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Adipocyte hyperplasia is characterized by an increase in adipocyte cell number and contributes significantly to the development of obesity. A key question regarding adipocyte hyperplasia concerns the mechanism by which preadipocytes undergo the transition from quiescence to proliferation. Here we examined molecular processes regulating the cell cycle inhibitor, p27, during adipocyte hyperplasia. Previous literature has shown p27 regulation by transcriptional and post-transcriptional mechanisms in various cell types. In neoplastic cells, the F-box protein, Skp2, can target phosphorylated p27 protein for degradation via the 26S proteasome. Studies presented here indicated cell cycle-dependent decreases in p27 occurring during early stages of adipocyte differentiation due to accelerated protein turnover. Further analyses revealed Cdk2-dependent phosphorylation and polyubiquitylation of p27 during S phase progression. Additionally, the 26S proteasome was found to be essential for the decrease in p27 and subsequent cell cycle progression. It was determined that Skp2 is transiently expressed during preadipocyte replication and is, at least, partly responsible for increased degradation of p27. These are novel data suggesting the presence and role of Skp2 during adipocyte hyperplasia.

It was demonstrated that expression of Skp2 is unique to preadipocyte proliferation and cannot be reproduced in fully differentiated, terminally growth-arrested adipocytes. Findings revealed transient and dramatic increases in Skp2 mRNA, preceding protein expression, due to promoter activation and not increased mRNA stability. These processes were significantly mediated by growth factor receptor signaling pathways involving PI3K and MAPK. Furthermore, inhibition of mTOR, a

downstream target of PI3K important for translational control, produced a significant reduction in Skp2 protein but not mRNA. Collectively, these data suggest Skp2 expression during adipocyte hyperplasia is regulated at multiple levels and dependent on kinase-driven signaling pathways.

The sesquiterpene lactone, helenalin, is a naturally occurring compound known to possess anti-proliferative and anti-inflammatory properties. This may be of benefit to the treatment of obesity as cells within adipose tissue have the capacity to proliferate and secrete pro-inflammatory cytokines. Studies presented here showed that helenalin prevents the increase in Skp2 mRNA by completely blocking Skp2 promoter activity. Further studies revealed helenalin's ability to prevent Akt phosphorylation, a key signaling molecule downstream of PI3K activation. Taken together, these findings provide valuable insight into molecular mechanisms regulating adipocyte hyperplasia.

REGULATION AND FUNCTION OF SKP2 IN MEDIATING P27 DEGRADATION DURING ADIPOCYTE HYPERPLASIA

by

Corinth Andrews Auld

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

SIGNIFICANCE OF RESEARCH

In 2002, the World Health Organization added obesity to its list of top ten preventable global health problems. In America alone, more than 60 million people (~1/4 of the population) are obese, a number that has doubled over the past ten years. The United States has one of the highest percentages of overweight and obese individuals when compared to other nations. In addition, almost a quarter of children in the U.S. are overweight or obese. These numbers and the fact that prevalence rates are continuing to rise are frightening due to the multitude of adverse health conditions associated with obesity (e.g., heart disease, stroke, diabetes, gall bladder disease, endometrial, breast, prostate and colon cancers). Hyperplastic obesity has been well-documented in humans, rats, mice, and guinea pigs and is largely triggered by dietary habits. Despite these early findings, how adipocyte proliferation occurs and is regulated is still unknown. The data presented here are significant because they may one day lead to novel strategies for prevention and treatment of obesity, which will improve life expectancy, quality-of-life, and health care costs (which reached over $90 billion in 2002). The results of these studies also have a significant impact on cancer research. A main target of our mechanistic approach, the F-box protein Skp2, is used as a marker of prognosis for certain types of cancers (i.e., elevated levels of Skp2 indicate poor prognosis). The elucidation of factors regulating Skp2 will enable the development of unique obesity and cancer therapies.

REVIEW OF LITERATURE

Hyperplastic Obesity. Studies on obesity and body composition have been conducted for over half a century. In 1962, Zingg et al. and Enesco et al. described increases in fat cell number as a contributor to increases in adipose tissue (Steinberg et al., 1962;Enesco and Labond, 1962). Hyperplastic obesity has been seen in rats, hamsters, and guinea pigs and has also been well-documented in humans (Pettersson et al., 1985;Hirsch and Batchelor, 1976). The accumulation of excess fat is largely controlled by dietary intake (i.e., caloric consumption, macronutrient profile of diet, etc.) and these earlier observations prompted other groups to evaluate the effect of nutritional status on increases in fat cell number. In studies of both young and adult rats, diets high in fat and sufficient in carbohydrates caused adipocyte hyperplasia (Mandenoff et al., 1982). Interestingly, rats made obese via a cafeteria-fed diet for 3 weeks are able to lose weight gained with appropriate changes in diet (Rothwell and Stock, 1997), however a cafeteria-fed diet for 13 weeks results in an irreversible weight gain (Rolls et al., 1980). This difference in the ability to "recover" from excessive weight gain was explained by a study done in 1982 by Mandenoff et al (Mandenoff et al., 1982). They found that up until 8 weeks of a cafeteria-fed diet, increases in weight gain were due to adipocyte hypertrophy and that between 8 and 20 weeks, changes were due to adipocyte hyperplasia. These studies and others demonstrate hypertrophy preceding hyperplasia in the development of diet-induced obesity. One of the most devastating aspects of hyperplastic obesity is that unlike cell size, increases in fat cell number are irreversible. Once an individual makes new fat cells, the size of these cells may change with weight gain or loss but the increased capacity for fat storage due to greater numbers is not reversible (Faust et al., 1978;Yang et al., 1990). Two studies done in

1978 showed that morbid cases of obesity involve at least two times the normal number of fat cells and that in rats, this can be seen after only 5 months of a high-fat diet (Faust et al., 1978;Bosello et al., 1978). Thus, hyperplastic adipose tissue has been associated with the poorest prognosis (Dizdar and Alyamac, 2004). Several studies have suggested a cell size prerequisite in order for proliferation to occur (Marques et al., 1998), however, it has been shown that in human obesity, adipocyte hyperplasia can be accompanied by normal fat cell size (Salans et al., 1973). This finding was coaborated by a study that showed a high-fat diet can induce a persistent or permanent obesity that is solely a result of adipocyte hyperplasia (Faust et al., 1978). Developmental stage is not a factor in the development of hyperplastic obesity as it has been seen in young (Hager et al., 1978;Lubetzki et al., 1980) and adult (Knittle and Hirsch, 1968;Hirsch and Batchelor, 1976) animals and humans. Interestingly, several groups have found that hibernating animals (i.e., dormice, woodchucks, and ground squirrels) appear to be protected from increases in adipocyte number (Mrosovsky et al., 1987;Young et al., 1982;Faust and Mrosovsky, 1987). Their weight gain is attributed to increases in adipocyte size and is hypothesized to be a result of some proliferation inhibitory factor or an extraordinarily large capacity of individual fat cells to store lipid. While hyperplastic obesity has been well-documented, there are very few studies examining mechanisms behind this increase in cell number and this tremendous gap in the knowledge base prevents prophylactic and treatment strategies targeting this type of obesity.

Adipocyte Differentiation. Cellular and molecular studies of adipogenesis have long been conducted using the “gold standard” model, 3T3-L1 cells. The murine 3T3-L1 lineage was originally isolated and identified by Howard Green (Todaro and Green, 1963;Green and Meuth, 1974;Green and Kehinde, 1976). These cells are fibroblast-like

and follow adipocyte conversion given appropriate hormonal stimulation. Before stimulation, preadipocytes are grown for two days after reaching confluency to ensure density-induced growth arrest (i.e., day 0). At this time, normal proliferation media (i.e., DMEM containing 10% calf serum) is replaced with adipogenic media consisting of DMEM, 10% fetal bovine serum, and MDI. The MDI cocktail includes 3-isobutyl-1-methylxanthine (MIX), the synthetic glucocorticoid dexamethasone, and insulin. Each component has very specific and important roles in the differentiation process, examples of which are to follow. MIX increases intracellular levels of cyclic AMP and leads to down-regulation of the transcription factor Sp1. When expressed in preadipocytes, Sp1 binds to the promoter of C/EBPα and blocks the C/EBP-response element, thereby preventing activation by C/EBPβ and C/EBPδ (Tang et al., 1999). The glucocorticoid dexamethasone is able to cause greater than 50-fold increases in C/EBPδ in 3T3-L1 adipocytes (MacDougald et al., 1994), providing a critical role during early stages of adipogenesis. The importance of C/EBP proteins is discussed in more detail below.

Insulin is used at pathophysiological concentrations known to activate the IGF1-receptor (IGF1R). The IGF1R to insulin receptor ratio in preadipocytes is 2:1 (Smith et al., 1988), thus much of the early signaling is downstream of IGF1R activation. Interestingly, while IGF1R expression remains unchanged throughout differentiation, the insulin receptor is increased 25-fold (Smith et al., 1988). The IGF1R is essential for adipogenesis and plays a major role in initiating clonal expansion. Clonal expansion is a very unique and brief, synchronized period of cell proliferation that occurs immediately after preadipocytes have been stimulated to differentiate and is required for adipocyte differentiation (Tang et al., 2003). Once cells go through one to two rounds of division, they complete the differentiation process and become terminally growth arrested. While

knowledge of distinct mechanisms regulating cell cycle control at this time is limited, the expression of particular cyclin-dependent kinase inhibitors has been examined. Morrison et al. identified changes in expression of $p27^{Kip1}$, $p21^{Cip1}$, and $p18^{INK4c}$ following stimulation with MDI (Morrison and Farmer, 1999). At day 0, p27 protein is highly expressed and then is dramatically decreased by 18 hours following the addition of MDI. In contrast, p21 protein is very low at day 0 and is increased in a biphasic manner after stimulation. The expression of cyclin-dependent kinase inhibitor p18 cannot be detected until day 3, at which time protein levels increase significantly. Levels of both p27 and p18 remain high throughout the differentiation process, however, p21 returns to day 0 levels by day 6. Determining the mechanisms by which these cell cycle proteins are regulated would provide valuable information about adipocyte proliferation and hyperplastic obesity.

E2F and retinoblastoma proteins play essential roles in cell cycle progression and are also highly regulated during adipocyte differentiation. The retinoblastoma (Rb) family includes pRb, p130, and p107, all of which bind E2F proteins. The E2F transcription factors bind to DNA as heterodimers with DP proteins (i.e., DP1 and 2) and regulate the expression of genes required for DNA replication and mitosis. E2F4 has been shown to be the predominant family member present during adipocyte differentiation, with protein levels detectable in day 0 quiescent cells through at least 5 days (Fajas et al., 2002). The role E2F4 plays during adipocyte differentiation may be largely regulated by the retinoblastoma protein bound to it. In growth-arrested T-cells, E2F4 is bound to p130. However, when these cells reenter the cell cycle there is a significant change as E2F4 binds to pRb and p107 (Moberg et al., 1996). The pattern seen in T-cells parallels what has been seen during adipocyte differentiation, where

induction of adipogenesis is accompanied by a very distinct switch in expression of p130 and p107. While p130 mRNA is present throughout adipocyte differentiation, protein levels are high on day 0, undetectable during clonal expansion, and then once again increased by day 3. The kinetics of p107 are exactly opposite as protein levels are transiently upregulated at day 1 and extremely low before and after this time (Richon et al., 1997). The very timely switch in expression of p130 and p107 surrounding this period of synchronized growth suggests opposing roles of these proteins during adipocyte differentiation.

The entire process of adipogenic conversion involves a very concerted up- and down-regulation of genes. The two major contributors to this process are the C/EBP family of transcription factors and the nuclear hormone receptor PPARγ. C/EBPβ and C/EBPδ are transiently expressed immediately following stimulation and play a critical role in initiating the course of differentiation. The expression of both C/EBPβ and C/EBPδ proteins is first seen within 4 hours of stimulation, however, DNA-binding activity cannot be detected until approximately 12-14 hours after stimulation (Tang and Lane, 1999). When DNA-binding of C/EBPβ was blocked during clonal expansion, there was a marked reduction in cell cycle markers (Zhang et al., 2004). In addition to the requirement for early cell cycle progression, C/EBPβ also binds to the promoters of both C/EBPα and PPARγ, inducing expression of these two master regulators of adipocyte differentiation (Christy et al., 1991;Tang et al., 2004). The role of C/EBPδ during adipogenesis is thought to be through synergy with C/EBPβ leading to initial PPARγ promoter activity and expression (Wu et al., 1996). This begins a positive feedback loop, as C/EBPα is able to bind to its own promoter as well as that of PPARγ (Tang et

al., 2004), and PPARγ is able to activate transcription of C/EBPα (Zuo et al., 2006). C/EBPα and PPARγ mRNA and protein levels are detectable by day 2 of adipocyte differentiation (Morrison and Farmer, 1999) and are known to be anti-mitotic suggesting a link between terminal growth arrest and adipocyte differentiation. Expression of C/EBPα and PPARγ is essential for adipogenesis (Rosen et al., 2002) and activation of critical genes such as GLUT-4 (Kaestner et al., 1990;Wu et al., 1998) and the insulin receptor (McKeon and Pham, 1991).

While adipose tissue was once regarded as a static, lipid storage tissue, it is now recognized as having very active endocrine function. Adipose tissue is comprised of preadipocytes, mature adipocytes, and macrophages. These cells work in concert to produce and secrete "adipokines" such as TNFα (Hotamisligil et al., 1993), IL-6 (Mohamed-Ali et al., 1998), and C-Reactive Protein (Ouchi et al., 2003). Release of these factors provides a link between obesity and inflammation associated with the metabolic syndrome. As mentioned previously, obesity can manifest through increases in adipocyte size and number. The lack of mechanistic research examining hyperplastic obesity has left many unanswered questions. Clonal expansion provides a perfect model in which to study preadipocyte proliferation as synchronization allows for the study of specific mechanisms.

Cell Cycle. Cell proliferation is highly regulated in order to prevent abnormal growth, copies of mutated or damaged genes, etc. There are four main sequential phases of the cell cycle: G_1, S, G_2, M. Cells are driven through this process by phosphorylation events initiated by the transient expression of cyclin proteins. Cyclins bind to and activate cyclin-dependent kinases (cdk), which are constitutively expressed

throughout the cell cycle. Figure 1.1 illustrates cell cycle phases and the cyclin/cdk complexes expressed during each phase. An initial early phase is a quiescent, non-proliferative state referred to as G_0. External mitogenic factors signal cells to exit G_0 and enter into G_1 phase. Cells are able to fluctuate between these two phases, however once the G_1-to-S phase restriction point is passed, environmental stimuli are no longer necessary and cells become fully committed to the cell cycle. Therefore, this restriction point is critical to cell cycle progression and is the first major cell cycle checkpoint. It is imperative that this transition be tightly controlled because it is during the following phase that DNA is replicated. One of the critical events that occur during this time is hyperphosphorylation of Rb proteins and subsequent release of E2F. The transcription factor E2F is then able to activate genes required for S phase entry and DNA replication. After duplicate copies of the genetic material are made, cells move out of S phase, through G_2 and into mitosis, during which time two identical daughter cells are made. Two of the three major cell cycle checkpoints occur at the end of G_1 and G_2 phases, as they are preparatory phases for DNA replication and mitosis, respectively. The third occurs immediately before the cell undergoes cytokinesis. As previously mentioned, cdk activity is essential for cell cycle progression. The activity of cyclin-dependent kinases is negatively regulated by cyclin-dependent kinase inhibitors. These proteins inhibit cdk activity by preventing interactions with cyclins or blocking their ATP binding site. There are two families of cyclin-dependent kinase inhibitors; INK4 and CIP/KIP, which function primarily to inhibit G_1 and S phase progression (Fig.1.1). The p27/Kip1 protein plays a pivotal role in cell cycle progression and must decrease in order for cells to pass the G_1-to-S phase restriction point.

Cyclin-Dependent Kinase Inhibitor, p27. The p27 gene was first cloned in 1994 by 3 separate groups (Polyak et al., 1994;Toyoshima and Hunter, 1994;Slingerland et al., 1994) and the protein crystal structure determined two years later (Russo et al., 1996). As knowledge about this protein increased, multiple roles emerged: regulator of drug resistance in solid tumors (St Croix et al., 1996), promoter of apoptosis (Katayose et al., 1997;Levkau et al., 1998), key player in oligodendrocyte and muscle differentiation (Durand et al., 1997;Zabludoff et al., 1998). By far, the most widely studied and noteworthy function of p27 is as a cell cycle inhibitor (Toyoshima and Hunter, 1994) and tumor suppressor (Morosetti et al., 1995). Once its role in cell cycle progression was established, a flurry of research was done examining p27 in human cancers which found it to be a useful prognostic tool (i.e., low levels of p27 correlate with poor prognosis or malignancy) for breast cancer (Porter et al., 1997;Tan et al., 1997), colorectal cancer (Fredersdorf et al., 1997), gastric carcinoma (Mori et al., 1997), non-small-cell lung carcinoma (Esposito et al., 1997), lymphoma (Sanchez-Beato et al., 1997), esophageal adenocarcinoma (Singh et al., 1998), prostate adenocarcinoma (Tsihlias et al., 1998), oral squamous cell carcinoma (Jordan et al., 1998), and malignant melanoma (Florenes et al., 1998). These observations spawned many studies on the regulation of p27. Interestingly, when over 500 tumors of different origins were analyzed, less than 5% had p27 gene mutations (Spirin et al., 1996). While p27 has been shown to be regulated at the level of transcription (e.g., methylation (Qian et al., 1998), FOXO family of transcription factors (Medema et al., 2000)), posttranscriptional modes of regulation have been even more extensively studied.

Research has shown that, within different cell types, p27 protein levels and activity can be controlled by various mechanisms. For example, ubiquitin/proteasome-

dependent degradation (Pagano et al., 1995), the KPC or Kip1 Ubiquitin-Promoting Complex (Kamura et al., 2004), phosphorylation and cellular localization (Reynisdottir and Massague, 1997;Fujita et al., 2002), and proteolytic processing (Levkau et al., 1998;Shirane et al., 1999) have all been implicated. The importance of p27 subcellular localization has become increasingly apparent in recent years. In 2001, Rodier et al. found that phosphorylation of p27 at Ser10 was required for nuclear export and a serine to alanine mutation sequestering p27 in the nucleus had no effect on interaction with the F-box protein Skp2 (S-phase kinase-associated protein) and subsequent ubiquitylation (Rodier et al., 2001). Phosphorylation of p27 at Ser10 was later found to be essential for Jab1/CRM1-mediated nuclear export (Tomoda et al., 2002;Ishida et al., 2002). Several other groups found that following Ser10 phosphorylation and cytosolic localization, Akt can phosphorylate p27 on Thr157, inhibiting association of p27 with the nuclear localization protein, importin α, providing further assurance of retainment in the cytosol (Liang et al., 2002;Shin et al., 2005;Viglietto et al., 2002). It has also been shown that p27 can localize to the cytosol by phosphorylation at Thr198 and binding to 14-3-3 proteins. Two kinases have been shown to phosphorylate p27 at this residue; Akt and an immediate downstream target of MAPKs, p90 ribosomal protein S6 kinases (Fujita et al., 2002;Fujita et al., 2003).

The importance of p27 and its regulation during cell cycle progression caused others to look at its expression in non-cancerous tissues. Morrison et al (Morrison and Farmer, 1999) first showed that p27 is highly regulated during clonal expansion of adipocyte differentiation. This period of obligatory proliferation and the regulated expression of p27 during this time provide a unique model for studying adipocyte hyperplasia. The importance of this is demonstrated by a study that shows p27 knockout

mice are obese due to adipocyte hyperplasia, resulting in a 2-fold greater number of fat cells when compared to wild-type counterparts (Naaz et al., 2004). These findings demonstrate urgency for mechanistic studies on the regulation of p27 in adipocytes. Both C/EBPβ and Calpain (Zhang et al., 2004;Patel and Lane, 2000) have been implicated in the decrease of p27 protein during clonal expansion. While not identified as a direct effect, preventing nuclear localization of C/EBPβ prevents p27 degradation and S phase progression during early stages of 3T3-L1 differentiation (Zhang et al., 2004). Patel et al. (Patel and Lane, 2000) demonstrated that induced expression of the endogenous calpain inhibitor, calpastatin, produced increased levels of p27 during clonal expansion. This has also been seen in choroidal melanoma cells where authors suggest the presence of a 25kDa cleaved product (Delmas et al., 2003). Taken together, these data imply multiple modes of p27 regulation during preadipocyte replication.

Ubiquitin/26S Proteasome System. The 26S proteasome/ubiquitylation process begins with an E1 ubiquitin activating enzyme that binds to and activates ubiquitin molecules in an ATP-dependent manner. Secondly, activated ubiquitin is transferred to an E2 ubiquitin conjugating enzyme that brings ubiquitin in close proximity to the E3 ubiquitin ligase, allowing the covalent addition of ubiquitin to a lysine residue(s) of a specific substrate (Hershko et al., 1983;Hershko and Ciechanover, 1998). Ubiquitin is a very small protein comprised of only 76 amino acids, with a mass of 8kDa. There is a requirement for the targeted substrate to be covalently linked to four ubiquitin molecules in order for recognition by the 26S proteasome (Thrower et al., 2000). This is a dynamic process within the cell as there are deubiquitylating enzymes present as well. As shown in Figure 1.2, Skp2-dependent ubiquitylation of p27 requires phosphorylation of p27 on Thr187 by Cyclin E-Cdk2 during late G_1 (Carrano et al., 1999;Tsvetkov et al.,

1999). Akt has also been shown to cause phosphorylation on this residue *in vitro* using human embryonic kidney 293T cell extract (Fujita et al., 2002). Once phosphorylated, p27 can bind to Cks1 and the F-box protein Skp2, the substrate recognition portion of the SCF^{SKP2} E3 ligase (Hao et al., 2005). Association with this ligase complex allows polyubiquitylation of p27, which is the target signal for rapid degradation by the 26S proteasome. The SCF^{Skp2} E3 Ligase is composed of 5 core proteins: Skp1, Roc1, Cul1, Skp2, and Cks1. The expression of Skp1, Roc1, and Cul1 has been shown to be quite stable during cell cycle progression (Lisztwan et al., 1998). However, Cul1 can be modified post-translationally by the addition of a protein, Nedd8, which has the ability to enhance activity of the SCF^{Skp2} E3 ligase. Skp2 can also be modified in a similar manner by the addition of a small protein, Cks1. Cks1 was first identified as a cell cycle regulator that interacts with cdks. Its function was not known until 2001 when Ganoth et al. revealed that Cks1 also binds to Skp2 and mediates Skp2-dependent p27 degradation (Ganoth et al., 2001). Interestingly, in 2004 Kamura et al. (Kamura et al., 2004) discovered a second F-box protein important for regulating p27 protein. The Kip1 Ubiquitin-Promoting Complex, or KPC, was first identified in Skp2-/- mouse embryonic fibroblasts and found to play an important role in degrading p27 in the cytoplasm. There is still much to be determined and understood regarding this novel E3 Ligase.

The F-box Protein, Skp2. Skp2 was first identified as a protein required for entry into S phase and highly expressed in transformed cells (Zhang et al., 1995). Its expression fluctuates throughout the cell cycle (Lisztwan et al., 1998) and regulation can occur at multiple levels. Limited studies have been done examining direct transcriptional regulation of Skp2. Imaki et al. (Imaki et al., 2003) was the first to characterize the Skp2 promoter and determine a positive regulatory element between -

207 and -103. They found that GABP (i.e., GA-binding protein) binds to that region and knockdown of this transcription factor results in a significant decrease in Skp2 promoter activity and mRNA expression. E2F1 has also been identified as a transcriptional regulator of Skp2 during proliferation of human fibroblasts and many tumor cell lines (Zhang and Wang, 2005). Most recently, the Forkhead Box M1 (FoxM1) has been shown to bind to and activate the promoters of Skp2 and its accessory protein Cks1, resulting in increased protein expression and function (Wang et al., 2005). Since then, FoxM1 has been shown to play a role in the proliferation and progression of human non-small cell lung cancer (Kim et al., 2006). Previous findings showing Skp2 overexpression in non-small cell lung cancer (Osoegawa et al., 2004) suggest a functional link between FoxM1 and tumor growth.

Research has shown post-translational regulation of Skp2 as well. Cyclin A/ Cdk2 complexes have been shown to phosphorylate Skp2 at Ser76 (Yam et al., 1999), however, the function of this modification is not fully understood. It is known that F-box proteins can participate in auto-ubiquitylation and studies suggest Skp2 is no exception. In 2000, *in vitro* observations revealed polyubiquitylation of Skp2 following addition of Cul1, Rbx1/Roc1, and Skp1 (Wirbelauer et al., 2000). Two primary E3 Ligases important for regulating cell cycle progression are SCF and APC/C (anaphase-promoting complex/cyclosome). Research by two separate groups demonstrate that Skp2 is a substrate for polyubiquitylation and subsequent degradation by the APC/C^{Cdh1} (Bashir et al., 2004;Wei et al., 2004). Thus, it is thought that the 26S proteasome plays a critical role in modulating Skp2 protein expression.

Several endogenous and exogenous factors that affect the expression of Skp2 have been identified. The prostacyclin mimetic, cicaprost, causes G_1 arrest and inhibits

Skp2 gene expression in smooth muscle cells (Stewart et al., 2004), although an exact mechanism by which this occurs is not known. Connexin43 is important for the formation of gap junctions and can also suppress tumor growth. Zhang et al. show that connexin43 promotes ubiquitylation and degradation of Skp2 independently of its ability to form gap junctions (Zhang et al., 2003c;Zhang et al., 2003b). In fibroblasts, Skp2 expression has been shown to be dependent on cell adhesion to the extracellular matrix and β1-integrin, a transmembrane receptor important for adhesion (Carrano and Pagano, 2001;Zhang et al., 2003a). It is worthy to note that there is crosstalk between integrin and IGF1R signaling (Shen et al., 2006) and β1-integrin may be required for IGF1R signaling (Goel et al., 2005). TGF-β signaling is known to inhibit cell cycle progression by increasing proteasome-dependent degradation of Skp2 in lung epithelial cells (Wang et al., 2004). Interestingly, there is also crosstalk between TGF-β, IGF1R, and integrin signaling (Huang and Huang, 2005). Another molecule involved in intracellular signaling that affects expression of Skp2 is PTEN. Overexpression of PTEN in human glioblastoma cells results in down-regulation of Skp2, suggesting a role for the PI3K pathway (Mamillapalli et al., 2001). Of particular interest to the study of hyperplastic obesity is the finding that the PPARγ ligand troglitazone, but not pioglitazone or ciglitazone, dramatically decreases Skp2 mRNA and protein expression in human hepatoma cells (Koga et al., 2003). Also, treatment of mammary carcinoma cells with specific fatty acid synthase inhibitors (i.e., siRNA or the anti-obesity drug Orlistat) produced significant reductions in Skp2 protein (Knowles et al., 2004). Finally, 5 μM all-trans retinoic acid completely blocked Skp2 protein expression in human KCNR neuroblastoma cells (Nakamura et al., 2003).

F-box proteins function within E3 Ligases by dictating substrate recognition and Skp2 is known to have multiple cellular targets. These substrates include p27 (Tsvetkov et al., 1999;Carrano et al., 1999;Sutterluty et al., 1999), E2F-1 (Marti et al., 1999), p130 (Tedesco et al., 2002), CDK9 (Kiernan et al., 2001), p57 (Kamura et al., 2003), p21 (Bornstein et al., 2003), free Cyclin E (Nakayama et al., 2000), FOXO1 (Huang et al., 2005a), and c-myc (von der et al., 2003). While Skp2 is known to cause rapid degradation of c-myc, it is interesting to note that Skp2 has also been shown to play an important role in c-myc-dependent gene expression. Skp2 can complex with c-myc and act as a transcriptional co-factor during S phase progression (von der et al., 2003). Due to the presence of ubiquitylated proteins at the promoters of Skp2/c-myc responsive genes, it is theorized that Skp2 promotes the degradation of repressors and/or co-repressors. This type of paradoxical relationship is also seen between Skp2 and E2F, as Skp2 causes proteasomal degradation of E2F1, but can also be a transcriptional target of E2F1. These mechanisms may be explained by the cell's need to tightly control DNA replication and mitosis.

The degradation of p27 is commonly considered the main function of Skp2 due to the critical role this regulation plays in cell cycle progression. Similar to p27, Skp2 expression is used as a prognostic tool (i.e., elevated levels of Skp2 are correlated with poor prognosis) for certain types of cancers such as oral squamous cell carcinoma (Kudo et al., 2001) colorectal cancer (Shapira et al., 2005), renal cell carcinoma (Langner et al., 2004), epithelial ovarian cancer (Hu and Liu, 2005), diffuse large B-cell lymphoma (Seki et al., 2003), soft tissue sarcomas (Oliveira et al., 2003), laryngeal squamous cell carcinomas (Dong et al., 2003), prostate cancer (Yang et al., 2002), melanoma (Li et al., 2004), and non-small-cell lung cancer (Osoegawa et al., 2004). In

addition, Nakayama et al. found that Skp2 knockout mice are two-thirds the size of their wild-type littermates (Nakayama et al., 2000). Body composition was not determined, however, the researchers reported that all external bodyparts (e.g., tail, ears) were normal which may suggest a decrease in adipose tissue contributing to the reduction in bodyweight. Furthermore, because of the lack of Skp2, one might propose that a potential decrease in adipose tissue would be due to decreased adipocyte number.

IGF1-Receptor Signaling. Growth factor receptors play a primary role in regulating cellular responses to environmental changes. The IGF-1 receptor (IGF1R) is constitutively expressed throughout adipocyte differentiation and is an essential, primary mediator of early signaling events following adipogenic stimulation (Smith et al., 1988). IGF1R signaling includes the activation of several kinase-driven cascades. Two such pathways are the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase pathways (PI3K), both of which have many downstream targets involved in cellular proliferation.

The MAPK pathway is initiated by autophosphorylation of the IGF1R triggered by binding of IGF1 or insulin. This causes activation of MAPK kinase kinases which leads to the phosphorylation of MAPK proteins. While there are three main MAPK proteins (i.e., ERK, p38, and JNK), ERK was the first to be identified and is the most important in regulating proliferation. The MAPKs p38 and JNK are primarily involved in mediating stress signals. The MAPKKK upstream of ERK is Raf. Raf phosphorylates and activates the MAPKK MEK 1/2 which in turn phosphorylates ERK 1/2 (i.e., the two isoforms of ERK). ERK exerts its effects on cell cycle progression in large part by upregulating Cyclin D1 (Lavoie et al., 1996;Weber et al., 1997), leading to critical downstream events such as pRb hyperphosphorylation.

Activation of the PI3K pathway also begins with autophosphorylation of the IGF1R and subsequent activation of PI3K. The two subunits (p85-regulatory and p110-catalytic) of PI3K are able to associate with the receptor and become phosphorylated. PI3K can then phosphorylate the phospholipid PIP_2, resulting in PIP_3 within the plasma membrane. The presence of PIP_3 causes constitutively phosphorylated PDK1 to dock on the plasma membrane. Perhaps the most widely studied downstream target of PDK1 is Akt/PKB, a kinase that also binds PIP_3 within the membrane via a PH domain allowing phosphorylation of Thr308 and Ser473 by PDK1 (Andjelkovic et al., 1999). It has since been determined that the protein kinase mTOR is also able to target Ser473 in adipocytes (Hresko and Mueckler, 2005). Once Akt is active, it phosphorylates targets in the cytoplasm (e.g., glycogen synthase kinase-3, GSK3-β) and nucleus (e.g., Foxo1 transcription factor) (Cross et al., 1995;Guo et al., 1999). The PI3K pathway can be inhibited by the endogenous phosphatase, PTEN. PTEN is able to remove a phosphate group from PIP_3, thereby preventing PDK1 from docking on the plasma membrane (Maehama and Dixon, 1998). However, when phosphorylated on serine 380 (S380), PTEN is inactivated and PIP_2 can be converted to PIP_3 by PI3K. The phosphorylation of PTEN is not dependent on external stimuli.

A major mode of regulating translational control within the cell is through the mTOR/Raptor pathway, which is downstream of PI3K. The protein kinase mTOR is phosphorylated following PI3K activation, leading to the inhibition of translation repressor 4E-BP1. Raptor is an mTOR-binding protein that helps facilitate recognition and phosphorylation of 4E-BP1 (Hara et al., 2002;Nojima et al., 2003). This initiates a cascade of events, beginning with the release of eIF4E, that promotes cap-dependent translation. The protein $p70^{S6K}$ is also downstream of mTOR and once activated, can

increase expression of proteins important for translational machinery (Burnett et al., 1998). Thus, mTOR exerts its effects on translational control by multiple mechanisms. It appears that mTOR plays a significant role in regulating adipogenesis as treatment of 3T3-L1 preadipocytes with the potent mTOR inhibitor rapamycin results in marked reductions in clonal expansion and C/EBPα (Yeh et al., 1995); expression of PPARγ and other adipogenic markers such as adipsin and aP2 (Cho et al., 2004); and insulin signaling, which was also seen in human adipocytes (Tremblay et al., 2005).

NFκB. NFκB is a downstream target of several signaling cascades (e.g., PI3K) and is known to regulate cell cycle progression (Kane et al., 1999). The NFκB family of transcription factors is composed of five proteins: RelA/p65, Rel B, c-Rel, p105/p50, and p100/p52. These proteins bind in homo- and heterodimers, with p65:p50 and p50:p50 being the most predominant forms found. NFκB dimers can have positive or negative effects on transcription depending on the combination (Chen and Greene, 2004). RelA/p65, Rel B, and c-Rel have transactivation domains and can upregulate the expression of many anti-apoptotic genes. Homodimers of p50 and p52 can also bind DNA but act to repress transcription. The IκB family of proteins can bind and inhibit these dimers by preventing nuclear localization and interaction with DNA. Research has shown that these inhibitory subunits can be removed by phosphorylation (initiated by Akt), polyubiquitylation and degradation by the 26S proteasome or sequestration by PI3K. In addition, p105 and p100 are members of the IκB family. Cleavage of these proteins by the 26S proteasome results in the formation of active NFκB proteins p50 and p52, respectively (Palombella et al., 1994).

RelA/p65 has warranted much attention due to its role in regulating such processes as proliferation, apoptosis, and inflammation. A great deal of research has been done to determine downstream targets of NFκB. The use of chemical inhibitors has become very popular, as several naturally occurring compounds have been identified. The most direct method of blocking p65 activity is by preventing DNA-binding. The sesquiterpene lactone, helenalin, is known to do this through alkylation of p65.

Helenalin. Helenalin is a biologically active compound that has long been used for external healing purposes. It was originally identified as one of the sesquiterpene lactones found in the yellow flower of arnica Montana L, belonging to the Compositae/Asteraceae family of flowering plants. Arnica has been used for the treatment of poor circulation, reduced immune function, and inflammatory conditions such as bruises, sprains, and rheumatic pain. Although The European Scientific Cooperative on Phytotherapy (ESCOP) approved the use of this flowering plant for external treatments, there are detrimental side effects associated with its use, including stomach discomfort, organ damage dermatitis, liver and kidney damage, and even death. Helenalin is just one of several sesquiterpene lactones found in this plant known to exhibit anti-inflammatory and analgesic effects. It has been used in molecular biology research as a potent inhibitor of NFκB p65 DNA-binding activity through direct alkylation of p65 (Lyss et al., 1998). Many biologically active compounds have multiple targets within the cell and emerging data imply this is also true for helenalin. In 2005, Huang et al. revealed helenalin's ability to alkylate telomerase (Huang et al., 2005b), an important enzyme in the progression of neoplastic growth. Helenalin's function as a potent alkylating agent suggests the possibility of additional targets within the cell.

Helenalin's anti-tumor properties have also been well-documented since the 1970's. In 1977, Lee et al. (Lee et al., 1977) describe helenalin's ability to inhibit nuclear DNA polymerase activity and increase cyclic AMP levels within Ehrlich ascites, observations consistent with a reversal of tumor growth. Interestingly, helenalin was also able to decrease HMG-CoA reductase activity and hypercholesteremic state. Helenalin's effect on proliferation and cholesterol synthesis makes it an interesting candidate for studies on hyperplastic obesity and adipogenesis.

STUDY OBJECTIVES

Chapter II: It has been shown that p27 protein decreases during early stages of adipocyte differentiation (i.e., clonal expansion). The F-box protein, Skp2, can target p27 for rapid degradation by the 26S proteasome in many types of cancer, thus leading to increased proliferation. In Chapter II, we demonstrate that the change in p27 expression during preadipocyte replication is due to a decrease in protein half-life. Subsequently, we show a transient upregulation of Skp2 during this time and examine Skp2's role in the decrease in p27.

Chapter III: We are the first to show Skp2 expression during adipogenesis. Determining how Skp2 is regulated in adipocytes is expected to provide valuable information for the study of hyperplastic obesity and the regulation of cell cycle in other cell/tissue types. We show that insulin is the primary component of the differentiation cocktail that causes the increase in Skp2. Furthermore, we examine downstream targets of insulin and IGF1R signaling.

Chapter IV: The sesquiterpene lactone, helenalin, is known to exhibit anti-inflammatory and anti-proliferative properties. However, its effect on adipogenesis and adipocyte hyperplasia has not yet been explored. Helenalin's primary function within the cell has long been thought to be inhibition of p65 DNA-binding by direct alkylation. In Chapter IV, we examine the effect of helenalin on Skp2 expression during early stages of adipogenesis. An effect on Akt signaling is also revealed.

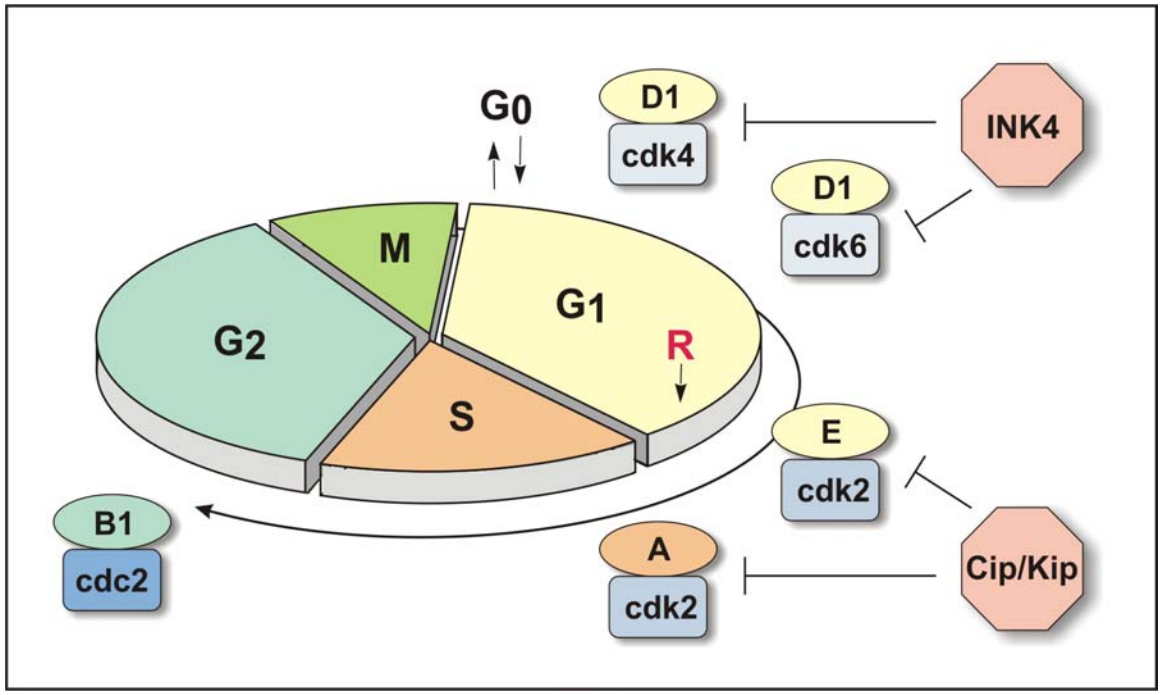

Figure 1.1. Mammalian Cell Cycle Model. Cell cycle progression is mediated by the sequential activation of cyclin-dependent kinases (cdks). Cdks (rectangles) are positively regulated by association with cyclin proteins (ovals) and negatively regulated by two classes of cyclin dependent kinase inhibitors (octagons). Cell cycle phases are illustrated as part of the pie chart, with a red "R" denoting the restriction point.

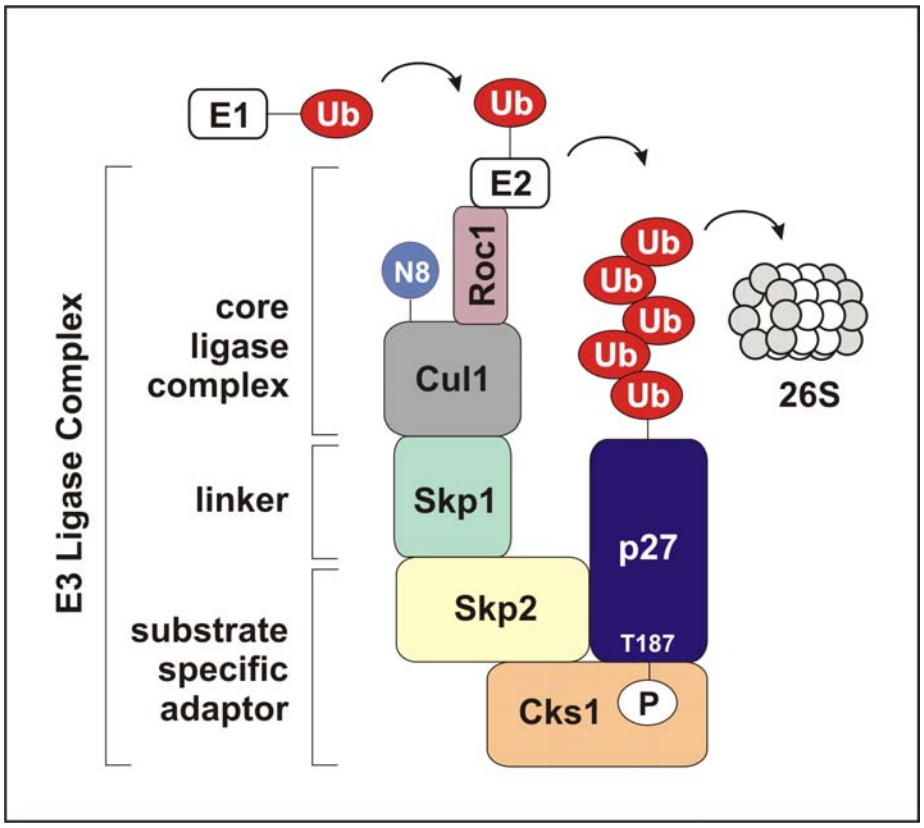

Figure 1.2. Model of SCF^{Skp2} E3 Ligase dependent degradation of p27. Phosphorylated p27 (Thr187) is bound by Skp2, Cks1, and the SCF E3 Ligase and targeted for polyubiquitylation and rapid degradation by the 26S proteasome.

REFERENCES

Andjelkovic,M., S.M.Maira, P.Cron, P.J.Parker, and B.A.Hemmings. 1999. Domain swapping used to investigate the mechanism of protein kinase B regulation by 3-phosphoinositide-dependent protein kinase 1 and Ser473 kinase. Mol. Cell Biol. 19: 5061-5072.

Bashir,T., N.V.Dorrello, V.Amador, D.Guardavaccaro, and M.Pagano. 2004. Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase. Nature 428: 190-193.

Bornstein,G., J.Bloom, D.Sitry-Shevah, K.Nakayama, M.Pagano, and A.Hershko. 2003. Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. J. Biol. Chem. 278: 25752-25757.

Bosello,O., F.Armellini, R.Ostuzzi, G.Bissoli, and L.A.Scuro. 1978. Behavior of adipose tissue cellularity in gross obesity. Minerva Med. 69: 3831-3833.

Burnett,P.E., R.K.Barrow, N.A.Cohen, S.H.Snyder, and D.M.Sabatini. 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc. Natl. Acad. Sci. U. S. A 95: 1432-1437.

Carrano,A.C., E.Eytan, A.Hershko, and M.Pagano. 1999. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. 1: 193-199.

Carrano,A.C. and M.Pagano. 2001. Role of the F-box protein Skp2 in adhesion-dependent cell cycle progression. J. Cell Biol. 153: 1381-1390.

Chen,L.F. and W.C.Greene. 2004. Shaping the nuclear action of NF-kappaB. Nat. Rev. Mol. Cell Biol. 5: 392-401.

Cho,H.J., J.Park, H.W.Lee, Y.S.Lee, and J.B.Kim. 2004. Regulation of adipocyte differentiation and insulin action with rapamycin. Biochem. Biophys. Res. Commun. 321: 942-948.

Christy,R.J., K.H.Kaestner, D.E.Geiman, and M.D.Lane. 1991. CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. Proc. Natl. Acad. Sci. U. S. A 88: 2593-2597.

Cross,D.A., D.R.Alessi, P.Cohen, M.Andjelkovich, and B.A.Hemmings. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378: 785-789.

Delmas,C., N.Aragou, S.Poussard, P.Cottin, J.M.Darbon, and S.Manenti. 2003. MAP kinase-dependent degradation of p27Kip1 by calpains in choroidal melanoma cells. Requirement of p27Kip1 nuclear export. J. Biol. Chem. 278: 12443-12451.Dizdar,O. and E.Alyamac. 2004. Obesity: an endocrine tumor? Med. Hypotheses 63: 790-792.

Dong,Y., L.Sui, Y.Watanabe, K.Sugimoto, and M.Tokuda. 2003. S-phase kinase-associated protein 2 expression in laryngeal squamous cell carcinomas and its prognostic implications. Oncol. Rep. 10: 321-325.

Durand,B., F.B.Gao, and M.Raff. 1997. Accumulation of the cyclin-dependent kinase inhibitor p27/Kip1 and the timing of oligodendrocyte differentiation. EMBO J. 16: 306-317.

Enesco,M. and C.P.Labond. 1962. Increase in cell number as a factor in the growth of the organ and tissue of the young male rat. J. Embryol. Exp. Morphol. 10: 530-562.

Esposito,V., A.Baldi, A.De Luca, A.M.Groger, M.Loda, G.G.Giordano, M.Caputi, F.Baldi, M.Pagano, and A.Giordano. 1997. Prognostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. Cancer Res. 57: 3381-3385.

Fajas,L., R.L.Landsberg, Y.Huss-Garcia, C.Sardet, J.A.Lees, and J.Auwerx. 2002. E2Fs regulate adipocyte differentiation. Dev. Cell 3: 39-49.

Faust,I.M., P.R.Johnson, J.S.Stern, and J.Hirsch. 1978. Diet-induced adipocyte number increase in adult rats: a new model of obesity. Am. J. Physiol 235: E279-E286.

Faust,I.M. and N.Mrosovsky. 1987. Resistance to adipocyte hyperplasia in ground squirrels given high-fat diets. Am. J. Physiol 253: R576-R579.

Florenes,V.A., G.M.Maelandsmo, R.S.Kerbel, J.M.Slingerland, J.M.Nesland, and R.Holm. 1998. Protein expression of the cell-cycle inhibitor p27Kip1 in malignant melanoma: inverse correlation with disease-free survival. Am. J. Pathol. 153: 305-312.

Fredersdorf,S., J.Burns, A.M.Milne, G.Packham, L.Fallis, C.E.Gillett, J.A.Royds, D.Peston, P.A.Hall, A.M.Hanby, D.M.Barnes, S.Shousha, M.J.O'Hare, and X.Lu. 1997. High level expression of p27(kip1) and cyclin D1 in some human breast cancer cells: inverse correlation between the expression of p27(kip1) and degree of malignancy in human breast and colorectal cancers. Proc. Natl. Acad. Sci. U. S. A 94: 6380-6385.

Fujita,N., S.Sato, K.Katayama, and T.Tsuruo. 2002. Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization. J. Biol. Chem. 277: 28706-28713.

Fujita,N., S.Sato, and T.Tsuruo. 2003. Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14-3-3 and cytoplasmic localization. J. Biol. Chem. 278: 49254-49260.

Ganoth,D., G.Bornstein, T.K.Ko, B.Larsen, M.Tyers, M.Pagano, and A.Hershko. 2001. The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. Nat. Cell Biol. 3: 321-324.

Goel,H.L., M.Breen, J.Zhang, I.Das, S.znavoorian-Cheshire, N.M.Greenberg, A.Elgavish, and L.R.Languino. 2005. beta1A integrin expression is required for type 1 insulin-like growth factor receptor mitogenic and transforming activities and localization to focal contacts. Cancer Res. 65: 6692-6700.

Green,H. and O.Kehinde. 1976. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell 7: 105-113.

Green,H. and M.Meuth. 1974. An established pre-adipose cell line and its differentiation in culture. Cell 3: 127-133.

Guo,S., G.Rena, S.Cichy, X.He, P.Cohen, and T.Unterman. 1999. Phosphorylation of serine 256 by protein kinase B disrupts transactivation by FKHR and mediates effects of insulin on insulin-like growth factor-binding protein-1 promoter activity through a conserved insulin response sequence. J. Biol. Chem. 274: 17184-17192.

Hager,A., L.Sjorstrom, B.Arvidsson, P.Bjorntorp, and U.Smith. 1978. Adipose tissue cellularity in obese school girls before and after dietary treatment. Am. J. Clin. Nutr. 31: 68-75.

Hao,B., N.Zheng, B.A.Schulman, G.Wu, J.J.Miller, M.Pagano, and N.P.Pavletich. 2005. Structural basis of the Cks1-dependent recognition of p27(Kip1) by the SCF(Skp2) ubiquitin ligase. Mol. Cell 20: 9-19.

Hara,K., Y.Maruki, X.Long, K.Yoshino, N.Oshiro, S.Hidayat, C.Tokunaga, J.Avruch, and K.Yonezawa. 2002. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 110: 177-189.

Hershko,A. and A.Ciechanover. 1998. The ubiquitin system. Annu. Rev. Biochem. 67: 425-479.

Hershko,A., H.Heller, S.Elias, and A.Ciechanover. 1983. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J. Biol. Chem. 258: 8206-8214.

Hirsch,J. and B.Batchelor. 1976. Adipose tissue cellularity in human obesity. Clin. Endocrinol. Metab 5: 299-311.

Hotamisligil,G.S., N.S.Shargill, and B.M.Spiegelman. 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259: 87-91.

Hresko,R.C. and M.Mueckler. 2005. mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. J. Biol. Chem. 280: 40406-40416.

Hu,Y.Q. and Y.J.Liu. 2005. Expressions of Cx43 and Skp2 in epithelial ovarian tumor and their clinical significances. Ai. Zheng. 24: 104-109.

Huang,H., K.M.Regan, F.Wang, D.Wang, D.I.Smith, J.M.van Deursen, and D.J.Tindall. 2005a. Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. Proc. Natl. Acad. Sci. U. S. A 102: 1649-1654.

Huang,P.R., Y.M.Yeh, and T.C.Wang. 2005b. Potent inhibition of human telomerase by helenalin. Cancer Lett. 227: 169-174.

Huang,S.S. and J.S.Huang. 2005. TGF-beta control of cell proliferation. J. Cell Biochem. 96: 447-462.

Imaki,H., K.Nakayama, S.Delehouzee, H.Handa, M.Kitagawa, T.Kamura, and K.I.Nakayama. 2003. Cell cycle-dependent regulation of the Skp2 promoter by GA-binding protein. Cancer Res. 63: 4607-4613.

Ishida,N., T.Hara, T.Kamura, M.Yoshida, K.Nakayama, and K.I.Nakayama. 2002. Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export. J. Biol. Chem. 277: 14355-14358.

Jordan,R.C., G.Bradley, and J.Slingerland. 1998. Reduced levels of the cell-cycle inhibitor p27Kip1 in epithelial dysplasia and carcinoma of the oral cavity. Am. J. Pathol. 152: 585-590.

Kaestner,K.H., R.J.Christy, and M.D.Lane. 1990. Mouse insulin-responsive glucose transporter gene: characterization of the gene and trans-activation by the CCAAT/enhancer binding protein. Proc. Natl. Acad. Sci. U. S. A 87: 251-255.

Kamura,T., T.Hara, S.Kotoshiba, M.Yada, N.Ishida, H.Imaki, S.Hatakeyama, K.Nakayama, and K.I.Nakayama. 2003. Degradation of p57Kip2 mediated by SCFSkp2-dependent ubiquitylation. Proc. Natl. Acad. Sci. U. S. A 100: 10231-10236.

Kamura,T., T.Hara, M.Matsumoto, N.Ishida, F.Okumura, S.Hatakeyama, M.Yoshida, K.Nakayama, and K.I.Nakayama. 2004. Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(Kip1) at G1 phase. Nat. Cell Biol. 6: 1229-1235.

Kane,L.P., V.S.Shapiro, D.Stokoe, and A.Weiss. 1999. Induction of NF-kappaB by the Akt/PKB kinase. Curr. Biol. 9: 601-604.

Katayose,Y., M.Kim, A.N.Rakkar, Z.Li, K.H.Cowan, and P.Seth. 1997. Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. Cancer Res. 57: 5441-5445.

Kiernan,R.E., S.Emiliani, K.Nakayama, A.Castro, J.C.Labbe, T.Lorca, K.K.Nakayama, and M.Benkirane. 2001. Interaction between cyclin T1 and SCF(SKP2) targets CDK9 for ubiquitination and degradation by the proteasome. Mol. Cell Biol. 21: 7956-7970.

Kim,I.M., T.Ackerson, S.Ramakrishna, M.Tretiakova, I.C.Wang, T.V.Kalin, M.L.Major, G.A.Gusarova, H.M.Yoder, R.H.Costa, and V.V.Kalinichenko. 2006. The Forkhead Box m1 Transcription Factor Stimulates the Proliferation of Tumor Cells during Development of Lung Cancer. Cancer Res. 66: 2153-2161.

Knittle,J.L. and J.Hirsch. 1968. Effect of early nutrition on the development of rat epididymal fat pads: cellularity and metabolism. J. Clin. Invest 47: 2091-2098.

Knowles,L.M., F.Axelrod, C.D.Browne, and J.W.Smith. 2004. A Fatty Acid Synthase Blockade Induces Tumor Cell-cycle Arrest by Down-regulating Skp2. J. Biol. Chem. 279: 30540-30545.

Koga,H., M.Harada, M.Ohtsubo, S.Shishido, H.Kumemura, S.Hanada, E.Taniguchi, K.Yamashita, R.Kumashiro, T.Ueno, and M.Sata. 2003. Troglitazone induces p27Kip1-associated cell-cycle arrest through down-regulating Skp2 in human hepatoma cells. Hepatology 37: 1086-1096.

Kudo,Y., S.Kitajima, S.Sato, M.Miyauchi, I.Ogawa, and T.Takata. 2001. High expression of S-phase kinase-interacting protein 2, human F-box protein, correlates with poor prognosis in oral squamous cell carcinomas. Cancer Res. 61: 7044-7047.

Langner,C., R.von Wasielewski, M.Ratschek, P.Rehak, and R.Zigeuner. 2004. Biological significance of p27 and Skp2 expression in renal cell carcinoma. A systematic analysis of primary and metastatic tumour tissues using a tissue microarray technique. Virchows Arch. 445: 631-636.

Lavoie,J.N., G.L'Allemain, A.Brunet, R.Muller, and J.Pouyssegur. 1996. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J. Biol. Chem. 271: 20608-20616.

Lee,K.H., I.H.Hall, E.C.Mar, C.O.Starnes, S.A.ElGebaly, T.G.Waddell, R.I.HADGRAFT, C.G.Ruffner, and I.Weidner. 1977. Sesquiterpene antitumor agents: inhibitors of cellular metabolism. Science 196: 533-536.

Levkau,B., H.Koyama, E.W.Raines, B.E.Clurman, B.Herren, K.Orth, J.M.Roberts, and R.Ross. 1998. Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade. Mol. Cell 1: 553-563.

Li,Q., M.Murphy, J.Ross, C.Sheehan, and J.A.Carlson. 2004. Skp2 and p27kip1 expression in melanocytic nevi and melanoma: an inverse relationship. J. Cutan. Pathol. 31: 633-642.

Liang,J., J.Zubovitz, T.Petrocelli, R.Kotchetkov, M.K.Connor, K.Han, J.H.Lee, S.Ciarallo, C.Catzavelos, R.Beniston, E.Franssen, and J.M.Slingerland. 2002. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. Nat. Med. 8: 1153-1160.

Lisztwan,J., A.Marti, H.Sutterluty, M.Gstaiger, C.Wirbelauer, and W.Krek. 1998. Association of human CUL-1 and ubiquitin-conjugating enzyme CDC34 with the F-box protein p45(SKP2): evidence for evolutionary conservation in the subunit composition of the CDC34-SCF pathway. EMBO J. 17: 368-383.

Lubetzki,J., C.Roussel, A.Mosse, J.Duprey, A.Warnet, and D.Bargy. 1980. Morphology of adipose tissue. Study in 102 obese subjects in reference to some clinical and biological criteria (author's transl). Pathol. Biol. (Paris) 28: 17-24.

Lyss,G., A.Knorre, T.J.Schmidt, H.L.Pahl, and I.Merfort. 1998. The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-kappaB by directly targeting p65. J. Biol. Chem. 273: 33508-33516.

MacDougald,O.A., P.Cornelius, F.T.Lin, S.S.Chen, and M.D.Lane. 1994. Glucocorticoids reciprocally regulate expression of the CCAAT/enhancer-binding protein alpha and delta genes in 3T3-L1 adipocytes and white adipose tissue. J. Biol. Chem. 269: 19041-19047.

Maehama,T. and J.E.Dixon. 1998. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. 273: 13375-13378.

Mamillapalli,R., N.Gavrilova, V.T.Mihaylova, L.M.Tsvetkov, H.Wu, H.Zhang, and H.Sun. 2001. PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27(KIP1) through the ubiquitin E3 ligase SCF(SKP2). Curr. Biol. 11: 263-267.

Mandenoff,A., T.Lenoir, and M.Apfelbaum. 1982. Tardy occurrence of adipocyte hyperplasia in cafeteria-fed rat. Am. J. Physiol 242: R349-R351.

Marques,B.G., D.B.Hausman, and R.J.Martin. 1998. Association of fat cell size and paracrine growth factors in development of hyperplastic obesity. Am. J. Physiol 275: R1898-R1908.

Marti,A., C.Wirbelauer, M.Scheffner, and W.Krek. 1999. Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. Nat. Cell Biol. 1: 14-19.

McKeon,C. and T.Pham. 1991. Transactivation of the human insulin receptor gene by the CAAT/enhancer binding protein. Biochem. Biophys. Res. Commun. 174: 721-728.

Medema,R.H., G.J.Kops, J.L.Bos, and B.M.Burgering. 2000. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature 404: 782-787.

Moberg,K., M.A.Starz, and J.A.Lees. 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol. Cell Biol. 16: 1436-1449.

Mohamed-Ali,V., J.H.Pinkney, and S.W.Coppack. 1998. Adipose tissue as an endocrine and paracrine organ. Int. J. Obes. Relat Metab Disord. 22: 1145-1158.

Mori,M., K.Mimori, T.Shiraishi, S.Tanaka, H.Ueo, K.Sugimachi, and T.Akiyoshi. 1997. p27 expression and gastric carcinoma. Nat. Med. 3: 593.

Morosetti,R., N.Kawamata, A.F.Gombart, C.W.Miller, Y.Hatta, T.Hirama, J.W.Said, M.Tomonaga, and H.P.Koeffler. 1995. Alterations of the p27KIP1 gene in non-Hodgkin's lymphomas and adult T-cell leukemia/lymphoma. Blood 86: 1924-1930.

Morrison,R.F. and S.R.Farmer. 1999. Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. J. Biol. Chem. 274: 17088-17097.

Mrosovsky,N., P.Nash, and I.M.Faust. 1987. Protection against fat cell hyperplasia in a hibernator, Glis glis. Am. J. Physiol 253: R580-R586.

Naaz,A., D.R.Holsberger, G.A.Iwamoto, A.Nelson, H.Kiyokawa, and P.S.Cooke. 2004. Loss of cyclin-dependent kinase inhibitors produces adipocyte hyperplasia and obesity. FASEB J. 18: 1925-1927.

Nakamura,M., T.Matsuo, J.Stauffer, L.Neckers, and C.J.Thiele. 2003. Retinoic acid decreases targeting of p27 for degradation via an N-myc-dependent decrease in p27 phosphorylation and an N-myc-independent decrease in Skp2. Cell Death. Differ. 10: 230-239.

Nakayama,K., H.Nagahama, Y.A.Minamishima, M.Matsumoto, I.Nakamichi, K.Kitagawa, M.Shirane, R.Tsunematsu, T.Tsukiyama, N.Ishida, M.Kitagawa, K.Nakayama, and S.Hatakeyama. 2000. Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. EMBO J. 19: 2069-2081.

Nojima,H., C.Tokunaga, S.Eguchi, N.Oshiro, S.Hidayat, K.Yoshino, K.Hara, N.Tanaka, J.Avruch, and K.Yonezawa. 2003. The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. J. Biol. Chem. 278: 15461-15464.

Oliveira,A.M., S.H.Okuno, A.G.Nascimento, and R.V.Lloyd. 2003. Skp2 protein expression in soft tissue sarcomas. J. Clin. Oncol. 21: 722-727.

Osoegawa,A., I.Yoshino, S.Tanaka, K.Sugio, T.Kameyama, M.Yamaguchi, and Y.Maehara. 2004. Regulation of p27 by S-phase kinase-associated protein 2 is associated with aggressiveness in non-small-cell lung cancer. J. Clin. Oncol. 22: 4165-4173.

Ouchi,N., S.Kihara, T.Funahashi, T.Nakamura, M.Nishida, M.Kumada, Y.Okamoto, K.Ohashi, H.Nagaretani, K.Kishida, H.Nishizawa, N.Maeda, H.Kobayashi, H.Hiraoka, and Y.Matsuzawa. 2003. Reciprocal association of C-reactive protein with adiponectin in blood stream and adipose tissue. Circulation 107: 671-674.

Pagano,M., S.W.Tam, A.M.Theodoras, P.Beer-Romero, G.Del Sal, V.Chau, P.R.Yew, G.F.Draetta, and M.Rolfe. 1995. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 269: 682-685.

Palombella,V.J., O.J.Rando, A.L.Goldberg, and T.Maniatis. 1994. The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell 78: 773-785.

Patel,Y.M. and M.D.Lane. 2000. Mitotic clonal expansion during preadipocyte differentiation: calpain-mediated turnover of p27. J. Biol. Chem. 275: 17653-17660.

Pettersson,P., R.Van, M.Karlsson, and P.Bjorntorp. 1985. Adipocyte precursor cells in obese and nonobese humans. Metabolism 34: 808-812.

Polyak,K., M.H.Lee, H.Erdjument-Bromage, A.Koff, J.M.Roberts, P.Tempst, and J.Massague. 1994. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 78: 59-66.

Porter,P.L., K.E.Malone, P.J.Heagerty, G.M.Alexander, L.A.Gatti, E.J.Firpo, J.R.Daling, and J.M.Roberts. 1997. Expression of cell-cycle regulators p27Kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. Nat. Med. 3: 222-225.

Qian,X., L.Jin, E.Kulig, and R.V.Lloyd. 1998. DNA methylation regulates p27kip1 expression in rodent pituitary cell lines. Am. J. Pathol. 153: 1475-1482.

Reynisdottir,I. and J.Massague. 1997. The subcellular locations of p15(Ink4b) and p27(Kip1) coordinate their inhibitory interactions with cdk4 and cdk2. Genes Dev. 11: 492-503.

Richon,V.M., R.E.Lyle, and R.E.McGehee, Jr. 1997. Regulation and expression of retinoblastoma proteins p107 and p130 during 3T3-L1 adipocyte differentiation. J. Biol. Chem. 272: 10117-10124.

Rodier,G., A.Montagnoli, L.Di Marcotullio, P.Coulombe, G.F.Draetta, M.Pagano, and S.Meloche. 2001. p27 cytoplasmic localization is regulated by phosphorylation on Ser10 and is not a prerequisite for its proteolysis. EMBO J. 20: 6672-6682.

Rolls,B.J., E.A.Rowe, and R.C.Turner. 1980. Persistent obesity in rats following a period of consumption of a mixed, high energy diet. J. Physiol 298: 415-427.

Rosen,E.D., C.H.Hsu, X.Wang, S.Sakai, M.W.Freeman, F.J.Gonzalez, and B.M.Spiegelman. 2002. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev. 16: 22-26.

Rothwell,N.J. and M.J.Stock. 1997. A role for brown adipose tissue in diet-induced thermogenesis. Obes. Res. 5: 650-656.

Russo,A.A., P.D.Jeffrey, A.K.Patten, J.Massague, and N.P.Pavletich. 1996. Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex. Nature 382: 325-331.

Salans,L.B., S.W.Cushman, and R.E.Weismann. 1973. Studies of human adipose tissue. Adipose cell size and number in nonobese and obese patients. J. Clin. Invest 52: 929-941.

Sanchez-Beato,M., A.I.Saez, J.C.Martinez-Montero, M.M.Sol, L.Sanchez-Verde, R.Villuendas, G.Troncone, and M.A.Piris. 1997. Cyclin-dependent kinase inhibitor p27KIP1 in lymphoid tissue: p27KIP1 expression is inversely proportional to the proliferative index. Am. J. Pathol. 151: 151-160.

Seki,R., T.Okamura, H.Koga, K.Yakushiji, M.Hashiguchi, K.Yoshimoto, H.Ogata, R.Imamura, Y.Nakashima, M.Kage, T.Ueno, and M.Sata. 2003. Prognostic significance of the F-box protein Skp2 expression in diffuse large B-cell lymphoma. Am. J. Hematol. 73: 230-235.

Shapira,M., O.Ben Izhak, S.Linn, B.Futerman, I.Minkov, and D.D.Hershko. 2005. The prognostic impact of the ubiquitin ligase subunits Skp2 and Cks1 in colorectal carcinoma. Cancer 103: 1336-1346.

Shen,M.R., Y.M.Hsu, K.F.Hsu, Y.F.Chen, M.J.Tang, and C.Y.Chou. 2006. Insulin-like growth factor 1 is a potent stimulator of cervical cancer cell invasiveness and proliferation which is modulated by {alpha}v{beta}3 integrin signaling. Carcinogenesis.

Shin,I., J.Rotty, F.Y.Wu, and C.L.Arteaga. 2005. Phosphorylation of p27Kip1 at Thr-157 interferes with its association with importin alpha during G1 and prevents nuclear re-entry. J. Biol. Chem. 280: 6055-6063.

Shirane,M., Y.Harumiya, N.Ishida, A.Hirai, C.Miyamoto, S.Hatakeyama, K.Nakayama, and M.Kitagawa. 1999. Down-regulation of p27(Kip1) by two mechanisms, ubiquitin-mediated degradation and proteolytic processing. J. Biol. Chem. 274: 13886-13893.

Singh,S.P., J.Lipman, H.Goldman, F.H.Ellis, Jr., L.Aizenman, M.G.Cangi, S.Signoretti, D.S.Chiaur, M.Pagano, and M.Loda. 1998. Loss or altered subcellular localization of p27 in Barrett's associated adenocarcinoma. Cancer Res. 58: 1730-1735.

Slingerland,J.M., L.Hengst, C.H.Pan, D.Alexander, M.R.Stampfer, and S.I.Reed. 1994. A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor beta-arrested epithelial cells. Mol. Cell Biol. 14: 3683-3694.

Smith,P.J., L.S.Wise, R.Berkowitz, C.Wan, and C.S.Rubin. 1988. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. J. Biol. Chem. 263: 9402-9408.

Spirin,K.S., J.F.Simpson, S.Takeuchi, N.Kawamata, C.W.Miller, and H.P.Koeffler. 1996. p27/Kip1 mutation found in breast cancer. Cancer Res. 56: 2400-2404.

St Croix,B., V.A.Florenes, J.W.Rak, M.Flanagan, N.Bhattacharya, J.M.Slingerland, and R.S.Kerbel. 1996. Impact of the cyclin-dependent kinase inhibitor p27Kip1 on resistance of tumor cells to anticancer agents. Nat. Med. 2: 1204-1210.

Steinberg,M.D., W.Zingg, and A.Angel. 1962. Studies of the number and volume of fat cells in adipose tissue. J. Pediatr. 61: 299-300.

Stewart,S.A., D.Kothapalli, Y.Yung, and R.K.Assoian. 2004. Antimitogenesis Linked to Regulation of Skp2 Gene Expression. J. Biol. Chem. 279: 29109-29113.

Sutterluty,H., E.Chatelain, A.Marti, C.Wirbelauer, M.Senften, U.Muller, and W.Krek. 1999. p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. Nat. Cell Biol. 1: 207-214.

Tan,P., B.Cady, M.Wanner, P.Worland, B.Cukor, C.Magi-Galluzzi, P.Lavin, G.Draetta, M.Pagano, and M.Loda. 1997. The cell cycle inhibitor p27 is an independent prognostic marker in small (T1a,b) invasive breast carcinomas. Cancer Res. 57: 1259-1263.

Tang,Q.Q., M.S.Jiang, and M.D.Lane. 1999. Repressive effect of Sp1 on the C/EBPalpha gene promoter: role in adipocyte differentiation. Mol. Cell Biol. 19: 4855-4865.

Tang,Q.Q. and M.D.Lane. 1999. Activation and centromeric localization of CCAAT/enhancer-binding proteins during the mitotic clonal expansion of adipocyte differentiation. Genes Dev. 13: 2231-2241.

Tang,Q.Q., T.C.Otto, and M.D.Lane. 2003. Mitotic clonal expansion: a synchronous process required for adipogenesis. Proc. Natl. Acad. Sci. U. S. A 100: 44-49.

Tang,Q.Q., J.W.Zhang, and L.M.Daniel. 2004. Sequential gene promoter interactions of C/EBPbeta, C/EBPalpha, and PPARgamma during adipogenesis. Biochem. Biophys. Res. Commun. 319: 235-239.

Tedesco,D., J.Lukas, and S.I.Reed. 2002. The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF(Skp2). Genes Dev. 16: 2946-2957.

Thrower,J.S., L.Hoffman, M.Rechsteiner, and C.M.Pickart. 2000. Recognition of the polyubiquitin proteolytic signal. EMBO J. 19: 94-102.

Todaro,G.J. and H.Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17: 299-313.

Tomoda,K., Y.Kubota, Y.Arata, S.Mori, M.Maeda, T.Tanaka, M.Yoshida, N.Yoneda-Kato, and J.Y.Kato. 2002. The cytoplasmic shuttling and subsequent degradation of p27Kip1 mediated by Jab1/CSN5 and the COP9 signalosome complex. J. Biol. Chem. 277: 2302-2310.

Toyoshima,H. and T.Hunter. 1994. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell 78: 67-74.

Tremblay,F., A.Gagnon, A.Veilleux, A.Sorisky, and A.Marette. 2005. Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology 146: 1328-1337.

Tsihlias,J., L.R.Kapusta, G.DeBoer, I.Morava-Protzner, I.Zbieranowski, N.Bhattacharya, G.C.Catzavelos, L.H.Klotz, and J.M.Slingerland. 1998. Loss of cyclin-dependent kinase inhibitor p27Kip1 is a novel prognostic factor in localized human prostate adenocarcinoma. Cancer Res. 58: 542-548.

Tsvetkov,L.M., K.H.Yeh, S.J.Lee, H.Sun, and H.Zhang. 1999. p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. Curr. Biol. 9: 661-664.

Viglietto,G., M.L.Motti, P.Bruni, R.M.Melillo, A.D'Alessio, D.Califano, F.Vinci, G.Chiappetta, P.Tsichlis, A.Bellacosa, A.Fusco, and M.Santoro. 2002. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. Nat. Med. 8: 1136-1144.

von der,L.N., S.Johansson, S.Wu, F.Bahram, A.Castell, C.Cetinkaya, P.Hydbring, I.Weidung, K.Nakayama, K.I.Nakayama, O.Soderberg, T.K.Kerppola, and L.G.Larsson. 2003. The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. Mol. Cell 11: 1189-1200.

Wang,I.C., Y.J.Chen, D.Hughes, V.Petrovic, M.L.Major, H.J.Park, Y.Tan, T.Ackerson, and R.H.Costa. 2005. Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. Mol. Cell Biol. 25: 10875-10894.

Wang,W., D.Ungermannova, J.Jin, J.W.Harper, and X.Liu. 2004. Negative regulation of SCFSkp2 ubiquitin ligase by TGF-beta signaling. Oncogene 23: 1064-1075.

Weber,J.D., D.M.Raben, P.J.Phillips, and J.J.Baldassare. 1997. Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. Biochem. J. 326 (Pt 1): 61-68.

Wei,W., N.G.Ayad, Y.Wan, G.J.Zhang, M.W.Kirschner, and W.G.Kaelin, Jr. 2004. Degradation of the SCF component Skp2 in cell-cycle phase G1 by the anaphase-promoting complex. Nature 428: 194-198.

Wirbelauer,C., H.Sutterluty, M.Blondel, M.Gstaiger, M.Peter, F.Reymond, and W.Krek. 2000. The F-box protein Skp2 is a ubiquitylation target of a Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. EMBO J. 19: 5362-5375.

Wu,Z., N.L.Bucher, and S.R.Farmer. 1996. Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids. Mol. Cell Biol. 16: 4128-4136.

Wu,Z., Y.Xie, R.F.Morrison, N.L.Bucher, and S.R.Farmer. 1998. PPARgamma induces the insulin-dependent glucose transporter GLUT4 in the absence of C/EBPalpha during the conversion of 3T3 fibroblasts into adipocytes. J. Clin. Invest 101: 22-32.

Yam,C.H., R.W.Ng, W.Y.Siu, A.W.Lau, and R.Y.Poon. 1999. Regulation of cyclin A-Cdk2 by SCF component Skp1 and F-box protein Skp2. Mol. Cell Biol. 19: 635-645.

Yang,G., G.Ayala, A.De Marzo, W.Tian, A.Frolov, T.M.Wheeler, T.C.Thompson, and J.W.Harper. 2002. Elevated Skp2 protein expression in human prostate cancer: association with loss of the cyclin-dependent kinase inhibitor p27 and PTEN and with reduced recurrence-free survival. Clin. Cancer Res. 8: 3419-3426.

Yang,M.U., E.Presta, and P.Bjorntorp. 1990. Refeeding after fasting in rats: effects of duration of starvation and refeeding on food efficiency in diet-induced obesity. Am. J. Clin. Nutr. 51: 970-978.

Yeh,W.C., B.E.Bierer, and S.L.McKnight. 1995. Rapamycin inhibits clonal expansion and adipogenic differentiation of 3T3-L1 cells. Proc. Natl. Acad. Sci. U. S. A 92: 11086-11090.

Young,R.A., L.B.Salans, and E.A.Sims. 1982. Adipose tissue cellularity in woodchucks: effects of season and captivity at an early age. J. Lipid Res. 23: 887-892.

Zabludoff,S.D., M.Csete, R.Wagner, X.Yu, and B.J.Wold. 1998. p27Kip1 is expressed transiently in developing myotomes and enhances myogenesis. Cell Growth Differ. 9: 1-11.

Zhang,H., R.Kobayashi, K.Galaktionov, and D.Beach. 1995. p19Skp1 and p45Skp2 are essential elements of the cyclin A-CDK2 S phase kinase. Cell 82: 915-925.

Zhang,H., I.Ozaki, T.Mizuta, T.Yoshimura, S.Matsuhashi, A.Hisatomi, J.Tadano, T.Sakai, and K.Yamamoto. 2003a. Mechanism of beta 1-integrin-mediated hepatoma cell growth involves p27 and S-phase kinase-associated protein 2. Hepatology 38: 305-313.

Zhang,J.W., Q.Q.Tang, C.Vinson, and M.D.Lane. 2004. Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes. Proc. Natl. Acad. Sci. U. S. A 101: 43-47.

Zhang,L. and C.Wang. 2005. F-box protein Skp2: a novel transcriptional target of E2F. Oncogene.

Zhang,Y.W., M.Kaneda, and I.Morita. 2003b. The gap junction-independent tumor-suppressing effect of connexin 43. J. Biol. Chem. 278: 44852-44856.

Zhang,Y.W., K.Nakayama, K.Nakayama, and I.Morita. 2003c. A novel route for connexin 43 to inhibit cell proliferation: negative regulation of S-phase kinase-associated protein (Skp 2). Cancer Res. 63: 1623-1630.

Zuo,Y., L.Qiang, and S.R.Farmer. 2006. Activation of C/EBPalpha expression by C/EBPbeta during adipogenesis requires a PPARgamma-associated repression of HDAC1 at the C/EBPalpha gene promoter. J. Biol. Chem.

CHAPTER II

SKP2-MEDIATED p27(KIP1) DEGRADATION DURING S/G_2 PHASE TRANSITION OF ADIPOCYTE HYPERPLASIA

ABSTRACT

Determining mechanisms responsible for preadipocyte replication is vital to understanding the etiology of hyperplastic obesity. Here we demonstrate that protein accumulation of the cyclin dependent kinase inhibitor, p27(Kip1), decreased modestly in mid-G_1, precipitously near the G_1/S transition, and maximally during G_2 phase progression of 3T3-L1 preadipocyte clonal expansion. The dramatic changes in p27 protein accumulation resulted from post-transcriptional mechanisms involving elevated protein degradation. Specific proteasome inhibitors, epoxomicin and lactacystin, completely abolished p27 degradation and S phase progression resulting in G_1 arrest. It is further demonstrated that p27 was phosphorylated on threonine 187 during S phase progression by Cdk2 and that phosphorylated p27 was polyubiquitylated and degraded. Data are also presented demonstrating increased protein accumulation of the F-box, Skp2, and accessory protein, Cks1, which peaked during G_2 phase coordinate with the maximal decrease in p27 protein levels. A direct role for Skp2 in p27 degradation was demonstrated with Skp2 specific RNA interference. It is also demonstrated that while specific inhibition of Cdk2 activity with Roscovitine completely blocked S phase progression and p27 (Thr187) phosphorylation and ubiquitylation, the loss of Cdk2 activity only partially prevented p27 degradation. Consistent with this observation, complete Skp2 knockdown did not completely prevent p27 degradation, suggesting that

other proteasome-dependent mechanisms contributed to p27 degradation during cell cycle progression. We also demonstrate that Skp2 knockdown had no effect on S phase progression suggesting that Skp2-mediated p27 degradation was not essential for G_1/S transition as quiescent preadipocytes reenter the cell cycle. Finally, evidence is presented suggesting that increased p27 inhibitory activity resulting from elevated p27 protein in the absence of Skp2 may have been neutralized by sequestration of p27 protein by Cyclin D1/Cdk4 complexes. Considering the kinetics of Cdk2 activation, p27 (Thr187) phosphorylation, and Skp2 and Cks1 protein accumulation, we propose that p27 phosphorylation and ubiquitylation by the SCF^{Skp2} E3 ligase is mechanistically responsible, in part for phase-specific p27 degradation during S and G_2 phase progression.

INTRODUCTION

Obesity develops through an expansion of adipose tissue mass resulting from enlargement of adipocyte size (hypertrophy) and cell number (hyperplasia). As mature adipocytes are terminally differentiated and resistant to cell division, adipocyte hyperplasia generally refers to proliferation and subsequent differentiation of adipocyte precursor cells or 'preadipocytes' (Hausman et al., 2001). Preadipocyte replication is accelerated at birth and puberty then slows to provide a constant ratio of preadipocytes to adipocytes in adulthood as long as body weight remains constant. Adipocyte hyperplasia, however, plays a critical role in the onset of obesity throughout all stages of childhood development and in adults under morbid conditions. Despite evidence indicating that the most severe forms of obesity with the poorest prognosis of treatment are associated adipocyte hyperplasia (Hirsch et al., 1989;Bjorntorp et al., 1982;Bonnet,

1981;Hausman et al., 2001), mechanisms regulating preadipocyte proliferation remain largely unknown.

A key question with regard to adipocyte hyperplasia concerns the mechanism by which preadipocytes undergo the transition from quiescence to proliferation. The decision of mammalian cells to replicate involves the convergence of mitogen-dependent signaling pathways on a series of cyclin-dependent kinases (Cdks) that function to mediate orderly cell cycle progression. Cells become mitogen-independent near the G_1/S phase transition following activation of Cdk2, subsequent phosphorylation of the retinoblastoma protein, and initiation of E2F transcriptional activity promoting S phase gene expression. As progression beyond this restriction point is independent of mitogen availability, activation of Cdk2 activity during late G_1 and S phase represents a pivotal and decisive point of autonomous cell cycle control.

Numerous lines of evidence have established the removal of p27 (Kip1) inhibitory activity as rate limiting for Cdk2 activation. Elevated p27 protein resulting from serum deprivation, density arrest, and terminal differentiation ensures cell cycle arrest in G_1 phase and subsequent entry into the G_0 state of quiescence. Under growth promoting conditions, p27 protein levels decrease modestly during mid-G_1 and precipitously near the G_1/S phase transition. Ectopic p27 expression unequivocally inhibits Cdk2 activity resulting in cell cycle arrest in G_1 (Polyak et al., 1994;Toyoshima and Hunter, 1994). Conversely, reduction of p27 protein increases the number of cells that enter S phase (Coats et al., 1996). Moreover, p27 gene ablation yields larger than normal mice with marked organomegaly as well as increased adipocyte number relative to wildtype mice (Naaz et al., 2004). Gene ablation of both p27 and p21(Cip1), another member of the Cip/Kip family of cyclin dependent kinase inhibitors (CKIs), results in a phenotype of

severe obesity, metabolic abnormalities, and more than a 5-fold increase in adipocyte number (Naaz et al., 2004).

While much attention has focused on regulation of p27 activity through changes in protein accumulation by post-transcriptional mechanisms involving protein degradation, the complexity of this process was underscored by recent reports suggesting that p27 protein proteolysis is regulated by distinct mechanisms during different phases of the cell cycle. During S and G_2 phases, the process of targeted degradation begins with Cdk2 phosphorylation of p27 (Sheaff et al., 1997;Vlach et al., 1997) on a threonine residue (Thr187) that promotes binding of nuclear SCF^{Skp2} (Skp1/Cul1/Rbx1/F-Box Protein; the superscript denotes the F-box protein) E3 ligase. Upon assembly, this complex mediates covalent modification of a polyubiquitin chain that targets p27 for degradation by the 26S proteasome (Carrano et al., 1999;Sutterluty et al., 1999;Tsvetkov et al., 1999). During G_1 phase progression, proteasome-dependent p27 degradation appears to involve the cytosolic ubiquitin E3 ligase, KPC (Kip1 Ubiquitination-Promoting Complex). In addition to being localized to a different compartment, KPC mediates ubiquitylation of p27 independent of Thr187 phosphorylation and Skp2 interaction (Kamura et al., 2004). Other p27 degradation mechanisms have also been described including proteolytic processing (Shirane et al., 1999) and degradation by non-lysosomal calpains (Delmas et al., 2003;Patel and Lane, 2000) and caspase-dependent pathways (Levkau et al., 1998;Loubat et al., 1999;Eymin et al., 1999). In addition, p27 levels can be regulated by transcriptional (Kops et al., 2002) and translational (Hengst and Reed, 1996) mechanisms. Furthermore, p27 activity can be modulated through sequestration by Cyclin D-Cdk4/6 complexes (Perez-Roger et al., 1999).

Considering the pivotal role p27 plays in cell cycle progression and the diversity of cell-type and condition-specific mechanisms regulating its inhibitory activity, we explored phase specific mechanisms regulating p27 protein accumulation during cell cycle progression of murine 3T3-L1 preadipocytes as a model of adipocyte hyperplasia. When grown to a state of density arrest, these quiescent preadipocytes synchronously reenter the cell cycle for 1-2 rounds of cell proliferation following exposure to a hormonal cocktail of methylisobutylxanthine, dexamethasone, and insulin (MDI). This period of cell cycle progression, referred to as 'mitotic clonal expansion', is a prerequisite for differentiation of this cell line and precedes the irreversible growth arrest that characterizes terminal differentiation. In this report, we demonstrate that p27 protein levels decrease during G_1-S-G_2 phase transitions of 3T3-L1 mitotic clonal expansion by elevated protein degradation mechanisms involving the 26S proteasome. In addition, data presented here document p27 phosphorylated on Thr187 by Cdk2 during S phase is targeted for ubiquitylation. Moreover, we demonstrate that preadipocyte replication is characterized by increased Skp2 and Cks1 protein levels with maximal accumulation occurring concomitantly with decreased p27 levels during S and G_2 phases of mitotic clonal expansion. Finally, using RNA interference for Skp2, we demonstrate a role for the SCF^{Skp2} E3 ligase in mediating p27 turnover during S and G_2 phases of the cell cycle.

MATERIALS AND METHODS

Materials - Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Cellgro by Mediatech. Bovine Serum, Fetal Bovine Serum (FBS), and Trypsin-EDTA were from Invitrogen Corporation. Propidium Iodide and RNase A was purchased from

Sigma. Chemical inhibitors were purchased from the following: Epoxomicin, Lactacystin (Boston Biochem), Cyclohexamide (Sigma), Roscovitine (Calbiochem), LY294002 (Promega), Leptomycin B (Sigma), PP1 (New England Biolabs). Enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Sciences.

Cell Culture and Differentiation - Murine 3T3-L1 preadipocytes were propagated in growth medium containing DMEM supplemented with 10% calf bovine serum as described previously (Morrison and Farmer, 1999). By standard differentiation protocol, preadipocytes were propagated in growth medium until reaching a state of density arrest at 2 days post-confluence. Growth medium was replaced at density arrest with differentiation medium comprised of DMEM supplemented with 10% fetal bovine serum (FBS), 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). The term "post-MDI" refers to the time elapsed since the addition of MDI to the culture medium. At 2 days post-MDI, medium was changed to DMEM supplemented with 10% FBS and 0.4 μM insulin. From 4 days post-MDI until harvest, maintenance medium containing DMEM supplemented only with 10% FBS was changed every 48 hrs. Throughout the study, "time 0" refers to density arrested cells immediately before chemical induction of differentiation with the addition of MDI to the culture medium. Experiments described herein were conducted within the period of differentiation spanning from density arrest (0 hr) through 32 hrs post-MDI. All experiments were repeated three to five times to validate results and ensure reliability.

Flow Cytometry - Cell cycle progression was assessed by flow cytometry. Briefly, cell monolayers were washed with phosphate-buffered saline (PBS), trypsinized, and

detached cells diluted in ice cold PBS to produce a single cell suspension. Cells were gently pelleted by centrifugation (300 x g, 5 min). Following centrifugation, PBS was decanted and cells were fixed and permeabilized by drop-wise addition of 70% ethanol at -20°C while vortexing. Fixed cells were incubated on ice for 30 minutes or stored at -20˚C for no more than one week before processing. Fixed cells were washed 2x with PBS and incubated in the dark for 30 minutes with 1ml propidium iodide staining solution containing 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A in PBS. DNA fluorescence was measured with a FACS Calibur Flow Cytometer (Becton Dickinson) equipped with a 488-nm argon laser. Width (FL2W) and area (FL2A) of PI fluorescence was recorded for at least 10,000 counts. DNA histograms were extracted from FL2W-FL2A dot plots after gating to eliminate aggregates. The percentage of cells in each phase of the cell cycle was analyzed using ModFit software (Verity).

Immunoblotting - Cell monolayers were washed with PBS and scraped into ice cold lysis buffer containing 1.0 M Tris, pH 7.4, 150 mM NaCl, 1% Triton X, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, and 10 mM N-ethylmaleimide (NEM). Lysis buffer was freshly supplemented with phosphatase inhibitors; 20mM β-glycerophosphate, 10mM NaFl, and 2 μM sodium vanadate, as well as protease inhibitors; 0.3 μM aprotonin, 21 μM leupeptin, E-64, 1 μM pepstatin, 50 μM phenanthroline, 0.5 μM phenylmethylsulfonyl fluoride. Cell lysates were clarified by centrifugation (13,000 x g, 10 min, 4°C) and protein concentration was determined by BCA assay (Pierce). Lysates were resuspended in loading buffer containing 0.25 M Tris, pH 6.8, 4% SDS, 10% glycerol, 10% dithiotreitol, 0.01% bromophenol blue, heated for 5 mins at 80°C, and placed on ice. Lysates were resolved on SDS-PAGE gels and transferred to polyvinylidene fluoride

membranes (Millipore). Following transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4°C and horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Immunoblots were developed with ECL and visualized by autoradiography CL-XPosure film (Pierce).

Immunoprecipitation – Cell monolayers were washed with PBS and scraped into ice cold precipitation buffer containing 10mM Tris pH 7.4, 150mM NaCl, 1% Triton X, 0.5% NP40, 1mM EDTA, 1mM EGTA, 10mM NEM. Phosphatase/protease inhibitors were freshly added as discussed above. Cell lysates were clarified by centrifugation (13,000 x g, 10 min, 4°C) and protein concentration was determined by BCA assay (Pierce). Lysates (500 μg) were incubated with primary antibody for 3 hr at 4°C with rotation. Protein G Magnetic Beads (New England Biolabs) were added to each sample and incubated for an additional 1 hr at 4°C. Immune complexes were collected by brief centrifugation (13,000 x g, 2 min, 4°C) and washed 3x with ice cold precipitation buffer. Complexes were resuspended in loading buffer, resolved on SDS-PAGE gels, and immunoblotted as discussed above.

RNA Isolation and Analysis - Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to manufacturer's instructions. Total RNA (1μg) was subjected to Reverse-Transcriptase Polymerase Chain Reaction using One-Step RT-PCR kit (QIAGEN) according to manufacturer's instruction. Briefly, RT-PCR reactions were carried out in the presence of the supplied buffer, dNTPs (400 μM), enzyme mix (reverse transcriptases and DNA polymerase), and RNase inhibitor (10 U). Gene specific primers were used at a final concentration of 0.6 μM and designed as follows: p27(Kip1) forward,

5′-CGCTTTTGTTCGGTTTTGTT-3′; p27 reverse, 5′-TTCGGAGCTGTTTACGTCTG-3′ (product size = 690 bp). QuantamRNA Classic and Classic II 18S primer/competimers (Ambion) were used in the reactions as internal standards at a final concentration of 0.6 μM according to manufacturer's instruction.

RNA interference (RNAi) - SMARTpools of short interfering RNAs (siRNAs) for non-targeting sequences and Skp2 specific sequences (Dharmacon) were transfected using electroporation (Amaxa). Briefly, 80% confluent monolayers were trypsinized and 2.5 x 10^6 cells resuspended in Nucleofector V solution (Amaxa) supplemented with siRNA oligos according to manufacturer's instructions. Cells were electroporated using program T20, transferred to pre-warmed growth medium and incubated at 37°C for no more than 10 minutes. Cells were than replated at a confluent density and fresh proliferation media replaced 24 hours post-transfection. Optimization with pmaxGFP (Amaxa) consistently resulted in 75% transfection efficiency.

RESULTS

Changes in p27 protein abundance relative to cell cycle phase transitions. While it is generally accepted that Cdk2 driven G_1/S phase transition is dependent on obligatory suppression of p27 activity, numerous phase-specific mechanisms regarding p27 inhibition have recently been described. To determine how p27 is regulated relative to cell cycle progression of replicating preadipocytes, we first determined the kinetics of phase transition with flow cytometric DNA analysis. Preadipocytes were propagated until reaching saturation density and maintained in growth medium containing 10% calf serum

until 2 days post-confluence. Density-arrested preadipocytes were stimulated to re-enter the cell cycle by switching to differentiation medium containing 10% FBS supplemented with MDI. Cells were harvested over time following exposure to MDI, stained with propidium iodide, and analyzed for DNA content. As illustrated in Figure 2.1A, all histograms displayed two DNA peaks in the 2n and 4n range representing G_0/G_1 and G_2/M cell populations, respectively. No significant apoptotic or polyploidy cells were noted. DNA histograms were analyzed with ModFit software (Becton Dickinson) to determine the distribution of cells in each phase of the cell cycle. As graphically illustrated in Figure 2.1B, 88% of density-arrested preadipocytes presented with G_0/G_1DNA content prior to stimulation (0hr post-MDI), with remaining 12% partitioned equally between S and G_2/M phases. Following exposure to MDI, cells progressively moved through G_1/S and S/G_2 phase transitions at 15 hrs and 19 hrs, respectively. Peak S (64%) and G_2/M (54%) phases were observed at 17 hrs and 21 hrs, respectively. Within 32 hrs following MDI treatment, 82% of the cell population again exhibited G_0/G_1 phase DNA content demonstrating the synchronous completion of one cell cycle. These estimations of the kinetics of phase transition of 3T3-L1 preadipocytes during clonal expansion are consistent with those obtained over time following subconfluent replating of density-arrested NIH3T3 fibroblasts (Shirane et al., 1999).

To analyze cell cycle protein abundance with respect to phase transition, total soluble protein was harvested under identical conditions employed in Figure 2.1A, resolved with SDS-Page, and immunoblotted with various anti-cyclin and p27 antibodies. The first lane of Figure 2.1C, representing the protein profile of proliferating preadipocytes (PPA) at ~70% confluence, illustrates that Cyclin D1 was the only protein examined showing modest accumulation under asynchronous, subconfluent growth

conditions. As cells reached two days post-confluence (0 hr), a divergent profile was observed with minimal and maximal accumulation of cyclin proteins and p27, respectively, providing further evidence that this condition represented a G_0/G_1 state of density-induced growth arrest. Following MDI stimulation, Cyclin D1 rapidly accumulated, peaked during mid-G_1 and progressively decreased during S and G_2/M phases. The onset of Cyclin A and Cyclin B1 accumulation marked the transition through S and G_2 phases, respectively, with both declining abruptly during late G_2/M phase. The abundant accumulation of p27 protein observed during density arrest progressively declined throughout the cell cycle with 72% and 55% of maximal levels remaining at 12 hrs (mid-G_1 phase) and 16 hrs (G_1/S transition) post-MDI, respectively. Peak decline in p27 protein abundance, however, was not observed until 22 hrs post-MDI coordinate with G_2/M phase progression with 35% of maximal abundance remaining. Comparing peak accumulation in cyclin protein levels following MDI stimulation with subconfluent proliferating preadipocytes (PPA) clearly illustrates the synchrony in which these density-arrested preadipocytes reentered and progressed through the cell cycle. Collectively, these data demonstrate that synchronous cell cycle progression correlates with a marked decline in p27 protein accumulation that began in mid-G_1 and peaked during G_2/M with a 65% decrease in p27 relative to maximal levels observed during density arrest.

Increased rate of p27 protein degradation. To determine the mechanism by which p27 protein abundance decreased during clonal expansion, total soluble protein and RNA were harvested at 0 hr and 20 hr post-MDI and subjected to immunoblotting and RT-PCR analysis of p27 protein and mRNA, respectively. As an internal control, 18S ribosomal RNA was determined concurrently in the same PCR reaction using

competimer technology. Densitometric analysis of representative findings depicted in Figure 2.2A is illustrated in Figure 2.2B. Stimulation of density-arrested 3T3-L1 preadipocytes with MDI resulted in a decrease in p27 protein accumulation to 35% of maximal abundance by 20 hrs with no change in mRNA demonstrating the involvement of a posttranscriptional event. To further categorize the mechanism, density-arrested cells were cultured with and without MDI for 18 hrs. At that point, protein synthesis was blocked with 30 μM cycloheximide. Total soluble protein was harvested over time and immunoblotted for p27 to examine the rate of protein decay. As shown in Figure 2.2C, MDI stimulation resulted in a 2-fold increase in p27 protein turnover ($t_{1/2}$) with 6.42 hrs and 3.25 hrs representing the half life of p27 during density arrest (G_0/G_1) and G_2/M phase, respectively. Estimations of p27 protein stability are consistent with other reports (Beniston and Campo, 2003). These data collectively demonstrate that the change in p27 protein abundance during cell cycle progression of replicating preadipocytes involves posttranscriptional events that include mechanisms mediating changes in protein degradation.

Proteasome-dependent p27 degradation. Multiple mechanisms involving p27 degradation during cell cycle progression have been described including proteolytic processing, as well as calpain and proteasome mediated degradation. To determine what role the proteasome plays in regulating p27 degradation during preadipocyte clonal expansion, we tested the effects of two potent, highly selective, irreversible, cell permeable, and structurally distinct proteasome inhibitors, epoxomicin and lactacystin. Both compounds inhibit all three major proteolytic activities of the 20S catalytic core of the 26S proteasome: chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide hydrolyzing (PGPH) activity (Meng et al., 1999;Kim et al., 1999;Fenteany et al., 1995).

Unlike many proteasome inhibitors, however, both epoxomicin and lactacystin do not inhibit other common proteases such as trypsin, chymotrypsin, papain, cathespin B, and calpain at concentrations up to 50 μM for epoxomicin (Sin et al., 1999) and 100 μM for lactacystin (Fenteany et al., 1995). The observation that the inhibitory concentration of epoxomicin was 25 times less than that for lactacystin (IC_{50} of 80 nM and 2 μM, respectively) is consistent with other reports demonstrating that epoxomicin is a more potent inhibitor of chymotrypsin-like activity than lactacystin (Meng et al., 1999). As proteasome proteolytic activity is known to play a permissive role in G_1-to-S phase progression, we first tested the effects of either inhibitor on S phase progression following MDI stimulation of density-arrested preadipocytes. As illustrated in Figure 2.3A, epoxomicin (1μM) or lactacystin (10μM), supplemented with MDI, completely abolished S phase progression 20hrs following MDI stimulation with greater than 91% of the cells not exiting the G_0/G_1 phase of the cell cycle. Cell morphology (not shown) and DNA histograms presented no evidence of apoptosis or cellular stress during this limited exposure of either inhibitor.

We subsequently tested both epoxomicin and lactacystin for their ability to suppress p27 degradation during preadipocyte replication. Density-arrested preadipocytes were stimulated with MDI in the absence or presence of either proteasome inhibitor over a range of concentrations given concomitantly with MDI. As shown in Figure 2.3B, both epoxomicin and lactacystin potently inhibited the degradation of p27 in a concentration dependent fashion. The effect was selective for degradation as a constitutively expressed protein, Skp1, was completely unaffected. Densitometric values of accumulated p27 resulting from each inhibitor were normalized to the level of p27 abundance at 20hrs post-MDI in the absence of inhibitor and logarithmically plotted

against inhibitor concentration (Figure 2.3C). The 50% inhibitory concentration (IC_{50}) was estimated at 80 nM for epoxomicin and 2 μM for lactacystin. Maximal suppression of p27 degradation by epoxomicin and lactacystin was estimated at 0.3 μM and 10 μM, respectively.

Ubiquitylation of p27 during Preadipocyte Replication. A polyubiquitin chain formed through isopeptide bonds involving the ε-amino group of internal lysine residues of four or more ubiquitin molecules and a protein substrate selectively targets the substrate to the 26S proteasome for degradation. As the decay in p27 protein is a function of cell cycle progression, proteasome specific inhibitors, such as epoxomicin and lactacystin, could prevent polyubiquitylated p27 degradation directly by blocking the catalytic activity of the 20S core or indirectly by blocking 'phase-specific' signaling necessary for ubiquitin ligase activity and/or other ubiquitin-unrelated proteolytic processes (e.g., calpain) directed toward p27. To determine a specific role for p27 ubiquitylation during preadipocyte replication, we examined the relative abundance and kinetics of accumulated unmodified p27 as well as ubiquitylated p27 (p27-Ub) following proteasome blockade during the course of cell cycle progression. As ubiquitin-modified proteins are difficult to detect due to rapid removal of the ubiquitin chain by endogenous de-ubiquitylating isopeptidases (Figure 2.4A), the isopeptidase inhibitor N-ethylmaleimide (NEM) was included in the lysis buffer of all samples to minimize p27 de-ubiquitylation during cell lysis.

Density-arrested preadipocytes were treated with medium containing MDI supplemented with and without 1 μM epoxomicin. Cell lysates were harvested at designated times post-MDI and immunoblotted for p27 and Skp1 (Figure 2.4B). In the absence of epoxomicin, p27 protein abundance decreased to 75%, 55%, and 35% of

unstimulated levels by 12 hrs (mid-G_1), 16hrs (G_1-to-S phase transition), and 20hrs (G_2/M phase) post-MDI, respectively. When comparing unstimulated (0hr) protein abundance with that observed for any time point post-MDI, it is clearly evident that epoxomicin completely abolished the decline in p27 protein abundance during all phases of cell cycle progression. Inhibition of p27 decay as early as 8 hrs post-MDI also demonstrates sensitivity to proteasomal blockade at a point during mid-G_1 significantly preceding G_1-to-S phase transition. Of additional interest, epoxomicin treatment not only prevented the decay in p27 protein, it also resulted in an increase in unmodified p27 protein accumulation above unstimulated levels (0hr) that plateaued by 12 hrs post-MDI and remained constant throughout 20 hrs of epoxomicin treatment. Finally, it is important to note that epoxomicin had no effect on Skp1 protein accumulation demonstrating the selectivity of proteasomal blockade toward p27 degradation.

Using the same experimental paradigm as described in Figure 2.4B, the kinetics and pattern of p27 ubiquitylation was examined by extended exposure of direct immunoblotting analysis of p27. As illustrated in Figure 2.4C, we consistently observed four or more p27 immunoreactive bands that migrated slower than unmodified p27 following epoxomicin treatment. These distinct bands (labeled with reverse text) likely represent ubiquitin modified p27 for the following reasons. Firstly, the intensity of the bands significantly and continuously increased with time following epoxomicin treatment of MDI-stimulated cells. Secondly, the mass of each band is consistent with the consecutive addition of 8 kDa ubiquitin moieties to unmodified p27 (i.e., 35, 43, 51, 59, and 67 kDa). Thirdly, these slower migrating bands were less evident in the absence of NEM in the lysis buffer (not shown). Fourthly, the *in vivo* ubiquitylation pattern reported here is consistent with numerous reports of *in vitro* p27 ubiquitylation under defined

conditions reported elsewhere. With these assumptions, ubiquitin-modified p27 was minimally visible by 8 hrs of MDI stimulation in the presence of proteasomal blockade and continued to increase unequivocally with each 4 hrs of epoxomicin exposure. Cell cycle progression in the absence of epoxomicin resulted in slight, but marked increased accumulation of ubiquitylated p27 protein by 20-24 hrs post-MDI. These data demonstrate that ubiquitylation is among the proteasome-dependent mechanisms regulating p27 degradation during preadipocyte replication.

Reciprocal accumulation of Skp2/Cks1 with p27 degradation. Skp2-dependent, as well as independent, mechanisms involving p27 ubiquitylation and proteasomal degradation have been described. To explore a role for Skp2 in p27 ubiquitylation during preadipocyte replication, we examined the protein abundance profile of various members of the SCF E3 ligase complex in the context of p27 protein levels and cell cycle phase transition. Total soluble protein was harvested over time following MDI stimulation of density-arrested preadipocytes and immunoblotted as illustrated in Figure 2.5. Under conditions of density arrest exhibiting abundant levels of p27 (i.e., 0 hr), we did not observe any appreciable accumulation of Skp2 or Cks1. Both proteins, however, accumulated concomitant with cell cycle progression. Skp2 protein was first evident during mid-G_1 and peaked in abundance at 22 hrs-post MDI during the S-G_2/M phase transition. Accumulation of Cks1 was delayed to early S phase and peaked along with Skp2 during early G_2/M phase progression. In contrast to these variable components, Skp1 exhibited no variability in abundance throughout the cell cycle. Similarly, no variability was noted in protein abundance of the other known members of the SCF complex, Cul1 and Roc1 (data not shown). In the context of p27 levels, Skp2 and Cks1 kinetically accumulated in a reciprocal manner with the increase

in p27 protein degradation. The highest abundance of Skp2 and Cks1 were observed with the lowest levels of p27 during G_2/M, 22 hrs post-MDI. These data strongly support the notion that Skp2 and Cks1 play a mechanistic role in p27 degradation. Furthermore, the precise timing of Skp2 and Cks1 accumulation is expected to play a permissive role in specific phase transition through temporal changes in p27 degradation.

Cdk2-dependent phosphorylation of p27 on residue Thr187. To further explore a role for the SCF^{Skp2} E3 ligase in p27 degradation, we examined the phosphorylation state of p27 on residue Thr187 which is known to be a prerequisite for p27 recognition by Skp2/Cks1. Total soluble protein was harvested at 0 hr and 18 hrs following MDI treatment and subjected to immunoprecipitation (IP) with a phosphospecific p27(T187) antibody. Comparing lanes 1 and 2 of Figure 2.6A illustrates that the abundance of p-p27(T187) was elevated during S-to-G_2/M phase transition (18 hrs post-MDI) relative to density-arrest (0 hr post-MDI). This pattern of accumulation was also observed with input protein (lanes 3 and 4), but to a lesser extent, demonstrating the effectiveness of the IP in concentrating the relative magnitude of protein accumulation. Input protein also showed a faster migrating p27 immunoreactive band with relative mass consistent with hypophosphorylated p27, suggesting minimal cross reactivity between Thr187 phosphorylated and non-phosphorylated forms of this protein. The observation that the faster migrating band decreased during S-to-G_2/M phase transition adds further support to the premise that this immunoreactive band represents the declining pool of p27 observed during cell cycle progression. These data are consistent with the notion that a phase-specific increase in p-p27(T187) leads to p27 ubiquitylation and subsequent degradation, decreasing total p27 during phase transition.

To confirm p27 phosphorylation, p-p27(T187) immunoprecipitates, harvested 18 hrs post-MDI, were treated prior to electrophoresis with or without Protein Phosphatase 1 (PP1) which displays selective phosphatase activity towards phosphoserine/threonine residues. Based on our working model that p-p27(T187) is a substrate for SCF E3 ligase activity, we also explored the premise that p-p27(T187) immunoreactive immunoprecipitates would migrate as successively slower bands with relative mass consistent with p27 ubiquitylation. As shown in Figure 2.6B, PP1 treatment dramatically decreased the abundance of multiple phosphorylated p27 reactive species that migrated slower in the gel as polyubiquitylated p27 (compare lanes 1 and 2). The effect of PP1 was most pronounced on reactive species with relative mass equivalent to p-p27(Ub_0), p-27(Ub_1), and p-p27(Ub_4) as illustrated.

We further explored the identity of the kinase responsible for phase-specific p27 phosphorylation. Density arrested preadipocytes were stimulated with and without MDI supplemented with specific inhibitors Roscovitine to block Cdk2 activity or LY294002 to block phosphatidylinositol 3-kinase (PI3K) and the downstream kinase Akt/PKB. Total soluble protein was harvested at 18 hrs following stimulation and immunoprecipitated for p-p27(T187). As depicted in Figure 2.6C, Roscovitine dramatically attenuated the level of all immunoreactive p-p27 immunoprecipitates (compare lane 1 and 2) as well as all immunoreactive species observed with input protein (compare lanes 4 and 5). In contrast, LY294002 had minimal effect on p-p27(T187) accumulation. These data strongly suggest that Cdk2 activity is responsible for p27 phosphorylation on residue Thr187 leading to ubiquitylation and degradation.

Inhibition of Cdk2 activity partially rescues p27 degradation. Proteasome-dependent degradation of p27 is known to occur through Thr187-dependent as well as

Thr187-independent mechanisms. To determine the contribution of Cdk2 in proteasome-mediated p27 degradation, density-arrested preadipocytes were stimulated with MDI supplemented with and without the specific inhibitors epoxomicin to block proteasome activity or roscovitine to block Cdk2 activity. Total soluble protein was harvested at 0 hr and 20 hrs post-MDI and immunoblotted for p27, Skp1 and Cyclin A as illustrated in Figure 2.7A. Consistent with other data presented in this report, p27 accumulation during G_2/M phase represented ~35% of that observed during density arrest. The dramatic increase in Cyclin A protein, which is known to accumulate acutely during S/G_2 phase, confirmed cell cycle progression with MDI stimulation. In the context of cell cycle progression, inhibition of proteasome catalytic activity with epoxomicin completely abolished the decrease in p27 as well as the increase in Cyclin A at 20 hrs post-MDI. While blocking Cdk2 activity with Roscovitine had similar effects on Cyclin A accumulation, the rescue of p27 degradation was incomplete with approximately 65% accumulation relative to density arrest (0 hr). Consistent with ablation of Cyclin A accumulation, both inhibitors equally prevented S phase progression and established G_0/G_1 arrest. No G_1 subpopulation of apoptotic cells were noted with either inhibitor. These data demonstrate that while Cdk2 activity is essential for S phase progression, it accounts for only part of proteasome-dependent p27 degradation.

Phase-specific Skp2 protein accumulation partially accounts for p27 degradation. To determine the contribution of Skp2 in p27 degradation, RNA interference (RNAi) was employed to knockdown Skp2 accumulation during cell cycle progression. Subconfluent preadipocytes were harvested and electroporated with two independent, sequence-distinct pools containing four short interfering RNAs (siRNAs) designed to specifically suppress murine Skp2 gene expression. To detect any off-target

effects caused by transfection, cells were also electroporated with a 'mock' pool of non-functional, non-targeting siRNAs. Following siRNA transfer, cells from both groups were replated at near confluency and stimulated with MDI at 2 days post-confluence (i.e., density arrest). As illustrated in Figure 2.8A, both Skp2 siRNA pools completely abolished Skp2 protein accumulation at 20 hrs post-MDI. The extent of the knockdown was not unexpected as these studies involved preventing protein accumulation versus knocking down the level of a constitutively expressed protein. Mock siRNA had no effect on Skp2 accumulation at 20 hrs post-MDI when compared to non-transfected cells (not shown). Both populations of siRNAs for Skp2 equally and partially rescued p27 accumulation to 61% (RNAi-1) and 59% (RNAi-2) of p27 levels observed at density arrest (0hr). Interestingly, this level of p27 rescue was nearly identical to the 65% rescue observed when Cdk2 activity and phospho-Thr187 dependent p27 ubiquitylation were blocked with Roscovitine (Figure 2.7A). Specificity was suggested as siRNA for Skp2 had no effect on Skp1 protein levels.

Considering the marked rescue of p27 degradation during S phase progression under conditions of Skp2 knockdown, we next determined the effect of Skp2 RNAi on phase transition. As illustrated in Figure 2.8A, we observed the expected accumulation of Cyclins D1, A, and B1 at 20 hrs post-MDI. Unexpectedly, however, Skp2 knockdown and resulting partial rescue of p27 degradation had no effect on protein accumulation of each cyclin suggesting that G_1-to-S phase transition was not obstructed. Under identical conditions, RNAi for Skp2 and non-targeting sequences (mock) presented with identical overlapping DNA histograms representing S-G_2/M phase progression at 20 hrs post-MDI (Figure 2.8B), suggesting that Skp2-mediated p27 degradation is not required for G_1-to-

S phase transition. Population doubling after one cell cycle was also similar between Skp2 and Mock siRNA treated cells (not shown).

Elevated interaction with between p27 and Cyclin D1/Cdk4 following Skp2 RNAi. We also observed that Skp2 knockdown modestly increased protein accumulation (~25%) of Cyclin D1 and Cdk4 relative to non-targeting siRNA (mock) treated cells at 20 hrs post-MDI (Figure 2.8A). As Cyclin D1/Cdk4 can bind and neutralize p27 inhibitory activity (Sherr and Roberts, 1999), we explored the possibility of increased protein-protein interaction between Cyclin D1/Cdk4 and p27. Skp2 and non-targeting siRNA pools were introduced into preadipocytes as described above. Lysates, collected from cells at 0 hr (density arrest) and 20 hrs post-MDI ($S\text{-}G_2/M$), were immunoprecipitated for p27 (polyclonal Ab) and immunoblotted for p27 (monoclonal Ab), Cdk2, Cyclin D1, and Cdk4 (Figure 2.9A). Similar p27 protein patterns were detected with p27 immunoprecipitates relative to input protein validating the IP procedure. Relative to density arrest (0hr), S/G_2 phase transition correlated with a 62% and 33% decrease in p27 protein with mock (compare lanes 3 vs 2) and Skp2 (compare lanes 4 vs 2) siRNAs, respectively.

For quantitation of protein-protein interaction, densitometric data of Cdk2, Cyclin D1, and Cdk4 were normalized to the level of immunoprecipitated p27 protein for each condition. These values were subsequently normalized to mock RNAi at 0 hr post-MDI and the results depicted in Figure 2.9B. Thus, values greater and lesser than one represent respective changes in p27 protein interaction relative to density arrest. The level of Cdk2-p27 protein interaction decreased by 24% and 19%, respectively, with mock versus Skp2 knockdown. This decrease in Cdk2-p27 protein interaction was consistent with expected elevated Cdk2 activity during this phase of the cell cycle (Gu et

al., 1992). Nearly identical Cdk2-p27 protein interaction between mock and Skp2 knockdown was also consistent with the observation that Skp2 knockdown produced no impediment to phase transition.

In contrast to the decrease in p27-Cdk2 interaction during S/G_2 phase transition, p27-Cyclin D1 and p27-Cdk4 protein interaction increased by 297% and 188%, respectively, with non-targeting RNAi. Under conditions of Skp2 knockdown, p27 interaction with Cyclin D1 and Cdk4 increased equivalently to 427% and 424%, respectively, relative to density arrest. These data support the premise that elevated p27 protein resulting from Skp2 knockdown was sequestered through elevated p27 interactions with Cyclin D1/Cdk4 during S/G_2 phase. These selective protein-protein interactions, presumably, suppressed the expected inhibition of Cdk2 activity associated with elevated p27 protein levels during S/G_2 transition under Skp2 knockdown conditions.

It was also noted that elevated protein-protein interactions resulting from Skp2 knockdown were not simply a function of elevated input protein. Under mock conditions, cell cycle progression at 20 hrs post-MDI correlated with a 45%, 43%, and 22% increase in Cdk2, Cyclin D1, and Cdk4 protein accumulation (input protein), respectively. Skp2 knockdown modestly increased S/G_2 accumulation for Cdk2, Cyclin D1, and Cdk4 to 61%, 64%, and 52%, respectively. Even considering the decrease in p27 protein during S/G_2, it is unlikely that differences in protein levels would account for a greater than 400% increase S/G_2 phase p27-Cyclin D1/Cdk4 interactions under conditions of Skp2 knockdown.

DISCUSSION

The observation that removal of p27 inhibitory activity is rate limiting for Cdk2 activation and that p27 proteins levels decrease during clonal expansion of density-arrested preadipocytes raises three important questions: 1) Does decreased p27 protein accumulation result from elevated protein turnover involving a proteasome-dependent mechanism(s)? 2) Is p27 phosphorylation by Cdk2 involved in p27 ubiquitylation by the SCF^{Skp2} E3 ligase during S and G_2 phases? and 3) Is Skp2-mediated p27 degradation essential for G_1/S phase transition? While previous reports have demonstrated that p27 protein levels decrease during 3T3-L1 preadipocyte clonal expansion (Morrison and Farmer, 1999;Patel and Lane, 2000;Tang et al., 2003;Zhang et al., 2004), only one study has reported that p27 is regulated by a calpain-dependent, proteasome-independent mechanism (Patel and Lane, 2000). In the current study we elaborated on mechanisms regulating p27 degradation by distinct mechanisms at different points in the cell cycle and determine the necessity for proteasome-mediated proteolysis for proliferation of preadipocytes during adipocyte hyperplasia.

To address the hypothesis that phase-specific mechanisms modulate p27 activity during cell cycle progression, we initially characterized the kinetics of protein fluctuation in precise reference to the timing of phase transitions. p27 protein levels decreased modestly in mid-G_1, decreasing further near the G_1/S phase transition, and maximally during G_2 phase. The early onset and extended period of p27 suppression as well as variant rates of protein fluctuation during specific points of the cell cycle strongly suggest the possibility that phase-specific mechanisms are likely involved as discussed below. The observation that p27 protein levels dramatically decreased over the course of G_1/S/G_2 phase transitions in the absence of any change in mRNA accumulation

demonstrates that transcriptional mechanisms, as well as any process involving p27 mRNA stability, are not involved during activation of preadipocyte cell cycle progression. We and others have determined that proliferative signals of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways synergistically mediate 3T3-L1 clonal expansion. A major target of PI3K signaling, Akt/PKB has been shown to directly phosphorylate and inactivate a family of Forkhead transcription factors consisting of Foxo4, Foxo1, and Foxo3a (Kops et al., 2002;Brunet et al., 1999;Rena et al., 1999). Others have further demonstrated that all three Forkhead family members inhibit cell cycle progression at the G_1/S transition, at least in part by controlling p27 gene expression (Medema et al., 2000;Nakamura et al., 2000;Brunet et al., 2001). In previous work, which focused on PPARγ mediated changes in p21 and p18 gene expression, it was reported that p27 mRNA levels decrease within two days of 3T3-L1 preadipocyte differentiation (Morrison and Farmer, 1999). While it is possible, even probable, that PI3K activation leads to a decrease in p27 mRNA at a subsequent point of differentiation (e.g., second cell cycle of clonal expansion), data presented here demonstrate that transcriptional mechanisms mediating changes in p27 gene expression are not involved in the initial G_1/S phase transition as preadipocytes undergo the transition from quiescence to proliferation.

In the absence of mRNA fluctuations, changes in p27 protein levels are regulated by translational mechanisms and/or processes mediating p27 degradation. We demonstrate in this report that cell cycle progression is marked by a 2-fold increase in the rate of p27 turnover during S/G_2 phase transition. While these data clearly demonstrate a role for regulated p27 proteolysis (e.g., calpain, proteasome, caspases), they do not rule out mechanisms involving translational control. Other studies have

reported that p27 protein levels decrease during cell cycle progression, in part through decreased p27 translation (Hengst and Reed, 1996). During the course of this investigation, we determined that rapamycin, a specific chemical inhibitor of the mTOR pathway, resulted in a modest increase in p27 protein levels during G_1/S phase transition (not shown). While this observation suggests a potential for p27 translational control, a direct mechanism was not determined. As we also found that Skp2 protein is sensitive to rapamycin treatment, it is plausible that mTOR blockade could lead to increased p27 protein accumulation, indirectly, through decreased p27 protein degradation. This level of regulation is often overlooked as a contributor to p27 regulation, therefore, further studies are needed to elucidate the role of translational control in p27 degradation during preadipocyte replication.

To address the question of whether p27 degradation is proteasome-dependent, we utilized two structurally distinct proteasome inhibitors, epoxomicin and lactacystin. The 26S proteasome complex is composed of a 20S catalytic core flanked by two outer 19S regulatory domains. The 20S core is a cylindrical-shaped complex made up of 28 subunits organized into four rings where the outer two rings contain seven different alpha subunits and the inner two rings contain seven different beta subunits. Epoxomicin covalently binds four of the beta subunits and potently inhibits the chymotrypsin-like activity of the proteasome without inhibiting any other tested protease (i.e., calpain, cathepsins, papain, trypsin, and chymotrypsin) at concentrations up to 50 μM (Meng et al., 1999). Lactacystin also binds covalently to a beta subunit of the 20S core and selectively inhibits all three proteasomal activities at different rates. Unlike epoxomicin, however, lactacystin also inhibits cathepsin A and tripeptidyl peptidase II, but without effect on other serine proteases (trypsin and chymotrypsin) and cysteine proteases

(calpain I, calpain II, papain, and cathepsin B) (Fenteany et al., 1994;Fenteany and Schreiber, 1998). Although widely used to study proteasome function, other non-specific inhibitors, such as peptide aldehydes (i.e., MG115 and MG132) and N-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN), also inhibit lysosomal (e.g., cathepsins) and calcium-activated proteases (e.g., calpains).

In this report, epoxomicin and lactacystin completely abolished the decrease in p27 protein associated with cell cycle progression with absolutely no effect on accumulation of the constitutively expressed protein, Skp1, strongly suggesting that the decrease in p27 protein was the result of increased degradation by the proteasome. While other studies using non-specific inhibitors have reached the same conclusion, these are the first data demonstrating proteasome specificity through the effect of epoxomicin on p27 degradation. As the decrease in p27 involves signaling events associated with cell cycle progression, it is conceivable that proteasome inhibitors could prevent the decrease in p27, indirectly, by inhibiting cell cycle events (e.g., Cdk2 activity) that signal the timely destruction of p27. To address this, we also present data demonstrating that epoxomicin treatment throughout G_1 phase increased ubiquitylated p27. The data provide direct evidence that p27 is polyubiquitylated during cell cycle progression of replicating preadipocytes. In the presence of epoxomicin, ubiquitylated p27 is not degraded by the proteasome.

Data are also presented clearly demonstrating that both epoxomicin and lactacystin unequivocally block S phase progression of clonal expansion resulting in G_1 phase arrest. While these data are consistent with other reports demonstrating that proteasome inhibitors effectively block cell proliferation, little is known concerning the mechanism of proteasome inhibitor mediated cell cycle arrest. From data presented in

this report, it could be speculated that preventing the decline in p27 protein levels in the presence of epoxomicin would prohibit Cdk2 activation during late G_1 phase, thereby inhibiting G_1/S phase transition. While elevated p27 protein levels are hypothesized to play a role, blocking the proteasome involves other cell cycle related events. For example, we have also determined that both epoxomicin and lactacystin treatment lead to a dramatic and sustained increase in p21 protein accumulation during mid-G_1 phase providing an additional mechanism supporting G_1 arrest resulting from proteasome blockade. In contrast to the data presented here, others have reported that while lactacystin (10 μM) was an effective inhibitor of subconfluent preadipocyte proliferation, this proteasome inhibitor had no effect 3T3-L1 clonal expansion (Patel and Lane, 2000). While ALLN, which blocks calpain and proteasome activities, effectively suppressed clonal expansion, no other specific proteasome inhibitor was used. The reason for the discrepancy between their findings and ours is unknown.

To address a phase-specific mechanism linking proteasome activity with p27 degradation, we present data demonstrating that 1) p27 protein is phosphorylated on Thr187 during S/G_2 phase, 2) phosphorylated p27 (Thr187) is polyubiquitylated, and 3) p27 phosphorylation is dependent on Cdk2 activity. Other studies have established that p27 can be phosphorylated on residue Thr187 by Cdk2, presumably when the amount of Cyclin E/A-Cdk2 complexes exceed the amount of available p27, triggering p27 degradation (Sheaff et al., 1997;Vlach et al., 1997;Muller et al., 1997;Malek et al., 2001). This pathway of p27 degradation is regulated in a phase specific manner as Cdk2 activation does not occur until late G_1 phase, with maximal activity through S and G_2. While activation of the PI3K/AKT pathway in early G_1 is essential for preadipocyte proliferation, data presented here suggest that this kinase is not responsible for p27

phosphorylation at Thr187. These are important observations as Akt has been shown to phosphorylate p27 at Thr187, Ser10, Thr198, and Thr157 (Fujita et al., 2002;Shin et al., 2002).

Our finding that phosphorylated p27 (Thr187) is also ubiquitylated during clonal expansion is consistent with recent studies establishing a role for Skp2 in ubiquitylation of p27 (Carrano et al., 1999;Sutterluty et al., 1999;Tsvetkov et al., 1999). The SCF^{Skp2} E3 ligase is a multiprotein complex whereby Roc1 and Skp1 interact with Cul1 through its C-terminus and N-terminus, respectively. Roc1 further recruits an E2 ubiquitin conjugating enzyme and Skp1 interacts with the F-box protein, Skp2. Unlike other SCF^{Skp2} substrates, interaction of Skp2 with p27 is dependent on p27 recognition by Cks1 which harbors the phosphorylated Thr187 binding site (Hao et al., 2005). Once assembled, this complex effectively positions p27 in the proper orientation for successive rounds of E2-mediated ubiquitin conjugation. In this report, we also demonstrate for the first time that maximal protein accumulation for Skp2 and Cks1 is restricted to S and G_2 phases of mitotic clonal expansion.

Considering the kinetics of 1) Cdk2 activation, 2) p27 (Thr187) phosphorylation, and 3) Skp2 and Cks1 protein accumulation, we propose that p27 phosphorylation and ubiquitylation by the SCF^{Skp2} E3 ligase is mechanistically responsible, in part for phase-specific p27 degradation during S and G_2 phase progression. While data presented in this report provide direct evidence confirming a role for Skp2 in p27 degradation through selective RNAi, several lines of evidence suggests that Skp2-mediated p27 ubiquitylation is only partially responsible for proteasome-mediated p27 degradation during preadipocyte replication. First, we demonstrate that specific proteasome inhibitors, such as epoxomicin and lactacystin, completely abolish any decrease in p27

protein levels during cell cycle progression. Specific blockade of Cdk2 activity with Roscovitine partially prevented p27 degradation. If proteasome-mediated p27 degradation was completely dependent on p27 phosphorylation by Cdk2, it would be expected that Roscovitine should completely prevent p27 degradation. Our observation that Skp2 knockdown partially prevented the decline in p27 supports the same premise that other proteasome-dependent mechanisms are also involved. Second, p27 protein levels began to decline during mid-G_1 at a point preceding Cdk2 activation and maximal accumulation of Skp2 and Cks1 protein.

Results from recent studies suggest that the SCF^{Skp2} pathway plays a role in proteasome-mediated p27 degradation during cell cycle progression by maintaining p27 protein at low levels during S and G_2 phases, but not for the decline in p27 protein that has been postulated as a prerequisite for G_1/S transition. Using a genetic knockin model replacing endogenous p27 with mutant p27(T187A) that cannot be phosphorylated, it was observed that both wildtype p27 as well as mutant p27 declined during G_1/S phase transition (Malek et al., 2001). In contrast to wildtype p27, p27(T187A) protein re-accumulated as cells completed G_1 and entered S phase suggesting that phosphorylation of p27 at Thr187 and subsequent ubiquitylation by SCF^{Skp2} E3 ligase activity functions to maintain p27 at low levels during S and G_2 phase transition.

To address the final question of whether Skp2-mediated p27 degradation is required for G_1/S phase as quiescent preadipocytes reenter the cell cycle, data presented here clearly demonstrate that while Skp2 RNAi markedly increased p27 protein accumulation, the near complete knockdown of Skp2 had absolutely no effect on S phase progression during synchronous cell cycle progression of preadipocyte mitotic clonal expansion. These observations are supported by other reports demonstrating that

Skp2 RNAi did not alter the length of the cell cycle nor the proportion of cells in S phase (Duan et al., 2001). Furthermore, other reports have demonstrated that lymphocytes from wildtype and Skp2 knockout animals have identical phase distribution profiles for up to three days after which cells accumulate in G_1 suggesting that elevated p27 protein resulting from the absence of Skp2 leads to G_1 arrest during subsequent cell cycles (Hara et al., 2001).

While no direct role for Skp2 has been demonstrated in Cyclin D1 ubiquitylation (Carrano et al., 1999;Nakayama et al., 2000;Ganiatsas et al., 2001), Skp2 deficient cells have been reported to have modest elevation in levels of Cyclin D1 (Nakayama et al., 2000). In this report, we also present initial evidence suggesting that the inhibitory action of p27 resulting from elevated p27 protein in the absence of Skp2 may be neutralized through sequestration by Cyclin D1/Cdk4 complexes. The amount of Cdk2 associated with p27 decreased during S phase and was nearly identical when comparing the presence or absence of Skp2 knockdown. Conversely, the amount of Cyclin D1 and Cdk4 associated with p27 dramatically increased during S phase progression under conditions of Skp2 knockdown, suggesting the possibility that Cdk2 activation is shielded from elevated p27 inhibitory activity through sequestration of p27 into Cyclin D1/Cdk4 complexes.

In summary, the data presented here support a model for different mechanisms regulating p27 protein degradation at different points in the cell cycle. While p27 degradation throughout cell cycle progression is proteasome-dependent, mechanisms eliminating p27 during late G_1 for Cdk2 activation and G_1/S transition appear to be independent of Skp2-mediated p27 degradation during S and G_2 phase progression. These data provide valuable insight into mechanisms mediating the transition of

preadipocytes from quiescence to proliferation during adipocyte hyperplasia and the onset of hyperplastic obesity.

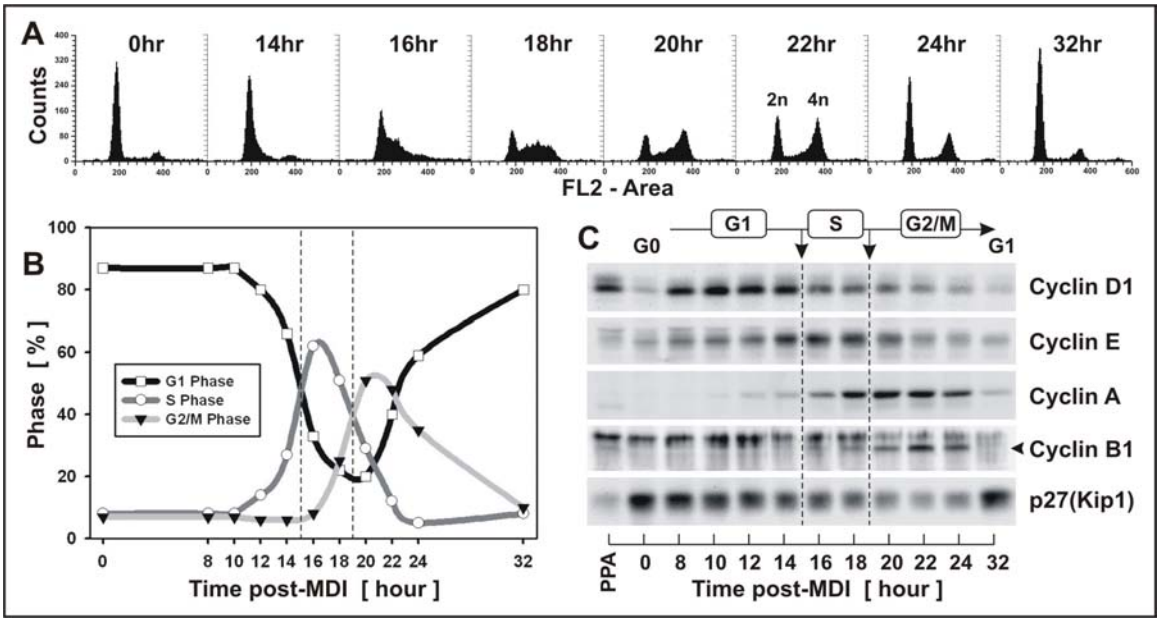

Figure 2.1. Changes in p27 protein accumulation relative to cell cycle phase transition. A. Density-arrested preadipocytes were harvested over time following exposure to MDI and stained with propidium iodide for DNA histograms generated with flow cytometric analysis. B. Cell cycle phase distribution was determined through mod-Fit analysis of DNA histograms and plotted over time following MDI exposure. Dotted lines through phase intersections approximate the kinetics of phase transitions. C. Total soluble protein was harvested at each time point following MDI exposure, separated by SDS-Page, and immunoblotted with designated antibodies. Protein from subconfluent proliferating preadipocytes (PPA) was included for assessment of synchrony.

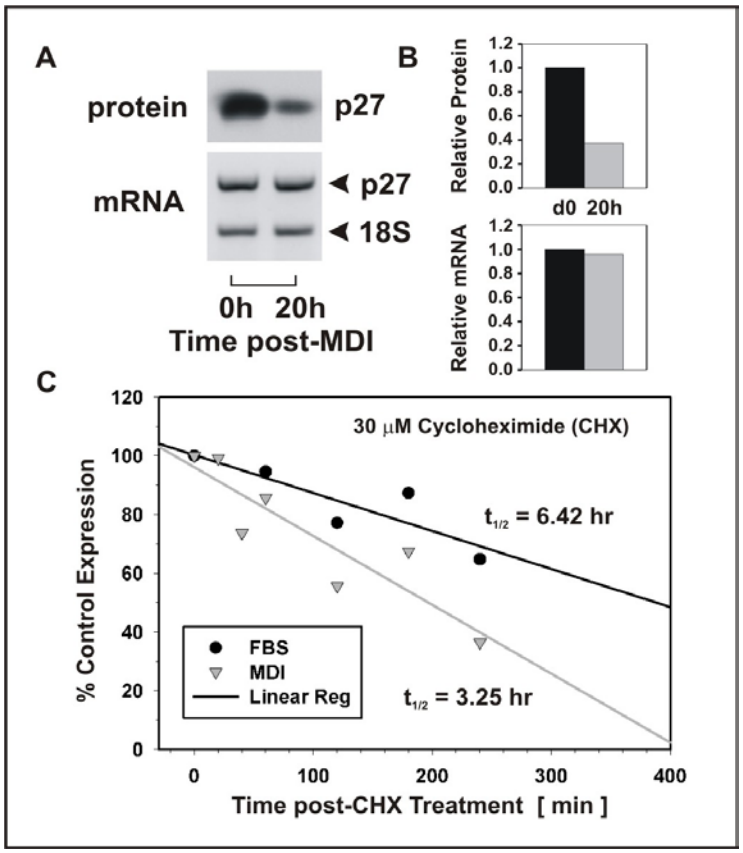

Figure 2.2. Posttranscriptional regulation of p27 during preadipocyte proliferation. A. Total soluble protein and RNA were harvested at 0 hr and 20 hr post-MDI and subjected immunoblotting and RT-PCR analysis of p27 protein and mRNA, respectively. B. Densitometric analysis of protein and RNA abundance at 0 hr and 20 hr post-MDI with p27 mRNA normalized to 18S mRNA. C. Density-arrested preadipocytes were cultured with and without MDI for 18 hrs. At that point, total soluble protein was harvested over time following treatment with 30 μM cycloheximide (CHX) and immunoblotted for p27. Linear regression of protein remaining over time post-CHX was used for estimation of protein half life ($T_{1/2}$).

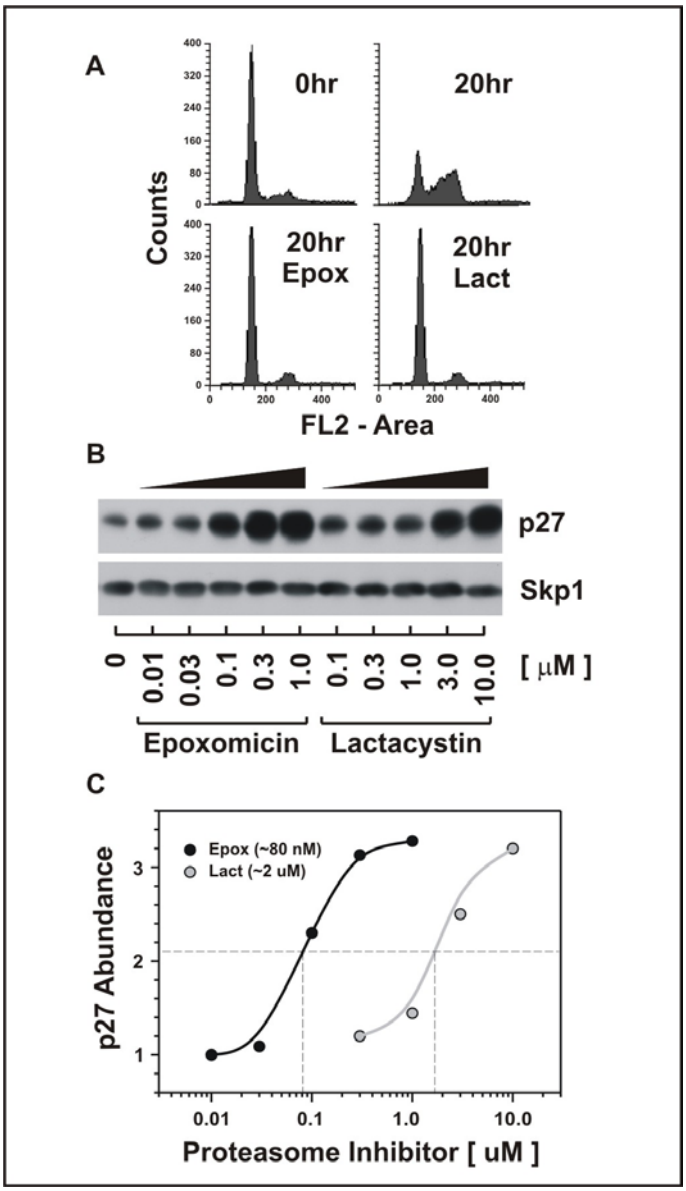

Figure 2.3. Proteasome-dependent inhibition of p27 degradation and S phase progression. A. Cells were harvested at 0 hr and 20 hr post-MDI treatment supplemented with or without Epoxomicin (Epox) or Lactacystin (Lact). Nuclei were stained and DNA histograms generated with flow cytometry. B. Density-arrested preadipocytes were stimulated with MDI for 18 hrs in the presence of increasing proteasome inhibitor concentration. Total cell lysates were harvested and immunoblotted for p27 and Skp2. C. Densitometric of p27 immunoblots were normalized to no treatment levels and plotted logarithmetically against proteasome inhibitor concentrations. Approximate IC_{50} values are illustrated.

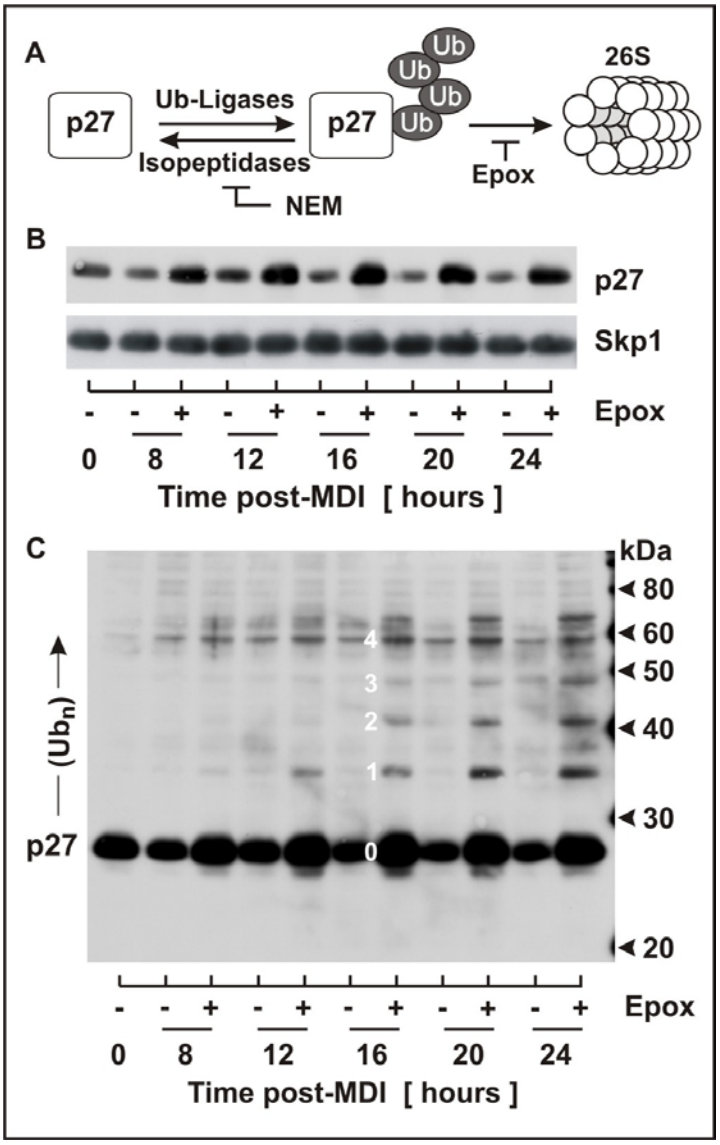

Figure 2.4. Ubiquitin-dependent p27 degradation. A. Diagram of processes regulating the accumulation of ubiquitylated p27 protein. All cell lysates were harvested in the presence of N-ethylmaleimide (NEM) to block de-ubiquitylating isopeptidase activity. B. Cell lysates were harvested from density-arrested preadipocytes at times indicated following MDI stimulation in the presence and absence of 1 μM epoxomicin (Epox) and immunoblotted as illustrated. C. Pattern of p27 ubiquitylation was examined by extended exposure of direct immunoblotting analysis of p27. Numbers on the right represent the mobility of molecular weight markers (kDa).

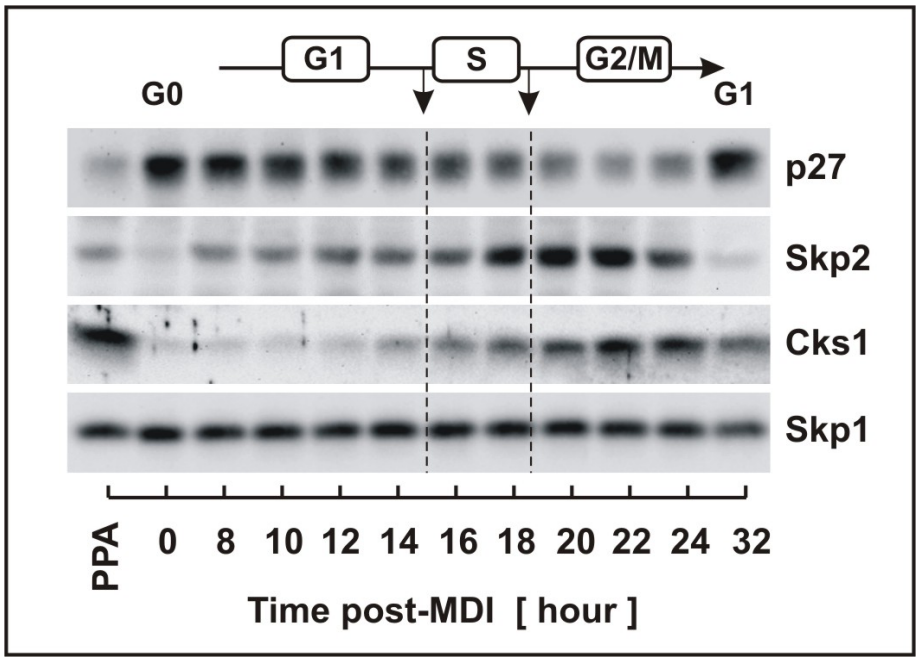

Figure 2.5. Maximal Skp2/Cks1 protein levels during S/G_2 phase progression. A. Total cell lysates were harvested from density-arrested preadipocytes at 0hr and 18hr post-MDI treatment, and immunoblotted for as illustrated. Dotted lines represent phase transitions as determined in Fig.1B.

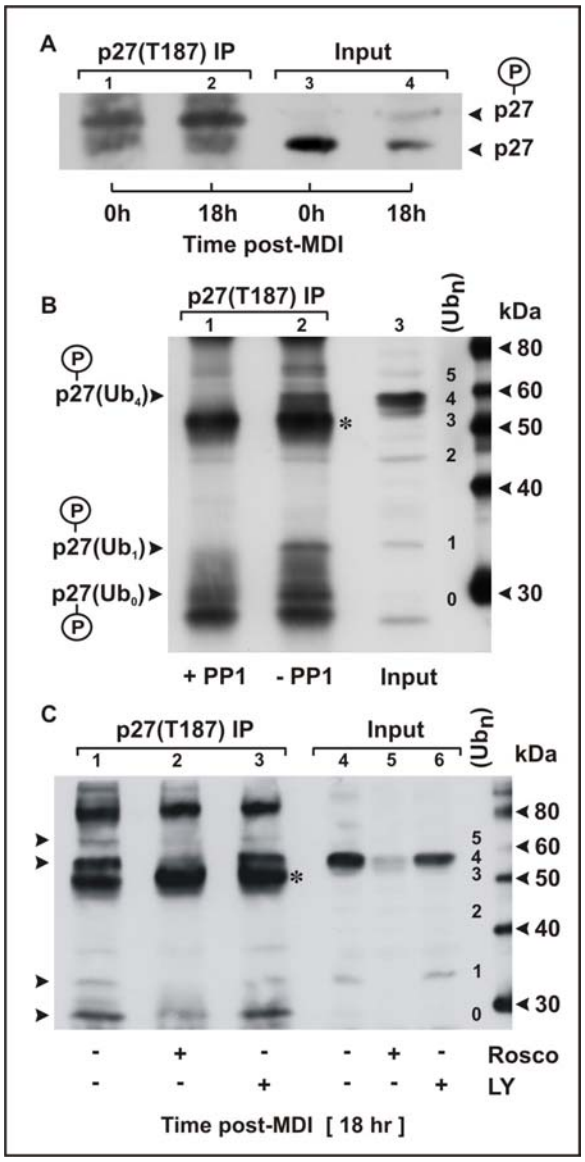

Figure 2.6. Ubiquitylated p27 phosphorylated on Thr187 by Cdk2. A. Total cell lysates were harvested from density-arrested preadipocytes at 0hr and 18hr post-MDI and immunoprecipitated (IP) with a polyclonal phosphospecific p27(T187) antibody. Immunoprecipitates were separated by SDS-Page electrophoresis and immunoblotted with a monoclonal p27 antibody. B. 18 hr post-MDI cell lysates were immunoprecipitated for phospho-p27(T187), treated with and without protein phosphatase-1 (PP1), and immunoblotted for p27. C. Total cell lysates from density-arrested preadipocytes, treated with MDI for 18 hrs in the presence or absence of 25μM Roscovitine (Rosco) or 10μM LY294002 (LY), were immunoprecipitated for phospho-p27(T187) and immunoblotted for p27. Input data were generated by immunoblotting 10% of protein used for immunoprecipitation. Asterisks denote IgG heavy chain. Numbers on the right represent the mobility of molecular weight markers (kDa).

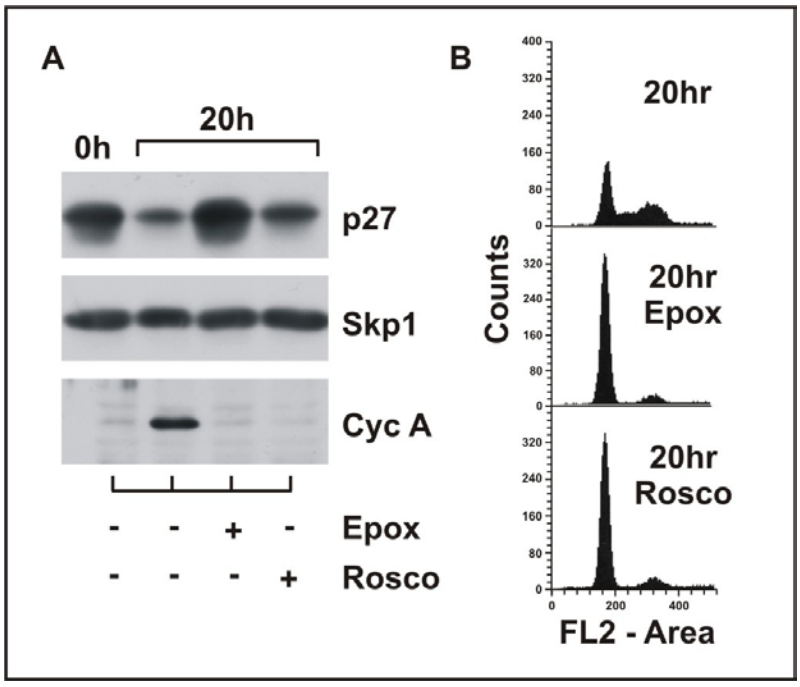

Figure 2.7. Cdk2 inhibition partially prevents p27 degradation. A. Total cell lysates, harvested from density-arrested preadipocytes at 0hr and 20hr post-MDI supplemented with and without 1μM Epoxomicin (Epox) or 25μM Roscovitine (Rosco), were separated by SDS-Page electrophoresis and immunoblotted for as illustrated. B. DNA histograms from cells treated as in panel A.

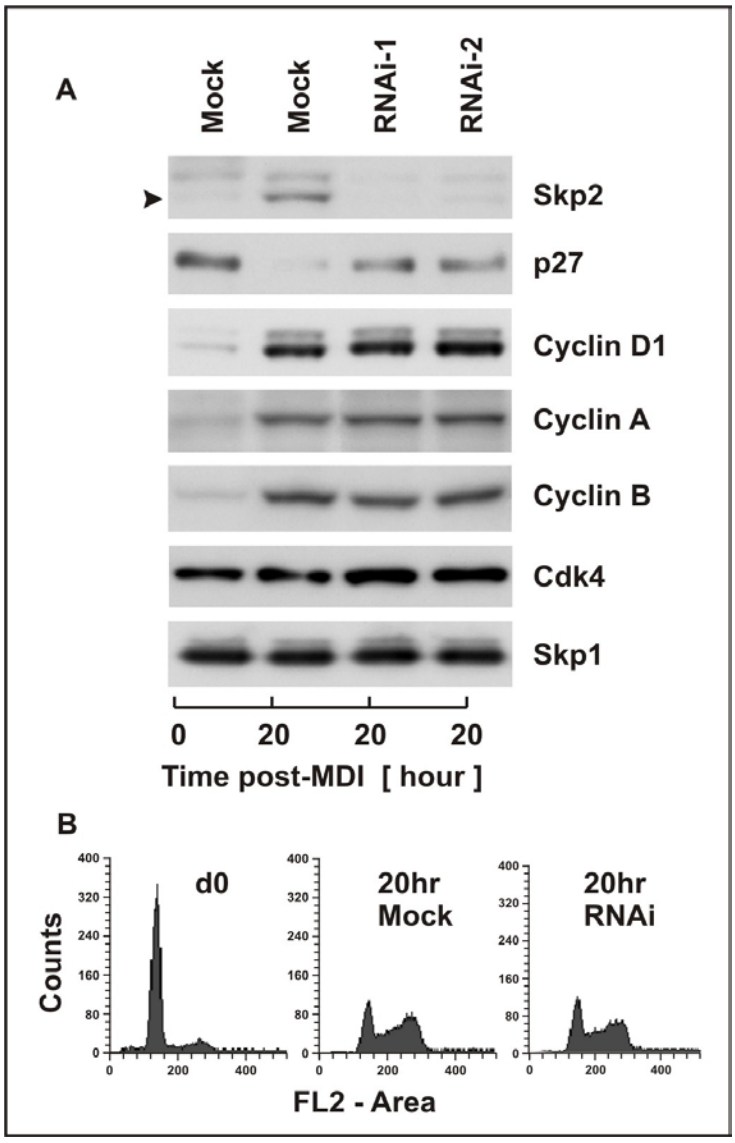

Figure 2.8. RNAi Skp2 knockdown partially prevents p27 degradation. A. Cells were transfected with non-targeting siRNA (Mock) and two different pools of siRNA for Skp2. Total cell lysates were harvested from density-arrested preadipocytes transfected with siRNA at 0hr and 20hr post-MDI, separated by SDS-Page electrophoresis and immunoblotted as illustrated. B. DNA histograms from cells treated as in panel A.

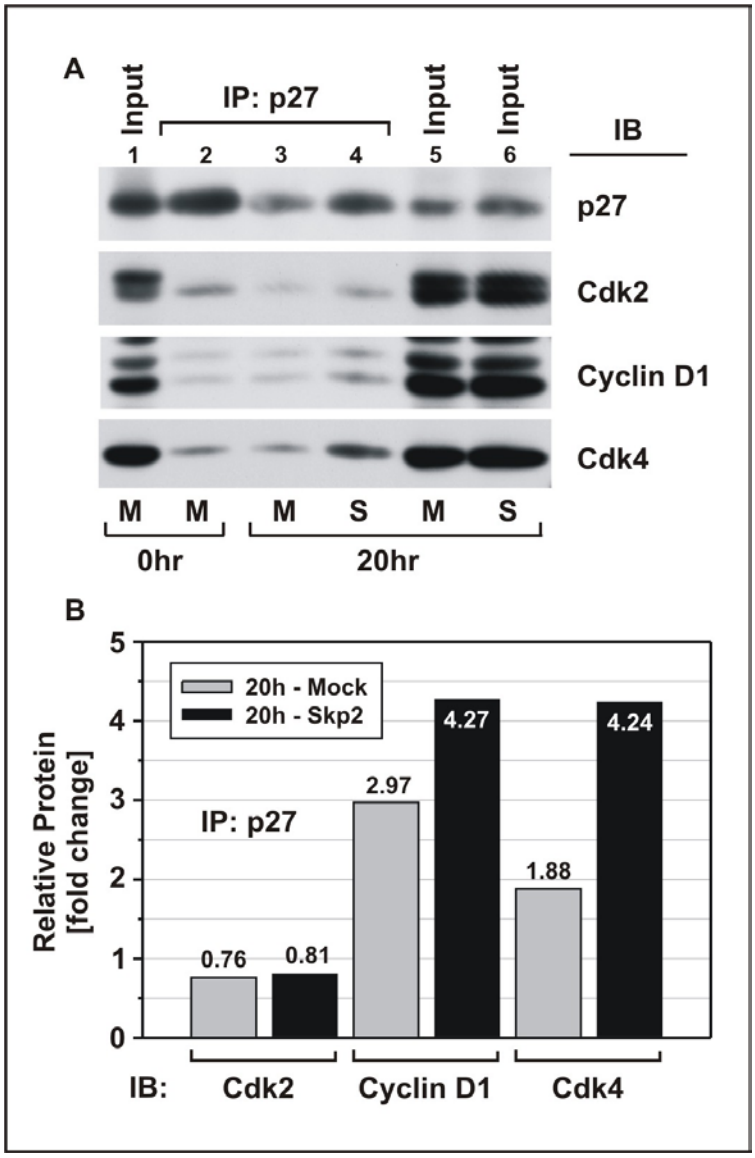

Figure 2.9. Increased p27 interaction with Cyclin D1/Cdk4 following Skp2 knockdown. A. Cells were transfected with non-targeting siRNA (Mock; M) or Skp2 siRNA (S). Total cell lysates, harvested at 0hr and 20 hr post-MDI, were immunoprecipitated (IP) for p27 and immunoblotted (IB) as illustrated. Input data were generated by immunoblotting 10% of protein used for immunoprecipitation. B. Densitometric data representing Cdk2, Cyclin D1, and Cdk4 were normalized to protein levels of p27 IP for each condition and to the level of p27 IP at 0hr post-MDI treated with non-targeting siRNA.

REFERENCES

Beniston,R.G. and M.S.Campo. 2003. Quercetin elevates p27(Kip1) and arrests both primary and HPV16 E6/E7 transformed human keratinocytes in G1. Oncogene 22: 5504-5514.

Bjorntorp,P., M.Karlsson, and P.Pettersson. 1982. Expansion of adipose tissue storage capacity at different ages in rats. Metabolism 31: 366-373.

Bonnet,F. 1981. Fat cell size and number in obese children. in Adipose Tissue in Children (ed. F.Bonnet), pp. 133-154. CRC Press.

Brunet,A., A.Bonni, M.J.Zigmond, M.Z.Lin, P.Juo, L.S.Hu, M.J.Anderson, K.C.Arden, J.Blenis, and M.E.Greenberg. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96: 857-868.

Brunet,A., J.Park, H.Tran, L.S.Hu, B.A.Hemmings, and M.E.Greenberg. 2001. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). Mol. Cell Biol. 21: 952-965.

Carrano,A.C., E.Eytan, A.Hershko, and M.Pagano. 1999. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. 1: 193-199.

Coats,S., W.M.Flanagan, J.Nourse, and J.M.Roberts. 1996. Requirement of p27Kip1 for restriction point control of the fibroblast cell cycle. Science 272: 877-880.

Delmas,C., N.Aragou, S.Poussard, P.Cottin, J.M.Darbon, and S.Manenti. 2003. MAP kinase-dependent degradation of p27Kip1 by calpains in choroidal melanoma cells. Requirement of p27Kip1 nuclear export. J. Biol. Chem. 278: 12443-12451.

Duan,H., L.M.Tsvetkov, Y.Liu, Y.Song, M.Swaroop, R.Wen, H.F.Kung, H.Zhang, and Y.Sun. 2001. Promotion of S-phase entry and cell growth under serum starvation by SAG/ROC2/Rbx2/Hrt2, an E3 ubiquitin ligase component: association with inhibition of p27 accumulation. Mol. Carcinog. 30: 37-46.

Eymin,B., O.Sordet, N.Droin, B.Munsch, M.Haugg, C.M.Van de, P.Vandenabeele, and E.Solary. 1999. Caspase-induced proteolysis of the cyclin-dependent kinase inhibitor p27Kip1 mediates its anti-apoptotic activity. Oncogene 18: 4839-4847.

Fenteany,G. and S.L.Schreiber. 1998. Lactacystin, proteasome function, and cell fate. J. Biol. Chem. 273: 8545-8548.

Fenteany,G., R.F.Standaert, W.S.Lane, S.Choi, E.J.Corey, and S.L.Schreiber. 1995. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science 268: 726-731.

Fenteany,G., R.F.Standaert, G.A.Reichard, E.J.Corey, and S.L.Schreiber. 1994. A beta-lactone related to lactacystin induces neurite outgrowth in a neuroblastoma cell line and inhibits cell cycle progression in an osteosarcoma cell line. Proc. Natl. Acad. Sci. U. S. A 91: 3358-3362.

Fujita,N., S.Sato, K.Katayama, and T.Tsuruo. 2002. Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization. J. Biol. Chem. 277: 28706-28713.

Ganiatsas,S., R.Dow, A.Thompson, B.Schulman, and D.Germain. 2001. A splice variant of Skp2 is retained in the cytoplasm and fails to direct cyclin D1 ubiquitination in the uterine cancer cell line SK-UT. Oncogene 20: 3641-3650.

Gu,Y., J.Rosenblatt, and D.O.Morgan. 1992. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. EMBO J. 11: 3995-4005.

Hao,B., N.Zheng, B.A.Schulman, G.Wu, J.J.Miller, M.Pagano, and N.P.Pavletich. 2005. Structural basis of the Cks1-dependent recognition of p27(Kip1) by the SCF(Skp2) ubiquitin ligase. Mol. Cell 20: 9-19.

Hara,T., T.Kamura, K.Nakayama, K.Oshikawa, S.Hatakeyama, and K.Nakayama. 2001. Degradation of p27(Kip1) at the G(0)-G(1) transition mediated by a Skp2-independent ubiquitination pathway. J. Biol. Chem. 276: 48937-48943.

Hausman,D.B., M.DiGirolamo, T.J.Bartness, G.J.Hausman, and R.J.Martin. 2001. The biology of white adipocyte proliferation. Obes. Rev. 2: 239-254.

Hengst,L. and S.I.Reed. 1996. Translational control of p27Kip1 accumulation during the cell cycle. Science 271: 1861-1864.

Hirsch,J., S.K.Fried, N.K.Edens, and R.L.Leibel. 1989. The fat cell. Med. Clin. North Am. 73: 83-96.

Kamura,T., T.Hara, M.Matsumoto, N.Ishida, F.Okumura, S.Hatakeyama, M.Yoshida, K.Nakayama, and K.I.Nakayama. 2004. Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(Kip1) at G1 phase. Nat. Cell Biol. 6: 1229-1235.

Kim,K.B., J.Myung, N.Sin, and C.M.Crews. 1999. Proteasome inhibition by the natural products epoxomicin and dihydroeponemycin: insights into specificity and potency. Bioorg. Med. Chem. Lett. 9: 3335-3340.

Kops,G.J., R.H.Medema, J.Glassford, M.A.Essers, P.F.Dijkers, P.J.Coffer, E.W.Lam, and B.M.Burgering. 2002. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol. Cell Biol. 22: 2025-2036.

Levkau,B., H.Koyama, E.W.Raines, B.E.Clurman, B.Herren, K.Orth, J.M.Roberts, and R.Ross. 1998. Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade. Mol. Cell 1: 553-563.

Loubat,A., N.Rochet, L.Turchi, R.Rezzonico, D.F.Far, P.Auberger, B.Rossi, and G.Ponzio. 1999. Evidence for a p23 caspase-cleaved form of p27[KIP1] involved in G1 growth arrest. Oncogene 18: 3324-3333.

Malek,N.P., H.Sundberg, S.McGrew, K.Nakayama, T.R.Kyriakides, J.M.Roberts, and T.R.Kyriakidis. 2001. A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase. Nature 413: 323-327.

Medema,R.H., G.J.Kops, J.L.Bos, and B.M.Burgering. 2000. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature 404: 782-787.

Meng,L., R.Mohan, B.H.Kwok, M.Elofsson, N.Sin, and C.M.Crews. 1999. Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. Proc. Natl. Acad. Sci. U. S. A 96: 10403-10408.

Morrison,R.F. and S.R.Farmer. 1999. Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. J. Biol. Chem. 274: 17088-17097.

Muller,D., C.Bouchard, B.Rudolph, P.Steiner, I.Stuckmann, R.Saffrich, W.Ansorge, W.Huttner, and M.Eilers. 1997. Cdk2-dependent phosphorylation of p27 facilitates its Myc-induced release from cyclin E/cdk2 complexes. Oncogene 15: 2561-2576.

Naaz,A., D.R.Holsberger, G.A.Iwamoto, A.Nelson, H.Kiyokawa, and P.S.Cooke. 2004. Loss of cyclin-dependent kinase inhibitors produces adipocyte hyperplasia and obesity. FASEB J. 18: 1925-1927.

Nakamura,N., S.Ramaswamy, F.Vazquez, S.Signoretti, M.Loda, and W.R.Sellers. 2000. Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. Mol. Cell Biol. 20: 8969-8982.

Nakayama,K., H.Nagahama, Y.A.Minamishima, M.Matsumoto, I.Nakamichi, K.Kitagawa, M.Shirane, R.Tsunematsu, T.Tsukiyama, N.Ishida, M.Kitagawa, K.Nakayama, and S.Hatakeyama. 2000. Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. EMBO J. 19: 2069-2081.

Patel,Y.M. and M.D.Lane. 2000. Mitotic clonal expansion during preadipocyte differentiation: calpain-mediated turnover of p27. J. Biol. Chem. 275: 17653-17660.

Perez-Roger,I., S.H.Kim, B.Griffiths, A.Sewing, and H.Land. 1999. Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). EMBO J. 18: 5310-5320.

Polyak,K., M.H.Lee, H.Erdjument-Bromage, A.Koff, J.M.Roberts, P.Tempst, and J.Massague. 1994. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 78: 59-66.

Rena,G., S.Guo, S.C.Cichy, T.G.Unterman, and P.Cohen. 1999. Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B. J. Biol. Chem. 274: 17179-17183.

Sheaff,R.J., M.Groudine, M.Gordon, J.M.Roberts, and B.E.Clurman. 1997. Cyclin E-CDK2 is a regulator of p27Kip1. Genes Dev. 11: 1464-1478.

Sherr,C.J. and J.M.Roberts. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. 13: 1501-1512.

Shin,I., F.M.Yakes, F.Rojo, N.Y.Shin, A.V.Bakin, J.Baselga, and C.L.Arteaga. 2002. PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nat. Med. 8: 1145-1152.

Shirane,M., Y.Harumiya, N.Ishida, A.Hirai, C.Miyamoto, S.Hatakeyama, K.Nakayama, and M.Kitagawa. 1999. Down-regulation of p27(Kip1) by two mechanisms, ubiquitin-mediated degradation and proteolytic processing. J. Biol. Chem. 274: 13886-13893.

Sin,N., K.B.Kim, M.Elofsson, L.Meng, H.Auth, B.H.Kwok, and C.M.Crews. 1999. Total synthesis of the potent proteasome inhibitor epoxomicin: a useful tool for understanding proteasome biology. Bioorg. Med. Chem. Lett. 9: 2283-2288.

Sutterluty,H., E.Chatelain, A.Marti, C.Wirbelauer, M.Senften, U.Muller, and W.Krek. 1999. p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. Nat. Cell Biol. 1: 207-214.

Tang,Q.Q., T.C.Otto, and M.D.Lane. 2003. Mitotic clonal expansion: a synchronous process required for adipogenesis. Proc. Natl. Acad. Sci. U. S. A 100: 44-49.

Toyoshima,H. and T.Hunter. 1994. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell 78: 67-74.

Tsvetkov,L.M., K.H.Yeh, S.J.Lee, H.Sun, and H.Zhang. 1999. p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. Curr. Biol. 9: 661-664.

Vlach,J., S.Hennecke, and B.Amati. 1997. Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. EMBO J. 16: 5334-5344.

Zhang,J.W., Q.Q.Tang, C.Vinson, and M.D.Lane. 2004. Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes. Proc. Natl. Acad. Sci. U. S. A 101: 43-47.

CHAPTER III

HORMONAL INDUCTION OF ADIPOGENESIS INDUCES SKP2 EXPRESSION THROUGH THE PI3K AND MAPK PATHWAYS

ABSTRACT

Hyperplastic obesity is particularly devastating because increased lipid accumulation is accompanied by increased adipocyte number, the latter of which is largely irreversible. Cellular mechanisms regulating the proliferation of these cells are poorly understood. The Ubiquitin-Proteasome System (UPS) has been shown to play an important role in adipocyte differentiation and we have shown previously its importance in regulating the cell-cycle inhibitor p27 during clonal expansion. This is mediated by the F-box protein, Skp2, of which nothing is known about its regulation during adipogenesis. Here, we describe the transient expression of Skp2 protein in 3T3-L1 preadipocytes during the early stages of adipocyte differentiation, a pattern that cannot be reproduced in fully mature adipocytes. Dramatic changes in mRNA are also present at this time and we show this to be due to increased promoter activity. Our studies further show that immediately following the addition of adipogenic media, both the PI3K and MAPK pathways increase Skp2 promoter activity. We have determined the responsive element(s) to be immediately upstream of the gene. In addition, our data suggests the possibility of translational control, downstream of PI3K. These are the first studies describing the regulation of the mammalian cell cycle protein Skp2 in 3T3-L1 cells, a potential mechanism for adipocyte hyperplasia.

INTRODUCTION

Obesity is a devastating condition due to its widespread occurrence in all populations regardless of race, age, gender, or socioeconomic status, and its negative effects on life expectancy and quality-of-life due to comorbidities such as heart disease, stroke, diabetes, and cancer. Early studies demonstrated that adipocyte hyperplasia is largely responsible for childhood (Hager et al., 1978), morbid (Pettersson et al., 1985), and diet-induced obesity (Mandenoff et al., 1982). A majority of the research-to-date has been focused on adipocyte hypertrophy, which has left many unanswered questions regarding adipocyte hyperplasia. There are two phases of hyperplastic obesity; the proliferation of preadipocytes and subsequent differentiation to form mature lipid-filled adipocytes. While much work has explored the process of adipocyte differentiation, little is known about the mechanisms regulating preadipocyte replication.

The F-box protein, Skp2 or S-phase kinase-associated protein, has been shown to promote cell cycle progression in neoplastic cells, in large part, by targeting the cyclin-dependent kinase inhibitor p27 for degradation. Skp2 is the substrate recognition component of a larger complex, the SCF^{Skp2} E3 Ligase, that targets specific proteins for polyubiquitylation and subsequent degradation by the 26S proteasome. Skp2 binds to the core of the E3 Ligase complex via its F-box motif, a region composed of approximately 40 amino acids common among all F-box proteins. Its peptide structure is further characterized by a region containing leucine-rich repeats (LRR). It is within this LRR region that Skp2 (as part of the SCF^{Skp2} E3 Ligase) binds Cks1 and p27, bringing the substrate, p27, in close proximity to ubiquitin-conjugating enzymes (Hao et al., 2005). In 1999, Morrison and Farmer first identified a decrease in p27 protein expression during early adipocyte differentiation while characterizing the switch between

proliferation and differentiation. Recently, Naaz et al. (Naaz et al., 2004) found that p27 knockout mice had 80% heavier fat pads than wildtype controls due to an increase in adipocyte number, not size (i.e., p27 KO animals had two times the number of fat cells). We are the first to show that Skp2 is transiently expressed during an early phase of adipocyte differentiation and that this increase plays a major role in regulating p27 (Chapter II). While much research has been devoted to the regulation of p27, less is known about how Skp2 is regulated. Determining mechanisms controlling Skp2 expression may provide important insight into the regulation of hyperplastic obesity.

Signal transduction pathways have been shown to play an important role in initiating cell cycle progression. Phosphatidylinositol-3 kinases (PI3Ks) exert their effects on proliferation through targets such as E2F (Brennan et al., 2002), NFκB (Shah et al., 2001), Forkhead transcription factors (Brunet et al., 1999;Kops et al., 2002), and mTOR (Gao et al., 2003). These effects can occur through PI3K directly or indirectly by its downstream targets, PDK1 and PKB/Akt. Activation of Akt also augments this process as it can lead to increased expression of the insulin-like growth factor 1 receptor (IGF1R) (Tanno et al., 2001). This is of particular interest as the IGF1R is a predominant cell-surface growth factor receptor expressed during early adipocyte differentiation. A second pivotal pathway is regulated by mitogen-activated protein kinases (MAPKs). The activation of this pathway leads to increased Cyclin D1 expression, Rb hyperphosphorylation, and subsequent downstream events (e.g., increases in Cyclin E/A – Cdk2 complexes) (Lavoie et al., 1996). The IGF1R is known to activate both pathways and is required for differentiation of 3T3-L1 preadipocytes (Smith et al., 1988). In this study we show significant regulation of Skp2 and its accessory protein Cks1 by insulin, at a concentration known to bind and activate the IGF1R. The

addition of the mitogenic cocktail MDI (methylisobutylxanthine, dexamethasone, and insulin) is the conventional way to induce adipocyte differentiation in these cells. Early stages of adipogenesis include a brief period of one to two rounds of synchronous cell division, which is obligatory for further differentiation as critical chromatin remodeling occurs and adipogenic precursor proteins are expressed.

The objective of this study was to identify the mechanism by which Skp2 expression is increased during adipocyte hyperplasia. We sought to determine the kinetics of Skp2 expression throughout adipocyte differentiation and identify the upstream events leading to its expression. In addition, we determined the level at which Skp2 regulation occurs. Our findings revealed that Skp2 promoter activity is increased in response to PI3K and MAPK activation during preadipocyte proliferation. In addition, Skp2 protein, but not mRNA, is responsive to Rapamycin, suggesting possible translational control. These data provide the first study of Skp2 regulation in adipocytes and provide a link between this cell cycle protein, early signaling events, and adipocyte hyperplasia.

MATERIALS AND METHODS

Materials - Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Cellgro by Mediatech. Bovine Serum, Fetal Bovine Serum (FBS), and Trypsin-EDTA were from Invitrogen Corporation. Propidium Iodide and RNase A was purchased from Sigma. Chemical inhibitors were purchased from the following: LY294002 and U1026 (Promega); Actinomycin D (Sigma); Rapamycin (Calbiochem). Antibodies used for western blot analysis were as follows: Skp2 (Zymed); Cks1, Skp1, Cyclin A, Cul1, and

C/EBPα (Santa Cruz Biotechnology). Enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Sciences.

Cell Culture and Differentiation - Murine 3T3-L1 preadipocytes were propagated in growth medium containing DMEM supplemented with 10% calf bovine serum as described previously (Morrison and Farmer, 1999). By standard differentiation protocol, preadipocytes were propagated in growth medium until reaching a state of density arrest at 2 days post-confluence. Growth medium was replaced at density arrest with differentiation medium comprised of DMEM supplemented with 10% fetal bovine serum (FBS), 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). The term "post-MDI" refers to the time elapsed since the addition of MDI to the culture medium. At 2 days post-MDI, medium was changed to DMEM supplemented with 10% FBS and 0.4 μM insulin. From 4 days post-MDI until harvest, maintenance medium containing DMEM supplemented only with 10% FBS was changed every 48 hrs. Throughout the study, "time 0" refers to density arrested cells immediately before chemical induction of differentiation with the addition of MDI to the culture medium. All experiments were repeated three to five times to validate results and ensure reliability.

Flow Cytometry - Cell cycle progression was assessed by flow cytometry. Briefly, cell monolayers were washed with phosphate-buffered saline (PBS), trypsinized, and detached cells diluted in ice cold PBS to produce a single cell suspension. Cells were gently pelleted by centrifugation (300 x g, 5 min). Following centrifugation, PBS was decanted and cells were fixed and permeabilized by drop-wise addition of 70% ethanol at -20°C while vortexing. Fixed cells were incubated on ice for 30 minutes or stored at

-20˚C for no more than one week before processing. Fixed cells were washed 2x with PBS and incubated in the dark for 30 minutes with 1ml propidium iodide staining solution containing 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A in PBS. DNA fluorescence was measured with a FACS Calibur Flow Cytometer (Becton Dickinson) equipped with a 488-nm argon laser. Width (FL2W) and area (FL2A) of PI fluorescence was recorded for at least 10,000 counts. DNA histograms were extracted from FL2W-FL2A dot plots after gating to eliminate aggregates.

Immunoblotting - Cell monolayers were washed with PBS and scraped into ice cold lysis buffer containing 1.0 M Tris, pH 7.4, 150 mM NaCl, 1% Triton X, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, and 10 mM N-ethylmaleimide (NEM). Lysis buffer was freshly supplemented with phosphatase inhibitors; 20 mM β-glycerophosphate, 10 mM NaF, and 2 μM sodium vanadate, as well as protease inhibitors; 0.3 μM aprotonin, 21 μM leupeptin, E-64, 1 μM pepstatin, 50 μM phenanthroline, 0.5 μM phenylmethylsulfonyl fluoride. Cell lysates were clarified by centrifugation (13,000 x g, 10 min, 4°C) and protein concentration was determined by BCA assay (Pierce). Lysates were resuspended in loading buffer containing 0.25 M Tris, pH 6.8, 4% SDS, 10% glycerol, 10% dithiotreitol, 0.01% bromophenol blue, heated for 5 mins at 80°C, and placed on ice. Lysates were resolved on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore). Following transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4°C and horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Immunoblots were developed with ECL and visualized by autoradiography CL-XPosure film (Pierce).

RNA Isolation and Analysis - Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to manufacturer's instructions. Total RNA (1μg) was subjected to Reverse-Transcriptase Polymerase Chain Reaction using One-Step RT-PCR kit (QIAGEN) according to manufacturer's instruction. Briefly, RT-PCR reactions were carried out in the presence of the supplied buffer, dNTPs (400 μM), enzyme mix (reverse transcriptases and DNA polymerase), and RNase inhibitor (10 U). Gene specific primers were used at a final concentration of 0.6 μM and designed as follows: Skp2 forward, 5′-GGCAAAGGGAGTGACAAAGA-3′; Skp2 reverse, 5′-TCAAAGCACCAGGAGAGATT-3′ (product size = 590 bp); Cks1 forward, 5′-TGTCTGAATCTGAATGGAGG-3′; Cks1 reverse, 5′-TGCTTCTGCCAAATGACTAA-3′ (product size = 381 bp). QuantamRNA Classic and Classic II 18S primer/competimers (Ambion) were used in the reactions as internal standards at a final concentration of 0.6 μM.

Plasmids – Promega's pGL3-basic vector was used to create mouse Skp2-pGL3 constructs for luciferase assays. Primers flanking the region of -2404 to +74 (2478bp) were used to amplify the murine Skp2 promoter from 3T3-L1 preadipocyte genomic DNA. Positive DNA fragments were gel purified. Vector and isolated genomic DNA were double-digested with Kpn I and Bgl II. The resulting DNA fragment was subcloned into the pGL3-basic vector. Nucleotide sequencing was conducted by SeqWright DNA Technology Services. This entire process was repeated twice to ensure reliability of results. Restriction digest reactions were done to make deletion constructs from this "full length" promoter (2.4kb) as follows: 2.2kb (-2248 to +74) insert using Kpn I and BbvC I; 1.5kb (-1573 to +74) using Kpn I and EcoR I; 1.0kb (-1073 to +74) using Kpn I and Nsi I; 0.4kb (-0.454 to +74) using Kpn I and Mlu I. 5' and 3' overhangs were flushed using T4

DNA Polymerase (Promega), resulting DNA fragments gel-purified, and blunt-end ligations were carried out using T4 DNA ligase (Promega). All vectors were transformed into JM109 competent cells (Promega) and screened for positive ligations using ampicillin-containing LB agar plates. DNA was isolated using the endotoxin-free Wizard PureFection Plasmid DNA Purification System (Promega). Restriction digests were performed to confirm positive clones.

Luciferase Reporter Assays – 3T3-L1 preadipocytes (2.0 x 10^6 cells) were transiently transfected with 5 μg of firefly reporter plasmid and 20.0 ng renilla-SV40 plasmid using Amaxa's Nucleofector (program T20), then incubated with pre-warmed proliferation media for no more than 10 minutes at 37°C. Cells were subsequently replated at a confluent density, fresh media replaced 24 hours post-transfection, and further cultured as indicated. Firefly and renilla luciferase were measured using the Dual-Glo Luciferase Assay System (Promega) and reported as relative firefly luciferase counts (firefly/renilla).

RESULTS

We have previously determined an upregulation of Skp2 protein and subsequent role in the regulation of p27 protein during clonal expansion. To examine Skp2 expression throughout the entire process of adipocyte differentiation, 3T3-L1 preadipocytes were stimulated with the differentiation cocktail, MDI, and protein abundance was determined by immunoblotting. As shown in Figure 3.1, Skp2 is not expressed at day 0, when cells are density-arrested. However, by 24 hours post-MDI, Skp2 protein expression increases transiently, returning to day 0 levels as the cells

begin to express adipogenic markers (e.g., C/EBPα) and become fully mature adipocytes. As well, other proteins essential for the function of the SCF^{Skp2} E3 Ligase are expressed during the same time. Skp1 and Cul1 are ubiquitously expressed throughout adipocyte differentiation, consistent with previous literature that suggests these components are not regulated at the level of protein expression. Interestingly, the expression pattern of Cks1 follows that of Skp2 precisely. This is extremely important as Cks1 has been shown to be required for Skp2-dependent degradation of p27 (Ganoth et al., 2001;Hao et al., 2005). During clonal expansion, an early stage of adipocyte differentiation, density-arrested preadipocytes synchronously reenter the cell cycle. This is demonstrated by the differential expression of Skp2 in proliferating preadipocytes (PPA), which are subconfluent and progressing through different stages of the cell cycle, and day 1 cells which have been arrested in G_0 by contact inhibition and driven back into the cell cycle very synchronously. These data show regulated expression of Skp2 and Cks1 during early stages of adipocyte differentiation.

To determine whether the upregulation of Skp2 protein is purely a result of adding the stimulation cocktail to these cells (i.e., a chemical effect) or if it is a phenomenon unique to cell cycle and clonal expansion, mature adipocytes were stimulated with MDI. As shown in Figure 3.2, Skp2 protein is transiently expressed during clonal expansion, 22 hours after the initial stimulation, but cannot be upregulated by administering the same chemical cocktail once the cells have fully differentiated (indicated by C/EBPα expression), and the same is true for its accessory protein Cks1. Thus, the mechanism(s) responsible for increased expression of Skp2, and its required accessory protein, are limited to preadipocyte proliferation.

There is limited information about the regulation of Skp2 in malignant cells and nothing is known about how it is regulated in adipocytes. To determine whether changes in Skp2 protein are accompanied by changes in mRNA during preadipocyte proliferation, 3T3-L1 cells were stimulated with MDI and samples harvested at indicated times post-stimulation. As shown in Figure 3.3, Skp2 protein increases slightly by 12 hours post-MDI and is highly expressed by 20 hours. This change in protein is preceded by a dramatic increase in Skp2 mRNA. Skp2 mRNA is extremely low at day 0 but is significantly increased by 12 hours following stimulation. The kinetics of Skp2 mRNA throughout differentiation match exactly that of Skp2 protein, as mRNA is increased at 12 and 20 hours during cell cycle progression and back down to basal levels during terminal differentiation (i.e., days 2 and 4). This suggests regulation at the level of transcription and/or mRNA stability but does not rule out further regulation by way of translational or post-translational control.

To identify the events leading to Skp2 expression, the effects of individual components of the stimulation cocktail were evaluated. Density-arrested 3T3-L1 preadipocytes were stimulated with MDI, individual components, or varying combinations of two components. Levels of Skp2 expression in the presence of these chemicals were compared to that of growth arrested cells (i.e., unstimulated condition – first lane in Figure 3.4). These studies revealed that while there is a synergistic effect of MDI together, insulin is the primary component responsible for Skp2 protein expression, as well as Cks1 and cell cycle progression, as shown by Cyclin A expression. Again, Skp1 is constitutively expressed, serving as a loading control. Protein levels were also examined from cells that received a media change with either calf serum (proliferation media) or fetal bovine serum (part of the differentiation media) 20 hours prior to

harvesting. This was done to determine whether the increase in Skp2 protein could be due to the replenishment of depleted growth factors via a media change with calf serum or a result of additional mitogens present in fetal bovine serum alone. We found no increase in Skp2 protein with either condition. Taken together, these data suggest that Skp2 induction is primarily mediated by insulin.

Insulin is known to play a role in cell cycle progression by activating different signaling cascades. Two such pathways are the PI3K and MAPK pathways which have been shown to activate downstream targets (e.g., c-myc, E2F, Cyclin D1) important for cell cycle progression. These kinase-driven pathways can work independently and/or synergistically, as there is crosstalk between the two cascades. To determine whether either pathway plays a role in regulating Skp2 expression during preadipocyte proliferation, cells were treated with the PI3K inhibitor LY294002, MAPK inhibitor U0126, or both. As shown in Figure 3.5A, inhibition of either pathway for 20 hours post-stimulation prevents maximal expression of Skp2 mRNA. While there is an additive effect of blocking both pathways, LY294002 has the greatest effect when comparing inhibition of either pathway alone. Cks1 mRNA is also significantly affected by LY294002 but only negligibly affected by U0126. However, there is a greater effect on Cks1 mRNA when both inhibitors are used simultaneously than with LY294002 alone. We examined protein levels for these same conditions (Figure 3.5B) and found that Skp2 protein followed the same pattern seen in mRNA, and to a slightly greater extent. Cks1 protein expression was negatively affected by LY294002 and also decreased in the presence of U0126. The two inhibitors in combination caused no further decrease than what was seen with LY294002 alone. To determine whether blocking these pathways also had an effect on cell cycle progression, we examined Cyclin A protein

expression, a marker of S phase, and found that both inhibitors decreased Cyclin A and did so synergistically, with LY294002 having the most potent effect alone. These data were further validated using flow cytometry. Figure 3.5C shows G_1 arrest in the presence of LY294002 and/or U0126. These findings indicate that PI3K and, to a lesser extent, MAPK signaling is required for the increase in Skp2 mRNA and protein levels during clonal expansion.

Because PI3K plays the primary role in regulating Skp2 and Cks1, we examined the phosphorylation status of critical kinases involved in this pathway to determine the time at which activation of downstream targets occurs. As shown in Figure 3.6, PDK1 is constitutively phosphorylated on Ser241 before and immediately following stimulation with slightly less phosphorylation at 20 hours. This pattern is consistent with previous findings that show serine-phosphorylated PDK1 present in unstimulated and IGF-1 stimulated 293 cells (Casamayor et al., 1999). While phosphorylation of PDK1 at S241 is necessary for activation, it is not sufficient. Activation requires association between PDK1 and PIP_3 in the plasma membrane, created by active PI3K. We show here that the primary downstream target of PDK1, Akt, is transiently phosphorylated at residues Ser473 and Thr308 beginning at 15-30 minutes post-stimulation, continuing through at least 8 hours. Phosphorylation of Akt is a strong indicator of activity, therefore, we examined the phosphorylation status of two of its targets, Foxo1 and GSK3β. Foxo1 is a transcription factor whose activity is blocked by cytosolic translocation upon phosphorylation by Akt at Ser256. Consistent with the kinetics of Akt phosphorylation, Foxo1 is phosphorylated at Ser256 30 minutes after stimulation through at least 8 hours and is no longer phosphorylated at this residue by 20 hours post-stimulation. Interestingly, the kinase GSK3β can also phosphorylate Foxo1 at this same residue,

however, cannot do so when phosphorylated at Ser9, as this inhibits GSK3β kinase activity. In Figure 3.6, we show constitutive phosphorylation of GSK3β at S9 before and immediately following the addition of MDI. Taken together, phosphorylation of GSK3β and Foxo1, simultaneously, suggests that Akt is active and is the kinase responsible for Foxo1 phosphorylation. The tumor suppressor PTEN inhibits this signaling cascade by reverting PIP_3 within the plasma membrane to PIP_2, hence blocking PDK1 and Akt activation, however, when phosphorylated at Ser380 is inactive. We found PTEN to be constitutively phosphorylated, further support of PI3K pathway activation.

We have shown increased levels of Skp2 mRNA in preadipocytes at 12 hours post-stimulation. To determine the kinetics of this increase in Skp2 mRNA, 3T3-L1 cells were stimulated and RNA collected every 2 hours until 12 hours post-MDI and then again at 34 hours, a time at which Skp2 mRNA should be low. Figure 3.7A shows that by 4 hours after stimulation with MDI, Skp2 mRNA begins to increase. There is a continual increase through 12 hours and then a dramatic decrease by 34 hours. This early increase in Skp2 mRNA is preceded by Akt phosphorylation, as it peaks at 1 hour (Figure 3.6.).

We have demonstrated the importance of the PI3K and MAPK pathways in the increase in Skp2 mRNA, and protein, but to determine how these signaling cascades regulate Skp2 mRNA in preadipocytes, we examined mRNA stability and promoter activity. To ascertain potential changes in Skp2 mRNA half-life, 3T3-L1 cells stimulated with FBS/MDI or maintained in FBS alone were treated with Actinomycin D (an inhibitor of mRNA synthesis) and mRNA harvested over a period of 5 hours beginning at 12 hours post-MDI. As illustrated in Figure 3.7B, no change in Skp2 mRNA stability during clonal expansion was observed. Because these data ruled out regulation at the level of

mRNA stability, we examined Skp2 promoter activity. To do this, 2.4kb upstream of the coding region of the Skp2 gene was cloned into a pGL3-basic luciferase reporter vector. Cells were transfected using Amaxa's Nucleofector and stimulated with MDI alone or MDI plus the pathway inhibitors to determine whether there is an increase in Skp2 promoter activity during preadipocyte replication and if so, whether the PI3K and MAPK pathways play a role in that increase. As shown in Figure 3.8, there is a greater than 2.5 fold increase in Skp2 promoter activity by 12 hours during clonal expansion as compared to activity at day 0. These data are identical to the kinetics of mRNA suggesting that the increase in Skp2 mRNA and protein is largely due to an increase in promoter activity. In the presence of LY294002, Skp2 promoter activity is inhibited by 35% and with U0126, activity is down by 20%. These inhibitors in combination had an additive effect, that is, Skp2 promoter activity was reduced by almost 50%. These findings show substantial regulation of Skp2 promoter activity by the PI3K and MAPK pathways, leading to an increase in Skp2 protein expression that promotes preadipocyte proliferation by contributing to p27 degradation.

After finding the Skp2 promoter to be responsive to MDI and inhibition of the PI3K and MAPK pathways, we determined in which region(s) these response elements were contained. To do this, restriction enzymes were used to make deletion constructs of the original 2.4kb promoter/pGL3 vector. The resulting promoter lengths were 2.4, 2.2, 1.5, 1.0, and 0.4kb (Figure 3.9A). As shown in Figure 3.9B, all constructs produce essentially the same amount of luciferase activity at day 0, however, the 0.476kb promoter is significantly less than 1.095kb. By 12 hours after stimulation with MDI, activity of all constructs increases by 100% with the 1.0kb having an even greater activity when compared to day 0. The patterns of expression for all constructs in the

presence of LY294002 and U0126 follow Skp2 mRNA kinetics precisely, that is LY294002 produces a significant decrease in luciferase activity and U0126 has a lesser effect. When these data were normalized to compare the amount of change from day 0, we observed no difference between constructs (Figure 3.9C). Taken together, these results indicate the presence of PI3K and MAPK responsive element(s) within the first 476bp of the Skp2 promoter.

When examining the effect of LY294002 and U0126 on Skp2 mRNA and protein, it was apparent that while the pattern was the same, the magnitude of change was slightly greater when looking at protein expression. This observation suggested Skp2 protein might also be regulated in addition to the increase in promoter activity. The PI3K pathway plays a major role in regulating translational control by activating the protein kinase mTOR. This activation can occur through any of the three major kinases (i.e., PI3K, PDK1, and Akt). To assess whether this pathway plays a role in Skp2 protein expression, cells were stimulated with MDI and treated with the specific mTOR inhibitor, Rapamycin. Skp2 mRNA and protein were collected. As shown in Figure 3.10, Rapamycin has no effect on Skp2 mRNA (panel A) but has a dramatic effect on protein (panel B) at 20 hours post-stimulation. These findings suggest the possibility that mTOR regulates the rate at which Skp2 protein is translated. Further studies are needed to determine whether this is indeed a direct effect.

DISCUSSION

The study of adipocyte hyperplasia has received little attention as great strides have been made in understanding late stages of adipocyte differentiation and hypertrophic obesity. Skp2 is overexpressed in many cancers and can be used as a prognostic tool when characterizing tumor stage (Kudo et al., 2001;Shapira et al., 2005;Langner et al., 2004;Hu and Liu, 2005;Seki et al., 2003;Oliveira et al., 2003;Dong et al., 2003;Yang et al., 2002;Li et al., 2004;Osoegawa et al., 2004). This regulation has been shown to be due to increases in transcription (Imaki et al., 2003;Zhang and Wang, 2005a) and posttranslational modification, via phosphorylation (Yam et al., 1999), ubiquitylation (Wei et al., 2004;Bashir et al., 2004) and possibly auto-ubiquitylation (Wirbelauer et al., 2000;Zhang et al., 2003) . In neoplastic cells, Skp2 has been shown to be upregulated by PI3K (Mamillapalli et al., 2001;Andreu et al., 2005) and Notch1 (Sarmento et al., 2005) signaling, and negatively regulated by TGFβ (Wang et al., 2004). To date, nothing is known about Skp2 regulation in adipose tissue. We show here that Skp2 is expressed transiently during preadipocyte proliferation in 3T3-L1 cells, primarily due to increases in transcription with the minimal promoter located within 500bp upstream of the Skp2 gene. These findings are consistent with a previous characterization of the Skp2 promoter in mouse embryonic fibroblasts (Imaki et al., 2003). Our promoter deletion studies revealed that this region includes an element which is responsive to PI3K and MAPK inhibition. While the presence of two distinct response elements within this region may exist, these pathways are known to overlap and a common element(s) is quite probable.

We have shown Skp2 mRNA begins to increase 4 hours after induction of adipocyte differentiation. This suggests a very early transcriptional event responsible for Skp2 promoter activity. In 2003, Imaki et.al. identified the GA-binding protein (GABP) as

an essential transcription factor for Skp2 promoter activity (Imaki et al., 2003). While GABP has been shown to play a role in mitochondrial thermogenesis in brown adipose tissue (Villena et al., 1998), its role in white adipose tissue proliferation and differentiation has yet to be determined. More recently, E2F1 has been shown to be a transcriptional regulator of Skp2 in human fibroblasts and tumor cell lines (Zhang and Wang, 2005b). We have identified two E2F consensus sequences within the 3T3-L1 murine Skp2 gene. The E2F proteins are expressed differentially throughout the cell cycle, with E2F-4 being the predominant form present in almost all phases of the cell cycle. It will be interesting to see whether E2F also plays a role in regulating Skp2 promoter activity during preadipocyte proliferation and identify the E2F protein(s) responsible for this action. The activity of E2F is primarily regulated by phosphorylation of the retinoblastoma protein (pRb), which increases late during G_1. Skp2 mRNA begins to increase at 4 hours following the addition of MDI, suggesting a regulation much earlier than pRb hyperphosphorylation. E2F-4 is a likely candidate for regulating Skp2 gene expression at this early time because it is known to be active during G_0-G_1 and its activity regulated by subcellular localization (Moberg et al., 1996;Verona et al., 1997) and its association with p130 and p107 (Moberg et al., 1996), two proteins that undergo a critical switch in expression during early stages of adipogenesis. It is also possible that E2F1 binds to the Skp2 promoter, during mid to late G1 phase, and augments expression rather than initiating promoter activity. It is crucial to understand how Skp2 is regulated during preadipocyte proliferation because of the impact on hyperplastic obesity. In addition to the paper by Naaz et al. showing increased adiposity and adipocyte number in p27 knockout mice (Naaz et al., 2004), Nakayama et al. showed that Skp2 knockout mice were two-thirds smaller than wildtype animals (Nakayama et

al., 2000). While body composition was not measured, the change in body size could be largely due to changes in adipose tissue due to the fact that they report no changes in external bodypart sizes.

The function of Skp2 as the substrate recognition portion of the SCF^{Skp2} E3 Ligase is dependent on expression of and interaction with the accessory protein, Cks1 (Spruck et al., 2001;Ganoth et al., 2001). We have shown that Cks1 is expressed in a coordinate manner with Skp2 and is also induced by insulin stimulation. Additionally, Cks1 protein cannot be increased during differentiation. This seems logical as the cell has completed the differentiation process and has likely put mechanisms in place to block any induction of cell cycle. While Cks1 is upregulated during clonal expansion, this increase is not as dramatic as what is seen with Skp2. Furthermore, Cks1 mRNA is regulated by PI3K alone and is not affected by the addition of U0126. However, mRNA levels are further decreased in the presence of both inhibitors suggesting synergy between the two signaling cascades. In contrast, Cks1 protein is regulated independently by both the PI3K and MAPK pathways. The regulation of Cks1 is important as it lends further insight into the activity of Skp2 and the SCF^{Skp2} E3 Ligase during the differentiation process. It appears that while some proteins (e.g., C/EBPβ, as discussed later) are expressed for some time before becoming active, this is not the case for Skp2. That is, Skp2 plays an important and targeted role in proliferation during early adipocyte differentiation and is expressed only during this time. Additionally, Skp1 and Cul1, required components of the E3 ligase, are constitutively expressed during differentiation. These core proteins can form E3 Ligase complexes with other F-box proteins (e.g., β-TrCP and Cyclin F) (Latres et al., 1999;Bai et al., 1996), a notable fact

as the importance of the 26S proteasome during adipocyte differentiation has become increasingly apparent in recent years.

Of particular interest is the effect of Rapamycin, potent inhibitor of mTOR, on Skp2 protein at 20 hours post-stimulation. Recently, mTOR was identified as a kinase responsible for Akt phosphorylation at Ser473, therefore contributing to Akt activation (Hresko and Mueckler, 2005;Sarbassov et al., 2005). However, we have shown decreased Skp2 mRNA and protein levels with the potent PI3K inhibitor LY294002 at 20 hours following stimulation, whereas inhibition of mTOR only had an effect on protein at this same time. This suggests that there is a redundant pathway involved in the presence of Rapamycin. Specifically, PDK1, once thought to only phosphorylate Akt at Thr308, has been shown to also target Ser473 (Balendran et al., 1999). Therefore, if phosphorylation of Akt at Ser473 by PDK1 occurs in preadipocytes, when mTOR is blocked (i.e., with Rapamycin) complete phosphorylation of Akt is not also blocked. This would indicate that there is a PI3K-dependent pathway, likely requiring Akt activation, leading to Skp2 promoter activation. In addition, there may be mTOR-dependent translational control of Skp2 during preadipocyte proliferation as well. 4E-BP1, one of the downstream targets of mTOR and a repressor of mRNA translation, has been shown to be phosphorylated transiently within 1 hour following insulin stimulation in 3T3-L1 preadipocytes allowing for increased translational control via this pathway. In addition, by day 4 of adipocyte differentiation, the time in which Skp2 levels decrease terminally, total 4E-BP1 protein expression increases dramatically (El Chaar et al., 2004), suggesting decreased translational control by this pathway. Moreover, we have shown maximal Skp2 mRNA expression by 10 hours following stimulation, however protein

levels do not peak until approximately 20 hours, further evidence of post-transcriptional control in addition to increased promoter activity.

Proliferation and differentiation are mutually exclusive processes and the link between the two during adipocyte differentiation is not fully understood. The differentiation process is highly regulated by the C/EBP family of transcription factors. C/EBPβ is expressed during early stages and leads to upregulation of the two master adipogenic proteins, C/EBPα and PPARγ. We have identified three putative C/EBPβ binding sites in the Skp2 promoter, however, while it has been shown to be essential for clonal expansion (Tang et al., 2005;Zhang et al., 2004), C/EBPβ DNA-binding activity is not present until 12 hours after stimulation with MDI (Tang and Lane, 1999), 8 hours after Skp2 mRNA begins to increase. In addition, C/EBPβ has been shown to be downstream of PI3K and MAPK signaling following PDGF stimulation (Tullai et al., 2004). Further studies are needed to determine an effect of C/EBPβ on Skp2 expression during preadipocyte proliferation. Interestingly, Skp2 expression cannot be induced during terminal adipocyte differentiation. We hypothesize that the presence of some adipogenic factor is required for the decrease in Skp2 because when re-introducing the stimulation media at d9, Skp2 expression is not affected and it is known that PI3K/Akt signaling is intact at this time (e.g., Glut4 trafficking). PPARγ has been shown to be anti-mitotic (Altiok et al., 1997) and, therefore, could potentially play a role in maintaining low levels of Skp2 during adipocyte differentiation. Interestingly, we have identified a potential PPAR/RXR binding site within the Skp2 promoter. The PPARγ ligand, troglitazone, but not other ligands, has been shown to decrease Skp2 protein expression in human hepatoma cells (Koga et al., 2003). The mechanism behind this may give insight into the regulation of low levels of Skp2 during late stages of

differentiation and terminal growth arrest. In conclusion, this is the first report of regulated Skp2 promoter activity during adipocyte differentiation and preadipocyte proliferation. These and future studies will provide valuable information into the mechanisms regulating early proliferation required for 3T3-L1 differentiation and hyperplastic obesity.

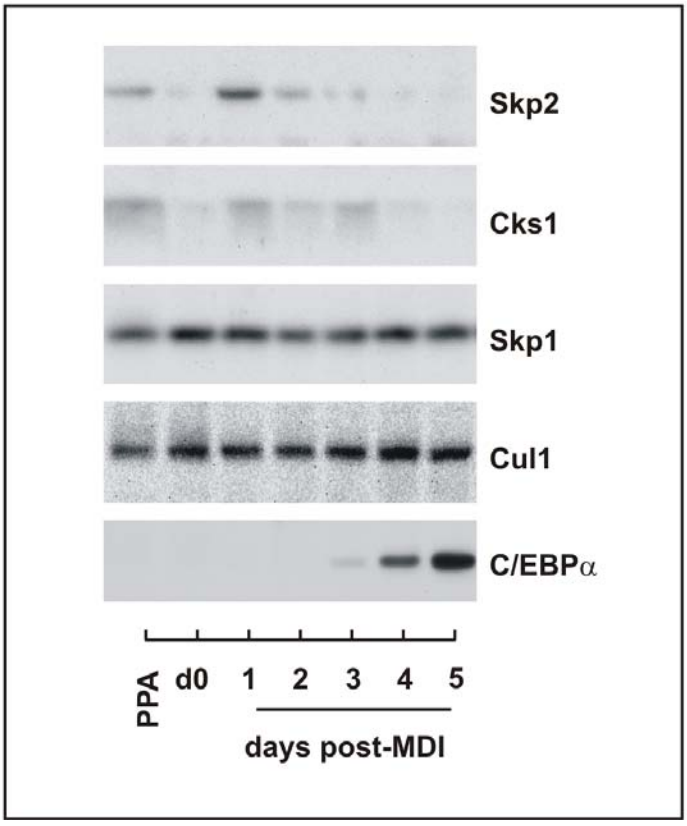

Figure 3.1. Expression of Skp2 and SCF^{Skp2} proteins through the course of adipocyte differentiation. 3T3-L1 preadipocytes were stimulated with MDI and whole cell lysates were collected every 2hr beginning at 8hr post-MDI. Unstimulated (d0) and subconfluent proliferating preadipocytes (PPA) samples were also harvested. One hundred micrograms of protein was used to detect protein expression patterns using western blot analysis.

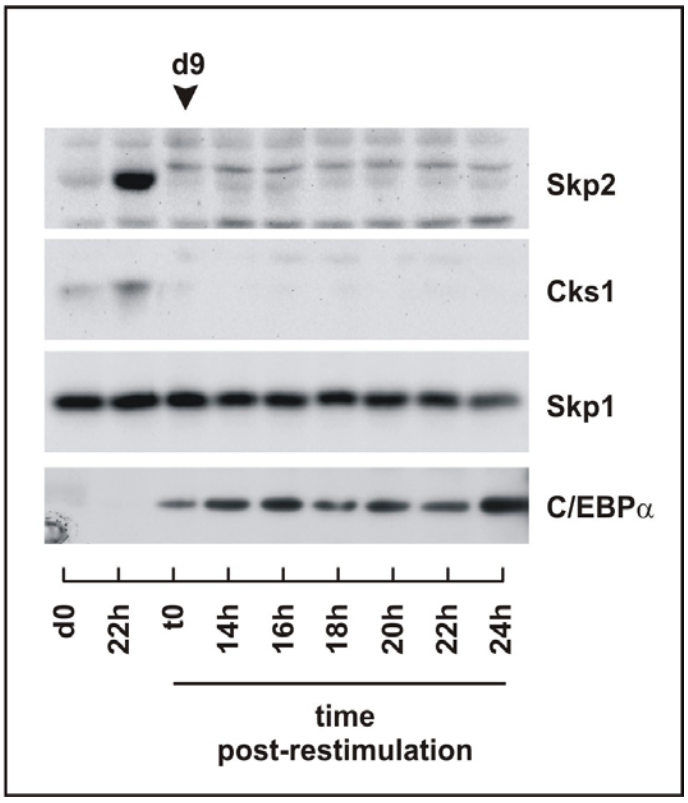

Figure 3.2. Skp2 dependence on cell cycle progression and subsequent inhibition during differentiation. 3T3-L1 preadipocytes were stimulated with MDI and differentiated for nine days, at which time the full adipogenic media (FBS/MDI) was administered to the cells and whole cell lysates were collected every two hours, beginning at 14hr following re-stimulation.

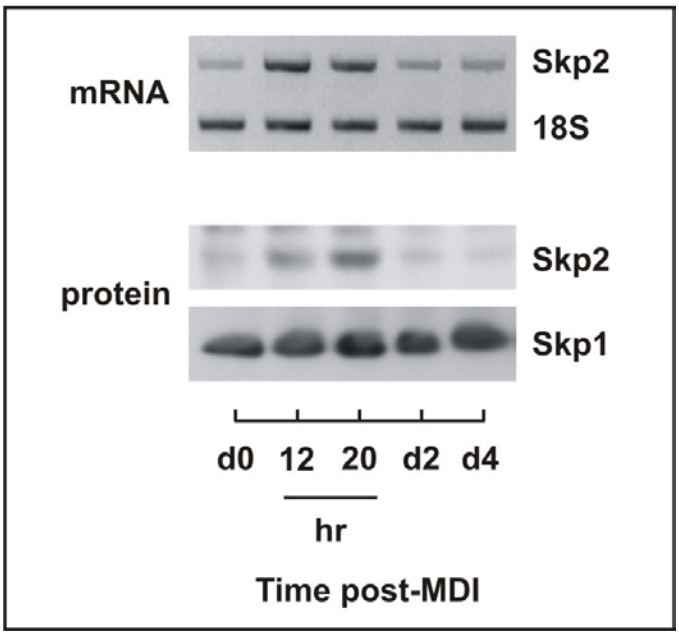

Figure 3.3. Skp2 protein expression during early stages of adipocyte differentiation is preceded by dramatic changes in mRNA. 3T3-L1 preadipocytes were stimulated with MDI and whole cell lysates and total RNA were harvested at 12hr, 20hr, 2 days, and 4 days after the addition of the differentiation cocktail. An unstimulated d0 sample was also collected. One hundred micrograms of protein was examined using western blot analysis. RNA was isolated and analyzed using RT-PCR.

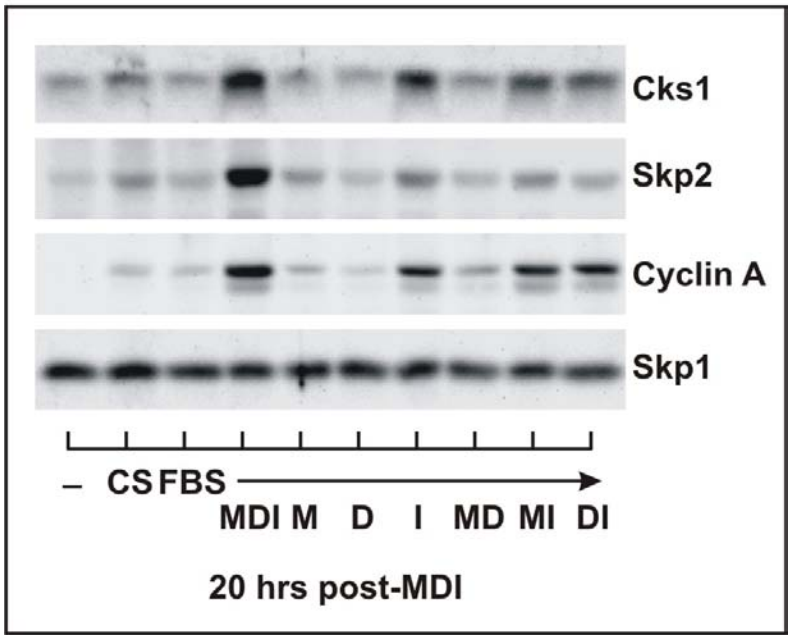

Figure 3.4. Insulin is required for maximal induction of Skp2, its accessory protein Cks1, and cell cycle progression. Cultured preadipocytes were stimulated with MDI, individual components, or combinations of these three components for 20hr. In addition, other cells had either: no media change, change to fresh calf serum (CS), or fetal bovine serum (FBS). Whole cell lysates were harvested and protein levels were determined by western blot analysis.

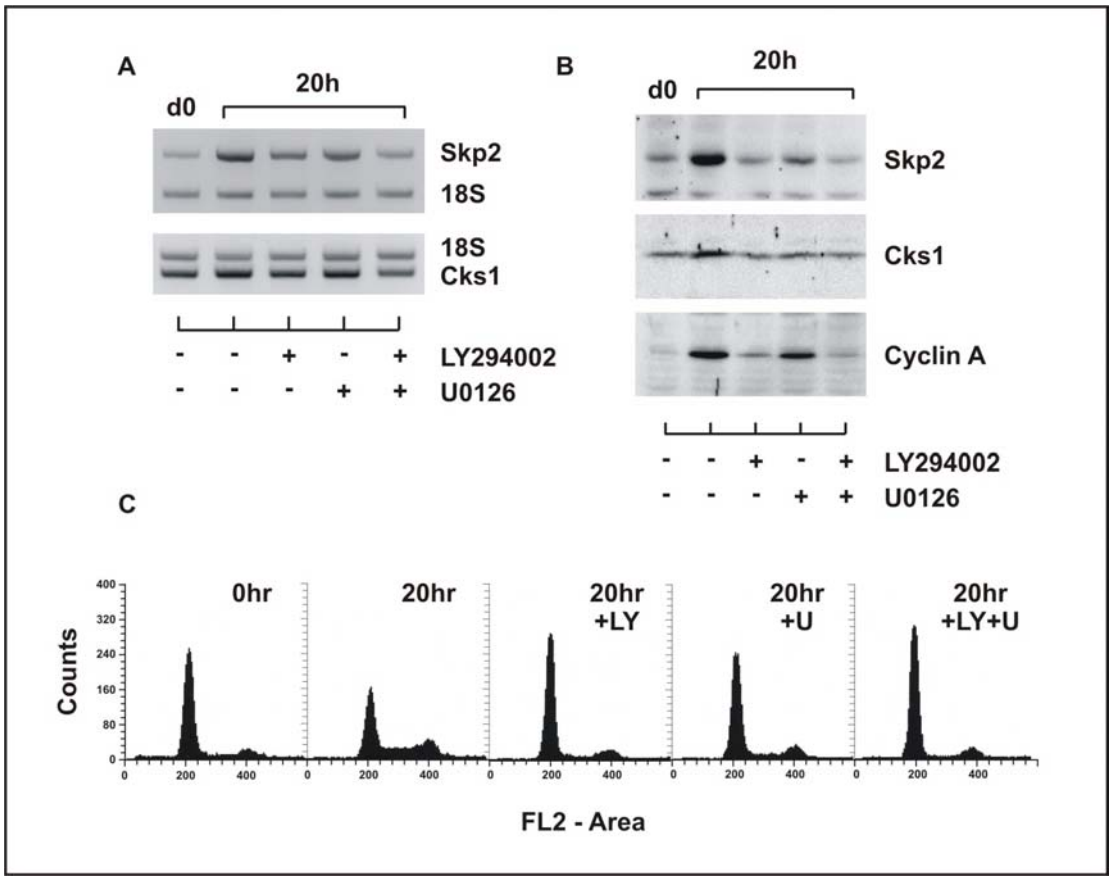

Figure 3.5. The PI3K and MAPK pathways play an important role in the increased abundance of Skp2 mRNA and protein. Cultured preadipocytes were stimulated with MDI in the presence of 10μM LY294002, 10μM U0126, or the two inhibitors combined. An unstimulated d0 sample was also used. Levels of mRNA (panel A) and protein (panel B) were examined using RT-PCR and western blot analysis. Flow cytometry was performed (Panel C) to evaluate effects on cell cycle progression.

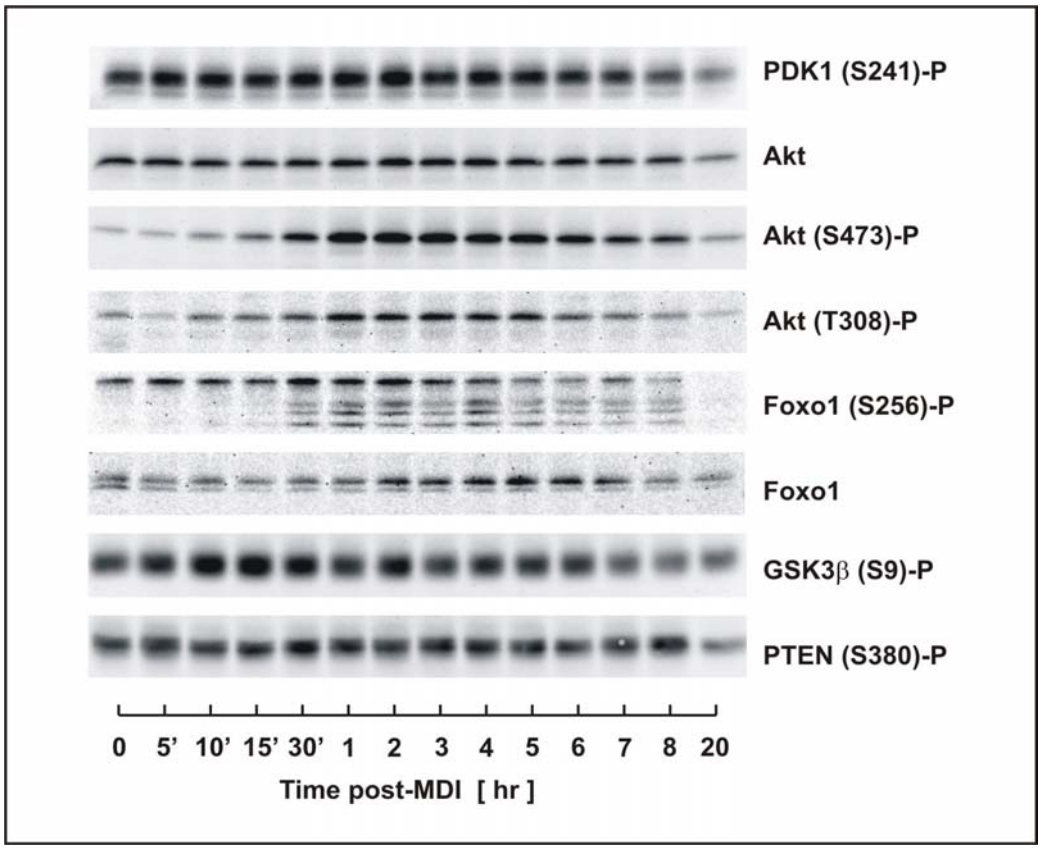

Figure 3.6. Downstream targets of PI3K are phosphorylated and activated immediately following stimulation with MDI. 3T3-L1 cells were stimulated with MDI and proteins harvested at times indicated post-MDI. Antibodies recognizing total and phosphorylated forms of individual proteins were used to evaluate activation of the PI3K pathway.

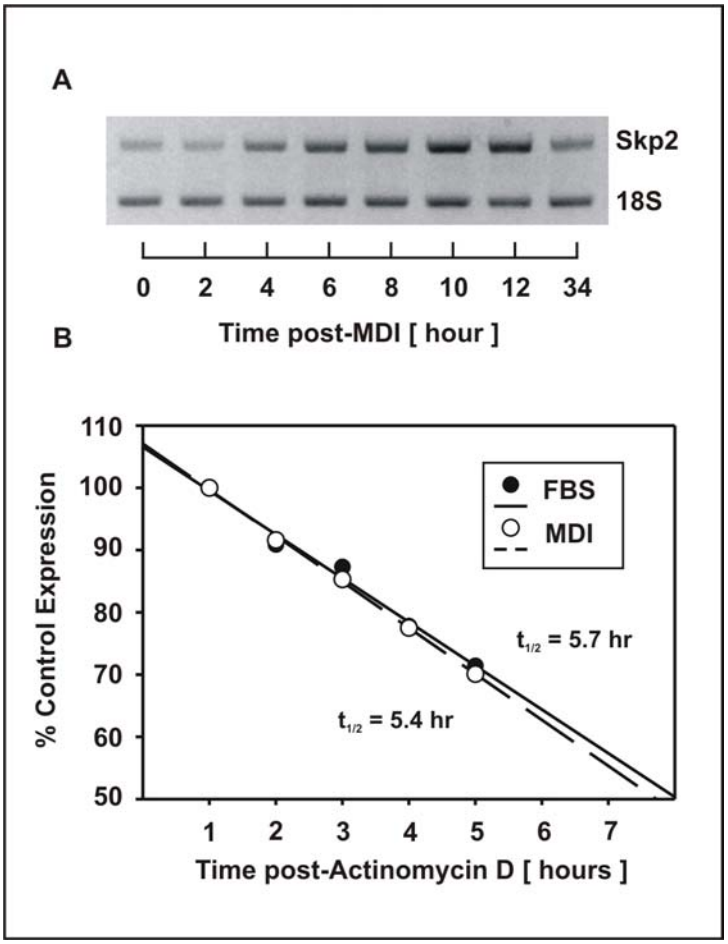

Figure 3.7. Changes in Skp2 mRNA occur early and are not due to changes in mRNA stability. A. Cultured preadipocytes were stimulated with MDI and RNA harvested along a short time course. mRNA was isolated and evaluated by RT-PCR. B. Cells were stimulated with FBS or FBS plus MDI. After 12 hours, 5μg/ml Actinomycin D was added to all plates and RNA harvested every hour for 5 hours. Samples were analyzed by RT-PCR.

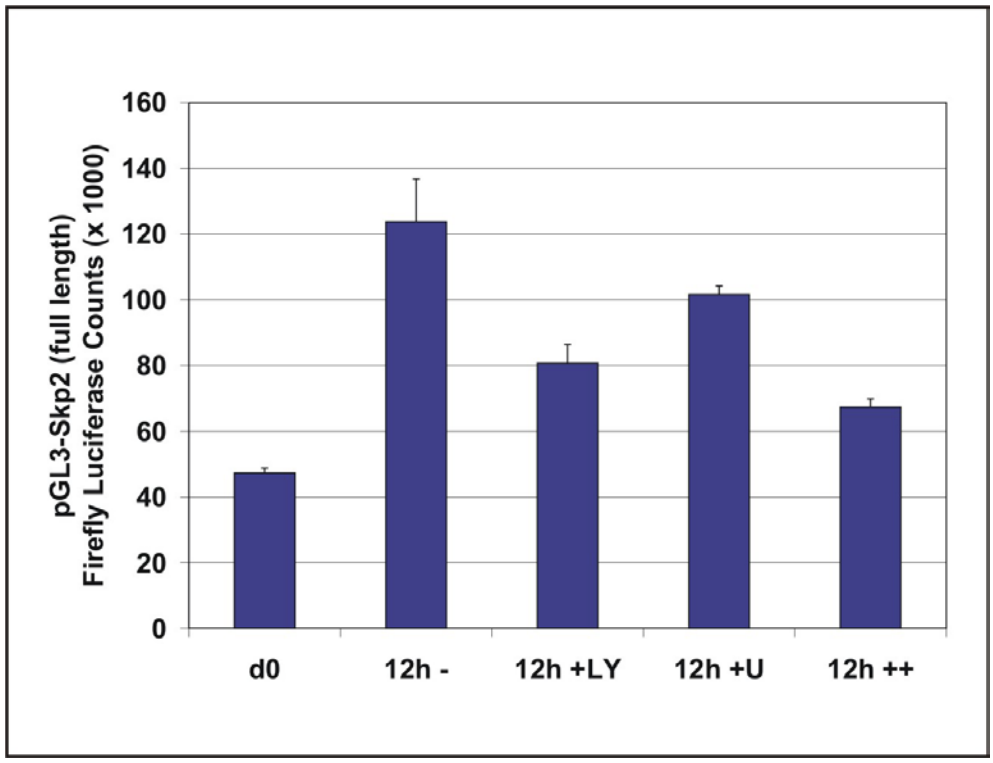

Figure 3.8. Skp2 promoter activity is increased following hormonal induction of adipogenesis. 3T3-L1 preadipocytes were transfected with 5.0 μg pGL3-Skp2 (full length promoter construct), grown to 2 days post-confluency, and stimulated with MDI, MDI + LY294002 (10μM), MDI + U0126 (10μM), or MDI + both inhibitors. Luciferase activity was measured 12 hours post-stimulation.

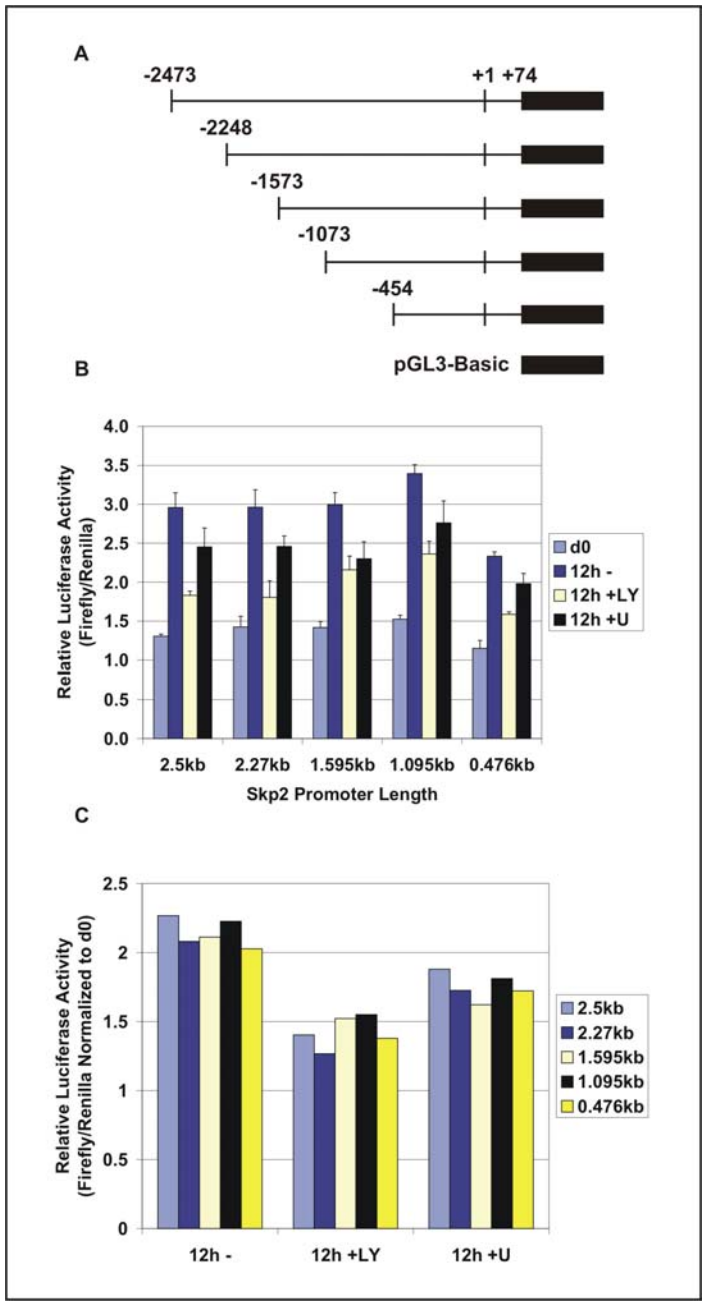

Figure 3.9. PI3K and MAPK responsive elements located within 500bp upstream of Skp2 gene. A. Schematic depiction of pGL3-Skp2 promoter deletion constructs. B and C. Preadipocytes were cotransfected with 5.0 μg of either pGL3-Skp2 full length (2.4kb), 2.2kb, 1.5kb, 1.0kb, or 0.4kb insert plus 20.0 ng renilla-SV40 as a transfection efficiency control. Cells were grown to 2 days post-confluency and stimulated with MDI, MDI + LY294002 (10μM), or MDI + U0126 (10μM). Luciferase activity was measured 12 hours post-stimulation.

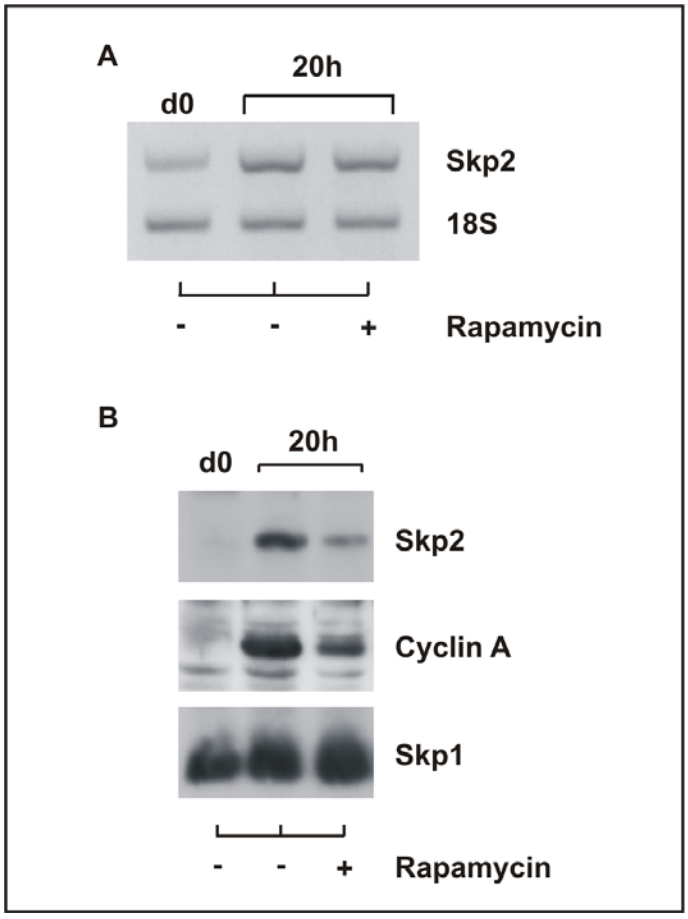

Figure 3.10. mTOR plays a role in regulating Skp2 protein expression during cell cycle progression. Cultured preadipocytes were stimulated with MDI with or without 10μM Rapamycin. A. mRNA was isolated and gene specific primers for Skp2 were used along with 18S control primers in RT-PCR reactions. B. Whole cell lysates were collected at d0 and 20hr following stimulation and protein levels evaluated by western blot analysis.

REFERENCES

Altiok,S., M.Xu, and B.M.Spiegelman. 1997. PPARgamma induces cell cycle withdrawal: inhibition of E2F/DP DNA-binding activity via down-regulation of PP2A. Genes Dev. 11: 1987-1998.

Andreu,E.J., E.Lledo, E.Poch, C.Ivorra, M.P.Albero, J.A.Martinez-Climent, C.Montiel-Duarte, J.Rifon, J.Perez-Calvo, C.Arbona, F.Prosper, and I.Perez-Roger. 2005. BCR-ABL induces the expression of Skp2 through the PI3K pathway to promote p27Kip1 degradation and proliferation of chronic myelogenous leukemia cells. Cancer Res. 65: 3264-3272.

Bai,C., P.Sen, K.Hofmann, L.Ma, M.Goebl, J.W.Harper, and S.J.Elledge. 1996. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell 86: 263-274.

Balendran,A., A.Casamayor, M.Deak, A.Paterson, P.Gaffney, R.Currie, C.P.Downes, and D.R.Alessi. 1999. PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. Curr. Biol. 9: 393-404.

Bashir,T., N.V.Dorrello, V.Amador, D.Guardavaccaro, and M.Pagano. 2004. Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase. Nature 428: 190-193.

Brennan,P., A.M.Mehl, M.Jones, and M.Rowe. 2002. Phosphatidylinositol 3-kinase is essential for the proliferation of lymphoblastoid cells. Oncogene 21: 1263-1271.

Brunet,A., A.Bonni, M.J.Zigmond, M.Z.Lin, P.Juo, L.S.Hu, M.J.Anderson, K.C.Arden, J.Blenis, and M.E.Greenberg. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96: 857-868.

Casamayor,A., N.A.Morrice, and D.R.Alessi. 1999. Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo. Biochem. J. 342 (Pt 2): 287-292.

Dong,Y., L.Sui, Y.Watanabe, K.Sugimoto, and M.Tokuda. 2003. S-phase kinase-associated protein 2 expression in laryngeal squamous cell carcinomas and its prognostic implications. Oncol. Rep. 10: 321-325.

El Chaar,D., A.Gagnon, and A.Sorisky. 2004. Inhibition of insulin signaling and adipogenesis by rapamycin: effect on phosphorylation of p70 S6 kinase vs eIF4E-BP1. Int. J. Obes. Relat Metab Disord. 28: 191-198.

Ganoth,D., G.Bornstein, T.K.Ko, B.Larsen, M.Tyers, M.Pagano, and A.Hershko. 2001. The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. Nat. Cell Biol. 3: 321-324.

Gao,N., Z.Zhang, B.H.Jiang, and X.Shi. 2003. Role of PI3K/AKT/mTOR signaling in the cell cycle progression of human prostate cancer. Biochem. Biophys. Res. Commun. 310: 1124-1132.

Hager,A., L.Sjorstrom, B.Arvidsson, P.Bjorntorp, and U.Smith. 1978. Adipose tissue cellularity in obese school girls before and after dietary treatment. Am. J. Clin. Nutr. 31: 68-75.

Hao,B., N.Zheng, B.A.Schulman, G.Wu, J.J.Miller, M.Pagano, and N.P.Pavletich. 2005. Structural basis of the Cks1-dependent recognition of p27(Kip1) by the SCF(Skp2) ubiquitin ligase. Mol. Cell 20: 9-19.

Hresko,R.C. and M.Mueckler. 2005. mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. J. Biol. Chem. 280: 40406-40416.

Hu,Y.Q. and Y.J.Liu. 2005. Expressions of Cx43 and Skp2 in epithelial ovarian tumor and their clinical significances. Ai. Zheng. 24: 104-109.

Imaki,H., K.Nakayama, S.Delehouzee, H.Handa, M.Kitagawa, T.Kamura, and K.I.Nakayama. 2003. Cell cycle-dependent regulation of the Skp2 promoter by GA-binding protein. Cancer Res. 63: 4607-4613.

Koga,H., M.Harada, M.Ohtsubo, S.Shishido, H.Kumemura, S.Hanada, E.Taniguchi, K.Yamashita, R.Kumashiro, T.Ueno, and M.Sata. 2003. Troglitazone induces p27Kip1-associated cell-cycle arrest through down-regulating Skp2 in human hepatoma cells. Hepatology 37: 1086-1096.

Kops,G.J., R.H.Medema, J.Glassford, M.A.Essers, P.F.Dijkers, P.J.Coffer, E.W.Lam, and B.M.Burgering. 2002. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol. Cell Biol. 22: 2025-2036.

Kudo,Y., S.Kitajima, S.Sato, M.Miyauchi, I.Ogawa, and T.Takata. 2001. High expression of S-phase kinase-interacting protein 2, human F-box protein, correlates with poor prognosis in oral squamous cell carcinomas. Cancer Res. 61: 7044-7047.

Langner,C., R.von Wasielewski, M.Ratschek, P.Rehak, and R.Zigeuner. 2004. Biological significance of p27 and Skp2 expression in renal cell carcinoma. A systematic analysis of primary and metastatic tumour tissues using a tissue microarray technique. Virchows Arch. 445: 631-636.

Latres,E., D.S.Chiaur, and M.Pagano. 1999. The human F box protein beta-Trcp associates with the Cul1/Skp1 complex and regulates the stability of beta-catenin. Oncogene 18: 849-854.

Lavoie,J.N., G.L'Allemain, A.Brunet, R.Muller, and J.Pouyssegur. 1996. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J. Biol. Chem. 271: 20608-20616.

Li,Q., M.Murphy, J.Ross, C.Sheehan, and J.A.Carlson. 2004. Skp2 and p27kip1 expression in melanocytic nevi and melanoma: an inverse relationship. J. Cutan. Pathol. 31: 633-642.

Mamillapalli,R., N.Gavrilova, V.T.Mihaylova, L.M.Tsvetkov, H.Wu, H.Zhang, and H.Sun. 2001. PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27(KIP1) through the ubiquitin E3 ligase SCF(SKP2). Curr. Biol. 11: 263-267.

Mandenoff,A., T.Lenoir, and M.Apfelbaum. 1982. Tardy occurrence of adipocyte hyperplasia in cafeteria-fed rat. Am. J. Physiol 242: R349-R351.

Moberg,K., M.A.Starz, and J.A.Lees. 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol. Cell Biol. 16: 1436-1449.

Morrison,R.F. and S.R.Farmer. 1999. Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. J. Biol. Chem. 274: 17088-17097.

Naaz,A., D.R.Holsberger, G.A.Iwamoto, A.Nelson, H.Kiyokawa, and P.S.Cooke. 2004. Loss of cyclin-dependent kinase inhibitors produces adipocyte hyperplasia and obesity. FASEB J. 18: 1925-1927.

Nakayama,K., H.Nagahama, Y.A.Minamishima, M.Matsumoto, I.Nakamichi, K.Kitagawa, M.Shirane, R.Tsunematsu, T.Tsukiyama, N.Ishida, M.Kitagawa, K.Nakayama, and S.Hatakeyama. 2000. Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. EMBO J. 19: 2069-2081.

Oliveira,A.M., S.H.Okuno, A.G.Nascimento, and R.V.Lloyd. 2003. Skp2 protein expression in soft tissue sarcomas. J. Clin. Oncol. 21: 722-727.

Osoegawa,A., I.Yoshino, S.Tanaka, K.Sugio, T.Kameyama, M.Yamaguchi, and Y.Maehara. 2004. Regulation of p27 by S-phase kinase-associated protein 2 is associated with aggressiveness in non-small-cell lung cancer. J. Clin. Oncol. 22: 4165-4173.

Pettersson,P., R.Van, M.Karlsson, and P.Bjorntorp. 1985. Adipocyte precursor cells in obese and nonobese humans. Metabolism 34: 808-812.

Sarbassov,D.D., D.A.Guertin, S.M.Ali, and D.M.Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307: 1098-1101.

Sarmento,L.M., H.Huang, A.Limon, W.Gordon, J.Fernandes, M.J.Tavares, L.Miele, A.A.Cardoso, M.Classon, and N.Carlesso. 2005. Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and p27 Kip1 degradation. J. Exp. Med. 202: 157-168.

Seki,R., T.Okamura, H.Koga, K.Yakushiji, M.Hashiguchi, K.Yoshimoto, H.Ogata, R.Imamura, Y.Nakashima, M.Kage, T.Ueno, and M.Sata. 2003. Prognostic significance of the F-box protein Skp2 expression in diffuse large B-cell lymphoma. Am. J. Hematol. 73: 230-235.

Shah,S.A., M.W.Potter, M.H.Hedeshian, R.D.Kim, R.S.Chari, and M.P.Callery. 2001. PI-3' kinase and NF-kappaB cross-signaling in human pancreatic cancer cells. J. Gastrointest. Surg. 5: 603-612.

Shapira,M., O.Ben Izhak, S.Linn, B.Futerman, I.Minkov, and D.D.Hershko. 2005. The prognostic impact of the ubiquitin ligase subunits Skp2 and Cks1 in colorectal carcinoma. Cancer 103: 1336-1346.

Smith,P.J., L.S.Wise, R.Berkowitz, C.Wan, and C.S.Rubin. 1988. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. J. Biol. Chem. 263: 9402-9408.

Spruck,C., H.Strohmaier, M.Watson, A.P.Smith, A.Ryan, T.W.Krek, and S.I.Reed. 2001. A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. Mol. Cell 7: 639-650.

Tang,Q.Q., M.Gronborg, H.Huang, J.W.Kim, T.C.Otto, A.Pandey, and M.D.Lane. 2005. Sequential phosphorylation of CCAAT enhancer-binding protein beta by MAPK and glycogen synthase kinase 3beta is required for adipogenesis. Proc. Natl. Acad. Sci. U. S. A 102: 9766-9771.

Tang,Q.Q. and M.D.Lane. 1999. Activation and centromeric localization of CCAAT/enhancer-binding proteins during the mitotic clonal expansion of adipocyte differentiation. Genes Dev. 13: 2231-2241.

Tanno,S., S.Tanno, Y.Mitsuuchi, D.A.Altomare, G.H.Xiao, and J.R.Testa. 2001. AKT activation up-regulates insulin-like growth factor I receptor expression and promotes invasiveness of human pancreatic cancer cells. Cancer Res. 61: 589-593.

Tullai,J.W., M.E.Schaffer, S.Mullenbrock, S.Kasif, and G.M.Cooper. 2004. Identification of transcription factor binding sites upstream of human genes regulated by the phosphatidylinositol 3-kinase and MEK/ERK signaling pathways. J. Biol. Chem. 279: 20167-20177.

Verona,R., K.Moberg, S.Estes, M.Starz, J.P.Vernon, and J.A.Lees. 1997. E2F activity is regulated by cell cycle-dependent changes in subcellular localization. Mol. Cell Biol. 17: 7268-7282.

Villena,J.A., O.Vinas, T.Mampel, R.Iglesias, M.Giralt, and F.Villarroya. 1998. Regulation of mitochondrial biogenesis in brown adipose tissue: nuclear respiratory factor-2/GA-binding protein is responsible for the transcriptional regulation of the gene for the mitochondrial ATP synthase beta subunit. Biochem. J. 331 (Pt 1): 121-127.

Wang,W., D.Ungermannova, J.Jin, J.W.Harper, and X.Liu. 2004. Negative regulation of SCFSkp2 ubiquitin ligase by TGF-beta signaling. Oncogene 23: 1064-1075.

Wei,W., N.G.Ayad, Y.Wan, G.J.Zhang, M.W.Kirschner, and W.G.Kaelin, Jr. 2004. Degradation of the SCF component Skp2 in cell-cycle phase G1 by the anaphase-promoting complex. Nature 428: 194-198.

Wirbelauer,C., H.Sutterluty, M.Blondel, M.Gstaiger, M.Peter, F.Reymond, and W.Krek. 2000. The F-box protein Skp2 is a ubiquitylation target of a Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. EMBO J. 19: 5362-5375.

Yam,C.H., R.W.Ng, W.Y.Siu, A.W.Lau, and R.Y.Poon. 1999. Regulation of cyclin A-Cdk2 by SCF component Skp1 and F-box protein Skp2. Mol. Cell Biol. 19: 635-645.

Yang,G., G.Ayala, A.De Marzo, W.Tian, A.Frolov, T.M.Wheeler, T.C.Thompson, and J.W.Harper. 2002. Elevated Skp2 protein expression in human prostate cancer: association with loss of the cyclin-dependent kinase inhibitor p27 and PTEN and with reduced recurrence-free survival. Clin. Cancer Res. 8: 3419-3426.

Zhang,J.W., Q.Q.Tang, C.Vinson, and M.D.Lane. 2004. Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes. Proc. Natl. Acad. Sci. U. S. A 101: 43-47.

Zhang,L. and C.Wang. 2005b. F-box protein Skp2: a novel transcriptional target of E2F. Oncogene.

Zhang,L. and C.Wang. 2005a. F-box protein Skp2: a novel transcriptional target of E2F. Oncogene.

Zhang,Y.W., K.Nakayama, K.Nakayama, and I.Morita. 2003. A novel route for connexin 43 to inhibit cell proliferation: negative regulation of S-phase kinase-associated protein (Skp 2). Cancer Res. 63: 1623-1630.

CHAPTER IV

NOVEL EFFECT OF HELENALIN ON AKT SIGNALING AND SKP2 EXPRESSION IN 3T3-L1 PREADIPOCYTES

ABSTRACT

Helenalin is a biologically active, naturally-occurring compound contained in the Compositae family of plants. It is known to have anti-inflammatory and anti-carcinogenic properties, yet mechanisms behind these actions are still being determined. We have previously shown that the F-box protein Skp2 is highly regulated during preadipocyte proliferation. Data presented here demonstrate that helenalin is a very potent inhibitor of Skp2 mRNA expression in 3T3-L1 preadipocytes when added with adipogenic media at day 0, before induction of Skp2. Additionally, when helenalin is added to cells 6 hours post-MDI, established Skp2 mRNA levels are reduced significantly. We have determined Skp2 mRNA to have a relatively short half-life (i.e., 5.5 hours), suggesting a rapid effect of helenalin. Further studies demonstrate helenalin's ability to completely block the increase in Skp2 promoter activity that occurs following MDI stimulation. The effect of helenalin on Skp2 expression occurs very early, within 3 hours following stimulation, and can be seen with changes in proliferation media alone. An examination of signaling events previously shown, by our lab, to be important for Skp2 upregulation revealed impaired Akt phosphorylation in the presence of helenalin. These studies are the first to show an effect of helenalin on Skp2 and growth factor receptor signaling during early stages of adipocyte differentation.

INTRODUCTION

The sesquiterpene lactone, helenalin, is a component found in the aerial portion of the flowering plant, arnica montana L. Arnica is a native plant of mountainous regions in Europe, specifically Russia, and has long been used as an anti-inflammatory treatment for external wounds (e.g., bruises) and was approved by The European Scientific Cooperative on Phytotherapy (ESCOP) to be used as such. One of the very early studies demonstrated that helenalin is an effective cardiotonic agent, improving cardiac strength in an *in vivo* animal model through increases in cyclic AMP (Itoigawa et al., 1987). However, helenalin is primarily used in cellular studies as an inhibitor of NFκB p65, a function that is important for its known anti-inflammatory effects. Hehner et al. suggests this occurs through inhibition of IKK activity and subsequent IκB degradation (Hehner et al., 1998;Hehner et al., 1998). Others demonstrate helenalin's ability to directly modify p65 via alkylation, thereby preventing p65 DNA-binding (Lyss et al., 1998).

Recent attention has been focused on determining the mechanism(s) by which helenalin exerts anti-tumor effects. Helenalin induces apoptosis at 10-50 μM concentrations in leukemia Jurkat T cells (Dirsch et al., 2001a;Dirsch et al., 2001b), a highly desired outcome of chemotherapeutic treatments. Interestingly, helenalin has also been shown to be a highly effective inhibitor of telomerase activity in cancer cells, primarily through alkylation and subsequent inactivation of the enzyme (Huang et al., 2005). In addition, several sesquiterpene lactones, including helenalin, possess powerful antioxidant activity, causing decreased lipid peroxidation (Jodynis-Liebert et al., 1999;Jodynis-Liebert et al., 2000).

These and other studies provide clear evidence that helenalin has multiple modes of action within the cell, aside from its commonly cited ability to inhibit NFκB DNA-binding. While helenalin is known to have anti-proliferative properties, a role in regulating cell cycle stage progression has yet to be investigated. Skp2 is an important positive regulator of proliferation and is overexpressed in many types of cancers. It functions as an F-box protein, the substrate recognition portion of the SCF^{Skp2} E3 Ligase. Skp2 is known to cause polyubiquitylation and subsequent degradation of negative cell cycle regulators p27 (Pagano et al., 1995;Carrano et al., 1999), p21 (Bornstein et al., 2003;Wang et al., 2005), p57 (Kamura et al., 2003), and p130 (Tedesco et al., 2002). Our lab has recently shown Skp2 to also be expressed during early stages of adipocyte differentiation when cells enter a short synchronous period of replication. Here, we demonstrate a very potent effect of helenalin on Skp2 expression and signaling events leading to induction of Skp2.

MATERIALS AND METHODS

Materials - Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Cellgro by Mediatech. Bovine Serum, Fetal Bovine Serum (FBS), and Trypsin-EDTA were from Invitrogen Corporation. Propidium Iodide and RNase A was purchased from Sigma. Chemical inhibitors were purchased from the following: Helenalin (Biomol) and Actinomycin D (Sigma). The following antibodies from Cell Signaling were used for western blot analysis: phospho-Akt (Ser473) and phospho-p44/42 (Thr202/Tyr204). Enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Sciences.

Cell Culture and Differentiation - Murine 3T3-L1 preadipocytes were propagated in growth medium containing DMEM supplemented with 10% calf bovine serum as described previously (Morrison and Farmer, 1999). By standard differentiation protocol, preadipocytes were propagated in growth medium until reaching a state of density arrest at 2 days post-confluence. Growth medium was replaced at density arrest with differentiation medium comprised of DMEM supplemented with 10% fetal bovine serum (FBS), 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). The term "post-MDI" refers to the time elapsed since the addition of MDI to the culture medium. At 2 days post-MDI, medium was changed to DMEM supplemented with 10% FBS and 0.4 μM insulin. From 4 days post-MDI until harvest, maintenance medium containing DMEM supplemented only with 10% FBS was changed every 48 hrs. Throughout the study, "time 0" refers to density arrested cells immediately before chemical induction of differentiation with the addition of MDI to the culture medium. Experiments described herein were conducted within the period of differentiation spanning from density arrest (0 hr) through 34 hrs post-MDI. All experiments were repeated three to five times to validate results and ensure reliability.

Protein Analysis - Cell monolayers were washed with PBS and scraped into ice cold lysis buffer containing 1.0 M Tris, pH 7.4, 150 mM NaCl, 1% Triton X, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, and 10 mM N-ethylmaleimide (NEM). Lysis buffer was freshly supplemented with phosphatase inhibitors; 20mM β-glycerophosphate, 10mM NaFl, and 2 μM sodium vanadate, as well as protease inhibitors; 0.3 μM aprotonin, 21 μM leupeptin, E-64, 1 μM pepstatin, 50 μM phenanthroline, 0.5 μM phenylmethylsulfonyl fluoride. Cell lysates were clarified by centrifugation (13,000 x g, 10 min, 4°C) and

protein concentration was determined by BCA assay (Pierce). Lysates were resuspended in loading buffer containing 0.25 M Tris, pH 6.8, 4% SDS, 10% glycerol, 10% dithiotreitol, 0.01% bromophenol blue, heated for 5 mins at 80°C, and placed on ice. Lysates were resolved on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore). Following transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4°C and horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Immunoblots were developed with ECL and visualized by autoradiography CL-XPosure film (Pierce).

RNA Isolation and Analysis - Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to manufacturer's instructions. Total RNA (1μg) was subjected to Reverse-Transcriptase Polymerase Chain Reaction using One-Step RT-PCR kit (QIAGEN) according to manufacturer's instruction. Briefly, RT-PCR reactions were carried out in the presence of the supplied buffer, dNTPs (400 μM), enzyme mix (reverse transcriptases and DNA polymerase), and RNase inhibitor (10 U). Gene specific primers were used at a final concentration of 0.6 μM and designed as follows: Skp2 forward, 5′-GGCAAAGGGAGTGACAAAGA-3′; Skp2 reverse, 5′-TCAAAGCACCAGGAGAGATT-3′ (product size = 590 bp). QuantamRNA Classic II 18S primer/competimers were used in the reactions as internal standards at a final concentration of 0.6μM.

Plasmids – Promega's pGL3-basic vector was used to create mouse Skp2-pGL3 constructs for luciferase assays. Primers flanking the region of -2404 to +74 (2478bp) were used to amplify the murine Skp2 promoter from 3T3-L1 preadipocyte genomic DNA. Positive DNA fragments were gel purified. Vector and isolated genomic DNA were

double-digested with Kpn I and Bgl II. The resulting DNA fragment was subcloned into the pGL3-basic vector. Nucleotide sequencing was conducted by SeqWright DNA Technology Services. This entire process was repeated twice to ensure reliability of results.

Luciferase Reporter Assays – 3T3-L1 preadipocytes (2.0 x 10^6 cells) were transiently transfected with 5 μg of firefly reporter plasmid using Amaxa's Nucleofector (program T20) and incubated with pre-warmed proliferation media for no more than 10 minutes at 37°C. Cells were then replated at a confluent density, fresh media replaced 24 hours post-transfection, and further cultured as indicated. Firefly luciferase was measured using the Dual-Glo Luciferase Assay System (Promega) and reported as firefly luciferase counts.

RESULTS

A potential effect of helenalin on Skp2 expression was examined by treating 3T3-L1 preadipocytes with 3, 5, or 8 μM helenalin at the time of adipogenic stimulation. Our previous experiments show that the increase in Skp2 protein during preadipocyte proliferation is preceded by an early and dramatic increase in Skp2 mRNA (Chapter III). Therefore, RNA was collected at d0 (i.e., 2 days post-confluency) when cells are density-arrested and then 12 hours with or without varying concentrations of helenalin. As shown in Figure 4.1, Helenalin decreases Skp2 mRNA at 12 hours and this effect is dose-dependent. A significant decrease can be seen with as little as 3 μM and because this compound is known to exhibit cytotoxic effects, all subsequent studies were carried

out using this lower concentration. It is important to note that this concentration is also below the IC_{50} which has been determined to be 6μM (Dirsch et al., 2001a).

Skp2 mRNA begins to increase by 4 hours following stimulation and is highly expressed at 12 hours (Figure 4.2A). Figure 4.1 shows an effect of helenalin when added before Skp2 mRNA increases (i.e., at time of stimulation). Helenalin was added at 6 hours post-stimulation to determine if increased levels of Skp2 mRNA could be reduced as well. 3T3-L1 cells were stimulated with MDI and helenalin added at that time or 6 hours post-stimulation. RNA was harvested 12 hours after the initial time of stimulation. We found that adding helenalin 6 hours after stimulation, a time at which Skp2 mRNA levels have already significantly increased, results in the same degree of inhibition as adding it with the stimulation media (Figure 4.2B). These findings suggest several possibilities. First, helenalin could be inhibiting something that occurs after 6 hours, therefore, when adding helenalin at stimulation, Skp2 mRNA would still increase until 6 hours but would then be prevented from any further increase after that. In this case, a long mRNA half-life would explain how adding helenalin at these two times would produce similar results. Another possibility is that the effect of helenalin occurs earlier than 6 hours post-MDI and Skp2 mRNA has a short half-life. Actinomycin D, a potent inhibitor of RNA synthesis, was used to determine Skp2 mRNA half-life. The results of these studies indicated Skp2 mRNA half-life to be approximately 5.5 hours (Chapter III). This is a relatively short half-life and is less than the amount of time cells were exposed to helenalin after adding it 6 hours post-MDI. Thus, it appears that helenalin may have a quick effect and Skp2 mRNA turnover is rapid enough to cause such decreased levels under these conditions.

To gain insight into how helenalin decreases Skp2, we determined the timeframe in which this effect occurs by examining mRNA levels at earlier time points following treatment. 3T3-L1 cells were stimulated plus or minus helenalin and RNA was collected at 3, 6, and 12 hours. As shown in Figure 4.3, we found that when added with the stimulation cocktail, helenalin blocks the induction of Skp2 mRNA by 6 hours and as early as 3 hours. Under normal conditions, Skp2 mRNA levels are slightly increased by 3 hours and this induction is completely inhibited by helenalin. These findings suggest helenalin blocks a very early step in the process of Skp2 gene expression.

Our lab has shown that during initial stages of 3T3-L1 preadipocyte differentiation, Skp2 protein expression is primarily driven by increases in promoter activity (Chapter III). Therefore, preadipocytes were transfected with a Skp2 promoter (2.4kb) luciferase reporter construct and grown to 2 days post-confluency. The resulting population of cells was stimulated with or without helenalin and Skp2 promoter activity was measured 12 hours after stimulation. As shown in Figure 4.4, helenalin completely blocks the induction of Skp2 promoter activity, as luciferase activity at 12 hours is no different from d0. Taken together, these data show that helenalin prevents the increase in Skp2 promoter activity and resulting mRNA expression.

We have formerly observed an increase in Skp2 promoter activity with a change in normal proliferation media (unpublished observations). While this is not sufficient to produce a subsequent increase in protein (Chapter III - Figure 3.4), we examined whether an increase in Skp2 mRNA occurs under the same conditions and if so, whether helenalin is also capable of blocking that increase. At d0, 3T3-L1 cells were exposed to a media change with calf serum (proliferation media) with or without helenalin, and RNA samples collected after 6 and 12 hours. As seen in Figure 4.5, a change in media did

result in elevated levels of Skp2 mRNA, although not to the degree seen with MDI stimulation (Figure 4.2A). Helenalin was indeed able to completely block this increase, suggesting its target is an intermediate step common to changes in proliferation and stimulation media.

Serum (calf and fetal bovine) can activate different signaling cascades through growth factor receptors, although not to the degree seen with insulin stimulation. Our lab previously demonstrated that the PI3K and MAPK pathways are required for Skp2 expression, with PI3K playing the predominant role. Therefore, cells were stimulated plus or minus helenalin and immunoblotting used to examine the phosphorylation state of Akt and ERK, important downstream targets of PI3K and MAPKK respectively. Under normal conditions, Akt is phosphorylated by 15 minutes after stimulation with MDI and can still be seen as late as 12 hours (Figure 4.6). ERK is also phosphorylated within 15 minutes of stimulation, a pattern that persists through at least 6 hours. We found that while helenalin had no effect on phospho-ERK, dramatic changes were seen in the phosphorylation of Akt on a residue critical for activity (Ser473). A decrease in Akt phosphorylation was observed as early as 3 hours, and this inhibition was further amplified by 6 and 12 hours. These data suggest a potent effect of helenalin on Akt signaling.

DISCUSSION

Dirsch et al. (Dirsch et al., 2001b) report a lag time of approximately 16 hours before signs of apoptosis appear in Jurkat T cells after treatment with helenalin, while others report neuronal death with 30 μM helenalin after only 6 hours (Chiarugi, 2002). The length of exposure in the studies presented in this paper was 12 hours and no

visible signs of toxicity were seen with 3 μM. However, we have seen very apparent signs of toxicity using 10 μM concentrations of helenalin in less than 12 hours (unpublished observations). It is important to note that the concentration at which we used helenalin is significantly lower than the IC_{50} (6 μM). Interestingly, healthy PHA-stimulated PBMC cells are resistant to the apoptotic effects of helenalin, at concentrations as much as 50 μM (Dirsch et al., 2001b), suggesting a cell-type specific and dose-dependent effect of helenalin on apoptotic signaling.

Skp2 promotes cell cycle progression during neoplastic growth and can be regulated by transcriptional and post-translational mechanisms. We have previously reported transient expression of Skp2 during mitotic clonal expansion of adipocyte differentiation, primarily due to promoter activation. Reduction of Skp2 levels with siRNA revealed a role for Skp2 in the degradation of p27 during proliferation, providing insight into the function of Skp2 in preadipocytes. Discerning mechanisms regulating preadipocyte replication such as this is essential for understanding the etiology of hyperplastic obesity. In results reported here, we show a very strong effect of helenalin on Skp2 expression. Helenalin is not only able to completely block the induction of Skp2 mRNA but prevents Skp2 promoter activity during early stages of adipocyte differentiation. While this regulation may also be mediated by changes in NFκB activity, we show that Akt signaling is impaired as helenalin almost completely blocks Akt phosphorylation following adipogenic stimulation. Previous work by our lab has shown that Skp2 expression during preadipocyte proliferation is dependent on activation of the PI3K pathway, suggesting the effect of helenalin on Skp2 expression is through changes in Akt signaling. Further studies are needed to discern whether helenalin is having a direct effect on Akt or whether the block in NFκB DNA-binding is somehow responsible.

We hypothesize that there is a direct effect of helenalin on Akt, or upstream kinases, due to the very rapid ability of helenalin to block Akt phosphorylation. As well, Akt is traditionally thought to be upstream of NFκB activation and a reversal in this process would certainly be novel and unexpected.

The cytotoxic effect of helenalin on tumor cells has been well-documented but how this happens is still under investigation. Data presented here suggest a block in growth factor receptor signaling due to the very early effect of helenalin and helenalin's ability to block the effects of serum on Skp2 mRNA expression. The PI3K/Akt pathway activates pro-mitotic survival signals following serum stimulation. The ability of this pathway to inhibit apoptosis is dependent on Akt phosphorylation and subsequent activation (Kennedy et al., 1997). Kennedy et al. showed that Akt blocks apoptosis by inhibiting Ced3/ICE-like activity, more commonly referred to as caspase activity (Kennedy et al., 1997). Interestingly, the broad-spectrum caspase inhibitor zVAD-fmk is able to abolish helenalin's ability to induce apoptosis in human leukemia Jurkat T cells (Dirsch et al., 2001b). Specifically, caspases 3 and 8 were shown to be involved in the process. We show that helenalin is an extremely potent inhibitor of Akt phosphorylation. This is a very specific effect as the phosphorylation of ERK remains unchanged. These findings suggest that inactivation of Akt signaling is the upstream event regulating helenalin's anti-mitotic caspase-dependent actions. In contrast to helenalin's early reduction of Akt phosphorylation, another sesquiterpene lactone, parthenolide, causes a dramatic increase in Akt phosphorylation following two to three hours of exposure (Chiarugi, 2002). Longer periods of exposure to parthenolide cause a decrease in Akt phosphorylation, similar to helenalin's early effect on Akt phosphorylation in 3T3-L1 reported here. Future studies are needed to evaluate helenalin's effect on events

upstream of Akt. However, it is very clear that helenalin's potent effects can be attributed to its ability to affect survival signals within the cell at many different levels (e.g., impaired Akt signaling, reduced p65 DNA-binding, and inhibition of telomerase activity).

Future studies are needed to examine the effect of helenalin on adipocyte differentiation. One might hypothesize a negative effect of helenalin on processes involved in adipocyte differentiation as it is known to block p65 DNA-binding and now shown to inhibit Akt phosphorylation. This line of investigation is also quite interesting knowing helenalin's most cited role as an anti-inflammatory agent. Obesity has recently been regarded as a chronic state of low-level inflammation, as adipocytes secrete pro-inflammatory cytokines such as TNFα (Hotamisligil et al., 1993;Hotamisligil et al., 1995) and IL-6 (Mohamed-Ali et al., 1998). Moreover, NFκB activity is highest during late stages of adipogenesis (Berg et al., 2004) and has been implicated in the release of cytokines from adipocytes (Skurk et al., 2004). We show that helenalin inhibits a key cell cycle regulator during preadipocyte hyperplasia, but perhaps this potent sesquiterpene lactone may also have effects on late stages of adipocyte differentiation and endocrine function.

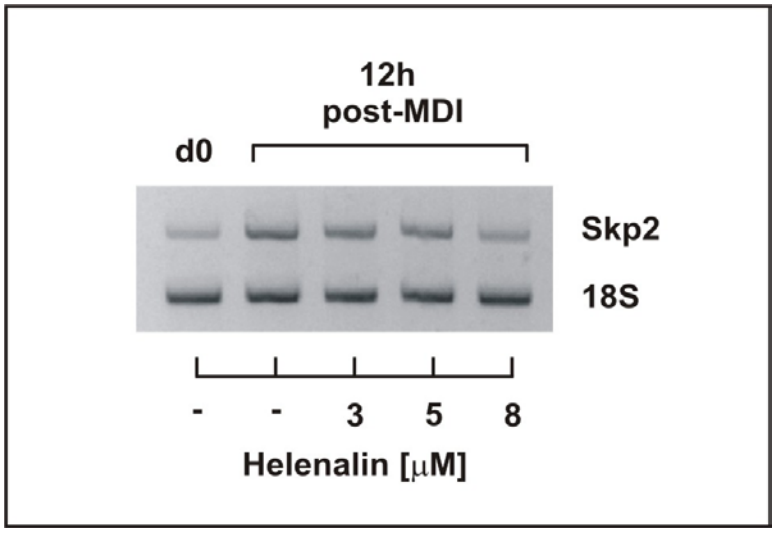

Figure 4.1. Helenalin decreases Skp2 mRNA expression in a dose-dependent manner. 3T3-L1 preadipocytes were grown to 2 days post-confluency and stimulated with 3, 5, or 8 μM helenalin. Total RNA was collected after 12 hours of stimulation. RNA was isolated and subjected to RT-PCR using gene-specific primers targeting Skp2. 18S primer/competimer technology was used as a control.

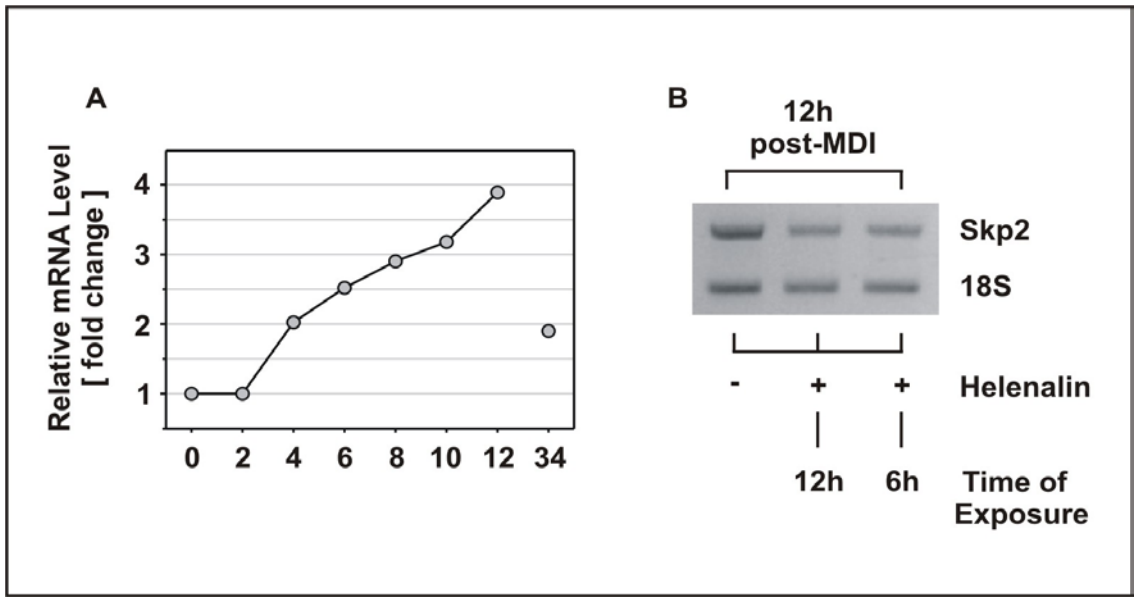

Figure 4.2. Helenalin is able to block the increase in Skp2 even after Skp2 has begun to be expressed. A. 3T3-L1 cells were stimulated with adipogenic media and RNA collected every 2 hours through 12 hours and then again at 34 hours. B. 3T3-L1 preadipocytes were stimulated and 3 μM helenalin added at time of stimulation or after 6 hours. Samples were collected 12 hours post-stimulation and Skp2 mRNA expression examined using RT-PCR.

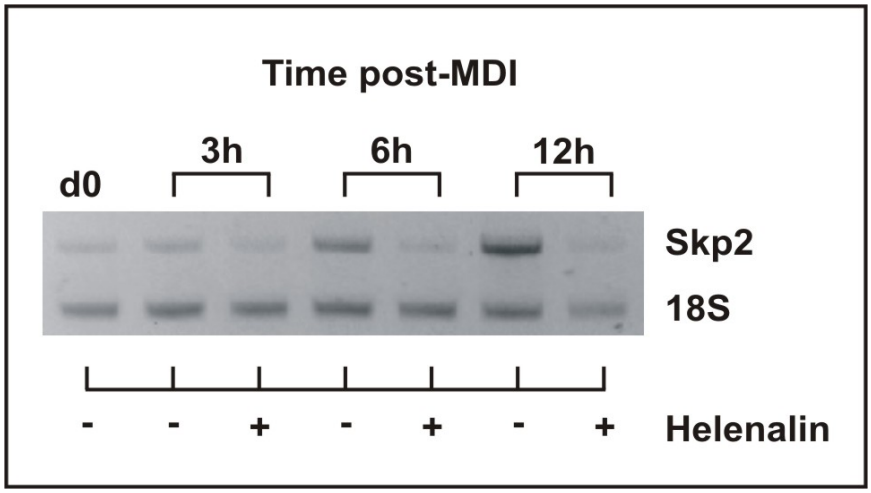

Figure 4.3. The decrease in Skp2 mRNA caused by helenalin occurs early. Preadipocytes were stimulated in the presence of 3 μM helenalin and RNA collected following 3, 6, and 12 hours. Skp2 mRNA expression was determined using RT-PCR.

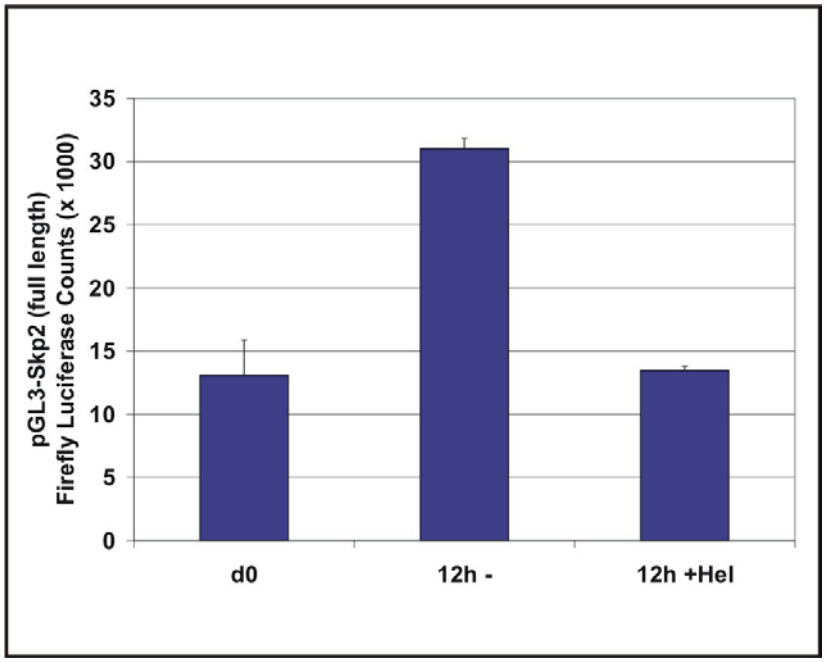

Figure 4.4. Helenalin prevents Skp2 promoter activity during preadipocyte hyperplasia. 3T3-L1 preadipocytes were transiently transfected with 5 μg of a pGL3-Skp2 promoter hybrid luciferase vector. Cells were stimulated with MDI plus or minus 3 μM helenalin and assayed 12 hours later for firefly luciferase activity.

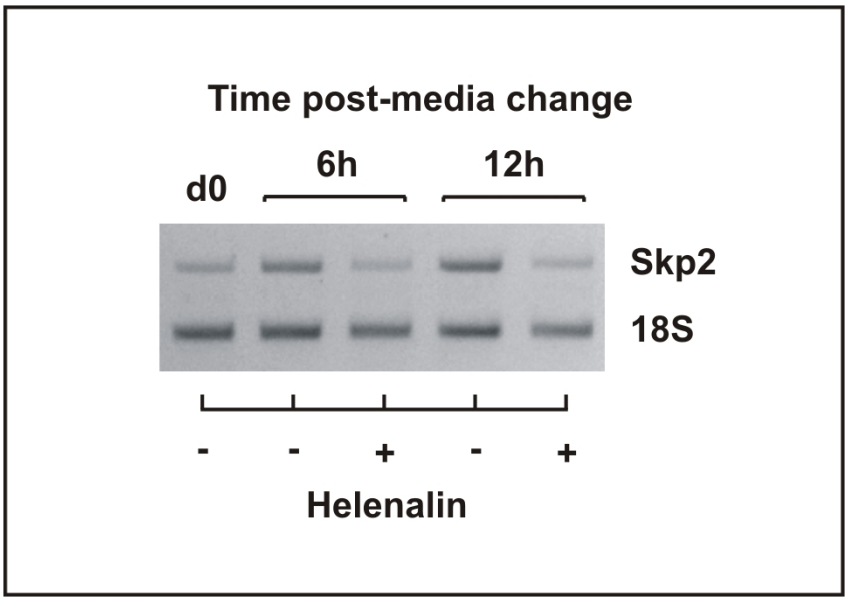

Figure 4.5. Skp2 mRNA increases with a change in proliferation media and helenalin is able to block this increase. A day 0 sample with no media change was collected as a control for Skp2 basal levels. Subsequently, cells received a media change with 10% calf serum in DMEM (proliferation media) plus or minus 3 μM helenalin. RNA was collected after 6 and 12 hours and RT-PCR performed.

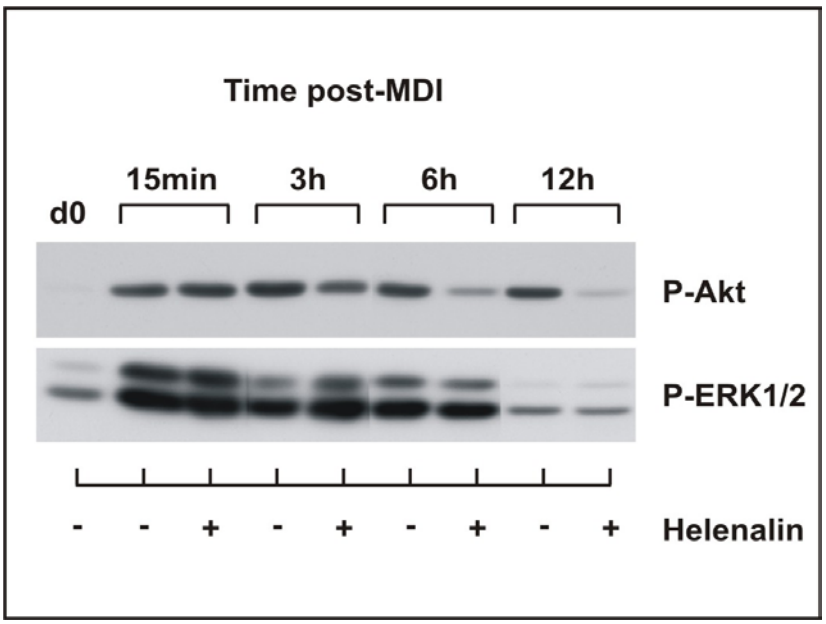

Figure 4.6. Helenalin blocks the phosphorylation of Akt but not ERK. Preadipocytes were stimulated with MDI plus or minus 3 μM helenalin and total cellular protein was harvested after 15 minutes, 3, 6, and 12 hours. An unstimulated d0 sample was also collected. Protein expression was examined by western blot analysis using indicated antibodies.

REFERENCES

Berg,A.H., Y.Lin, M.P.Lisanti, and P.E.Scherer. 2004. Adipocyte differentiation induces dynamic changes in NF-kappaB expression and activity. Am. J. Physiol Endocrinol. Metab 287: E1178-E1188.

Bornstein,G., J.Bloom, D.Sitry-Shevah, K.Nakayama, M.Pagano, and A.Hershko. 2003. Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. J. Biol. Chem. 278: 25752-25757.

Carrano,A.C., E.Eytan, A.Hershko, and M.Pagano. 1999. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol. 1: 193-199.

Chiarugi,A. 2002. Characterization of the molecular events following impairment of NF-kappaB-driven transcription in neurons. Brain Res. Mol. Brain Res. 109: 179-188.

Dirsch,V.M., H.Stuppner, and A.M.Vollmar. 2001a. Cytotoxic sesquiterpene lactones mediate their death-inducing effect in leukemia T cells by triggering apoptosis. Planta Med. 67: 557-559.

Dirsch,V.M., H.Stuppner, and A.M.Vollmar. 2001b. Helenalin triggers a CD95 death receptor-independent apoptosis that is not affected by overexpression of Bcl-x(L) or Bcl-2. Cancer Res. 61: 5817-5823.

Hehner,S.P., M.Heinrich, P.M.Bork, M.Vogt, F.Ratter, V.Lehmann, K.Schulze-Osthoff, W.Droge, and M.L.Schmitz. 1998. Sesquiterpene lactones specifically inhibit activation of NF-kappa B by preventing the degradation of I kappa B-alpha and I kappa B-beta. J. Biol. Chem. 273: 1288-1297.

Hotamisligil,G.S., P.Arner, J.F.Caro, R.L.Atkinson, and B.M.Spiegelman. 1995. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J. Clin. Invest 95: 2409-2415.

Hotamisligil,G.S., N.S.Shargill, and B.M.Spiegelman. 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259: 87-91.

Huang,P.R., Y.M.Yeh, and T.C.Wang. 2005. Potent inhibition of human telomerase by helenalin. Cancer Lett. 227: 169-174.

Itoigawa,M., K.Takeya, H.Furukawa, and K.Ito. 1987. Mode of cardiotonic action of helenalin, a sesquiterpene lactone, on guinea pig ventricular myocardium. J. Cardiovasc. Pharmacol. 9: 193-201.

Jodynis-Liebert,J., M.Murias, and E.Bloszyk. 1999. Effect of several sesquiterpene lactones on lipid peroxidation and glutathione level. Planta Med. 65: 320-324.

Jodynis-Liebert,J., M.Murias, and E.Bloszyk. 2000. Effect of sesquiterpene lactones on antioxidant enzymes and some drug-metabolizing enzymes in rat liver and kidney. Planta Med. 66: 199-205.

Kamura,T., T.Hara, S.Kotoshiba, M.Yada, N.Ishida, H.Imaki, S.Hatakeyama, K.Nakayama, and K.I.Nakayama. 2003. Degradation of p57Kip2 mediated by SCFSkp2-dependent ubiquitylation. Proc. Natl. Acad. Sci. U. S. A 100: 10231-10236.

Kennedy,S.G., A.J.Wagner, S.D.Conzen, J.Jordan, A.Bellacosa, P.N.Tsichlis, and N.Hay. 1997. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev. 11: 701-713.

Lyss,G., A.Knorre, T.J.Schmidt, H.L.Pahl, and I.Merfort. 1998. The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-kappaB by directly targeting p65. J. Biol. Chem. 273: 33508-33516.

Mohamed-Ali,V., J.H.Pinkney, and S.W.Coppack. 1998. Adipose tissue as an endocrine and paracrine organ. Int. J. Obes. Relat Metab Disord. 22: 1145-1158.

Morrison,R.F. and S.R.Farmer. 1999. Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. J. Biol. Chem. 274: 17088-17097.

Pagano,M., S.W.Tam, A.M.Theodoras, P.Beer-Romero, G.Del Sal, V.Chau, P.R.Yew, G.F.Draetta, and M.Rolfe. 1995. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 269: 682-685.

Skurk,T., H.van, V, and H.Hauner. 2004. Angiotensin II stimulates the release of interleukin-6 and interleukin-8 from cultured human adipocytes by activation of NF-kappaB. Arterioscler. Thromb. Vasc. Biol. 24: 1199-1203.

Tedesco,D., J.Lukas, and S.I.Reed. 2002. The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF(Skp2). Genes Dev. 16: 2946-2957.

Wang,W., L.Nacusi, R.J.Sheaff, and X.Liu. 2005. Ubiquitination of p21Cip1/WAF1 by SCFSkp2: substrate requirement and ubiquitination site selection. Biochemistry 44: 14553-14564.

CHAPTER V

EPILOGUE

Data presented in the previous chapters, along with other findings made by our lab, have opened many avenues of questioning. The importance of Skp2 in cancerous and non-cancerous cell proliferation, as well as disease states, provides a strong basis for research focusing on this cell cycle regulator. Preliminary data and potential areas of focus will be discussed here.

Our studies have revealed PI3K-dependent regulation of Skp2 during adipocyte hyperplasia. However, the inhibitor we used targets PI3K, thereby preventing activity of all downstream targets. The canonical method by which PI3K signaling is transduced is through phosphorylation and activation of PDK1 and Akt. A very interesting and important addition to this story would be to test the effect of specific PDK1 and Akt inhibitors on Skp2 because of the many targets and parallel pathways downstream of PI3K. PI3K has downstream targets besides PDK1 and these additional studies would determine the extent to which PDK1 is required. While PDK1 directly phosphorylates Akt, it has other substrates as well. Expanding our knowledge about essential players within this pathway will help guide future experiments about the regulation of Skp2.

We have seen a dramatic decrease in Skp2 mRNA and protein in the presence of potent 26S proteasome inhibitors, epoxomicin and lactacystin. We have also seen a block in Skp2 promoter activity by epoxomicin. In previous work, we demonstrated that PI3K plays an essential role in Skp2 expression. Taken together, these two separate findings implicate a PI3K, proteasome-dependent mechanism. NFκB p65 is a

downstream target of both PI3K and MAPK signaling (Sizemore et al., 1999;Park and Levitt, 1993) that requires proteasomal degradation for activation (Palombella et al.1994). We have found that treating 3T3-L1 preadipocytes with the potent p65 DNA-binding inhibitor, helenalin, at stimulation completely blocks the increase in Skp2 (Chapter IV). While we have also shown that helenalin is a potent inhibitor of Akt signaling, its effect on NFκB is direct and the potential for NFκB to regulate Skp2 cannot be ignored as NFκB p65 is known to play an important role in cell cycle progression. It is interesting to note that while not an exact match, we have found three potential NFκB binding sites within the Skp2 promoter. Studies should not be limited to p65 or to the traditional NFκB transcription-activating complexes. The reasoning for this is that in 2001, Kurland et al. found that p50 homodimers, primarily thought to be repressors of transcription, bound to and activated transcription of the proto-oncogene *bcl-2* in murine B-cell lymphoma cells (Kurland et al., 2001). Furthermore, the effect of p65 on gene expression can depend on association with other key factors. This was clearly demonstrated by Ashburner et al. when they showed a direct interaction between p65 and HDAC proteins resulting in repression of NFκB target genes (Ashburner et al., 2001). Thus, the potential involvement of all NFκB family members should be evaluated.

We have also identified several putative C/EBPβ binding sites within the Skp2 promoter of preadipocytes, however, the role of C/EBPβ in Skp2 expression has never been examined. C/EBPβ is known to be essential for clonal expansion and while protein levels are upregulated very early after MDI stimulation, DNA-binding is not present until cells have entered G_1 phase. This would suggest that if C/EBPβ plays a role in Skp2 expression, it would enhance transcription rather than initiate it. In line with this theory,

is our finding that while insulin is the primary component responsible for the increase in Skp2 during clonal expansion, there is enhanced expression when all components are combined. MIX and dexamethasone are known to exert effects on C/EBPs, which could explain their ability to increase Skp2 expression above that seen with insulin alone. To investigate whether C/EBPβ aids in or is required for Skp2 expression, experiments preventing DNA-protein interactions would be appropriate. Several techniques that could be employed include using C/EBPβ siRNA during early stages of differentiation, deleting regions of the Skp2 promoter containing C/EBPβ binding sites, or inhibiting C/EBPβ nuclear localization or DNA-binding (e.g., expressing C/EBPβ with sequence mutations). Further studies would involve determining a direct interaction between C/EBPβ and the Skp2 promoter in 3T3-L1 preadipocytes.

Our finding that Rapamycin blocks Skp2 protein, but not mRNA, expression at 20 hours post-MDI stimulation suggests translational control may play a role in Skp2 expression at this time. Rapamycin is a potent inhibitor of mTOR and downstream translational control (Oshiro et al., 2004). It is known that Skp2 protein can be regulated post-translationally by the ubiquitin/ proteasome system, however, translational control of Skp2 has not been studied. We have found no change in Skp2 mRNA stability during clonal expansion (Chapter III), which rules out direct regulation by proteins such as HuR, an important regulator of mRNA stability throughout adipogenesis (Gantt et al., 2005). Regulation of Skp2 protein expression at the level of translation initiation would be a very novel finding.

The importance of identifying factors that decrease Skp2 cannot be overstated as Skp2 is overexpressed in many cancers and has now been shown to be highly regulated during adipocyte hyperplasia. Our initial finding that re-stimulation of mature adipocytes

with MDI is not able to cause an increase in Skp2 protein, as it does in preadipocytes, suggests the importance of differentiation-specific factors in maintaining low levels of Skp2 during this time. Koga et al. have shown that treatment of human hepatoma cells with the synthetic PPARγ ligand troglitazone causes significant decreases in Skp2 mRNA and protein (Koga et al., 2003). PPARγ is one of two master regulators of adipocyte differentiation and has been shown to be anti-mitotic, therefore, it is highly possible that PPARγ plays a role in regulating Skp2 in 3T3-L1 cells. To test this hypothesis, Skp2 gene expression should be examined under the same re-stimulation conditions discussed earlier. This would provide insight as to whether low levels of Skp2 are primarily regulated at the level of gene or protein expression. While there may be an increase in Skp2 gene expression with re-stimulation, we expect no change due to the fact that these cells are terminally growth-arrested, a feature which is critically important for differentiation to occur (Shao and Lazar, 1997).

PPARγ is a nuclear hormone receptor that heterodimerizes with the retinoid-X receptor (RXR) to regulate transcription of many genes during adipogenesis. We have identified a putative PPAR/RXR binding site within the Skp2 promoter of 3T3-L1 cells. Taken together, a direct inhibition of Skp2 promoter activity by PPAR/RXR binding may be possible. There are several studies that would address this question. First, Skp2 expression at all levels should be examined in the presence of siRNA targeting PPARγ under re-stimulation conditions. It may also prove advantageous to include C/EBPα siRNA alone and in combination with PPARγ siRNA to see if the synergy between these two transcription factors plays a role in low levels of Skp2. If PPARγ does indeed regulate Skp2 expression, one might expect to see increases in Skp2 promoter activity

following re-stimulation with PPARγ siRNA, however, because regulation by other factors and at other levels is likely, an increase in Skp2 protein may not be seen.

We show the importance of PI3K signaling for the expression of Skp2 and know from many previously published studies that Akt signaling is still intact and plays a critical role during adipocyte differentiation, a time when Skp2 expression is at its lowest. Therefore, it seems likely that decreased Skp2 gene expression during late stages of adipogenesis is not only due to the absence of transcriptional activators, but the presence of repressors as well. While direct transcriptional inhibition by PPARγ has not been published, one cannot rule out this possibility. Therefore it may be prudent to consider whether PPARγ plays a role in repressing Skp2 promoter activity. Interestingly, all-trans retinoic acid is able to block Skp2 expression in human neuroblastoma cells (Nakamura et al., 2003). All-trans retinoic acid (RA) binds to its receptor, RAR, which heterodimerizes with RXR to regulate transcription. The common element in these two stories is RXR and perhaps ectopic expression of PPARγ in neuroblastoma cells would have the same effect on Skp2 as RA treatment. These questions warrant further investigation. In 1997, Schwarz et al. showed retinoic acid inhibits adipocyte differentiation by preventing C/EBPβ-mediated transcription (Schwarz et al., 1997). The link between this and RA's effect on Skp2 expression provides further support for an effect of C/EBPβ on Skp2 as discussed above.

In addition to studying the regulation of Skp2, it will also be important to examine other potential targets of Skp2 during adipocyte hyperplasia. The ability to achieve very specific and potent knockdown of Skp2 using siRNA in these cells will help tremendously in doing this. A substrate that has particular relevance to adipogenesis is the retinoblastoma protein p130. Skp2 has been shown to target p130 for ubiquitylation and

degradation in human fibroblasts (Tedesco et al., 2002). There is a very dramatic decrease in p130 protein at day 1 of adipocyte differentiation, with no change in mRNA levels (Richon et al., 1997). Furthermore, the kinetics of p130 expression during clonal expansion are the exact inverse of Skp2 protein expression, suggesting a role for Skp2.

Our lab has also made a very novel discovery regarding histone deacetylase activity and Skp2 expression. Chromatin modifications play an essential role in regulating eukaryotic gene expression and include phosphorylation, methylation, sumoylation, and acetylation. Histone acetylation and deacetylation play a significant role in regulating gene expression and cellular processes such as cell cycle, differentiation, and apoptosis. Histone deacetylases (HDACs) remove acetyl groups from histones resulting in more tightly wound nucleosomes and decreased transcription. There are four classes of HDACs: Class I (HDAC1, 2, 3, and 8); Class II (HDAC 4, 5, 6, 7, 9, and 10); Class III (Sirtuins); and maize HD2. The antifungal antibiotic, Trichostatin A (TSA), inhibits Class I and II HDACs and causes cell cycle arrest in both G_1 and G_2 phases (Yoshida and Beppu, 1988). We have seen opposing effects of TSA on Skp2 expression in 3T3-L1 cells. In density-arrested cells, Skp2 mRNA and protein levels are low. We have found that TSA causes a significant increase in mRNA but not protein at this time. This increase is not seen in fully differentiated cells suggesting a more dominant mechanism by which Skp2 gene expression is suppressed in fully mature adipocytes. These data indicate that HDACs play a role in maintaining low levels of Skp2 at this time. Conversely, during G_2 phase when Skp2 levels are maximal, TSA almost completely blocks induction of both mRNA and protein. This demonstrates a requirement of HDAC activity for Skp2 expression. The amount of inhibition seen is directly proportional to the amount of time cells were exposed to TSA following MDI

stimulation, which suggests HDACs are functioning early in the process of Skp2 upregulation.

There are no published papers to date studying acetylation of the Skp2 promoter, making these very novel findings. The effect of TSA during density-arrest implies a direct effect of HDAC inhibition. We have also treated cells with two other HDAC inhibitors, sodium butyrate and Nicotinamide, to narrow involvement of specific classes of HDACs. Sodium butyrate (NaB), like TSA, targets Classes I and II, while Nicotinamide blocks activity of the third class (i.e., Sirtuins). Skp2 mRNA is increased at day 0 with TSA and sodium butyrate but not Nicotinamide, establishing a functional role of Class I and/or Class II HDACs. Further studies are needed to examine whether regions of the Skp2 promoter are indeed deacetylated at this time and to determine the specific HDAC(s) involved.

We have also seen an effect of TSA and NaB, but not NAM, on Skp2 expression during clonal expansion suggesting an early and indirect role of Class I and II HDACs. Interestingly, TSA and NaB have the ability to block proteasome activity by down-regulating the expression of several critical subunits (Place et al., 2005). As mentioned previously, we have seen a dramatic decrease in Skp2 mRNA and protein expression with specific inhibitors of the 26S proteasome. It is entirely possible that the mechanism by which TSA affects Skp2 expression during the G_2 phase of adipocyte hyperplasia is through blocking the activity of the 26S proteasome. The ramifications for this indirect action are great, as 26S proteasome activity is critical for the expression and activity of many endogenous proteins. NFκB inactivation is one example of an indirect effect of TSA inhibiting the 26S proteasome and gene expression (Place et al., 2005). This, in combination with the effect of helenalin on Skp2 expression, provides one line of

research to examine the ability of TSA to decrease Skp2 expression during early stages of adipogenesis.

The data presented in Chapters II through IV have added significantly to the limited body of knowledge on the regulation of adipocyte hyperplasia. The discovery of Skp2 expression in 3T3-L1 cells and the synchronized period of growth in which these studies were conducted provide a wonderful model for studying this cell cycle regulator. These findings have also provided ample preliminary data for potential lines of research on this topic. The benefit of future studies on Skp2 regulation will not only aid in the molecular understanding of hyperplastic obesity but cancer as well.

REFERENCES

Ashburner,B.P., S.D.Westerheide, and A.S.Baldwin, Jr. 2001. The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Mol. Cell Biol. 21: 7065-7077.

Gantt,K., J.Cherry, R.Tenney, V.Karschner, and P.H.Pekala. 2005. An early event in adipogenesis, the nuclear selection of the CCAAT enhancer-binding protein {beta} (C/EBP{beta}) mRNA by HuR and its translocation to the cytosol. J. Biol. Chem. 280: 24768-24774.

Koga,H., M.Harada, M.Ohtsubo, S.Shishido, H.Kumemura, S.Hanada, E.Taniguchi, K.Yamashita, R.Kumashiro, T.Ueno, and M.Sata. 2003. Troglitazone induces p27Kip1-associated cell-cycle arrest through down-regulating Skp2 in human hepatoma cells. Hepatology 37: 1086-1096.

Kurland,J.F., R.Kodym, M.D.Story, K.B.Spurgers, T.J.McDonnell, and R.E.Meyn. 2001. NF-kappaB1 (p50) homodimers contribute to transcription of the bcl-2 oncogene. J. Biol. Chem. 276: 45380-45386.

Nakamura,M., T.Matsuo, J.Stauffer, L.Neckers, and C.J.Thiele. 2003. Retinoic acid decreases targeting of p27 for degradation via an N-myc-dependent decrease in p27 phosphorylation and an N-myc-independent decrease in Skp2. Cell Death. Differ. 10: 230-239.

Oshiro,N., K.Yoshino, S.Hidayat, C.Tokunaga, K.Hara, S.Eguchi, J.Avruch, and K.Yonezawa. 2004. Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function. Genes Cells 9: 359-366.

Palombella,V.J., O.J.Rando, A.L.Goldberg, and T.Maniatis. 1994. The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell 78: 773-785.

Park,J.H. and L.Levitt. 1993. Overexpression of mitogen-activated protein kinase (ERK1) enhances T-cell cytokine gene expression: role of AP1, NF-AT, and NF-KB. Blood 82: 2470-2477.

Place,R.F., E.J.Noonan, and C.Giardina. 2005. HDAC inhibition prevents NF-kappa B activation by suppressing proteasome activity: down-regulation of proteasome subunit expression stabilizes I kappa B alpha. Biochem. Pharmacol. 70: 394-406.

Richon,V.M., R.E.Lyle, and R.E.McGehee, Jr. 1997. Regulation and expression of retinoblastoma proteins p107 and p130 during 3T3-L1 adipocyte differentiation. J. Biol. Chem. 272: 10117-10124.

Schwarz,E.J., M.J.Reginato, D.Shao, S.L.Krakow, and M.A.Lazar. 1997. Retinoic acid blocks adipogenesis by inhibiting C/EBPbeta-mediated transcription. Mol. Cell Biol. 17: 1552-1561.

Shao,D. and M.A.Lazar. 1997. Peroxisome proliferator activated receptor gamma, CCAAT/enhancer-binding protein alpha, and cell cycle status regulate the commitment to adipocyte differentiation. J. Biol. Chem. 272: 21473-21478.

Sizemore,N., S.Leung, and G.R.Stark. 1999. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Mol. Cell Biol. 19: 4798-4805.

Tedesco,D., J.Lukas, and S.I.Reed. 2002. The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF(Skp2). Genes Dev. 16: 2946-2957.

Yoshida,M. and T.Beppu. 1988. Reversible arrest of proliferation of rat 3Y1 fibroblasts in both the G1 and G2 phases by trichostatin A. Exp. Cell Res. 177: 122-131.