transforms into a completely different but normal cell type (Tosh and Slack, 2002). Himms-Hagen et al., used 5-bromodeoxyuridine (BrdU) to track the proliferating cell population upon β3-adrenergic agonist injection into mice (Himms-Hagen et al., 2000). They found that the multilocular cells, a characteristic of brown adipocytes that were formed in retroperitoneal WAT (rWAT) lacked expression of BrdU. Thus, they concluded that the multilocular cells found in the rWAT adipose depot transdifferentiated from white adipocytes and not from the proliferation and subsequent differentiation of stem cells. To further support the hypothesis of transdifferentiation it has been shown that brown adipocytes specific to the BAT depot share a common

precursor with skeletal muscle expressing myf5, whereas white adipose depots do not contain cells that had expressed this marker (Seale et al., 2008; Timmons et al., 2007). This suggests that brown adipocytes that arise in WAT do so from pre-existing white adipocytes.

Barbatelli et al. also suggest that the appearance of beige adipocytes in white adipose tissue is due to transdifferentiation (Barbatelli et al., 2010). They show that upon injection of the β1-adrenergic agonist, xamoterol, into mice there is an increase in the number proliferating cells within subcutaneous adipose depots. However, β3-adrenergic agonist, CL316,243, caused no change in the number of proliferating cells. They assumed that the β3-adrenergic agonist that causes the formation of brown adipocytes in WAT is transdifferentiating the white adipocytes into beige adipocytes, since there is no change in the proliferating cell population. However, the assumption that the lack of change in the number of proliferating cells is indicative of a lack of activity in the stem cell population could be false. It is possible that the stem cells may be replenishing at the same rate as differentiation. Although, these examples propose the possibility that transdifferentiation takes place, there is contradictory data supporting the idea of de novo beige adipocyte formation.

Using a downstream target of β3-adrenergic agonist, prostaglandin E2 (PGE_2) or carbaprostacyclin ($cPGI_2$), Vegiopoulos et al. showed that stem cells are activated during beige adipocyte formation. In their experiments, upon addition of PGE_2 or $cPGI_2$ into cultured SV fraction cells, there was a significant increase in the formation of brown-like adipocytes (Vegiopoulos et al., 2010). Furthermore, the addition of $cPGI_2$ to a FACS

enriched population of preadipocytes, resulted in a drastic increase in brown-like adipocytes. Thus these results indirectly demonstrate that β3-adrenergic agonist, CL316,243, is not involved in transdifferentiation but is exerting its effect at the level of the stem cell. Very recently, an elegant study by Wu et al., showed that there is in fact a beige pre-adipocyte found in WAT strongly supporting the theory of de novo beige adipocyte formation (Wu et al., 2012). They found that this beige stem cell was able to give rise to brown-like adipocytes that had a different gene expression profile than white and the classical brown adipocytes found in BAT depots. In agreement, Walden et al. hypothesized that mouse adipose depots can be divided into three categories: classical brown adipose depots, brite or subcutaneous depots and 'genuine' white depots (visceral) (Walden et al., 2011). In this regard, this study shows disparate p107 gene expression levels in the subcutaneous compared to the visceral depots that strongly implicate p107 down regulation with the appearance of beige adipocytes (Figure 5B and 5C). .

In this study, injection of CL316,243 into wild type Balb/c mice resulted in a significant decrease in the expression of p107 in the inguinal subcutaneous adipose depot (Figure 6B). As p107 down regulation results in brown adipocyte formation in vitro and as it is expressed in the SV fraction (Figure 5C), it strongly suggests that the presence of beige adipocytes after CL316,243 treatment is due to de novo adipocyte differentiation and not transdifferentiation. Although, this study does not refute that transdifferentiation may take place, it suggests another possible mechanism for beige adipocyte formation, through the activation and differentiation of stem cells. An important experiment to substantiate the role of p107 in de novo formation of beige adipocytes would require

FACS to enrich the population of non-committed MSCs in the SV fraction to determine if the knock down of p107 in these cells would result in brown adipocyte formation.

PRDM16 is a transcriptional co-activator required for brown adipocyte formation (Kajimura et al., 2010; Seale et al., 2007). This study is the first to show that PRDM16 regulates p107 transcription for the commitment of MSCs to the brown adipocyte lineage. There is an inverse relationship between p107 and PRDM16 expression levels in the subcutaneous adipose depots that is extremely large in BAT depots without any expression of p107 (Figures 5B, 5C and 5D). Importantly, this study demonstrated that PRDM16 down regulated p107 levels; whereas, knockdown of p107 had no effect on PRDM16 expression, suggesting p107 is downstream of PRDM16 function (Figure 15B and 15C). PRDM16 controls p107 at the level of promoter regulation of transcriptional activity (Figures 16C). This is corroborated by unpublished results from Deanna Porras, a member of the Scimè lab, that show by chromatin immunoprecipitation (ChIP) assays that PRDM16 binds at the promoter of p107 within 900 bp proximal to the start site (Supplementary Figure 1).

PRDM16 has two zinc-finger domains through which is can bind to DNA (Nishikata et al., 2003). PRDM16 completely loses its ability to bind DNA with a mutation at amino acid 998 arginine for a glutamine (R998Q) (Seale et al., 2007). However, PRDM16-R998Q continues to activate the brown adipocyte transcription program (Seale et al., 2007). In this study, PRDM16-R998Q was shown to retain the ability to down regulate p107 transcription from a promoter fragment 900 bp proximal to

the start site (Figure 17A). Interestingly, this suggests that PRDM16 is binding at the p107 promoter in a complex with a DNA binding protein (Figure 17B).

The DNA binding protein is not an E2F family member since mutated E2F binding domains (EBDs) on the p107 900 bp promoter fragment does not rescue the down regulation by PRDM16 (Figure 18). Hence more research is required to determine what protein is acting in a complex with PRDM16 to down regulate p107 expression (Figure 17B). The unidentified DNA binding protein might be c/ebpβ. Early studies of genetically altered mice have revealed that deletions in c/ebpβ caused defects in adipose tissue development (Tanaka et al., 1997; Wang et al., 1995). Early in vitro studies revealed that c/ebpβ rapidly increased upon stimulation of adipocyte differentiation and is more important during commitment of stem cells (Cao et al., 1991). More recently, it was shown that c/ebpβ bound to PRDM16 is involved in the switch of myoblasts and pre-adipocytes to the brown adipocyte lineage (Kajimura et al., 2009, 2010). There are two suspected c/ebpβ binding sites within the 900 bp proximal p107 promoter according to a freely accessible data base that identifies putative transcription factor binding sites, Genomatix (Supplementary Table 1). Hence, c/ebpβ is a possible candidate for the protein X in the PRDM16 complex which regulates the p107 promoter (Figure 17B).

Studies in cultured cells and mouse models have revealed that PPARγ plays a central role in the differentiation of both white and brown adipocytes (Barak et al., 1999; Koppen and Kalkhoven, 2010; Nedergaard et al., 2005; Rosen et al., 1999, 2002; Tai et al., 1996). As mentioned the PRDM16-c/ebpβ complex initiates the commitment step of the MSC to form brown adipocytes by activating PPARγ (Figure 23) (Kajimura et al.,

2010). PPARγ is also known to bind PRDM16 (Seale et al., 2008). Although, PPARγ is not the primary determinant in brown versus white commitment, once bound with PRDM16 it activates the brown adipogenic program (Seale et al., 2007). Again, a search of the Genomatix data base identified two putative binding domains for PPARγ within the 900 bp p107 promoter fragment (Supplementary Table 1). Hence, either PPARγ or c/ebpβ proteins could be protein X that interacts with PRDM16 on the p107 promoter (Figure 17B). Luciferase assays and ChIP analysis experiments need to be performed with PPARγ and c/ebpβ in order to confirm which binding partner is involved in the regulation of p107 expression.

Although, this research provides evidence that p107 down regulation is involved in the commitment to the brown adipocyte lineage and the de novo formation of brown adipocytes through the PRDM16 pathway, it still leaves to question how p107 functions to block brown adipocyte formation. It is known that both quiescent and differentiated cells have very little and no expression of p107 compared to G1 phase of the cell cycle which has high levels of p107 (Classon and Dyson, 2001). G1 is important because it is during this stage that the cell makes important decisions to proliferate, commit or differentiate (Loyer et al., 1996; Zavitz and Zipursky, 1997). The main role for p107 is to repress gene promoters involved in cell cycle progression (Zhu et al., 1993). It is thought that the shortening of the G1-phase is most likely caused by the down regulation of several regulators of the cell cycle including p107 (Pinto et al., 2008). Vanderluit et al. in an elegant study showed that a shortened G1 phase due to loss of p107 regulates the transition from a progenitor cell to a committed neuroblast (Vanderluit et al., 2007).

Analogously, this might represent the rationale for the abundant BAT in newborn infants due to the shorter G1 cell cycle phase that is apparent during development (Burdon et al., 2002; Hu et al., 2012; Lean et al., 1986; Savatier et al., 1994). Thus, the knockdown or complete absence of p107 expression may result in shortening G1 phase allowing the cells to commit to become brown adipocytes.

Another possibility for how p107 exerts its function could be through the interaction of factors that block brown or promote white adipocyte formation. This study is the first to show that PRDM16 function can be overridden by the overexpression of p107. Indeed p107 is able to block the up-regulation of the pro-thermogenic proteins UCP-1, Cidea, Elovl3 in cells despite the constant expression of PRDM16 (Figures 20B, 22B and 23). This suggests that p107 down regulation is necessary for PRDM16 to bring onboard the thermogenic program. Although, p107 cannot bind DNA, it has been shown to complex with E2F proteins which do bind DNA (Wirt and Sage, 2010). Intriguing to this line of thought, is the fact that early in G1 phase of the cell cycle, p107 in a complex with E2Fs binds histone deacetylases (HDAC) and remodels chromatin causing the repression of gene transcription (Kennedy et al., 2000; Macaluso et al., 2006; Zini et al., 2001). Inactivating specific genes might allow the stem cell to commit to a particular lineage, in this scenario a white or brown adipocyte depending on the availability of p107. Thus is it possible that the p107/E2F/HDAC complex has a binding site on the promoters of these pro thermogenic genes and is repressing their transcription through chromatin remodeling early on in the cell cycle. Hence, it is conceivable that when signals are sent during the commitment of the stem cell to a brown adipocyte, p107 is down regulated in

order to prevent the remodeling of the chromatin on the pro-thermogenic promoters. Hence, this would provide an explanation as to why p107 would be required to exert its effect at an early time point during the commitment of the stem cell and would be of little importance in an already differentiated adipocyte. However, it is unknown whether p107 is directly or indirectly exerting its transcriptional effect of pro-thermogenic genes through the attenuation of E2F activity, chromatin remodeling, or an unknown intermediate transcription factor. To ascertain if a p107 complex is on the promoter of the pro-thermogenic genes, UCP-1, Cidea and Elovl3, luciferase assays and ChIP analysis need to be performed on their promoters with the addition of p107.

This study and others show that the KD or complete lack of p107 results in the up-regulation of PGC-1α, indicating that the expression of p107 may repress its transcription (Figures 12C and 8C) (Scimè et al., 2005). However, interestingly this study shows that the overexpression of p107, unlike the other pro-thermogenic factors tested, is unable to down regulate PGC-1α expression when in the presence of PRDM16 (Figure 20B and 22B). Thus suggesting that, although, the p107/E2F/HDAC complex may be remodeling the chromatin at the PGC-1α promoter; PRDM16 is able to override the effect of p107 on the promoter when both proteins are expressed simultaneously.

Other experiments required for this research include testing p107 expression with the addition of factors known to promote brown adipocyte differentiation including pRB BMP7 and Orexin. pRB down regulation has been shown to be involved in brown adipocyte differentiation, however whether its role is during the differentiation or commitment phase is still unclear (Dali-Youcef et al., 2007; Chen et al., 1996; Hansen,

1999; Hansen et al., 2004b; Higgins et al., 1996; Scimè et al., 2005). Although, it has been shown that the p107 KO mice have decreased levels of pRB, experiments examining a direct relationship between pRB and p107 have not been performed (Scimè et al., 2005). The addition of BMP7 to C3H10T1/2 cells has been shown to bind BMPR1A which activates the Smad signalling cascade promoting brown adipocyte differentiation in vitro and in vivo (Tseng et al., 2008). Moreover, Orexin has also been shown to promote brown adipocyte formation by activating the Smad pathway through BMPR1A (Seale, 2011; Sellayah et al., 2011; Tupone et al., 2011). Interestingly, p107 has been shown to interact with Smad3 (Chen et al., 2002) and it would be of interest to instigate experiments with the addition of BMP7 and Orexin in MEFs and MSCs to find if they down regulate p107 gene expression or inactivate protein activity through hyperphosphorylation.

Overall, the pathway involved in beige adipocyte commitment is important in order to manipulate adipose tissue in humans and promote weight loss. Humans have a highly variable amount of BAT which is strongly correlated to leanness (Ouellet et al., 2012; van Marken Lichtenbelt et al., 2009; Vijgen et al., 2011). Through the use of MEFs, this study has shown the possibility of implanting p107 KO cells in order to form new brown-like adipocytes (Figure 8). One might envision that transplants of stem cells collected from human adipose tissue committed to the brown adipocyte lineage will be used as an obesity therapy for individuals unable to undergo bariatric surgery. Although, successful transplants forming brown-like adipocytes resulted from p107 KO MEFs, further tests for the functionality of the newly formed adipose depots are required. These

experiments include testing for whole body resistance to weight gain and insulin sensitivity. This would involve using obese mice or mice on a high fat diet, injecting the p107 KO MEFs subcutaneously at various sites and testing for weight loss in the obese mouse and resistance to weight gain in the other scenario. These mice would then be tested for insulin sensitivity using a glucose tolerance test. Furthermore, the p107 KD is required to be tested in hMADS to confirm that this phenomenon occurs in humans. Experiments to test this idea would involve injecting p107 KD hMADS cells into nude mice to confirm that they can form a fat pad and again as above test these mice for resistance to weight gain and insulin sensitivity. Through more research the true potential of injecting p107 KO stem cells into humans in order to combat obesity is a very real possibility.

All together, the data presented gives credence to the hypothesis of de novo formation of brown adipocytes through the mobilization of MSCs in WAT. It also identifies p107 as a key player in the PRDM16 pathway and its role in the switch between brown and white adipocyte lineage commitment. Finally, it unlocks another piece of the puzzle in understanding brown adipogenesis and how the pathway can be manipulated as an obesity counter-measure.

Supplementary Data

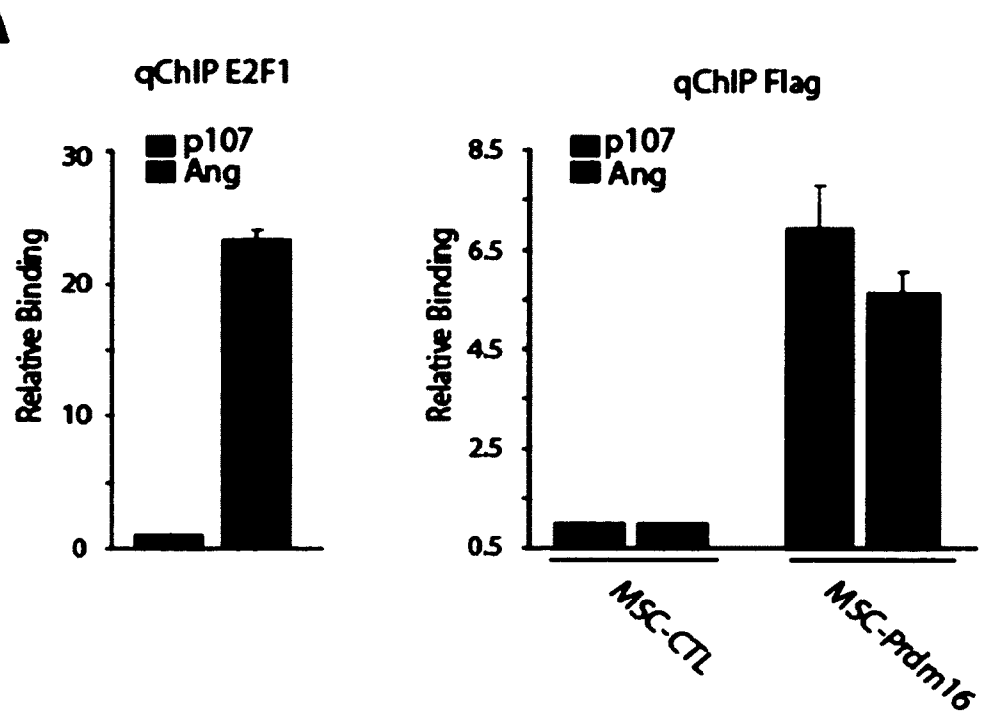
Supplementary Table 1.

Transcription Factor	900 bp proximal promoter binding	Affinity
PPARγ	620-642	0.86
PPARγ	841-863	0.839
C/ebpβ	310-324	0.949
C/ebpβ	436-450	0.966

Supplementary Table 1. Putative DNA binding regions for PPARγ and C/ebpβ on the p107 promoter

Putative binding sites with affinity scores (Genomatix) for PPARγ and C/ebpβ, potential protein "X" candidates, on the p107 promoter within 900 bp proximal to the start site.

Supplementary Figure 1



Supplementary Figure 1. ChIP analysis of PRDM16 on the p107 promoter

Quantitative chromatin immunoprecipitation (qChIP) analysis for Flag tagged Prdm16 in different MSC-CTL and MSC-Prdm16 cell lines, and for E2F1 in MSC-Prdm16 cells amplifying with primers for the promoters of p107 (-214 to -32) or positive control angiotensinogen (-127 to +23). (n=3)

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