# The Mechanism By Which TCERG1 Inhibits the Growth Arrest Activity of C/EBPα

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## ABSTRACT

Transcription elongation regulator 1 (TCERG1) is a nuclear protein involved in transcriptional elongation and splicing events, suggesting these two activities may be connected. Moreover, TCERG1 was recently identified as a novel interactor and co-repressor of CCAAT/Enhancer Binding Protein α (C/EBPα) transcriptional activity, suggesting TCERG1 has additional biological roles. Interestingly, TCERG1 also inhibits the growth arrest activity of C/EBPα. Additionally, the original clone found to interact with C/EBPα consisted of only the amino-terminal domain of TCERG1 and functional analysis of this clone indicated that it retained the ability to repress both C/EBPα-mediated growth arrest and transcriptional activity. Furthermore, a TCERG1 mutant whose amino-terminal region was deleted was unable to interact with or repress the transcriptional and growth arrest activities of C/EBPα, suggesting the functional domain(s) lie elsewhere. In this study, domains of TCERG1 were examined for the ability to inhibit C/EBPα-mediated growth arrest and the mechanism whereby this effect occurs. By exploiting fluorescent properties of expressed proteins fused with green fluorescent protein, the extent to which each TCERG1 mutant was able to reverse C/EBPα-mediated growth arrest of cultured cells was assessed. Our analyses suggest that the inhibitory activity of TCERG1 lies within the amino-terminal region and may involve WWI and WWII domains within this region. Additionally, laser scanning confocal microscopy (LCSM) was used to visualize the subnuclear localization of fluorescent proteins fused to TCERG1 and C/EBPα. When expressed alone, TCERG1 localized to splicing factor-rich nuclear speckles while C/EBPα was found to reside in discrete punctate foci, both localization patterns being distinct and different from each other. Results from co-localization studies after co-expressing both proteins indicate an alteration in the subnuclear distribution of TCERG1. Furthermore, TCERG1 co-localizes with C/EBPα, suggesting a possible mechanism whereby TCERG1 inhibits the growth arrest and transcriptional activities mediated by C/EBPα.

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*"It takes courage to push yourself to places you have never been before… to test your limits… to break through barriers. And the day came when the risk to remain tight inside the bud was more painful than the risk it took to blossom."*

***– Anais Nin (1903-1977)***

To my father,
with whom I wish I could
share this achievement

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| | |
|---|---|
| bZIP | Basic Region-Leucine Zipper |
| C/EBPα | CCAAT/Enhancer-Binding Protein Alpha |
| CBP | CREB-Binding Protein |
| cdk | Cyclin-Dependent Kinase |
| CMV | Cytomegalovirus |
| COS7 | African Green Monkey Kidney Cells |
| CTD | Carboxy Terminal Repeat Domain |
| DBD | DNA Binding Domain |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylene-diamine tetraacetic acid disodium salt |
| EGFP | Enhanced Green Fluorescent Protein |
| GIR | Growth Inhibitory Region |
| HA | Hemagglutinin |
| HEK293T | Human Embryonic Kidney 293T |
| HEPES | N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) |
| HIV-1 LTR | Human Immunodeficiency Virus-1 Long Terminal Repeat |
| HRP | Horseradish Peroxidase |
| kDa | Kilodalton |
| LB | Luria-Bertani |
| LIP | Liver Inhibitor Protein |
| LSCM | Laser Scanning Confocal Microscopy |
| NLS | Nuclear Localization Signal |
| O.D. | Optical Density |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| PFA | Paraformaldehyde |
| PIPES | Piperazine-N,N'-bis[2-ethanesulfonic acid] |
| Pit-1 | Pituitary-Specific Positive Transcription Factor 1 |
| PTEF-b | Positive Transcription Elongation Factor-b |
| Rb | Retinoblastoma Protein |
| RNAPII | RNA Polymerase II |
| SD | Standard Deviation |
| SDS | Sodium Dodecyl Sulphate |
| SV40 | Simian Virus 40 |
| SWI/SNF | SWItch/Sucrose Non-Fermentable |
| TAD | Transactivation Domain |
| TAE | Tris-Acetate EDTA Buffer |
| TB | Terrific Broth |
| TBP | TATA-Binding Protein |
| TBS | Tris-Buffered Saline |
| TBST | Tris-Buffered Saline + 0.001% Tween-20 |
| TCERG1 | Transcription Elongation Regulator 1 |
| TE | Transcriptional Element |
| Tris | Tris-[hydroxymethyl]-aminomethane |

## 1. INTRODUCTION AND OVERVIEW

The biological roles of transcription factors and the genes that they regulate have been a long-term focus for many researchers. In particular, CCAAT/Enhancer Binding Protein α (C/EBPα) has been studied extensively in its relation to two distinct activities mediated by different domains: transcriptional and anti-proliferative activity (Lekstrom-Himes and Xanthopoulos, 1998; Harris *et al.*, 2001; Wang *et al.*, 2001). Although C/EBPα is characterized as a master regulator of several important tissue-specific genes, how this activity is achieved in addition to the regulation of its anti-proliferative effects has been a subject of debate for many years (reviewed in Darlington *et al.*, 1995; Johnson, 2005). Hence, McFie *et al*. (2006), sought to identify a potential co-factor and/or regulator of C/EBPα activity. Using a yeast two-hybrid screening approach with a human liver cDNA library and the transactivation domain of C/EBPα (amino acids 6-217) as bait, a single novel interactor was identified as transcription elongation regulator 1 (TCERG1) (McFie *et al.*, 2006). TCERG1 was subsequently characterized for its role or function in relation to C/EBPα activity and the mechanism by which it occurs. Although TCERG1 has been described as a negative-acting elongation factor that may bridge transcriptional and splicing events, most of the information gained from various studies is indirect and relies on conclusions based on other factors involved in these activities (Sune *et al.*, 1997; Shimada *et al.*, 1999; Sune and Garcia-Blanco, 1999; Bohne *et al.*, 2000; Carty *et al.*, 2000; Goldstrohm *et al.*, 2001; Smith *et al.*, 2004; Pearson *et al.*, 2008). Therefore, McFie *et al.* (2006) attempted to assign a biological role for TCERG1 in a way that could be directly linked to its function(s). Using various biochemical techniques, results indicated that TCERG1 interacts with and acts as an inhibitor of C/EBPα transcriptional activity (McFie *et al.*, 2006). Chromatin immunoprecipitation analyses of mouse liver nuclei showed that TCERG1 was present with C/EBPα at gene promoters repressed by C/EBPα, such as hepatocyte nuclear factor-6, but not at promoters subject to C/EBPα-mediated transactivation, like the phosphoenolpyruvate carboxykinase promoter (McFie *et al.*, 2006). This interesting result led to the proposal that TCERG1 may be involved in further repression of promoters down-regulated by C/EBPα. Further characterization of TCERG1 revealed that it had no intrinsic repressor activity since a Gal4-TCERG1 fusion was unable to repress transcriptional activation of a reporter gene under the control of an SV40 promoter linked to a

number of Gal4 DNA binding sites. Thus, the inhibition of transcription by TCERG1 may be specific to promoters responsive to C/EBPα transcriptional activity.

TCERG1 function was next examined in the context of C/EBPα-mediated growth arrest of cultured cells. Previous studies showed that a short domain, consisting of amino acids 175-217 in the amino terminus of C/EBPα, is involved in mediating the anti-proliferative activity of C/EBPα (Hendricks-Taylor and Darlington, 1995; Wang *et al.*, 2001). This region alone is able to inhibit cellular proliferation of cultured cells by inhibiting the activities of regulators of cell cycle progression, cyclin-dependent kinase (cdk) 2 and cdk4 (Wang *et al.*, 2001). In a recent study, researchers were able to show that upon overexpression of TCERG1, cellular arrest caused by C/EBPα is relieved (McFie *et al.*, 2006). Moreover, the amino-terminal domain of TCERG1 was sufficient to inhibit C/EBPα activity, suggesting that important functional domains required for the reversal of growth arrest are located in this region. Further analysis by chromatin immunoprecipitation demonstrated that the amino-terminal region of TCERG1 retained the ability to interact with C/EBPα.

Despite the recent evidence gathered by McFie *et al*. (2006), it is still not understood which motifs of TCERG1 are implicated in the inhibitory activities of C/EBPα and the mechanism whereby this inhibition occurs. The focus of my studies was to determine which functional domain(s) of TCERG1 are involved in the inhibition of cellular proliferation mediated by C/EBPα and to propose a model for the mechanism by which this occurs.

## 2. REVIEW OF THE LITERATURE

### 2.1 CCAAT/Enhancer Binding Protein Family

CCAAT/Enhancer Binding Proteins (C/EBPs) belong to a class of transcription factors that function as master regulators of several cellular responses (reviewed in Lekstrom-Himes and Xanthopoulos, 1998; Ramji and Foka, 2002). To date, six isoforms have been identified: C/EBPα, -β, -γ, -δ, -ε, and -ζ, each designated by the Greek letter for the order in which each was discovered (Cao *et al.*, 1991; Ramji and Foka, 2002). Some *c/ebp* genes give rise to several transcripts due to differential splicing and others produce polypeptides of different sizes as a result of translation from alternative AUG start sites (Ramji and Foka, 2002). C/EBPs bind to the DNA consensus sequence: RTTGCGYAAY, where R is A or G and Y is C or T, although variations of this motif have been identified (Osada *et al.*, 1996). All C/EBP isoforms share significant conservation, >90% identity, in the amino acid sequence found in the carboxy-terminal domain. This domain consists of two important functional regions collectively termed the bZIP domain: a basic amino acid-rich region responsible for DNA binding and a leucine zipper motif that is involved in dimerization with other C/EBP molecules. Dimerization between two C/EBP molecules via the leucine zipper is a prerequisite for DNA binding (Agre *et al.*, 1989). C/EBPs can form homo- and heterodimers with other C/EBPs or bZIP proteins depending on the type of tissue and cellular state as expression of C/EBPs may be altered during certain physiological conditions. Heptad repeats consisting of leucine and other hydrophobic residues in the leucine zipper region form an α-helical arrangement through interactions with a second heptad repeat, generating a coiled-coil structure in parallel orientation (Landschulz *et al.*, 1988b; Vinson *et al.*, 1993; Podust *et al.*, 2001). The resulting dimer forms a helical structure resembling a pair of scissors, each blade representing the basic region from each monomer, which becomes associated with the DNA binding consensus sequence found in the DNA major groove (Podust *et al.*, 2001; Tahirov *et al.*, 2001). In general, the amino-terminal domain of all C/EBPs is divergent but some share components of the transactivation domains (TAD) found within this region. C/EBPγ and C/EBPβ liver inhibitor protein (LIP) isoform lack a TAD, and thus form non-functional heterodimers that can

bind to DNA but are incapable of transcriptional activity (Descombes and Schibler, 1991). C/EBPζ also acts as an inhibitor due to the presence of proline residues in the basic region that lead to the disruption of the α-helical arrangement; thus heterodimers can no longer bind to the DNA consensus sequence (Ron and Habener, 1992). Taken together, the majority of C/EBPs function to activate specific genes in a variety of cellular responses and tissues, while others function to repress or inhibit transcriptional activity by forming inactive heterodimers with other C/EBP isoforms.

C/EBPs participate in the regulation of a variety of biological functions involving the expression of hormones, mitogens, cytokines, nutrients and toxins, amongst many others (Darlington *et al.*, 1995; Lekstrom-Himes and Xanthopoulos, 1998; Roesler, 2001; Ramji and Foka, 2002). Additionally, some non-transcriptional roles exerted by C/EBPs involve cellular proliferation, differentiation and inflammation response (reviewed in Lekstrom-Himes and Xanthopoulos, 1998; Ramji and Foka, 2002). Many of the responses elicited by C/EBPs are dependent on the state of the cell and the tissue in which this activity occurs. For example, several C/EBP members are involved in the differentiation process of several tissues including hepatocytes and adipocytes, which have been studied most extensively (Christy *et al.*, 1989; Cao *et al.*, 1991; Mischoulon *et al.*, 1992; Darlington *et al.*, 1998). When cells are subjected to hormone stimulation, expression of C/EBPβ and -δ are induced first, then activate transcription of *c/ebpα* later during the differentiation process (Cao *et al.*, 1991; Christy *et al.*, 1991; Umek *et al.*, 1991; Mischoulon *et al.*, 1992; Legraverend *et al.*, 1993). As levels of C/EBPα rise significantly, it acts as a master regulator of tissue-specific genes possessing C/EBP binding sites in their promoter regions (MacDougald *et al.*, 1995). Elegant studies by several groups of researchers have shown that C/EBPα alone is both required and sufficient to trigger differentiation of pre-adipocytes even in the absence of stimulants for differentiation initiation (Samuelsson *et al.*, 1991; Freytag and Geddes, 1992; Freytag *et al.*, 1994; Lin and Lane, 1994; Lane *et al.*, 1996). Collectively, these studies and others used a variety of techniques to show that induction of C/EBPα is adequate to promote differentiation of several cell types (Freytag *et al.*, 1994; Lin and Lane, 1994). Finally, terminal differentiation and maintenance of quiescence is thought to be largely under the control of C/EBPα (Christy *et al.*, 1989; Mischoulon *et al.*, 1992; Legraverend *et al.*, 1993; Lane *et al.*, 1996; Jiang and Lane, 2000). The main C/EBP isoform under investigation in this thesis is C/EBPα and is discussed in more detail below.

### 2.1.1 Structure and Biological Roles of C/EBPα

C/EBPα, which consists of 358 amino acids, was the first C/EBP family member to be cloned, and has since been extensively characterized (Figure 1) (Landschulz *et al.*, 1988a; Birkenmeier *et al.*, 1989; Cao *et al.*, 1991; Darlington *et al.*, 1995, 1998; Lekstrom-Himes and Xanthopoulos, 1998; Gheorghiu *et al.*, 2001; Roesler, 2001). C/EBPα was originally identified as a heat-stable factor found in rat liver nuclei that interacted with the CCAAT-box motif found upstream of several viral promoters in addition to core homology enhancer regions (Landschulz *et al.*, 1988a). Like all C/EBP transcription factors, C/EBPα contains a highly conserved carboxy-terminal basic region and leucine-zipper motif, collectively termed the bZIP domain, that is involved in dimerization and DNA binding (Figure 1) (Landschulz *et al.*, 1988a, 1988b). Expression of C/EBPα occurs in a variety of tissues such as adipose, liver, intestine, lung, adrenal gland, peripheral-blood mononuclear cells and placenta tissues, with the highest levels of expression present in the differentiated cells of adipose and liver tissue (Antonson and Xanthopoulos, 1995). The translation of the mRNA encoding C/EBPα generates two polypeptides of 30 and 42 kDa, $p30^{C/EBP\alpha}$ and $p42^{C/EBP\alpha}$, respectively, due to alternate AUG translational start sites, with the former having lower transactivation potential (Ossipow *et al.*, 1993). Much of what is known about the biological importance of C/EBPα was discovered using murine knock-out models (Darlington *et al.*, 1995; Wang *et al.*, 1995). Analyses of *c/ebpα*$^{-/-}$ knock-out mice appear phenotypically normal at birth but die soon after from severe hypoglycemia (Darlington *et al.*, 1995). In addition, within the first two hours after birth, glycogen stores are not detected due to the drastic reduction in glycogen synthase activity and mRNA levels of key gluconeogenic enzymes. Thus, no glucose can be synthesized from hepatic stores or be maintained at normal levels in the blood. C/EBPα-deficient mice also have greatly reduced lipid stores in the form of white and brown adipose tissue. Collectively, these studies presented by Darlington *et al*. (1995) using *c/ebpα*$^{-/-}$ knock-out mice have contributed greatly to our understanding of the vital roles C/EBPα plays in post-natal development, metabolic processes and the transcriptional regulation of genes which it governs.

Extensive studies have revealed that C/EBPα is a very important nuclear protein with two distinct activities mediated by different domains (Lekstrom-Himes and Xanthopoulos, 1998; Harris *et al.*, 2001; Wang *et al.*, 2001). The transcriptional activity of C/EBPα occurs

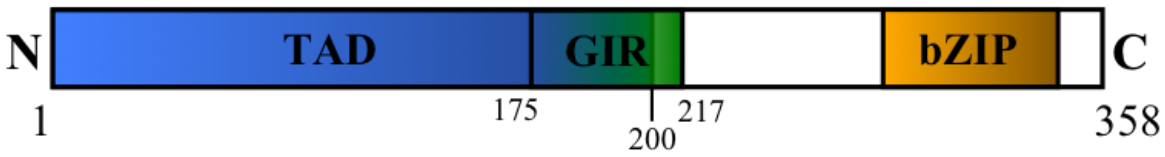


**Figure 1. CCAAT/Enhancer-Binding Protein α (C/EBPα) is a Transcription Factor with Two Distinct and Separate Functions.**
C/EBPα consists of 358 amino acids and functions as a transcription factor by first forming dimers through the leucine zipper, followed by DNA binding mediated by the basic region and leucine zipper (collectively, bZIP). Subsequently, C/EBPα regulates transcriptional activity via transactivation elements found within the first 200 residues, which makes up the transactivation domain (TAD). C/EBPα also functions to inhibit cellular proliferation by interacting with proteins critical for cell cycle progression through the growth inhibitory region (GIR; amino acids 175-217).

through C/EBP-dependent dimerization and DNA binding via the bZIP domain, and subsequently operates to activate and repress many important and tissue-specific genes (reviewed in Darlington *et al.*, 1995; Lekstrom-Himes and Xanthopoulos, 1998; Roesler, 2001; Ramji and Foka, 2002). The second function of C/EBPα involves cellular growth arrest and differentiation of certain cell types. This activity was first suggested by Umek *et al*. (1991), to involve the amino-terminal domain of C/EBPα. Since then, a short region spanning amino acids 175-217 within the amino terminus has been identified and termed the growth inhibitory region (GIR, Figure 1) (Hendricks-Taylor and Darlington, 1995; Wang *et al.*, 2001). In a study presented by Wang *et al*. (2001), results from *in vitro* glutathione-S-transferase-pulldown assays and later confirmed by *in vivo* assays, showed that critical components for cell cycle progression, cdk2 and cdk4, bind to a short region found within the GIR of C/EBPα. This interaction occurs via the kinase activity domains of each cdk, thereby preventing association with their respective cyclins. Finally, Wang *et al*. (2001) examined a variety of cultured cells for transcriptional targets regulated by C/EBPα in a growth arrest-dependent manner, but none were identified. This suggests that the transcriptional activity of C/EBPα is not required for inhibiting cellular proliferation and that C/EBPα-mediated growth arrest and transcriptional activity can operate independently of each other.

#### 2.1.1.1 Mechanism and Regulation of C/EBPα Transcriptional Activity

C/EBPα is a transcription factor that is involved in the regulation of tissue-specific genes that play critical roles in energy metabolism (reviewed in Darlington *et al.*, 1995; Roesler, 2001). Transcriptional activity occurs via three transactivation elements (TE I-III) located within the first 200 residues of the amino-terminal region (Figure 1) (Friedman *et al.*, 1989). TE I and II interact with components of the basal RNA polymerase II (RNAPII) transcriptional machinery, TATA-binding protein (TBP) and TFIIB, to mediate transactivation (Nerlov and Ziff, 1995). However, TE III has been characterized as a negative regulatory domain (Nerlov and Ziff, 1994).

Several co-activators of C/EBPα transcriptional activity have been identified. These include p300/CREB-binding protein (CBP) histone acetyltransferases, TBP and retinoblastoma tumor-suppressor protein (Rb) (Chen *et al.*, 1996; Erickson *et al.*, 2001; Wang and Timchenko, 2005). These two co-activators bind to regions found in the TE I region of C/EBPα and enhance the DNA binding and transactivation potential of C/EBPα (Chen *et al.*, 1996; Charles *et al.*, 2001; Erickson *et al.*, 2001). C/EBPα can also recruit the chromatin remodeling complex SWItch/Sucrose Non-Fermentable (SWI/SNF) to the proximity of the promoter region by interacting with the Brahma (Brm) subunit of this complex (Pedersen *et al.*, 2001; Muller *et al.*, 2004). Recent evidence suggests that sumoylation may be involved in the regulation of C/EBPα activity (Khanna-Gupta, 2008). Sumoylation of lysine 159 found in the TE III negative regulatory region of C/EBPα has been implicated in the decreased ability of C/EBPα to transactivate liver- and myeloid-specific genes. Moreover, reporter assays conducted in erythroleukemia cells demonstrate that overexpression of $p30^{C/EBP\alpha}$ causes increased sumoylation of $p42^{C/EBP\alpha}$, thereby inhibiting its transcriptional activity. Finally, downregulation of C/EBPα occurs in cells stimulated with insulin or epidermal growth factor, which leads to an increased turnover rate of C/EBPα transcripts (Christy *et al.*, 1991; Samuelsson *et al.*, 1991; Mischoulon *et al.*, 1992; Legraverend *et al.*, 1993; MacDougald *et al.*, 1995; Lane *et al.*, 1996). Thus, by altering C/EBPα mRNA levels, the rate of transcription is affected, leading to lower levels of C/EBPα expression. Insulin also affects the regulation of C/EBPα by indirectly activating serine/threonine protein phosphatase 1 and 2A, which dephosphorylate C/EBPα, abrogating its DNA-binding activity (MacDougald *et al.*, 1995).

#### 2.1.1.2 Regulation of C/EBPα Growth Arrest Activity and Differentiation

A very different but no less important role for C/EBPα involves differentiation of several cell types and the process of cellular regeneration (Birkenmeier *et al.*, 1989; Christy *et al.*, 1991; Ramji and Foka, 2002). In the late 1980's, scientists showed that C/EBPα was expressed in the tissues of adult livers and was found exclusively in differentiated hepatocytes and adipoctyes (Birkenmeier *et al.*, 1989; Friedman *et al.*, 1989). In 1991, C/EBPα function was first related to cellular growth arrest and terminal differentiation of pre-adipocytes (Christy *et al.*, 1991; Samuelsson *et al.*, 1991; Umek *et al.*, 1991). Since then, several lines of evidence from studies in several cell types have suggested that C/EBPα function has an inverse relationship to cellular proliferation and mitotic clonal expansion (Ramji and Foka, 2002; Johnson, 2005). For example, C/EBPα is extensively linked to the regeneration of liver tissue after a partial hepatectomy, whereby 60-70% of hepatic tissue is surgically removed (Mischoulon *et al.*, 1992; Flodby *et al.*, 1996). Under these conditions, differentiated cells acquire the ability to re-enter the cell cycle by undergoing a reorganization of the regulatory mechanisms involved in the maintenance of cellular fate. This process involves the temporal expression of specific factors required to initiate this process (Mischoulon *et al.*, 1992; Greenbaum *et al.*, 1995; Diehl, 1998). In the first 24 hours after a partial hepatectomy, the abundance of C/EBP family members -β and -δ mRNA levels increase and cyclin D, a cell cycle regulatory factor, is induced (Mischoulon *et al.*, 1992; Greenbaum *et al.*, 1995; Wang *et al.*, 2001). Subsequently, DNA binding of C/EBPβ and -δ to the promoter region of *c/ebpα* is enhanced, resulting in the upregulation of C/EBPα expression. Once expressed, C/EBPα regulates its own expression in order to maintain the terminally differentiated state of the cell (Christy *et al.*, 1991; Mischoulon *et al.*, 1992; Legraverend *et al.*, 1993; MacDougald *et al.*, 1995; Timchenko *et al.*, 1995). Regulation of the expression of C/EBPα by itself was demonstrated in an elegant study by Lin and Lane (1994). In this study, a missense mutation was introduced into the *c/ebpα* gene such that the translational start site for the 30 kDa isoform was eliminated, thus only $p42^{C/EBP\alpha}$ could be expressed. Upon the expression of the mutated C/EBPα in cells, subsequent levels of endogenous $p30^{C/EBP\alpha}$ and $p42^{C/EBP\alpha}$ transcripts could be detected regardless of differentiation status, suggesting that C/EBPα is involved in the regulation of its own expression. Moreover, when cells were stimulated to differentiate, the

onset of endogenous C/EBPα expression occurred one day later compared to cells overexpressing C/EBPα and induced to differentiate concurrently. The extent of the involvement of C/EBPα in the maintenance of the differentiated state was demonstrated in C/EBPα$^{-/-}$ livers, which have increased proliferation rate (Flodby *et al.*, 1996). Moreover, Wang *et al*. (1995) showed that transgenic mice containing null mutations in the *c/ebpα* gene display a dramatic increase in hepatocyte differentiation at birth. The expression and regulation of C/EBPα occur in a similar manner in cultured pre-adipocytes under conditions where differentiation is stimulated (Christy *et al.*, 1989, 1991; Cao *et al.*, 1991). The sequence of events that drive cells into the process of and maintenance of either proliferation or differentiation is complex and involves many levels of regulation. It is often found that many of the factors involved in these events play reciprocal roles between the different cellular fates, enhancing the expression of some genes, while repressing others. Although it has been well established that several C/EBP isoforms are involved in the maintenance of terminally differentiated quiescent cells, the intricacies involved in the entire process still remain to be fully elucidated.

It has been established that C/EBPα can inhibit the proliferation of a variety of tissues and cultured cells (Wang *et al.*, 2001; Johnson, 2005; McFie *et al.*, 2006). Several different models have been proposed for the mechanism of growth arrest depending on the state of the cell and tissue in which it occurs (Figure 2) (Johnson, 2005; Wang and Timchenko, 2005; Wang *et al.*, 2006). C/EBPα can interact with many components important for cell cycle progression in each model including cdk2, cdk4, E2F transcription factor, Rb, and the Brm subunit of the SWI/SNF chromatin remodeling complex (Timchenko *et al.*, 1997, 1999b; Pedersen *et al.*, 2001; Porse *et al.*, 2001; Wang *et al.*, 2001, 2002a, 2004; Iakova *et al.*, 2003; Muller *et al.*, 2004). For example, in early to mid-G1 phase, growth arrest occurs because of C/EBPα-mediated induction of the cdk inhibitor p21 expression, leading to the formation of stable p21/C/EBPα complexes (Figure 2 upper left, green arrow). This in turn causes the inhibition of cdk/cyclin kinase activity that normally functions to disrupt Rb/E2f inhibitory complexes (Timchenko *et al.*, 1996, 1999a, 1999b). Thus, E2F remains bound to Rb and is unable to bind to the promoter regions of S-phase specific genes and activate transcription. Alternatively, C/EBPα interacts with the kinase activity domains of cdk2 and cdk4, resulting in the inhibition of their kinase activity (Figure 2 upper left, red blocked arrow) (Wang *et al*.,

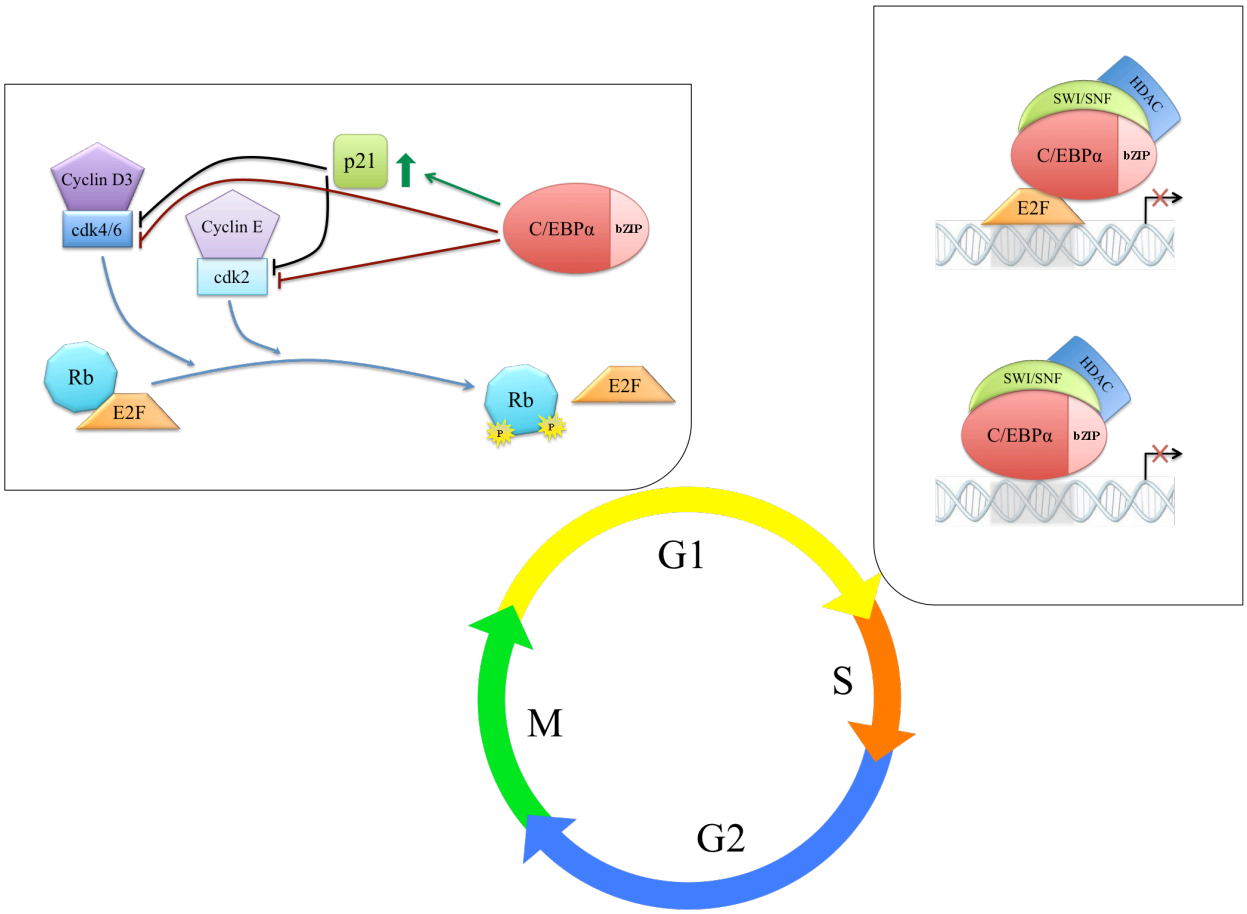


**Figure 2. Model for C/EBPα-Mediated Growth Arrest.**
C/EBPα-mediated growth arrest occurs as a result of one or more different levels of regulatory activity depending on the stage of the cell cycle. In G1, cyclin-dependent activation of cdk4/6 and later cdk2 leads to the phosphorylation of Rb (blue arrows, upper left). This results in its dissociation from transcription factor E2F, which is important for the transcriptional regulation of S-phase genes. Alternatively, C/EBPα may also cause growth arrest by direct interactions with cell cycle regulators or by affecting the transcriptional activity of cell cycle-specific genes at promoter sites. C/EBPα can act through p21, which itself inhibits the activities of cdks, or by direct binding to cdk2 and cdk4 in G1 phase. C/EBPα may also be recruited to E2F-dependent promoters via interactions with E2F and/or histone deacetylase/SWI/SNF chromatin remodeling complex or by directly binding to E2F sites in the promoter region leading to transcriptional inhibition of S-phase genes (right side). This image has been modified from Johnson, 2005 and based on evidence provided or reviewed by Timchenko *et al.*, 1999a, 1999b; Slomiany *et al.*, 2000; Pedersen *et al.*, 2001; Porse *et al.*, 2001; Wang *et al.*, 2001, 2006; Muller *et al.*, 2004; Wang and Timchenko, 2005.

2001). Studies show that a C/EBPα mutant unable to inhibit cdk2 cannot cause growth arrest, but mutants defective in DNA binding still retain anti-proliferative activity, suggesting that the first mechanism of growth arrest may be a compensatory tool in conditions where DNA binding of C/EBPα is altered (Harris *et al.*, 2001). In a third scenario, when cells approach S-phase, C/EBPα acquires the ability to interact with and recruit the SWI/SNF chromatin remodeling complex and histone deacetylases to promoter sites regulated in an E2F-dependent manner (Figure 2 upper right) (Slomiany *et al.*, 2000; Pedersen *et al.*, 2001; Porse *et al.*, 2001; Iakova *et al.*, 2003). Once localized to the promoter region, C/EBPα associates with E2F, thereby inhibiting E2F-mediated transcriptional activation. This type of regulation has been observed in adipose tissue (Dyson, 1998). However, evidence from a different study implicates the second model of growth arrest by the inhibition of cdk2/cyclin complexes through interactions with C/EBPα as the main mechanism of growth arrest in the liver (Wang *et al.*, 2001). This demonstrates that several mechanisms of C/EBPα anti-proliferative effects exist and may be specific to certain tissue types. Finally, studies have also shown that C/EBPα can bind to promoter regions containing E2F binding sites such as the dihydrofolate reductase promoter in the absence of E2F (Figure 2 lower right) (Slomiany *et al.*, 2000; Harris *et al.*, 2001; Wang and Timchenko, 2005). This type of transrepression mediated by C/EBPα has been proposed as the mechanism for the inhibition of hepatocyte proliferation in livers of old mice (Wang *et al.*, 2006).

It can be challenging to examine the mechanisms of C/EBPα anti-proliferative activity since even low levels of C/EBPα expression results in growth arrest and can lead to apoptosis (McFie *et al.*, 2006, my observations). However, sumoylation of Lys159 in the TE III has been shown to block the growth arrest activity of C/EBPα by preventing interactions with critical subunits of the SWI/SNF chromatin remodeling complex important for regulating E2F-dependent genes (Khanna-Gupta, 2008). Similar inhibition of C/EBPα has been observed when serine 193 is dephosphorylated, a process which occurs after cells have been stimulated with insulin (Wang and Timchenko, 2005). These results were confirmed when Ser193 was replaced with threonine, which cannot be phosphorylated by the specific kinase activated from insulin-mediated stimulation. Additionally, insulin inhibits C/EBPα activity by enhancing the turnover rate of C/EBPα mRNA, leading to the downregulation of C/EBPα expression and its activity (MacDougald *et al.*, 1995).

## 2.2 Transcription Elongation Regulator 1

### 2.2.1 Structure of TCERG1

TCERG1, formally known as co-activator of 150 kDa or CA150, was first discovered as a nuclear protein involved in Tat-activated transcription of the human immunodeficiency virus-1 long terminal repeat (HIV-1 LTR) promoter through interactions with Tat-specific factor 1 (Sune *et al.*, 1997). TCERG1 is a large nuclear protein consisting of 1098 amino acids and is approximately 150 kDa (Figure 3). A second variant of TCERG1 in humans also exists that is missing 21 internal residues due to the excision of an in-frame exon resulting in a protein of 1077 amino acids in length. However, the full-length form that has been the subject of most studies. Highly conserved across species from *C. elegans* to *H. sapiens*, TCERG1 is also expressed in all human tissues albeit at different levels (Bohne *et al.*, 2000). Several studies examining the role of TCERG1 in brain tissue, where it is highly expressed, have suggested an involvement in Huntington's disease pathogenesis (Holbert *et al.*, 2001; Chattopadhyay *et al.*, 2003; Arango *et al.*, 2006). The amino-terminal domain of TCERG1 contains three WW domains that each consists of 20-23 residues, containing several aromatic amino acids flanked by conserved tryptophan residues (WW) (Figure 3). These domains are involved in protein-protein interactions with proline-containing sequences found in proteins such as splicing factor-1 (Goldstrohm *et al.*, 2001). In addition, the amino-terminal domain contains a glutamine-alanine (QA)-repeat, unique among mammalian proteins, but whose function is currently unknown, and a putative nuclear localization signal (NLS). The carboxy terminus of TCERG1 includes six FF domains, in which two conserved phenylalanine (FF) residues are separated by ~35 amino acids, that also mediate protein-protein interactions. These modules have been shown to bind the phosphorylated carboxy-terminal repeat domain (CTD) of RNAPII through weak interactions contributed by multiple FF domains of TCERG1 (Sune *et al.*, 1997; Carty *et al.*, 2000). Several lines of evidence have suggested that interactions provided by the FF and WW domains of TCERG1 act as a bridge between negative regulation of transcriptional elongation and pre-mRNA splicing events, respectively (Carty *et al.*, 2000; Goldstrohm *et al.*, 2001; Lin *et al.*, 2004; Smith *et al.*, 2004).

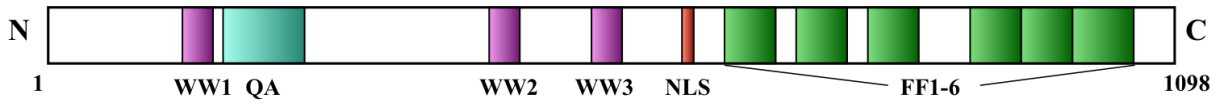


**Figure 3. Transcription Elongation Regulator 1 (TCERG1) is a Constitutively Expressed Nuclear Protein of 150 kDa.**
TCERG1 consists of 1098 amino acids with several functional domains. In the amino terminal domain lie three domains (purple) that each consist of 23 amino acids flanked by tryptophan residues that bind to proline-rich sequences, termed WW domains (purple). In addition, there is a unique glutamine-alanine repeat (QA, teal) with no known functional role. A putative nuclear localization signal (NLS, red) lies between the amino and carboxy terminal domains. The carboxy terminal region contains six FF domains designated by sequences of amino acids flanked by phenylalanine residues that are also involved in protein-protein interactions.

### 2.2.2 Functional Roles of TCERG1

Currently, the functional characterization of TCERG1 is focused on its role in transcriptional and elongation events. TCERG1 regulates transcription of HIV-1 LTR in part by affecting elongation efficiency of RNAPII complexes (Sune and Garcia-Blanco, 1999). This conclusion was supported by the results obtained from a collection of experiments. First, ectopic expression of TCERG1 represses HIV-1 LTR basal and Tat-activated transcription *in vivo* and is dependent on the presence of a TATA-box. Moreover, this activity is specific to the TATA-box of HIV-1 LTR and not other viral promoters such as CMV, early SV40, RSV, or HSVTK. However, transrepression by TCERG1 can be inhibited in the presence of high levels of transcription factor TBP, although the mechanism of inhibition is indirect since TBP neither interacts with nor alters the levels of TCERG1 expression. Second, using an RT-PCR based assay to quantify the production of short and long transcripts, overexpression of TCERG1 resulted in a marked 5-fold decrease in the amount of long transcripts in comparison to short transcripts. Collectively, these results lead Sune and Garcia-Blanco (1999) to propose that TCERG1 forms complexes with unidentified components of a negative-acting elongation complex, causing inefficient elongation of transcripts. Hence, it is unknown if TCERG1 affects RNAPII directly or during the formation of an elongation-competent RNAPII complex. If the latter is true, then TCERG1 may interact with this complex, leading to a disruption of the RNAPII holoenzyme or critical components associated with it. In fact, Carty *et al*. (2000) were

able to show by Far-Western analysis that TCERG1 binds to the phospho-CTD of RNAPII which is competent for elongation. Moreover, TCERG1 exclusively binds to the hyperphosphorylated elongation form of RNAPII and not to its unphosphorylated counterpart, shown by co-immunoprecipitation analysis (Carty *et al.*, 2000). Findings presented by Sune *et al*. (1997) and Goldstrohm *et al.* (2001) demonstrated the ability of TCERG1 to associate with positive transcription elongation factor-b (PTEF-b), a nuclear protein whose major function is in elongation. Collectively, these studies support the hypothesis that TCERG1 is involved in the downregulation of the elongation process of pre-mRNA production. Finally, studies by Carty *et al*. led to the functional characterization of the FF domains in TCERG1. Results of Far-Western analyses indicated that FF2 and FF5 were required for binding to phospho-CTD of RNAPII and were subsequently classified as protein binding domains (Carty *et al.*, 2000).

Since the identification of TCERG1, researchers have embarked on a quest to find a direct biological role for this elusive nuclear protein. Results of *in situ* RNA analysis in mouse embryos showed that TCERG1 could be involved in limb development since elevated levels of TCERG1 were detected in zones with high mitotic activity in the appendages. Additionally, other studies have provided strong evidence for a role for TCERG1 in Huntington's disease based on the presence of high levels of TCERG1 in neuronal aggregates in the brain tissue of patients with this disease (Holbert *et al.*, 2001). Moreover, increased levels of TCERG1 and accumulation of aggregates correlates with the increase in grade of the disease, suggesting an underlying function in Huntington's disease pathogenesis (Holbert *et al.*, 2001). Finally, McFie *et al.* (2006) has recently discovered a new biological role for TCERG1 with respect to the inhibition of C/EBPα-mediated transcriptional and growth arrest activity. The exploration into the regulation of C/EBPα activity mediated by TCERG1 thus far is discussed below and forms the foundation from which my thesis is based.

### 2.2.3 TCERG1 Acts as an Inhibitor of C/EBPα Activity

Recently, McFie *et al.* (2006) used a yeast two-hybrid approach to identify novel interacting co-factors of C/EBPα using a C/EBPα molecule consisting of amino acids 6-217. Furthermore, the mutated C/EBPα transcriptional activity was inactivated by the introduction of three point mutations in order to decrease the potential background transcriptional activation

mediated by this domain. Upon screening of a human liver cDNA library, one clone was isolated and identified as TCERG1. Additional experiments, such as chromatin immunoprecipitation assays, gave further evidence to support the interaction between these two proteins. Furthermore, yeast two-hybrid analysis demonstrated a strong genetic interaction between these two proteins. TCERG1 and another C/EBP isoform, C/EBPβ, were also shown to interact, suggesting TCERG1 may be able to function with other C/EBP molecules. Subsequently, reporter gene assays also showed that TCERG1 is able to repress the transactivation activities of C/EBPα and C/EBPβ in a dose-dependent manner. Moreover, TCERG1 demonstrated a dominant effect over the enhanced transactivation potential of C/EBPβ in the presence of co-activator p300 by significantly inhibiting transcriptional activity.

To investigate if TCERG1 was present on C/EBPα-specific promoter sites, chromatin immunoprecipitation analyses were used to examine two different types of promoters regulated by C/EBPα: one that is activated and one that is repressed by C/EBPα, the PEPCK and the hepatocyte nuclear factor-6 promoter, respectively (McFie *et al.*, 2006). Interestingly, TCERG1 was only detected at the hepatocyte nuclear factor-6 promoter repressed by C/EBPα, suggesting that TCERG1 was recruited to this promoter and may be involved in regulating C/EBPα transrepression of this promoter.

TCERG1 was also assessed for any involvement in the growth arrest activity of C/EBPα. When overexpressed, TCERG1 showed the remarkable ability to reverse the anti-proliferative effects of C/EBPα in cultured cells (McFie *et al.*, 2006). Upon further examination, TCERG1 could neither interact with nor inhibit the growth arrest activities of a mutant containing only the GIR (amino acids 175-217, see Figure 1) of C/EBPα, suggesting the interaction and/or functional domain(s) lie elsewhere in C/EBPα. Moreover, since the original clone obtained from the yeast two-hybrid screen contained amino acids 89-480 of the amino-terminal domain of TCERG1, a truncation mutant consisting of amino acids 641-1098 of TCERG1 was constructed under the suspicion that the amino-terminal domain was involved in mediating inhibition of C/EBPα anti-proliferative activity. This mutant did not interact with or act as a co-repressor of any C/EBPα activities relating to growth arrest or transcriptional activity, leading to the preliminary conclusion that the amino-terminal domain of TCERG1 contained the important functional domains required for interaction with C/EBPα and for mediating its repressive effects on C/EBPα activity.

## 2.3 Subnuclear Compartmentalization

Recent evidence suggests that subnuclear localization is involved in the regulation of gene expression (Misteli *et al.,* 1997; Matera, 1999; Mintz and Spector, 2000; Phair and Misteli, 2000; Hendzel *et al*., 2001; Misteli, 2001, 2007, 2008a; Carmo-Fonseca, 2002; Iborra and Cook, 2002; Wang *et al*., 2002b; Corry and Underhill, 2005; Gorski *et al*., 2006; Dernburg and Misteli, 2007). Furthermore, the nucleus contains many functionally different non-overlapping compartments that facilitate several different types of activity, including transcriptional and splicing events. Data also suggest that the proteins involved in these processes are stored in one region within the nucleus and recruited to others where activities, such as transcriptional activity, occur. Therefore, subnuclear localization may play a role in the inhibition of C/EBPα activity mediated by TCERG1.

### 2.3.1 Characterization of Nuclear Bodies

With the advent of new imaging technology, extensive research has been conducted in an effort to better understand the complexity of the nucleus (Iborra *et al*., 2003). Several lines of evidence have revealed that the nucleus consists of various subcompartments that are arranged into distinct non-membranous domains, termed nuclear bodies or nuclear factories (reviewed in Matera, 1999; Misteli, 2001; Wang *et al*., 2002a). Each range in size from 0.1 to several micrometers and are generally distinguished by their unique distribution pattern. Although most nuclear bodies have not been fully characterized, their constituents and the functional roles that accompany the different proteins they contain have generally defined each type of nuclear body. Thus, assigning biological functions to each type of subnuclear structure has created a new area of research devoted to investigating the dynamic environment within the nucleus.

It has been suggested that interactions between nuclear bodies and other foci are dynamic and can change in response to cellular signals and/or other factors (Matera, 1999; Wang *et al.*, 2002b; Dernburg and Misteli, 2007). Moreover, the establishment of nuclear architecture is critical for bringing nuclear factors and protein complexes within close proximity, permitting the coupling of events, such as transcription and pre-mRNA processing,

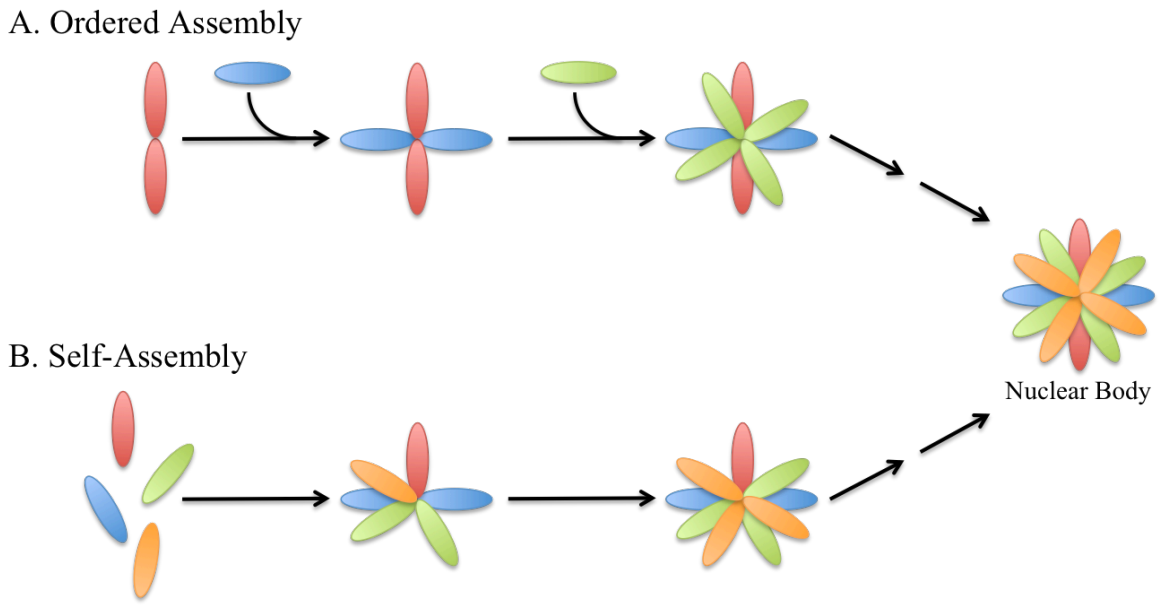


**Figure 4. Models of Nuclear Body Assembly.**
A. Nuclear bodies form by the association of its components in an ordered and sequential fashion. Each new constituent becomes associated based on the nuclear factors already present. B. Formation of nuclear bodies occurs by random assembly of related nuclear factors, thereby generating a concentration of nuclear factors. This image has been modified from Misteli, 2008a.

or to segregate nuclear structures as a means of global regulation. Two models have been proposed for the purpose of higher-ordered nuclear architecture (Figure 4) (Misteli, 2001, 2007, 2008a; Kaiser *et al.*, 2008). One model suggests that the nucleus contains several compartmentalized structures that are formed in an ordered and sequential way, and serve as stable storage sites for various nuclear factors (Figure 4A) (Misteli, 2001, 2007, 2008a). The second model proposes that nuclear bodies self-assemble through random associations of freely moving molecules (Figure 4B) (Misteli, 2001; Kaiser *et al.*, 2008). Furthermore, it has been proposed that the integrity of nuclear body stability may be influenced by several factors and the interactions between molecules are generally transient in nature. Recently, an elegant study was published that followed the *de novo* formation of Cajal bodies, which contain various types of ribonucleoproteins (Kaiser *et al.*, 2008). In this study, the kinetics of Cajal body formation was measured by limiting the concentrations of proteins known to localize in these nuclear subcompartments. Results suggest that the formation of Cajal bodies support the self-assembly

model. However, assembly and establishment of other types of nuclear bodies may occur through an organized mechanism that involves pre-established subnuclear architecture. Regardless of the nuclear body formation mechanism, the formation event itself can be influenced by a number of factors including cellular signals, post-translational modification of its constituents, energy dependence, cell cycle regulation, or even in a tissue-specific manner (Matera, 1999; Misteli, 2001; Wang *et al.*, 2001). Moreover, the speed at which each molecule can diffuse unobstructed through the nucleoplasm in the presence of other nuclear bodies and chromatin structure and the strength with which each bind other components of the newly formed structure, contribute to the rate of nuclear body formation. Collectively, nuclear bodies are thought to be highly dynamic in order to co-ordinate the flux of certain factors to and from sites of activity.

### 2.3.2 Role of Nuclear Bodies in Gene Regulation

The formation and stability of nuclear bodies have been described as dynamic events that involve rapid exchange of factors to either assemble functional stations or release components into the nucleoplasm so they may transverse to sites of activity (Gorski *et al.*, 2006; Misteli, 2007, 2008a). Moreover, the establishment of subnuclear organization provides an efficient way to concentrate nuclear factors in a controlled environment that can be dedicated to a specific function (Hendzel *et al.*, 2001).

Although chromatin mobility in interphase nuclei is to some extent limited and its architecture is highly structured, chromatin can undergo re-organization in the proper conditions. The accessibility and regulation of chromatin structure, often determined by the status of histone acetylation, provide the driving force for the subsequent efflux and assembly of regulatory protein complexes involved in the coordination of transcription and pre-mRNA splicing events. The importance of chromatin structure in the formation and stability of subnuclear compartments is critical during interphase of the cell cycle in order to establish tight regulation of the expression of many genes. For example, examination of histone modifying enzymes during mitosis, using quantitative *in situ* imaging techniques, revealed that histone acetyltransferases and histone deacetylases move from disassembled interchromosomal spaces back into distinct foci as cells enter early G1 phase, concomitant with the reactivation of their

respective activities (Kruhlak *et al.*, 2001).

Chromatin structure also plays a role in regulating cell cycle arrest and differentiation. Current literature supports the view that differentiation-specific transcription factors can inhibit transcription of cell cycle genes by targeting histone deacetylases to promoter sites, permitting transcriptional repression by the histone-modifying enzyme (reviewed in Cheung *et al.*, 2000). For example, C/EBPα associates with p300/CBP, which has histone deacetlyase activity, and with the Brm subunit of SWI/SNF chromatin remodeling complex in order to regulate specific genes during growth arrest and differentiation (Erickson *et al.*, 2001; Pedersen *et al.*, 2001). Secondly, transcription factors may recruit histone acetyltransferase complexes in order to silence the expression of other genes in order to regulate cellular fate (Cheung *et al.*, 2000).

Several types of nuclear bodies have been implicated as sites of transcriptional activity, while others provide a stable but transcriptionally inactive site for protein complex assembly or for storage. Moreover, some transcription factors may associate with different subnuclear domains depending on the state of the cell. For example, Parfenov and colleagues (2003) used a variety of imaging tools to demonstrate that the transcription factor Oct-4 is found at dense fibrillar components of the nucleolus and is associated with RNAPII at perichromatin fibrils and interchromatin granules during the transcriptionally active stage of the mouse oocyte nucleus. However, in transcriptionally inert oocytes, Oct-4 becomes localized to perichromatin granules, nucleolus-like bodies and Cajal bodies (Parfenov *et al.*, 2003). Alternatively, regulation of nuclear factor may occur in response to cellular signals in response to a stimulus (reviewed in Corry and Underhill, 2005). It has been demonstrated that heat shock factors are activated in response to cellular stress by inducing the expression of proteins involved in the maintenance of intracellular homeostasis. In these conditions, transient subnuclear domains are formed, termed nuclear stress granules, where proteins responsible for cellular maintenance accumulate and may form protein complexes and/or work together to activate transcription of specific genes.

### 2.3.3 Subnuclear Characterization of C/EBPα and TCERG1

Several studies have examined the subnuclear compartmentalization of C/EBPα in a variety of cells lines (Tang and Lane, 1999; Schaufele *et al.*, 2001, 2003; Zhang *et al.*, 2001;

Liu *et al.*, 2002, 2007; Day *et al.*, 2003; Enwright *et al.*, 2003; Demarco *et al.*, 2006). The pattern of subnuclear distribution of C/EBPα in murine-derived cell types is defined by the presence of 20-50 distinct punctate foci localized near centromeres of interphase heterochromatin (Tang and Lane, 1999; Schaufele *et al.*, 2001). In studies looking at the subnuclear localization of C/EBPs during adipocyte differentiation, it was found that when C/EBPβ is first expressed, it is localized throughout the nucleoplasm (Tang and Lane, 1999). Within 12 to 16 hours of expression, C/EBPβ acquired DNA-binding activity and was subsequently localized to peri-centromeric regions, while C/EBPδ remained dispersed throughout the non-nucleolar regions of the nucleoplasm (Tang and Lane, 1999). Moreover, the ability to redistribute to peri-centromeric regions was thought to be dependent on phosphorylation of C/EBPβ, which was later confirmed by immunofluorescence using phospho-specific antibodies directed to sites targeted by mitogen-activated protein kinase in cells treated with growth hormone (Tang and Lane, 1999; Piwien Pilipuk *et al.*, 2003). Cell fractionation and *in situ* immunofluorescence studies demonstrated that there was a delay between C/EBPβ expression, sequestration to peri-centromeric regions and DNA binding to the promoter region of *c/ebpα* (Tang and Lane, 1999). The DNA binding capacity is also thought to be as a result of the phosphorylation C/EBPβ that occurs shortly after the induction of its expression. Observations reported for C/EBPα were identical to C/EBPβ, suggesting that both isoforms undergo similar mechanisms involved in peri-centromeric relocalization in a DNA binding-dependent manner (Tang and Lane, 1999). This was one of the initial reports describing and relating the subnuclear localization of C/EBPs, post-translational modification and DNA binding activity (Tang and Lane, 1999). Since then, evidence suggests that mouse major α-satellite DNA repeat sequences found within transcriptionally inert peri-centromeric regions are responsible for the sequestration of C/EBPα to these domains (Liu *et al.*, 2007). Furthermore, examination a C/EBPα molecule containing only the bZIP domain was sufficient to target the molecule to peri-centromeric heterochromatin, yet a mutant whose leucine zipper was removed could no longer associate with these subnuclear structures (Schaufele *et al.*, 2001; Liu *et al.*, 2002). Interestingly, an amino acid substitution of valine to alanine at position 296 resulted in the abrogation of DNA binding to α-satellite sequences found at peri-centromeric regions and was instead distributed in the interchromatin space generally associated with transcriptional activity (Schaufele *et al.*, 2001; Liu *et al.*, 2007). This inability to localize to

peri-centromeric regions also led to increased levels of C/EBPα-mediated transcriptional activity of certain promoters localized in interchromatin space (Liu *et al.*, 2007). Thus, the DNA-binding domain of C/EBPα is both sufficient and necessary for proper targeting to peri-centromeric heterochromatin in GHFT1-5 and 3T3-l1 cell lines (Schaufele *et al.*, 2001; Liu *et al.*, 2002, 2007). Additional studies by Liu *et al*. (2007) demonstrated that pituitary-specific positive transcription factor-1 (Pit-1) sequestered C/EBPα away from peri-centromeric domains to sites occupied by Pit-1 in a dispersed nucleoplasmic pattern containing euchromatin. Thus, peri-centromeric subnuclear compartments have been attributed to act as stable storage sites where C/EBPα is concentrated and can be released or recruited to areas of active transcription. Additional support for this hypothesis came from reports by Day *et al.* (2003) and Enwright *et al.* (2003) who demonstrated that DNA binding by C/EBPα at α-satellite DNA is a prerequisite to its recruitment by Pit-1 and their subsequent cooperative transcriptional activity at pituitary-specific promoters. This evidence suggests that C/EBPα is maintained and stored in peri-centromeric regions prior to recruitment and cooperative activation of specific promoters by Pit-1. Moreover, a truncation mutant of C/EBPα missing the TAD is able to interact with Pit-1 but both C/EBPα and Pit-1 remain associated with peri-centromeric regions. Further characterization of the interactions between Pit-1 and C/EBPα using fluorescence resonance energy transfer analysis to visualize living GHFT1-5 pituitary cells suggest that protein domains that specify re-localization are separate from those involved in the direct functional and stable positioning within the subnuclear architecture. This hypothesis recapitulates the data demonstrating that DNA binding activity and localization to inactive heterochromatin by C/EBPα is required for interactions with Pit-1 and for the ensuing redistribution of Pit-1/C/EBPα complexes to euchromatic regions containing active transcription. Similar requirements for DNA-binding and phosphorylation have been described for proper peri-centromeric heterochromatin localization of Ikaros, a transcription factor expressed in hematopoietic cells that regulates the expression of lymphocyte-specific genes (Brown *et al.*, 1997; Cobb *et al.*, 2000; Gurel *et al.*, 2008). The DNA-binding and dimerization domains of Ikaros were essential for proper localization in several different cell lines, suggesting that the requirements for subnuclear compartmentalization is an intrinsic property of Ikaros, and that the pattern of distribution is not limited to lymphocyte cells (Cobb *et al.*, 2000). Subsequent characterization of Ikaros subnuclear localization revealed that phosphorylation was important

for the ability of Ikaros to acquire peri-centromeric occupancy and that reversible phosphorylation of specific residues controls subnuclear localization and regulation of Ikaros transcriptional activity during the differentiation of thymocytes (Gurel *et al.*, 2008). Data collected from studies examining the relationship between peri-centromeric localization of Ikaros and the regulation of its function appear to be analogous to observations made with C/EBPα and, thus, have provided insight for a functional role for subnuclear localization in the control of C/EBPα activity.

Recent studies have demonstrated that certain nuclear co-factors can be recruited to peri-centromeric regions in a C/EBPα-dependent manner (Schaufele *et al.*, 2001, 2003). This suggests that these sites may also act as assembly stations for the formation of protein complexes that can then be recruited to various regions of active transcription. CBP and TBP, two enhancers of C/EBPα transcriptional activity, are generally localized in a reticular pattern throughout the nucleus of GHFT1-5 cells (Schaufele *et al.*, 2001). However, when each is co-expressed with C/EBPα, CBP and TBP rapidly become associated with peri-centromeric regions. Moreover, redistributon of CBP and TBP requires binding through the TAD of C/EBPα, which reaffirms the concept that protein domains specifying co-localization are distinct from those that direct functional positioning of protein complexes within the nuclear substructure, as proposed by Day and colleagues (2003). Finally, it has been demonstrated that C/EBPα regulates the levels of acetylated histone H3 present at peri-centromeric regions (Zhang *et al.*, 2001). Increased levels of histone H3 acetylation are dependent on the transcriptional integrity of C/EBPα (Zhang *et al.*, 2001). This was demonstrated using two C/EBPα mutants: one whose TAD was deleted, and the other inactivated (Schaufele *et al.*, 2001) by forming a fusion protein with the carboxy-terminal domain of EGFP (Zhang *et al.*, 2001). The amount of acetylated H3 remained at levels similar to cells not expressing C/EBPα to when each C/EBPα mutant was expressed (Zhang *et al.*, 2001). It is known that C/EBPα interacts with and recruits co-regulators that modify chromatin structure and histone acetylation, such as CBP and p300 (Erickson *et al.*, 2001), which can alter the accessibility of genes to regulatory proteins involved in mediation of transcriptional activation or repression. Thus, control over the expression of certain genes found in proximity to peri-centromeric regions may be subject to C/EBPα-dependent regulation and may be important during cellular growth arrest or terminal differentiation. Collectively, whether acting as a stable storage

facility, as a site for protein complex assembly, or in the regulation of chromatin structure, subnuclear compartmentalization of C/EBPα appears to be an important component in the regulation and function of C/EBPα-mediated activities.

Recent studies have examined the dynamics of other nuclear bodies, such as the splicing factor-rich nuclear speckles. Data suggests that nuclear speckles serve as storage sites for nuclear factors involved in transcription and pre-mRNA splicing, such as RNApII and SC35, respectively (Bregman *et al*., 1995; Mortillaro *et al*., 1996; Miteli *et al.*, 1997; Melcak *et al.*, 2000; Mintz and Spector, 2000; Hall *et al.*, 2006). Moreover, pinin/DRS, a cell adhesion-related protein and mRNA processing factor that primarily localizes within nuclear speckles, was recently found to drastically redistribute to the nucleoplasm and cytosol upon the overexpression of the transcription factor C-terminal binding protein 1 (Alpatov *et al.*, 2004). Additionally, pinin/DRS was shown to relieve C-terminal binding protein 1-mediated transcriptional repression at certain promoters by a functional interaction between the two proteins. These studies and others provide mounting evidence for the functional association between nuclear proteins involved in mRNA processing, transcriptional activity and alterations in nuclear localization (Carty *et al.*, 2000; Goldstrohm *et al.*, 2001; Alpatov *et al.*, 2004). Therefore, since TCERG1 is thought to act as a bridge between transcription and splicing, subnuclear localization may play a role in TCERG1-mediated inhibition of C/EBPα activity.

As the characterization of TCERG1 continues to be pursued, a singular study has examined the specific subnuclear distribution of this elusive nuclear factor (Sanchez-Alvarez *et al.*, 2006). Using indirect immunostaining techniques, the spatial distribution of endogenous TCERG1 in HeLa and non-transformed IMR90 fibroblast cell lines has been recently described by Sanchez-Alvarez *et al.* (Sanchez-Alvarez *et al.*, 2006). In this study, several experiments were performed to establish that TCERG1 accumulates within two non-nucleolar populations: mainly in splicing factor-rich nuclear speckles and at lower levels heterogeneously dispersed throughout the nucleoplsm. Subsequent deletion analysis of TCERG1 suggested that the FF domains found within in the carboxy-terminal domain are important for proper nuclear speckle localization. This finding is supported by the fact that TCERG1 associates with the hyperphosphorylated carboxy-terminal repeat of RNAPII, which is also a component of nuclear speckles (Bregman *et al.*, 1995; Mortillaro *et al.*, 1996; Carty *et al*., 2000). Finally, Arango *et al.* (2006) were able to show that the $(QA)_{38}$ repeat was important for TCERG1 nuclear

localization in the nuclei of primary rat striatal cells using immunofluorescence and LSCM, although this requirement has not been examined in other cell lines. Therefore, it is possible that function(s) provided by different domains are important for nuclear localization of TCERG1 in a tissue-dependent manner.

### 2.3.4 Nuclear Localization is Important for Transcription Factor Activity

Given that TCERG1 and C/EBPα localize within different and distinct subnuclear compartments, LSCM can be used to examine any alterations in subnuclear localization that may occur upon their co-expression. It is unknown if subnuclear localization is involved in the TCERG1-mediated inhibition of C/EBPα activity, however, if such a mechanism exists, several scenarios can be predicted. First, C/EBPα activity may be inhibited by a mechanism mediated by TCERG1, either by direct or indirect association, resulting in restricted mobility or accessibility of C/EBPα to various subnuclear domains where it functions. Additionally, interactions between C/EBPα and TCERG1 may prevent association with co-activators, such as CBP and TBP, by limiting the number of free C/EBPα molecules able to form active protein complexes. Both mechanisms would result in the confinement and accumulation of C/EBPα within inactive nuclear domains, thereby detaining C/EBPα, and preventing access to all sites of activity altogether. Moreover, the concept of a nuclear retention mechanism has been described for several nuclear factors found within the nucleolus and with RNAPII in response to alterations in physiological cellular conditions or by chemical inhibitors, respectively (Misteli *et al.*, 1997; Gorski *et al.*, 2006). Although the aforementioned hypothetical models are with respect to inhibition of C/EBPα activity, a similar mechanism involving protein mobility and subnuclear structure has been described for the co-operative transcriptional activation of C/EBPα and Pit-1 in GHFT1-5 pituitary cells (Demarco *et al.*, 2006). In this model, the assembly of the protein complex, Pit-1/C/EBPα, is stabilized by the dominant chromatin-binding activities of Pit-1, leading to decreased C/EBPα mobility. This permits stable association of the protein complex with a particular nuclear subdomain, such as transcriptionally active euchromatin. This hypothesis was further emphasized by the analysis of a single missense mutation in Pit-1 that abrogated the dominant chromatin-binding activity, resulting in the formation of protein complexes with C/EBPα restricted to peri-centromeric

regions. A third mechanism of inhibition may involve the recruitment of C/EBPα to an entirely different nuclear body where there is no C/EBPα activity. As a result, access to factors involved in recruiting or activating C/EBPα transcriptional activity would be prohibited. This mechanism of inhibition has been observed with C/EBPδ, which is involved in differentiation and growth arrest of mouse mammary epithelial cells (O'Rourke *et al.*, 1999; Zhou *et al.*, 2008). In co-localization studies examined by LSCM, Zhou *et al.* (2008) were able to demonstrate that a potent inhibitor of C/EBPδ activity, protein inhibitor of STAT-y (PIASy), repressed the activities of C/EBPδ in part by sequestering it to the nuclear periphery of HC11 mouse mammary epithelial cells. PIASy also inhibits the activities of transcription factors by sumoylation, reducing DNA binding, and recruiting histone deacetylases (Kim *et al.*, 2002; Schmidt and Muller, 2003; Zhou *et al.*, 2008). However, re-localization of C/EBPδ by direct interaction with PIASy occurs independent of other forms of inhibition, suggesting translocation of C/EBPδ alone may be sufficient to inhibit its activity (Zhou *et al.*, 2008). Furthermore, it was suggested that inhibition of C/EBPδ activity could also be a consequence of interaction or association with additional transcription factors found at the nuclear periphery, a region considered to be transcriptionally inert (Shaklai *et al.*, 2007; Zhou *et al.*, 2008). Finally, "scratch" assays were performed to examine the effect of PIASy expression on the biological role of C/EBPδ-mediated control of cellular proliferation and migration (Zhou *et al.*, 2008). These *in vitro* assays are designed such that a small area of confluent monolayer of HC11 cultured cells was removed using a pipette tip (scratch), thereby inducing the proliferation and migration of cells into the open area. Cells transiently transfected with C/EBPδ and PIASy demonstrated enhanced ability to repopulate "scratch" regions, versus cells expressing only C/EBPδ. Collectively, these studies suggest that PIASy inhibits the transcriptional and growth arrest activity of C/EBPδ in HC11 mammary epithelial cells by sequestering C/EBPδ to the transcriptionally inactive nuclear periphery. Thus, it is possible that other C/EBP isoforms can be negatively regulated by a similar mechanism, such as the inhibition of C/EBPα by TCERG1.

Splicing factor-rich nuclear speckles are thought to be storage sites for various splicing factors in addition to other factors required for transcription, such as hyperphosphorylated RNAPII (Bregman *et al*., 1995). Studies by Misteli *et al.* (1997) demonstrated that components of nuclear speckles have the ability to translocate to various sites of active transcription. Futhermore, it has been demonstrated that splicing factor SC35 can diffuse out of nuclear

speckles into transcriptionally active regions at peri-chromatin fibrils and can participate in pre-mRNA splicing (Melcák *et al*., 2000). Based on these observations, it is possible that TCERG1 may translocate to areas surrounding or occupied by C/EBPα where it can bind to and inhibit C/EBPα activity responsible for arresting cellular proliferation and/or transcriptional activation. Additional evidence to support the hypothesis for the re-distribution of TCERG1 by C/EBPα is based on a recent study showing the ability of C/EBPα to recruit subnuclear factors, CBP and TBP, to peri-centromeric regions in mouse progenitor pituitary GHTF1-5 cells (Schaufele *et al*., 2001). This demonstrates that C/EBPα has the ability to recruit transcriptional co-factors to subnuclear compartments occupied by C/EBPα. Moreover, this function may not be limited to co-activators and may include the recruitment of negatively acting factors like TCERG1 as well.

## 2.4 Hypothesis

TCERG1 inhibits the growth arrest activities of C/EBPα through functional motif(s) that lie exclusively in the amino-terminal region of TCERG1 and the mechanism by which this occurs involves subnuclear localization.

## 2.5 Objectives and Rationale

A. To identify the region(s) of TCERG1 responsible for inhibiting C/EBPα-mediated growth arrest in cultured cells using growth arrest assays.

Recently data demontrated that TCERG1 inhibits C/EBPα-mediated growth arrest activity in several cell lines (McFie *et al.*, 2006). Additional evidence suggests that TCERG1 interacts with C/EBPα through domains found within the amino-terminal domain. Thus, mutants of TCERG1 were generated and tested in growth arrest assays with cells expressing C/EBPα in an effort to map the regions responsible for mediating TCERG1 inhibitory activity. A transfection-based proliferation assay in combination with fluorescence technology allows the observation and quantification of cells expressing fluorescent fusion proteins to TCERG1

and C/EBPα. Each TCERG1 mutant was examined for its ability to reverse growth arrest of cultured cells co-expressing C/EBPα-EGFP.

B. To determine the nuclear distribution of both C/EBPα-EGFP and mCherry-TCERG1 using LSCM when co-expressed in cultured cells as a possible mechanism for inhibition of C/EBPα by TCERG1.

Evidence from a variety of studies suggests that components of several nuclear bodies have the ability to translocate to areas of active transcription and/or sites where splicing events occur (Misteli *et al.*, 1997, 2008b; Melcak *et al.*, 2000; Phair and Misteli, 2000; Carmo-Fonseca, 2002; Faro-Trindade and Cook, 2006; Gorski *et al.*, 2006). Additionally, it has been shown that some nuclear factors can be recruited to other nuclear structures as a means of inhibition (Finlan *et al.*, 2008; Zhou *et al.*, 2008). Moreover, it has been demonstrated that C/EBPα can recruit co-activators to peri-centromeric regions in GHFT1-5 progenitor pituitary cells where transcriptionally active protein complexes may be formed (Schaufele *et al*., 2001). Therefore, using LSCM (Matera, 1999; Misteli, 2001; Wang *et al.*, 2002b; Iborra *et al.*, 2003), the relationship of the inhibitory function of TCERG1 and spatial distribution between TCERG1 and C/EBPα was characterized. Different fluorescent protein fusions to TCERG1 and C/EBPα were co-expressed in two cell lines and examined for any alterations in their subnuclear localization patterns relative to conditions where each were expressed alone.

# 3. MATERIALS AND METHODS

## 3.1 Reagents

All reagents used were of analytical grade or higher. Names of reagents and suppliers are listed in Table 1. Addresses for each supplier are subsequently listed in Table 2.

**Table 1: List of Reagents and Suppliers.**

| Reagent | Supplier |
|---|---|
| **Common Reagents** | |
| 100 base pair (N3231), 1 kilobase pair (N3232) DNA ladder | NEB |
| Calf Intestinal Alkaline Phosphatase (M0290) | NEB |
| DNA-Modifying Enzymes | NEB |
| Paraformaldehyde | BDH |
| Polyethylenimine | Polysciences |
| T4 DNA Ligase (15224) | Invitrogen |
| T4 Polynucleotide Kinase (M0201) | NEB |

| | |
|---|---|
| **Bacterial Culture Reagents** | |
| Ampicillin | Unison Biotek |
| Kanamycin | ICN |
| NM522 Competent *Escherichia coli* | Stratagene |
| NZ-Amine A® from Bovine Milk | Sigma-Aldrich |
| XL-10 Gold® Ultracompetent *Escherichia coli* | Stratagene |

| | |
|---|---|
| **Commerical Kits** | |
| QIAquick® Gel Extraction Kit | Qiagen |
| QIAquick® PCR Purification Kit | Qiagen |

| | |
|---|---|
| **Cell Culture Reagents** | |
| Antibiotic-Antimycotic (100x) | Invitrogen |
| Dulbecco's Modified Eagle's Medium - Low Glucose (D6046) | Sigma-Aldrich |
| Fetal Bovine Serum (Canadian Origin) | PAA |
| Trypsin, 0.25 % (1x), EDTA•4 Na | Invitrogen |

| **Reagents for Immunofluorescence** | |
|---|---|
| ColorpHast® pH Test Stripe Non-Bleeding pH 0-14 | EMD |
| pmCherry-C1 Mammalian Expression Vector | Clontech |
| Prolong® Gold Antifade Reagent with 4',6-diamidino-2-phenylindole | Invitrogen |
| Vista Vision Cover Glasses No. 1.5 | VWR |

| **Protein Analysis Reagents** | |
|---|---|
| 30% Acrylamide/Bis Solution (29:1) | Bio-Rad |
| Autoradiography Film | Denville Scientific |
| PageRuler™ Prestained Protein Ladder Plus (SM1811) | Fermentas |
| Western Lightning™ Chemiluminescence Reagent Plus Kit | Perkin Elmer |

**Table 2: List of Names and Addresses of Reagent Suppliers.**

| **Supplier** | **Address** |
|---|---|
| Abcam | Cambridge, Massachusetts, USA |
| BDH | Saskatoon, Saskatchewan, Canada |
| Bethyl Labs | Montgomery, Texas, USA |
| Bio-Rad | Hercules, California, USA |
| Clontech | Mountain View, California, USA |
| Denville Scientific | Metuchen, New Jersey, USA |
| EMD | Madison, Wisconsin, USA |
| Fermentas | Burlington, Ontario, Canada |
| ICN | St. Laurent, Quebec, Canada |
| Invitrogen | Burlington, Ontario, Canada |
| NEB | Mississauga, Ontario, Canada |
| PAA | Etobicoke, Ontario, Canada |
| Perkin Elmer | Boston, Massachusetts, USA |
| Polysciences | Warrington, Pennsylvania, USA |
| Qiagen | Mississauga, Ontario, Canada |
| Santa Cruz | Santa Cruz, California, USA |
| Sigma-Aldrich | Oakville, Ontario, Canada |
| Stratagene | La Jolla, California, USA |
| Unison Biotek | Scarborough, Ontario, Canada |
| VWR | Mississauga, Ontario, Canada |

### 3.2 Bacterial Strains and Media Preparations

*E. coli* NM522 and XL10-Gold® ultracompetent *E. coli* cells (Stratagene, California, USA) were used as the host for the amplification of plasmid DNA.

Luria-Bertani (LB) agar plates containing either kanamycin or ampicillin were used to propagate both *E. coli* strains. In general, LB plates were prepared by combining 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl and 15 g bacto-agar in 1 L double distilled water ($ddH_2O$) and subsequently sterilized by autoclaving for 20 min at 15 lb/sq. in. Once cooled to 45-50°C, kanamycin was added with a final concentration of 50 µg/mL or ampicillin to 100 µg/mL, then poured into 100 mm Petri dishes and stored at 4˚C until used.

LB liquid medium used to prepare competent *E. coli* NM522 cells was prepared as indicated above with the exception that no agar or antibiotics were added. Appropriate antibiotics were added when required for the propagation of bacterial cells.

NZY broth used in the transformation protocol of XL10-Gold® ultracompetent *E. coli* cells (Stratagene) was made from 10 g NZ-Amine A® casein hydrolysate from bovine milk, 5 g yeast extract, and 5 g NaCl in 1 L $ddH_2O$. The mixture was adjusted to pH 7.5 and then autoclaved. Prior to use, NZY broth was supplemented with the addition of sterile 0.004% (w/v) glucose, 12.5 mM $MgCl_2$ and 12.5 mM $MgSO_4$.

For small and large quantity plasmid DNA isolation, a single *E. coli* colony was inoculated in complete Terrific Broth (TB) medium. TB was prepared by combining 12 g of bacto-tryptone, 24 g of bacto-yeast extract and 4 mL glycerol in 900 mL $ddH_20$ and then autoclaved. Once cooled, 100 mL potassium-salt solution (0.17 M $KH_2PO_4$ and 0.72 M $K_2HPO_4$) and the appropriate antibiotics were added.

### 3.3 Molecular Cloning

Protocols in this section were based in part on those described in Sambrook *et al*. (1989).

### 3.3.1 Preparation of Competent Bacterial Cells

Two mL of LB were inoculated with a single colony of *E. coli* and incubated overnight at 37˚C with agitation. The culture was transferred to a flask containing 200 mL LB and incubated at 37˚C with agitation for 1-2 h until $O.D._{590}$ reached 0.375. The bacterial culture was transferred into pre-chilled 50 mL sterile conical tubes and incubated on ice for 10 min. Following incubation, cultures were centrifuged at 7000 X *g* for 10 min to pellet the cells. The supernatant was decanted and cells resuspended in 10 mL cold $CaCl_2$ wash solution (60 mM $CaCl_2$, 15% glycerol, 10 mM 2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid, pH 7.0). Samples were again subjected to centrifugation and resuspended in 10 mL $CaCl_2$ wash solution and then placed on ice for 30 min. Cells were then centrifuged at 7000 X *g*, the supernatant removed, and the pellet resuspended in 2 mL $CaCl_2$ solution. The competent bacteria were aliquoted into sterile 1.5 mL cryovials and stored at -80˚C.

### 3.3.2 Transformation of Competent Bacterial Cells

*E. coli* NM522 cell stocks were thawed on ice. In a pre-chilled 14 mL polypropylene tube, 100 µL *E. coli* cells plus 2 µL DNA ligation reaction were gently mixed and placed on ice for 30 min. Following incubation, the transformation mixture was heat-pulsed at 42˚C for 2 min and then returned to ice for 2 min. The transformation mixture was spread onto LB plates containing the appropriate antibiotic, inverted and incubated overnight at 37˚C.

Transformation of XL10-Gold® ultracompetent *E. coli* cells was performed as described in the protocol provided by the manufacturer.

### 3.3.3 Small Scale Plasmid DNA Preparations

The protocol used to isolate small scale plasmid DNA was adopted from a protocol by Lee and Rasheed (1990). Single *E. coli* colonies were selected and individually inoculated into 5 mL complete TB containing the appropriate antibiotic. For amplification of plasmids with kanamycin resistance, cultures were grown in complete TB for 1 h with agitation at 37˚C before

kanamycin was added. In the final step, all plasmids were resuspended in 10 mM Tris-HCl and 1 mM EDTA, pH 8.0.

### 3.3.4 Large Scale Plasmid DNA Preparations

Plasmid DNA was extracted from lysed *E. coli* cells as described by Sambrook *et al*. (1989). A single *E.coli* colony was inoculated in 5 mL TB and incubated at 37˚C with agitation for 6-18 hr. The bacterial culture was then added to 200 mL complete TB containing the appropriate antibiotic and incubated with agitation overnight at 37˚C. The final pellet containing plasmid DNA was resuspended in 10 mM Tris-HCl and 1 mM EDTA, pH 8.0, to a final concentration of 1.0 μg/μL. Plasmids were deemed to be of sufficient purity when the $O.D._{260/280}$ ratio was between 1.7 and 1.8.

### 3.3.5 Plasmids

A series of TCERG1 mutants each consisting of the following amino acids: 32-1098, 32-668, 32-293, 281-1098, and 641-1098 were constructed previously in our laboratory (Pam McFie) by ligating the coding region of each into the BglII/EcoRI site of $pHA_3$. $pHA_3$ contains three tandem hemagglutinin ($HA_3$) epitopes cloned into pRc-CMV (Invitrogen) 5' to the insert of interest so that the resulting protein contains three HA epitopes fused to the amino-terminal region. $pHA_3$ was kindly provided by D. Anderson (Saskatoon Cancer Centre). Additionally, a simian virus 40 (SV40) large T-antigen NLS (Ohno and Shimura, 1996) was cloned in frame into the BglII restriction site at the amino terminus of TCERG1 truncation mutants 641-1098 and 32-293 to facilitate nuclear localization of these mutants whose endogenous NLS was deleted. The generation of $pHA_3$ 641-1098 and $pHA_3$ 32-293 is described in more detail in section 3.3.5.3.

The following describes the generation of pmCherry-TCERG1 mutants used in this study. pmCherry-C1 mammalian expression vector encodes a monomeric red fluorescent protein derived from *Discosoma striata* (DsRed) and has been modified to reduce cellular toxicity in addition to increased stability relative to other variants of DsRed (Shaner *et al.*, 2004). The excitation and emission maxima for mCherry are 587 nm and 610 nm, respectively.

Vectors containing mCherry-TCERG1 mutants were constructed by isolating the BglII/HindIII (32-668, 32-293) or BglII/EcoRI (32-1098, 281-1098, 641-1098) fragment from $pHA_3$-TCERG1 mutants then ligated in-frame into pmCherry. Both pmCherry-TCERG1 32-668 and 32-293 retained the $HA_3$ tag as well as the SV40 NLS in 32-293 (see section 3.3.5.3 for generating $pHA_3$-TCERG1 32-293). Each TCERG1 mutant generated was subjected to diagnostic restriction analysis and then sent to PBI-NRC (Saskatoon, Saskatchewan, Canada) for DNA sequence verification.

For both growth arrest and co-localization studies, a vector encoding enhanced green fluorescent protein (EGFP) was used to assist in visualization. pEGFP-N1 (Clontech) contains a multiple cloning site for a cDNA insert of interest in-frame with the amino-terminal end of EGFP (Yang *et al.*, 1996). EGFP is a red-shifted derivative of wild-type GFP from *Aequorea victoria* with two distinguishing alterations. A double amino acid substitution of Phe to Leu at position 64 and Ser to Thr at position 65 results in a significant increase in the fluorescence intensity relative to wild-type GFP (Cormack *et al.*, 1996). Additionally, higher levels of expression of EGFP in mammalian systems were achieved by human codon optimization. The excitation and emission maxima for EGFP are 488 nm and 507 nm, respectively. pEGFP-N1 and pEGFP-N1-C/EBPα were a gift from R. Day (Indiana University School of Medicine). pEGFP-N1-C/EBPα has been renamed as pC/EBPα-EGFP since EGFP is fused to the carboxy-terminal end of C/EBPα . pC/EBPα-EGFP also contains a FLAG epitope fused to the amino-terminus of C/EBPα-EGFP for detection purposes if necessary.

Additional vectors used in co-localization studies are described as follows: pEGFP-LAP encodes EGFP fused to the amino-terminal region of liver activating protein (LAP) isoform of C/EBPβ and was a gift from C. Asselin (Université de Sherbrooke). pEGFP-Sp1, a gift from O. Rohr (Université de Strasbourg), was generated by cloning the Sp1 cDNA into the XhoI/HindIII sites of pEGFP-C1 (Clontech).

#### 3.3.5.1 Restriction Digest of Plasmid DNA

All DNA-modifying enzymes were purchased from New England Biolabs (NEB) unless otherwise stated. In a total volume of 20 µL, 2 µg plasmid DNA, 10 U of each restriction enzyme, and 2 µL appropriate 10X buffer were added, mixed and incubated at 37°C for 1-2 h.

#### 3.3.5.2 DNA Fragment Isolation and Verification

In order to verify and isolate plasmid DNA fragments, DNA was subjected to agarose gel electrophoresis after digestion with restriction enzymes. DNA samples were resuspended in 2 µL of 6X sample loading buffer (25 mM bromophenol blue, 25 mM xylene cyanol FF, 30% glycerol) before loading into a 1% agarose gel containing 1X TAE (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0) and 1 µg/mL ethidium bromide. A 1 kilobase pair or 100 base pair DNA ladder was also loaded as appropriate. Electrophoresis was carried out in an apparatus containing 1X TAE buffer for 1 h at 100 volts. When required, the DNA fragment of appropriate size was excised and purified using a QIAquick® Gel Extraction Kit as described by the manufacturer.

#### 3.3.5.3 Generation of Nuclear Localization Signal TCERG1 Mutants

The following protocols describe the generation of TCERG1 32-293 and 641-1098 truncation mutants in the $pHA_3$ expression plasmid.

##### 3.3.5.3.1 Dephosphorylation of Plasmid DNA

Previously digested plasmid DNA was purified using a QIAquick® Gel Extraction Kit. In a 500 µL microcentrifuge tube, 5 µg of plasmid DNA was resuspended in a solution containing 1X NEB buffer 3, 10 U calf intestinal alkaline phosphatase in a total volume of 20 µL and incubated in a 37°C water bath for 1 h. The resulting plasmid DNA was purified using a QIAquick® PCR Purification Kit as described by the manufacturer.

##### 3.3.5.3.2 Preparation of the SV40-NLS Oligonucleotide Fragment

The SV40 large-T antigen NLS (Ohno and Shimura, 1996) was generated from complementary oligonucleotides: forward 5'-GATCCCCTAAGAAGAAGAGGAAAGTCA-3' and reverse 5'-GATCTGACTTTCCTCTTCTTCTTAGGG-3' (Invitrogen) which code for amino acids PKKKRKV. Proper orientation of the NLS was established by the ligation of five

nucleotides (underlined) of the forward oligonucleotide with the 3' BglII site of the vector. This was achieved by the introduction of a single nucleotide point mutation in the BglII site, effectively removing it. The SV40-NLS oligonucleotides were first annealed by placing 10 µg of each primer diluted with 0.1X TE in a final volume of 100 µL, incubated in a water bath at 95°C for 5 min then slowly cooled to room temperature (RT). Next, 400 ng of annealed oligonucleotide was then phosphorylated with 10 U T4 polynucleotide kinase, 1X T4 polynucleotide kinase buffer and 10 mM ATP in a final volume of 10 µL. The sample was incubated at 37°C for 30 min, followed by 5 min at 70°C to inactivate T4 polynucleotide kinase.

#### 3.3.5.3.3 Ligation of TCERG1 NLS Mutants into $pHA_3$ and pmCherry-C1

Approximately 10-30 fmol of vector and 30-90 fmol double-stranded oligonucleotide were added to 10 U T4 ligase, and 4 µL 5X buffer, adjusted to 20 µL with $ddH_2O$ and incubated at RT overnight. Two microlitres of the ligation reaction was transformed into either competent *E. coli* NM522 or XL10-Gold® ultracompetent *E. coli* as described above.

Expression vectors containing TCERG1 32-293 and 641-1098 were verified using restriction enzyme analysis and subjected to agarose gel electrophoresis performed on a 3% agarose gel with the same conditions as stated previously. A 3% agarose gel was used to observe small differences in DNA fragment sizes corresponding to the SV40 NLS 27 base pair fragment. Selected clones were sent to PBI-NRC (Saskatoon, Saskatchewan, Canada) for DNA sequence verification. Each TCERG1 mutant contained one SV40 large T-antigen NLS with the exception of pmCherry-TCERG1 641-1098 which had two tandem repeats of the NLS.

## 3.4 Mammalian Cell Culture

Human embryonic kidney 293T (HEK293T) cells, containing the gene for simian virus large T-antigen, were kindly provided by D. Muruve (University of Calgary). African green monkey kidney cells (COS7) were provided by S. Stone (University of Saskatchewan). All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with

16.65 mM glucose and 5% fetal bovine serum at 37°C with 5% $CO_2$. Cells were passaged twice a week at 10-20% confluency and maintained in 100 mm tissue culture plates. Mammalian cell stocks were prepared by trypsinization and resuspended in 5 mL media, followed by centrifugation at 1500 X *g* for 5 min at 4°C. The supernatant was removed, the cell pellet washed with 1X PBS, then resuspended in 1 mL of serum-containing DMEM with 10% DMSO. The cell suspension was then incubated for 1 h each at 4°C, -20°C, and -80°C before storing in liquid nitrogen.

### 3.5 Growth Arrest Assay

COS7 cells were subcultured at 20% confluency into 60 mm tissue culture plates and transfected the following day. In a 14 mL polypropylene tube containing 215 µL of 150 mM NaCl and 10 µg total DNA, 30 µL of 0.1% polyethylenimine was added and gently mixed immediately for 10 sec followed by a 10 min incubation at RT. The transfection mixture was then added drop-wise to tissue culture plates containing cells and incubated at 37°C with 5% $CO_2$. Media was replaced 4 h post-transfection with fresh serum-containing media. The following day, cells were resuspended first in a small volume of trypsin, and then in increasing volumes of serum-containing medium to ensure the majority of the cells were well separated. Cells were then seeded at 50% confluency into duplicate 60 mm tissue culture plates and incubated at 37°C with 5% $CO_2$ for 24-48 h until visualized. Between 140 and 160 individual green fluorescing cell colonies were assessed using an Olympus IX70 inverted fluorescent microscope (Olympus, Ontario, Canada). Each fluorescing cell colony was categorized as either single cells exhibiting growth arrest or as clusters of two or more actively proliferating cells. Images were captured using an imaging device coupled to the microscope and modified using SPOT advanced software (Diagnostic Instruments, Inc., Sterling Heights, Michigan, USA). All data generated was obtained using a 9:1 plasmid ratio of $pHA_3$-TCERG1 mutants to pC/EBPα-EGFP. This ratio was achieved by expressing different plasmid ratios of $pHA_3$-TCERG1 32-1098 to pC/EBPα-EGFP from 2:1 to 9:1 in COS7 cells until reversal of growth arrest was demonstrated to levels similar to previous reports (McFie *et al.*, 2006).

### 3.6 Protein Expression Determination by Western Blot Analysis

The following protocols are for the extraction and quantification of cellular protein and subsequent analysis using Western Blot procedure.

#### 3.6.1 Preparation of COS7 Cytosolic and Nuclear Extracts

The protocol described refers to cells propagated in 100 mm tissue culture plates. Transfected COS7 cells were washed once with cold 1X PBS before adding 1 mL cold low-salt buffer (20 mM HEPES, pH 7.5, 20% glycerol, 10 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1% Triton X-100). Cells were harvested and homogenized using a Dounce homogenizer. The cell lysates was transferred to a 14 mL polypropylene tube and pelleted by centrifugation at 7000 X *g* for 5 min. The supernatant containing the cytosolic extract was removed to a fresh pre-chilled 14 mL tube and set aside. The cell pellet was resuspended in 400 µL high-salt lysis buffer (20 mM HEPES, pH 7.5, 20% glycerol, 500 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 1 mM DTT, and 0.1% Triton X-100) and was incubated on ice for 15-30 min followed by a 5-10 min incubation at RT. The cell suspension was centrifuged at 1000 X *g* for 10 min at 4°C and the supernatant containing the nuclear extract was transferred to a fresh pre-chilled 1.5 mL ultracentrifuge tube. All cellular extracts were quantified for protein concentration and prepared for SDS-PAGE electrophoresis as described in section 3.6.2 and 3.6.3.

#### 3.6.2 Protein Quantification of Cellular Extracts

For protein quantification, the Bio-Rad Protein Assay (Bio-Rad) was performed according to the microassay procedure supplied with the reagents. Typically, one part concentrated dye reagent was diluted with four parts $ddH_2O$. In 1 mL of 1X dye reagent, 1 µL of cytosolic or nuclear extract was added, incubated at RT for 5 min then read at $O.D._{595}$ using a spectrophotometer. A protein standard curve was performed using BSA ranging from 0-10 µg/mL for determining sample protein concentration. Samples were prepared for analysis by Western Blot by diluting them to 1 µg/µL in 5X SDS loading buffer (250 mM Tris, pH 6.8,

10% SDS, 50% glycerol, 1% bromophenol blue, and 25% β-mercaptoethanol), heating at 95°C for 5 min to denature proteins and placing them on ice before storing at -80°C.

### 3.6.3 SDS Polyacrylamide Gel Electrophoresis

All protein samples were resolved on a 9% SDS polyacrylamide gel consisting of N,N'-methylene-bis-acrylamide (29:1), 375 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.1% ammonium persulfate, and 0.0004% N,N,N',N'-tetramethylethanediamine, with a 4% stacking gel (N,N'-methylene-bis-acrylamide (29:1), 130 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.1% ammonium persulfate, and 0.001% N,N,N',N'-tetramethylethanediamine). A molecular weight marker, PageRuler™ prestained protein ladder Plus, and approximately 15-20 μg denatured protein extracts were loaded onto the SDS-PAGE gel and subjected to electrophoresis at 125 volts for 90 minutes using an Bio-Rad Mini Protean® II Apparatus (Bio-Rad). The running buffer was composed of 25 mM Tris-HCl, 192 mM glycine, and 0.1% (w/v) SDS.

The resolved samples were then transferred onto a polyvinylidene fluoride membrane using a Bio-Rad Mini Trans-Blot® transfer cell filled with transfer buffer, pH 8.3 (48 mM Tris-HCl, 39 mM glycine, 0.037% (w/v) SDS, and 20% methanol) and run at 75 volts for 3 hours or 40 volts overnight at 4˚C. The membrane was then removed and incubated in blocking buffer made with 5% blotting grade blocker non-fat milk/1X TBST, pH 7.5 (137 mM NaCl, 2.7 mM KCl, 19 mM Tris-HCl and 0.001% Tween-20) for 1 hour at RT with agitation. The membrane was then incubated with the appropriate primary antibody diluted in 5% milk/1X TBST overnight at 4˚C to facilitate hybridization. The following morning the membrane was washed three times with 5% milk/1X TBST for 10 minutes each and subsequently incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted in 5% milk/1X TBST for 1 hour at RT with gentle agitation. Following incubation, the membrane was washed once each with 5% milk/1X TBST, 1X TBST, and 1X Tris-buffered saline, pH 7.5 (137 mM NaCl, 2.7 mM KCl and 19 mM Tris-HCl) for 10 minutes before preparing for detection. The membrane bound antibody-protein complexes were detected using Western Lightning Chemiluminescence Reagent Plus kit as described by the manufacturer and exposed on autoradiography film for an appropriate time at room temperature. For a complete list of antibodies and the dilutions used, see Table 3.

## 3.7 Laser Scanning Confocal Microscopy

### 3.7.1 Transient Transfection and Microscope Slide Preparation

HEK293T and COS7 cells were subcultured at 10-20 % and were transiently transfected using polyethylenimine as described in section 3.5 with the exception that cells were not resuspended in increasing volumes of serum-containing medium. Cells were subsequently subcultured 1:2 12-24 h post-transfection in 6-well plates containing sterilized no. 1 1/2 glass cover slips. Cells were maintained at 37°C with 5% $CO_2$ for approximately 24 h before washing once with 1X PBS and fixed using a 4% (w/v) paraformaldehyde (PFA) solution (see next paragraph for preparation) for 15 min at RT. Cells were washed three times with 1X PBS for 5 min. Cover slips were air-dried and mounted onto microscope slides using Prolong® Gold Antifade mounting media reagent plus 4',6-diamidino-2-phenylindole and stored in the dark at RT for 24 h. For experiments requiring immunofluorescence, cells were permeabilized using 0.2-0.6 % Triton X-100 for 5-10 min at RT following fixation, and then washed twice with 1X PBS for 5 min. Cells were blocked for 15 min at RT with 1 % (w/v) bovine serum albumin/1X PBS solution, followed by the addition of primary antibody diluted to 1:1000 using blocking solution and incubated at RT for 1 h. Cover slips were then washed three times with 1X PBS for 5 min, then incubated with the appropriate Alexa Fluor® conjugated secondary antibody diluted to 1:200 using blocking solution for 45 min at RT. Cover slips were washed five times with 1X PBS, air-dried and mounted as indicated above. A complete list of antibodies and their applications used in this study are provided in Table 3.

The 4% (w/v) PFA fixation solution was prepared as follows. Four grams of PFA were mixed with approximately 60 mL of 1X PBS, and then placed on a heating block while stirring until the solution reached 60˚C. The heat source was turned off and 10 N NaOH was added drop wise until the solution turned clear. As the solution cooled, 1 N HCl was added drop wise until the PFA solution reached pH 7.0, which was assessed using ColorpHast® pH Test Stripe Non-Bleeding pH 0-14 test strips. The final solution was made up to 100 mL using 1X PBS, sterilized using a 0.22 μm filter and stored at 4˚C up to one month.

**Table 3: List of Antibodies Used for Western Blot Analysis and Immunofluorescence.**

Each antibody was diluted as appropriate for its application. Catalog numbers and suppliers are also indicated. WB indicates Western Blot analysis; IF indicates immunofluorescence.

| Name | Dilution | Application | Supplier |
|---|---|---|---|
| Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) *highly cross-adsorbed* (A11029) | 1:200 | IF | Invitrogen |
| Alexa Fluor® 594 Goat Anti-Mouse IgG (H+L) *highly cross-adsorbed* (A11032) | 1:200 | IF | Invitrogen |
| Donkey Anti-Mouse IgG-HRP (sc-2314) | 1:10000 | WB | Santa Cruz |
| Goat Anti-Rabbit IgG-HRP (sc-2004) | 1:10000 | WB | Santa Cruz |
| Living Colors® Rabbit Anti-DsRed (632496) | 1:1000 | WB | Clontech |
| Mouse Anti-Hemagglutinin Antibody (sc-7392) | 1:1000 | WB | Santa Cruz |
| Mouse Anti-SC35 (phospho) Nuclear Speckle Marker (ab11826) | 1:1000 | IF | Abcam |
| Mouse Anti-β-Actin (sc-47778) | 1:2000 | WB | Santa Cruz |
| Rabbit Anti-C/EBPα (sc-61) | 1:1000 | WB | Santa Cruz |
| Rabbit Anti-CA150 (A300-360A) | 1:2000 | WB | Bethyl Labs |
| Rabbit Anti-TBP (sc-204) | 1:1000 | WB | Santa Cruz |

### 3.7.2 Image Acquisition and Analysis

Prepared slides were imaged using an Olympus Fluoview 300 laser scanning confocal microscope (D. Mousseau, University of Saskatchewan) equipped with a blue Argon (488 nm, 10 mW) and green Helium Neon (543 nm, 1 mW) laser. Briefly, slides were inverted (cover slip side down), mounted using immersion oil at 60x objective (Plan-Apochromat 60XO/1.4), and viewed under fluorescence with the appropriate filter until a cell was selected for imaging. Once chosen, image acquisition was performed using 3-dimensional XYZ optical scanning through the thickness of the sample. Pinhole diameter (aperture, 100 μm), laser power, photomultiplier tube, gain, and offset were set optimally to decrease background signals in the acquired image. Additionally, sequential scanning, first green, then red, was selected for certain images to improve the resolution and quality of the red signal. Once an appropriate Z-section scan was chosen, an image was acquired using Kalman filtering (set at 6) at a slow scan speed (4.57 s/scan) for the best resolution.

# 4. RESULTS

## 4.1 TCERG1 Reverses Growth Arrest Activity of C/EBPα

TCERG1 is a modular protein containing multiple functional domains, therefore, it follows that one or more of these domains could be involved in TCERG1-mediated inhibition of C/EBPα activity. Since TCERG1 has the ability to inhibit all activities of C/EBPα, both transcriptional and growth arrest, it is important to resolve which domains of TCERG1 are responsible for the inhibition of C/EBPα activity. Recent results obtained using a proliferation assay examining a TCERG1 mutant consisting of only the carboxy-terminal domain (amino acids 641-1098) revealed that this domain lacks the ability to reverse growth arrest of cells, suggesting the functional or interaction domain(s) lies elsewhere (McFie *et al.*, 2006).

To further characterize the functional domain(s) that reside in TCERG1 responsible for reversing C/EBPα anti-proliferative activity, an assay was designed to both qualitatively and quantitatively assess TCERG1 inhibitory activity in COS7 cells. The identification of growth-arrested or actively proliferating cells was provided by the ectopic expression of EGFP, which can be observed in live cells using a fluorescence microscope. Cells were visualized three days post-transfection and those expressing EGFP fluoresced throughout the cell, while cells expressing EGFP fused to the carboxy-terminal domain of C/EBPα, C/EBPα-EGFP, could be identified by the presence of green fluorescence restricted to the nuclei. Although this type of assay was fairly challenging to develop, once established, the results were reproducible. The transfection efficiency ranged from 40-60% using chemical methods, determined by comparison between green filter and bright field imaging using a fluorescence microscope. Also, controlling the initial dilution of cells and ensuring the majority were well separated single-cell colonies at the time of transfection dramatically decreased the occurrence of false positives (data not shown). Finally, the advantage of this method is that only transfected and subsequent fluorescing cells are assayed unlike many commercially available proliferation kits that assay all cells regardless of transfection efficiency or initial confluency of cells, the latter often required at 80-90%.

Growth arrest assays were initially performed in COS7 cells expressing TCERG1 and C/EBPα-EGFP individually to establish this assay in this cell line. COS7 cells were chosen for several reasons; they express lower levels of endogenous TCERG1 relative to other cell lines previously examined in our laboratory (data not shown), cells proliferate in monolayers, and lastly, the cellular morphology of the cells make it easy to identify clusters of proliferating cells. The cDNA obtained for TCERG1 was originally isolated from a vector coding for amino acids 32-1098 provided by M. Garcia-Blanco (Duke University), who also determined that the first 31 amino acids of TCERG1 were dispensable (Sune *et al.*, 1997). Therefore, TCERG1 32-1098 was cloned in-frame into the $pHA_3$ vector containing three-tandem hemagglutinin ($HA_3$) epitopes fused to the amino terminal end of TCERG1. In the case of C/EBPα, it has been previously demonstrated that EGFP fused to the amino terminal end of C/EBPα (EGFP-C/EBPα) could still mediate growth arrest but was transcriptionally inactive (Schaufele *et al.*, 2001). Therefore, the complementary C/EBPα carboxy-terminal end fusion to EGFP (C/EBPα-EGFP), which retains all C/EBPα activities (Schaufele *et al.*, 2001), was selected for all subsequent studies.

COS7 cells were transiently transfected with appropriate vectors and were visualized three days post-transfection using a fluorescence microscope for cells expressing EGFP or C/EBPα-EGFP in the presence and absence of TCERG1 32-1098. Figure 5 contains representative images obtained from growth arrest assays. The first image in each panel represents the phase contrast view (greyscale), followed by an image obtained using a filter for green fluorescence. The last image represents a merged image of the first two to verify the presence of clustered or single-cell colonies. For each assay, a range of 140-160 individual cell colonies were counted per plate and categorized as single cells under growth arrest (Figure 5B) or proliferating cells characterized by the presence of colonies of two or more cells, respectively (Figure 5A and C). A graphical representation of the results is found in Figure 6. In control assays, where EGFP alone was expressed, numerous clustered cells were identified and accounted for 71% of the cell colonies assessed. Next, COS7 cells were transfected with plasmids encoding either C/EBPα-EGFP or TCERG1 32-1098 to show the effect of each protein on the proliferative capacity of the cells. When C/EBPα-EGFP was expressed alone, only 18% of the colonies assessed were in clusters, demonstrating growth arrest of cells. In contrast, COS7 cells overexpressing TCERG1 32-1098 were actively proliferating and

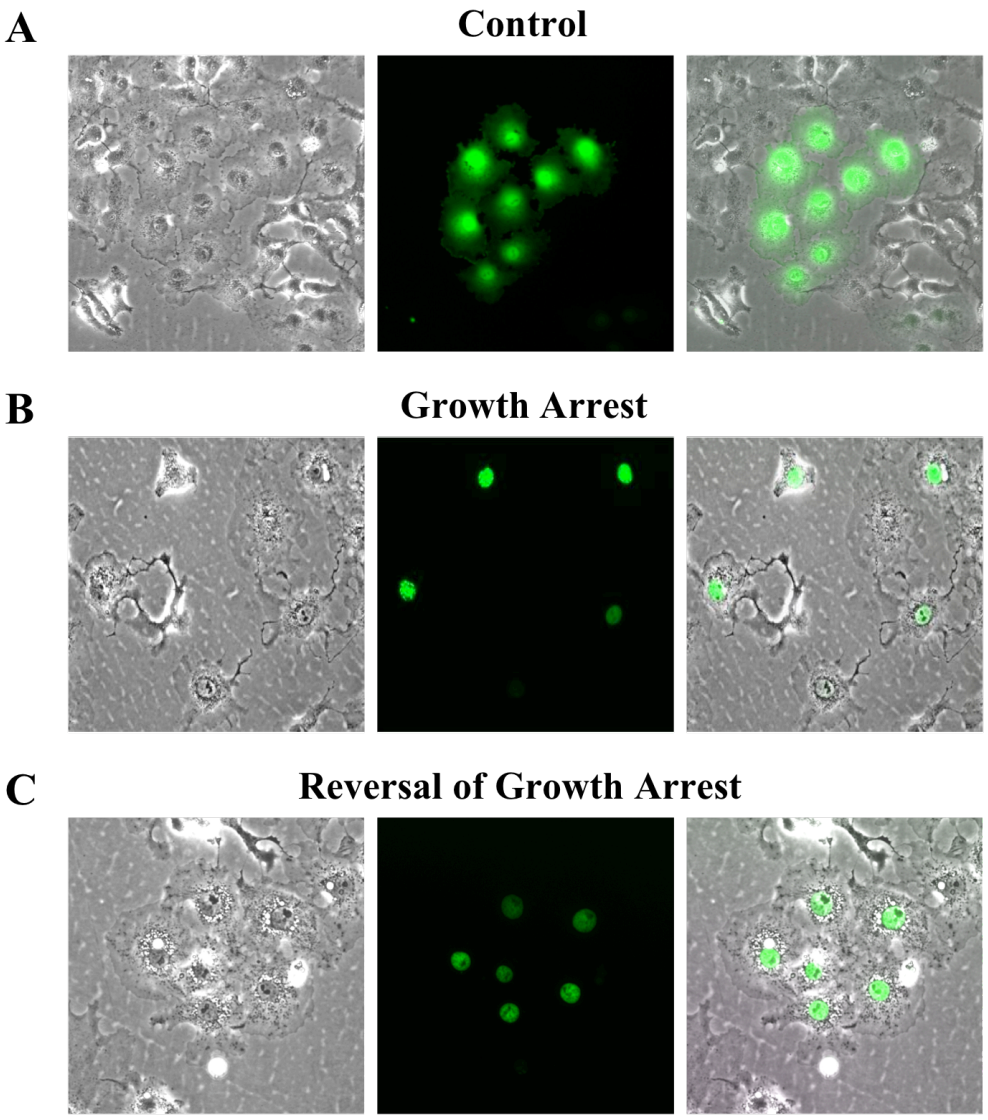


**Figure 5. Growth Arrest Assays to Examine the Ability of TCERG1 to Reverse C/EBPα-Mediated Growth Arrest.**

COS7 cells were transiently transfected to express EGFP, C/EBPα-EGFP, or C/EBPα-EGFP plus TCERG1. Cells were observed three days post-transfection using an inverted fluorescent microscope to qualitatively assess the proliferative capacity of cells as under growth arrest or in proliferating clusters. Each panel from left to right consists of images using phase contrast, a green fluorescent filter, and the corresponding merged image. Images were captured with a digital camera and modified using SPOT Advanced software. (A) Proliferation is observed as a cluster of cells that are visualized when TCERG1 is co-expressed with EGFP (green cells). (B) Cells are observed as single cells under growth arrest upon the expression of C/EBPα-EGFP (green nuclei) and in conditions where TCERG1 is unable to reverse C/EBPα-EGFP-mediated growth arrest. (C) Reversal of C/EBPα-EGFP-mediated growth arrest in the presence of TCERG1 is observed as a cluster of green nuclei indicative of proliferation and confirmed by phase contrast imaging.

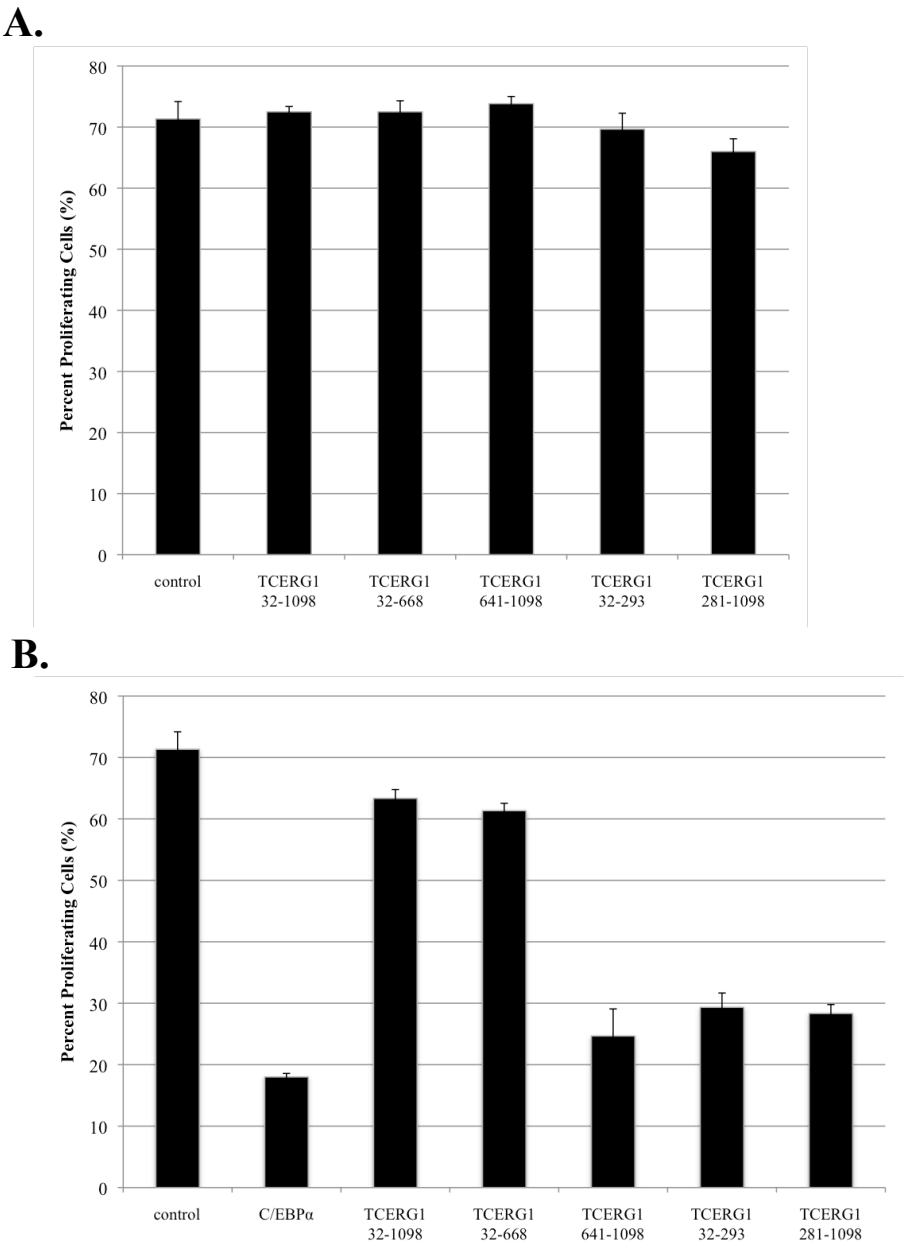


**Figure 6. The Amino-Terminal Domain of TCERG1 Reverses Growth Arrest Caused by C/EBPα.**

A. COS7 cells were transiently transfected with an expression vector for EGFP (control) alone and with various TCERG1 truncation mutants as indicated. Between 140-160 individual green-fluorescing colonies of two or more cells were visually identified and counted using a fluorescence microscope and are shown as a percentage. B. The percentage of proliferating cells expressing each TCERG1 truncation mutant in the presence of C/EBPα-EGFP. Control indicates the percentage of cells proliferating when EGFP is expressed alone. The values shown are the means ± standard error of at least three independent assays performed in duplicate.

contained 73% of colonies of clustered cells, similar to results obtained in control conditions (Figure 6A).

Once the above conditions were established, cells were transiently co-transfected with vectors expressing C/EBPα-EGFP and TCERG1 32-1098. Consistent with previous studies, TCERG1 32-1098 was able to reverse C/EBPα-EGFP-mediated growth arrest of cells (McFie *et al.*, 2006) indicated by the presence of 63% proliferating cell colonies, a difference of 45% relative to cells demonstrating growth arrest (Figure 6B, compare TCERG1 32-1098 to C/EBPα). These results confirm the ability of TCERG1 to reverse C/EBPα anti-proliferative activity in COS7 cells.

### 4.1.1 Generation of TCERG1 Truncation Mutants to Identify Region(s) Responsible for Inhibition of C/EBPα Growth Arrest Activity

To determine which domain(s) within TCERG1 is responsible for the inhibition of growth-arrest by C/EBPα, several truncation mutants of TCERG1 were generated. Each mutant was designed with careful consideration, with the deletion of specific modular domains suspected to be involved in the inhibition of growth arrest caused by C/EBPα. An illustration of each TCERG1 mutant is provided in Figure 7 indicating the corresponding amino acids and the domains retained in each construct. All mutants were cloned into a mammalian expression vector that linked three HA epitopes onto the amino-terminal end of the expressed protein. In the case of TCERG1 641-1098 and 32-293, a nuclear localization signal (NLS) from the SV40 large T-antigen was engineered onto the amino terminal end of TCERG1 immediately following the $HA_3$ epitopes since the endogenous NLS was deleted.

Initially, protein expression for each TCERG1 truncation mutant was determined by Western Blot analysis prior to testing in growth arrest assays to ensure that the protein was being expressed and was of the expected size. COS7 cells were transiently transfected with equal amounts of TCERG1 expression plasmid, cytosolic and nuclear extracts were prepared and analyzed using Western Blot analysis. Each TCERG1 mutant was detected using antibodies to the hemagglutinin epitope fused to the amino-terminal end of the protein. All TCERG1 mutants were detected in the nuclear fraction; however, TCERG1 32-668, 641-1098 and 32-293 were also present in the cytosolic extracts (Figure 8). The cytoplasmic population

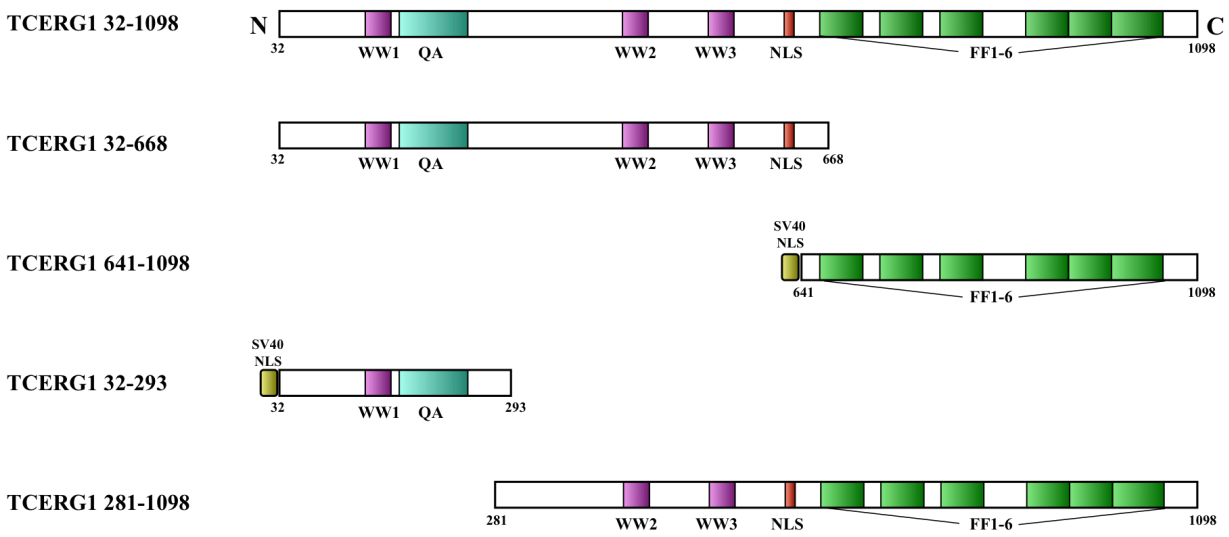


**Figure 7. TCERG1 Truncation Mutants Used in This Study.**
TCERG1 is a 1098 amino acid nuclear protein. The amino terminus contains three WW domains (purple), implicated in protein-protein interactions, and a unique glutamine-alanine $(QA)_{38}$ repeat (teal) of unknown function. The carboxy terminal domain consists of six FF domains (green), which, like WW domains, are involved in protein-protein interactions. TCERG1 also has a putative nuclear localization signal (NLS, red) located between the amino and carboxy terminal domains. The amino acids contained in each mutant are stated on the left in parentheses and indicated in each illustration. An SV40 large T-antigen NLS (yellow) was cloned in-frame to the amino terminus of TCERG1 641-1098 and 32-293 to replace the endogenous NLS that has been deleted as a result of its construction.

of the latter two mutants could be partially explained by the loss of endogenous NLS and the fact that the SV40 NLS was not completely efficient or sufficient for nuclear targeting. It is possible that certain regions have been removed that contribute to proper nuclear localization, thus resulting in a cytoplasmic pool. However, using LSCM techniques to visualize TCERG1 mutants fused to a red fluorescent protein, TCERG1 641-1098 was the only mutant that could be observed both in the cytoplasm and within the nucleus, while 32-668 and 32-293 were exclusively localized within the nucleus (Figure 8, bottom panel). Inspection of levels of TCERG1 641-1098 and 32-293 protein detected by Western Blot analysis indicated that these two mutants were expressed at higher levels than 32-1098. Due to the difference in expression levels, approximately half of the amount of plasmid for these two mutants was transfected in COS7 cells to account for protein expression variation. Finally, all mutants migrated to a molecular weight comparable to the theoretical size predicted using Internet software

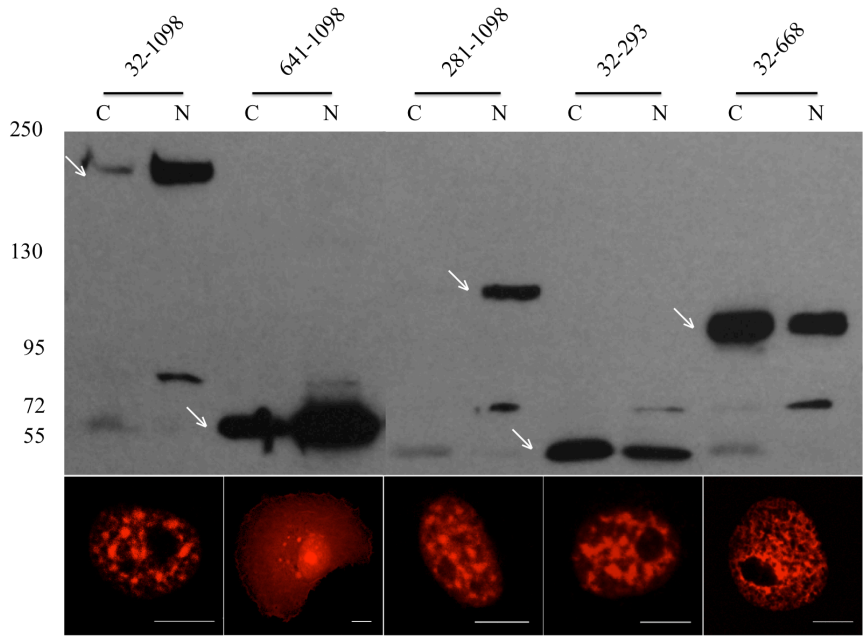


**Figure 8. Protein Expression Profiles of $HA_3$-TCERG1 Mutants in COS7 Cells.**
COS7 cells were transfected with each $HA_3$-TCERG1 truncation mutant and cytosolic (C) and nuclear (N) extracts were prepared 24 h post-transfection. Approximately 12.5 μg extract was loaded into and resolved in a 4% stacking/9% resolving SDS-polyacrylamide gel. TCERG1 mutants (indicated at the top) were detected using antibodies to the hemagglutinin epitope fused to the amino-terminal end of each mutant. An arrow is used to mark the band corresponding to each each mutant as appropriate. A molecular weight marker, in kilodaltons, is indicated on the left. The bottom panel of images represents the localization of each TCERG1 expressed as a fusion protein to mCherry, which emits red fluorescence. Images of COS7 nuclei, with the exception of TCERG1 641-1098 (entire cell), were captured using LSCM. The magnification of each image varied depending on the size of the cell or nucleus. Scale bar: 10 μm.

(Walker, 2005). The estimated size of each protein is as follows, in the order of appearance in the Western blot: 32-1098, 180 kDa; 641-1098, 65 kDa; 281-1098, 120 kDa; 32-293, 40 kDa; 32-668, 100 kDa. The predicted sizes of each protein including the $HA_3$ epitope and NLS where appropriate are as follows: TCERG1 32-1098, 125 kDa; 641-1098, 60.6 kDa; 281-1098, 95.5 kDa; 32-293, 33.5 kDa; 32-668, 73.5 kDa. It is important to note that based on the amino acid sequence alone of full length TCERG1, software predicted the protein to be 124 kDa, even though TCERG1 was originally characterized and identified as a 150 kDa protein (Sune *et al.*, 1997). Thus, the difference in size between the actual and predicted molecular weights could be a result of post-translational modification.

### 4.1.2 Amino-Terminal Domain of TCERG1 is Required to Reverse Growth-Arrested Cells

Next, growth arrest assays were performed to examine the effect of various TCERG1 truncation mutants for their ability to inhibit C/EBPα-mediated growth-arrested COS7 cells in an attempt to identify the region(s) within TCERG1 responsible for the inhibitory activity. The initial TCERG1 mutants examined in growth arrest assays were based on previous studies postulating that the amino terminal portion of TCERG1 was important for reversing C/EBPα-mediated growth-arrested cells (McFie *et al.*, 2006). Therefore, a mutant lacking the amino terminal domain was generated and designated TCERG1 641-1098 (Figure 7) in order to corroborate previous results showing that a similar mutant lacks inhibitory activity (McFie *et al.*, 2006). In addition to this, a mutant whose carboxy-terminus was deleted, TCERG1 32-668, was also generated and examined on the premise that this mutant would retain the functional domain(s) required for TCERG1 inhibitory activity. Both TCERG1 mutants were examined for the ability to reverse C/EBPα-mediated growth arrest of COS7 cells. Results of each assay are summarized in Figure 6. All assays were initially performed with each TCERG1 mutant alone as a control to assess whether the expression of the TCERG1 mutant had any effect on the proliferative capacity of COS7 cells. Initial test conditions in cells expressing TCERG1 641-1098 or TCERG1 32-668 resulted in the identification of 73-74% proliferating cell colonies, comparable to TCERG1 32-1098 (73%; Figure 6A). This indicated that there were no apoptotic or anti-proliferative effects in COS7 cells related to the expression of each mutant. Next, cells were transiently transfected to co-express each TCERG1 mutant and C/EBPα-EGFP and were visually assessed three days later. Similar to previous observations (McFie *et al.*, 2006), results of the growth arrest assays confirmed that the carboxy-terminal portion of TCERG1 (641-1098) was unable to reverse growth arrest as 75% individual cell colonies remained single celled (Figure 6B). Furthermore, in assays examining TCERG1 32-668, 61% of cells were identified as clusters, levels similar to that achieved by TCERG1 32-1098 (Figure 6B). These results further support the hypothesis that the functional portion of TCERG1, with respect to reversing the anti-proliferative activity of C/EBPα, lies within the amino terminal domain.

In order to investigate which domains within the amino terminus may contribute to this activity, two additional mutants were generated. TCERG1 32-293 contains the first WW domain and the unique QA repeat, and TCERG1 281-1098 contains WW2 and WW3 in addition to all six FF domains (Figure 7). Control experiments in conditions where each mutant was expressed in the absence of C/EBPα-EGFP showed no effect on the proliferative capacity of COS7 cells relative to EGFP or TCERG1 32-1098, with 66-70% cell colonies identified in clusters (Figure 6A). Growth arrest assays were then performed in the presence of C/EBPα-EGFP and results indicated that TCERG1 32-293 and 281-1098 were unable to successfully revert growth-arrested cells to a proliferative capacity as indicated by 29% and 28% clustered cells, respectively (Figure 6B). These findings were similar to that observed with TCERG1 641-1098, which lacks the amino-terminal region entirely. This suggested that these mutants lack the domain(s) within the amino-terminus necessary for inhibitory activity and that multiple domains in this region may be required for this activity. Therefore, based on the collective results from growth arrest assays, amino acids 32-668 are required and sufficient for the inhibition of growth arrest of COS7 cells, and multiple domains in this region may be involved.

### 4.2 Spatial Distribution of TCERG1 and C/EBPα in COS7 Cells

The discovery that the nucleus is a complex organelle consisting of subcompartments arranged into discrete functional domains has created a new area of research devoted to investigating the dynamic environment within the nucleus (Matera, 1999; Misteli, 2001; Wang *et al.*, 2002b; Corry and Underhill, 2005). As such, evidence from various elegant studies suggests that components of many nuclear bodies have the ability to redistribute to areas of active transcription and/or sites where splicing events occur (Bregman *et al*., 1995; Misteli *et al*, 1997; Melcak *et al*, 2000; Phair and Misteli, 2000; Misteli 2001, 2008b; Corry and Underhill, 2005; Demarco *et al*, 2006; Gorski *et al*, 2006). This theory led to the hypothesis that TCERG1 may translocate to sites occupied by C/EBPα, such as in transcription factories, where it can act to prevent C/EBPα transcriptional activity. Likewise, TCERG1 could sequester C/EBPα away from transcriptionally active sites to splicing factor-rich nuclear speckles. Thirdly, TCERG1 may recruit C/EBPα to other nuclear bodies where C/EBPα has no

activity, thereby altering the localization of both proteins. Finally, TCERG1 may relocalize to functional compartments that act as storage sites for C/EBPα, thereby preventing translocation of C/EBPα to transcriptionally active sites or domains required for C/EBPα-mediated activity. Therefore, we set out to examine if the ectopic expression of TCERG1 had any effect on the subnuclear distribution of C/EBPα or vise versa.

Fluorescent fusion proteins and immunostaining techniques using fluorophores in combination with LSCM are widely used to examine localization of proteins in cells (Iborra *et al.*, 2003). Selecting fluorophores with non-overlapping excitation and emission spectra allows one to visually characterize alterations in localization patterns. Furthermore, images acquired using z-axis optical scanning techniques provide fine detail that can detect subtle differences in the spatial distribution of different proteins. In the studies presented here, utilization of fluorescent fusion proteins was chosen as the preferred method of subnuclear localization analysis instead of immunostaining strategies for several reasons. First, all previous studies examining the subnuclear localization of C/EBPα were performed with fusion proteins (Tang and Lane, 1999; Schaufele *et al.*, 2001, 2003; Zhang *et al.*, 2001; Liu *et al.*, 2002; Day *et al.*, 2003; Enwright *et al.*, 2003; Demarco *et al.*, 2006). Second, indirect immunostaining techniques using secondary antibodies conjugated with fluorophores can result in non-specific binding and subsequent background signal that can generate images that are not well defined or are difficult to detect (data not shown). Additionally, antibody dilution for fine particle and co-localization studies require considerable fine-tuning (Mintz and Spector, 2000). Although using fluorescent fusion proteins leads to lower levels of expression relative to non-fluorophore-tagged proteins, newly accumulated protein deposits can be observed by a direct means that is not possible with immunofluorescence, which relies on the indirect detection of the protein of interest.

The expression plasmid encoding C/EBPα-EGFP used in growth arrest assays was also used for co-localization studies. EGFP has an excitation/emission spectrum of 488 nm and 507 nm, respectively. mCherry, a red fluorescent protein, was selected as the contrasting fusion protein for TCERG1 and its mutants. mCherry is a variation of *Discosoma striata* (DsRed) that is monomeric and has several modifications to enhance protein expression (Shaner *et al.*, 2004). It has an excitation/emission spectrum of 587 nm and 610 nm, respectively. Each TCERG1 mutant was cloned in-frame such that mCherry was fused to the amino-terminal region of the

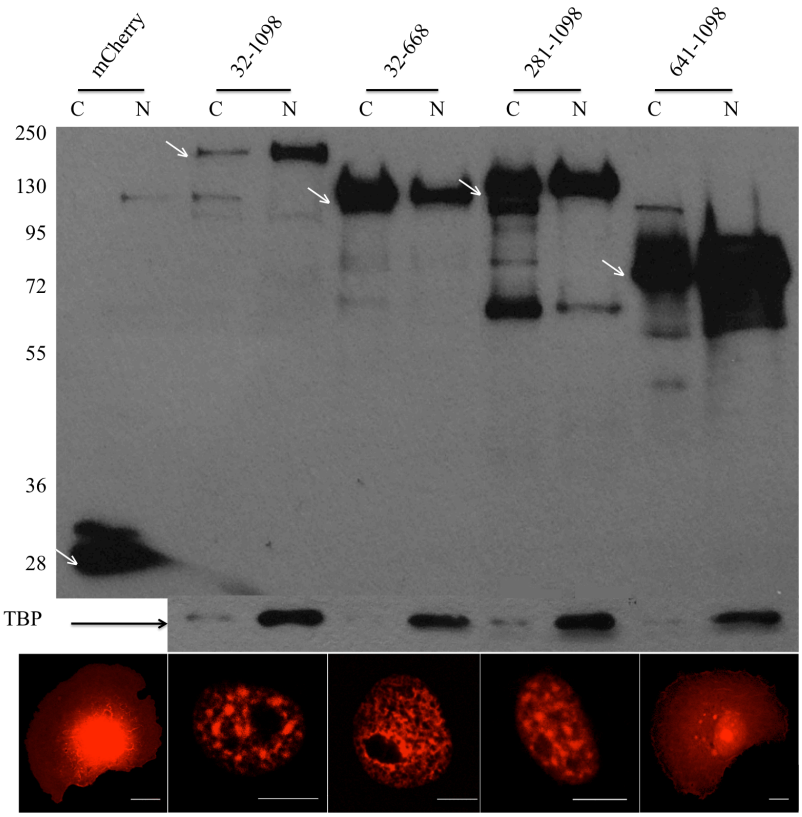


**Figure 9. Expression Analysis of mCherry-TCERG1 Mutants.**
COS7 cells were transfected with mCherry or mCherry-TCERG1 truncation mutant and extracts were prepared 24 hours post-transfection. The expression of each mCherry-TCERG1 truncation mutant (indicated above) was detected by immunoblot analysis of prepared cytosolic (C) and nuclear (N) extracts (15 μg). Protein extracts were detected using an antibody to DsRed which also recognizes mCherry. TBP (43 kDa) was also detected to serve as a loading control for the presence of nuclear proteins. Arrows indicate the overexpressed mCherry-TCERG1 proteins where appropriate and molecular weight markers, in kilodaltons, are shown. Estimated protein sizes are as follows: mCherry, 30 kDa; TCERG1 32-1098, 180 kDa; 32-668, 110 kDa; 281-1098, 120 kDa; 641-1098, 80 kDa. The bottom panel shows images obtained by LSCM of COS7 cells expressing the corresponding mCherry or mCherry-TCERG1 mutant. mCherry and mCherry-TCERG1 641-1098 represent whole cells, and TCERG1 32-1098, 32-668 and 281-1098 are detected in the nuclei of COS7 cells. Scale bars have been equalized to 10μm to account for different magnifications.

resulting polypeptide. The integrity of the open reading frame for each mutant was verified by DNA sequencing and each mutant was expressed in COS7 cells to assess protein expression.

Cytosolic and nuclear extracts were prepared, resolved by Western Blot analysis and detected using an antibody to DsRed (Figure 9). mCherry is approximately 30 kDa and was

primarily expressed in the cytosol whereas mCherry-TCERG1 32-1098 (actual size: 180 kDa, predicted size: 148 kDa) was predominantly expressed in the nucleus. The expression of the transcription factor TBP was used as a control for nuclear protein verification. The expression of the remaining mCherry-TCERG1 mutants: 32-668, 120 kDa ; 641-1098, 90 kDa; and 281-1098, 140 kDa (predicted sizes: 96 kDa, 84.5 kDa, and 122 kDa, respectively), with the exception of 32-293 which was not detected, were also present in the cytosolic fraction. However, apart from mCherry-TCERG1 641-1098, no fluorescence could be observed in the cytoplasm of cells when visualized by LSCM, including 32-293 (Figure 9, bottom panel). Finally, although modifications have been made to improve levels of mCherry-fusion protein expression (Shaner *et al.*, 2004), it may have played a factor in the expression and stability of mCherry-TCERG1 32-1098, resulting in lower levels of expression relative to the other mutants. The size and length of the fusion protein may also play a factor in its reduced expression. Therefore, the quantity of plasmid DNA transfected for each TCERG1 truncation mutant for co-localization studies was adjusted such that each mutant was similarly expressed relative to TCERG1 32-1098.

It has been established that C/EBPα and TCERG1 localize to different compartments within the nucleus, although both populate non-nucleolar regions (Tang and Lane, 1999; Zhang *et al.*, 2001; Sanchez-Alvarez *et al.*, 2006). Endogenous and ectopic expression of C/EBPα generally localize to distinct punctate foci occupied by heterochromatin, as demonstrated in a variety of murine-derived cell lines (Tang and Lane, 1999; Schaufele *et al.*, 2001; Zhang *et al.*, 2001). Conversely, TCERG1 localizes to a functionally and structurally different subnuclear compartment termed splicing factor-rich nuclear speckles (Sanchez-Alvarez *et al.*, 2006), characterized by the functional constituents localized within these discrete domains (Fu and Maniatis, 1990). Additionally, a small population of TCERG1 has been observed throughout non-nucleolar regions of the nucleoplasm (Sanchez-Alvarez *et al.*, 2006). Therefore, it was essential to show that both C/EBPα and TCERG1 localize to their respective nuclear bodies in COS7 cells, as this is the first time these studies were performed in this cell type. COS7 cells were first co-transfected with vectors encoding fluorescent proteins, fixed, and prepared for visualization using LSCM. In control experiments expressing either EGFP or mCherry, each protein localized diffusely throughout the cell with no apparent pattern (Figure 10A). In contrast, C/EBPα-EGFP and mCherry-TCERG1 32-1098 appeared to localize to different but

distinguishable patterns within the nucleus. C/EBPα-EGFP generally localized to large globular foci that number between 20-50 per cell and was absent from the nucleoli. Additionally, it should be noted that in COS7 cells, C/EBPα-EGFP adopted about five distinct subnuclear patterns, which could be attributed to various stages of the cell cycle since the cells were not synchronized (see section 4.2.1.1). The pattern shown in Figure 10A appeared in approximately 75% of the cells observed in this study, and therefore was chosen as the primary localization of C/EBPα-EGFP in COS7 cells. In contrast, mCherry-TCERG1 32-1098 principally localized to less distinct, relatively large subnuclear foci that were granular in nature, reminiscent of nuclear speckles (Figure 10A). Additionally, there was a less concentrated, more dispersed population throughout the nucleoplasm but was excluded from the nucleolus, consistent with observations in other cell lines (Sanchez-Alvarez *et al.*, 2006). Since there has only been one publication examining the subnuclear localization of TCERG1, it was necessary to determine if the pattern observed in COS7 cells corresponded to nuclear speckles. SC35 is well established as a nuclear speckle marker (Fu and Maniatis, 1990; Hall *et al.*, 2006), therefore if TCERG1 indeed localized to these nuclear subcompartments, considerable overlap between the two should be observed. Thus, cells were transiently transfected with pmCherry-TCERG1 32-1098 and prepared for indirect immunofluorescence to detect the endogenous SC35 prior to imaging. The subnuclear speckle pattern was observed for both SC35 (green) and TCERG1 32-1098 (red) and was confirmed by the complete overlap between the two, demonstrated by areas of yellow colour (Figure 10B, merge). These results suggest that TCERG1 localizes to splicing factor-rich nuclear speckles in COS7 cells.

### 4.2.1 TCERG1 Co-Localizes with C/EBPα

If the inhibition of C/EBPα by TCERG1 occurs through a general mechanism involving nuclear retention or by inducing a redistribution of C/EBPα from sites of active transcription, LSCM can be used as a tool to examine the subnuclear distribution patterns when both TCERG1 and C/EBPα are co-expressed. Therefore, to investigate if the localization pattern of either protein could be influenced by the co-expression of the other, cells were transiently co-transfected with expression vectors for C/EBPα-EGFP and mCherry-TCERG1 32-1098 and prepared for imaging using LSCM. Remarkably, the subnuclear pattern of mCherry-TCERG1

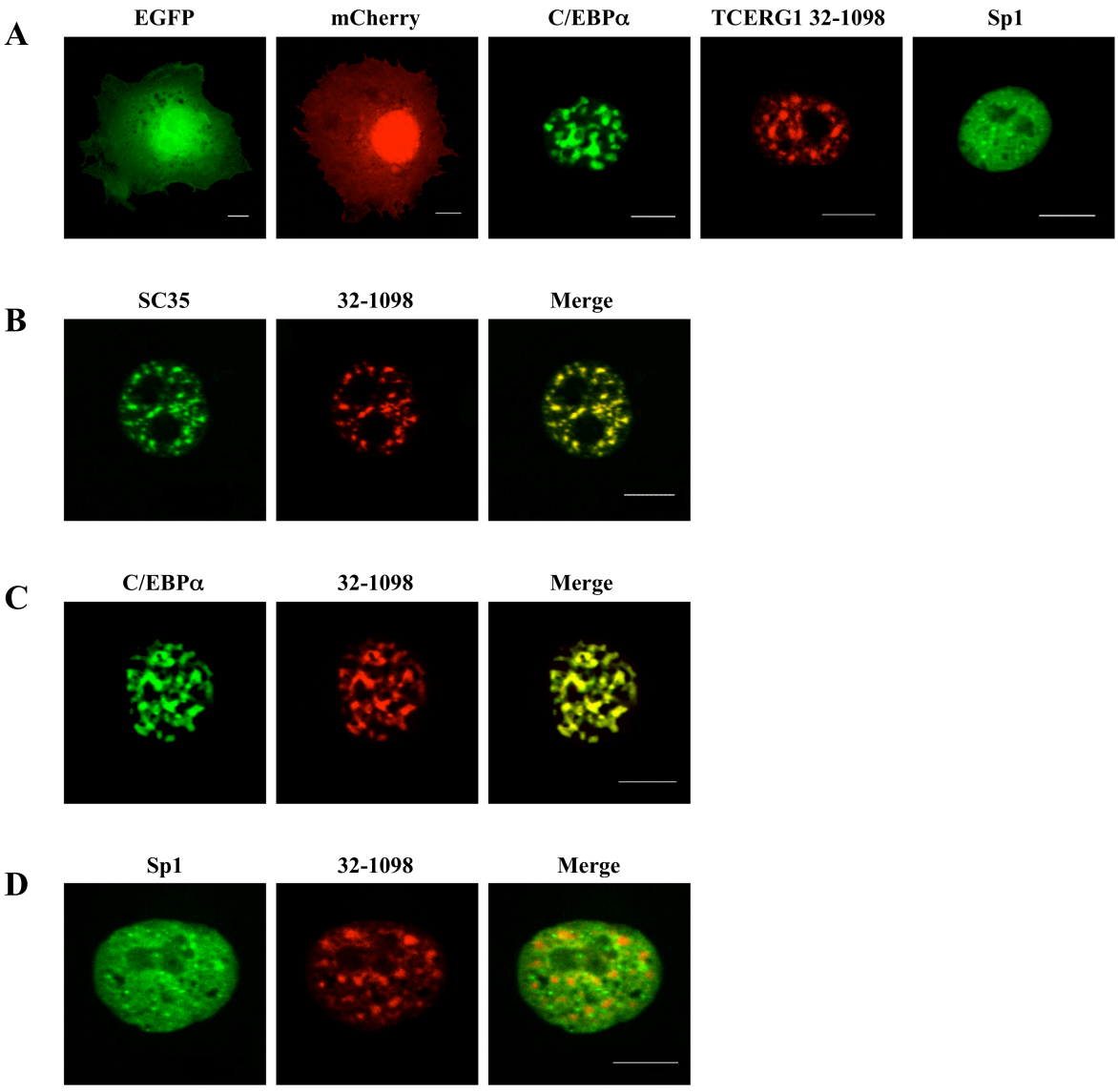


**Figure 10. TCERG1 Co-Localizes with C/EBPα in COS7 Cells.**
COS7 cells were transiently transfected with vectors expressing either enhanced green fluorescent (EGFP, green) or mCherry (red) proteins and visualized using an Olympus FluoView 300 laser scanning confocal microscope under 60x oil immersion. Images were collected by Z-sectioning using software provided with the microscope. A 10 μm scale is indicated where appropriate and have been adjusted accordingly based on magnification. With the exception of EGFP and mCherry, all images represent COS7 cell nuclei (A) Localization of EGFP, mCherry, C/EBPα-EGFP, mCherry-TCERG1 32-1098, and EGFP-Sp1 controls. (B) Detection of SC35, a nuclear speckle marker, using conjugate Alexa Fluor® 488 antibody (green) and its overlap with mCherry-TCERG1 32-1098 (merge, shown in yellow). (C) Co-expression of C/EBPα-EGFP and mCherry-TCERG1 32-1098. (D) Expression of mCherry-TCERG1 32-1098 and EGFP-Sp1, a non-specific transcription factor for TCERG1 activity (McFie *et al.*, 2006).

32-1098 was drastically altered and localized to distinct globular punctate foci. Moreover, the localization of TCERG1 completely overlapped with C/EBPα-EGFP indicated by regions of yellow, strongly suggesting that these two proteins co-localized (Figure 10C, merge). To confirm the specificity of the co-localization between C/EBPα-EGFP and mCherry-TCERG1, COS7 cells were transiently transfected with plasmids to express EGFP fused to Sp1, a transcription factor previously shown not to interact with TCERG1 (McFie *et al.*, 2006), in the absence and presence of mCherry-TCERG1 32-1098. Ectopic expression of EGFP-Sp1 predominantly localized in a heterogeneous non-nucleolar pattern with a subpopulation concentrated in 5-20 small punctate foci (Figure 10A), similar to results obtained by Moorefield *et al.* (2006) in COS1 cells. Next, cells co-expressing EGFP-Sp1 and mCherry-TCERG1 32-1098 were assessed for alterations in spatial distribution of either protein. Images obtained from LSCM indicated that EGFP-Sp1 had no effect on the subnuclear localization of mCherry-TCERG1 or vise versa, and that TCERG1 remained localized to splicing factor-rich nuclear speckles (Figure 10D). These results provided strong evidence to show that the redistribution of mCherry-TCERG1 32-1098 to distinct punctate foci occupied by C/EBPα-EGFP occurs specifically upon the co-expression of C/EBPα-EGFP. Finally, different amounts of EGFP-C/EBPα and mCherry-TCERG1 32-1098 were co-transfected individually and together, and although there was some cell-to-cell variation, the level of protein expression did not affect subnuclear distribution of either protein (data not shown). This demonstrates that the concentration of TCERG1 and C/EBPα with respect to protein mobility and accessibility was not a factor in the co-localization studies.

#### 4.2.1.1 TCERG1 Localizes to Areas Occupied by C/EBPα Regardless of Initial C/EBPα Pattern

During the investigation of the subnuclear localization of C/EBPα by LSCM, it was discovered that C/EBPα adopted five distinct patterns within the nucleus of COS7 cells (Figure 11). Of the five, C/EBPα primarily localized in well-defined globular punctate foci in roughly 75% of the cells, and was dispersed throughout the nucleoplasm in about 10-15% of cells visualized (Figure 11A and B). In the remaining 10-15% of cells expressing C/EBPα, three distinct patterns were observed: stippled, dispersed with areas of non-uniform accumulation,

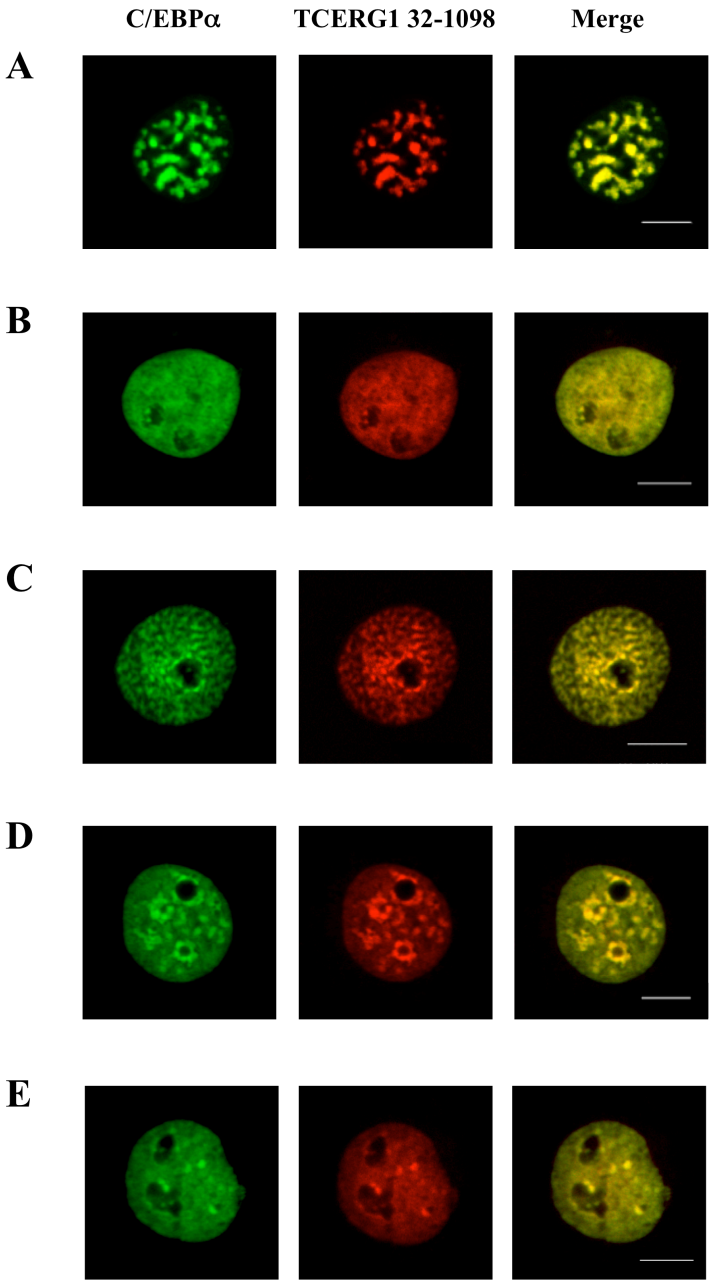


**Figure 11. TCERG1 Co-Localizes with C/EBPα Regardless of Subnuclear Distribution.** COS7 cells were transfected with expression vectors encoding C/EBPα-EGFP and mCherry-TCERG1 32-1098. Cells were fixed and prepared for visualization using a laser scanning confocal microscope as described in materials and methods. C/EBPα (green) displays several different subnuclear localization patterns in unsynchronized COS7 cells (A-E). TCERG1 32-1098 (red) is shown in the middle panel and the following merged image of C/EBPα and TCERG1 where overlap is indicated by the presence of a yellow colour, shown on the right. Scale bars have been normalized to 10 μm to account for different magnifications. could provide evidence for the domain(s) essential for co-localization with C/EBPα.

and diffuse with 5-10 small punctate foci (Figure 11C-E, respectively). Due to the different localization patterns adopted by C/EBPα-EGFP, it was questioned if the marked redistribution of TCERG1 was a result of a particular subnuclear occupancy of C/EBPα-EGFP. Remarkably, regardless of the subnuclear residence of C/EBPα-EGFP, mCherry-TCERG1 32-1098 appeared to co-localize with C/EBPα as evidenced by the abundant yellow colour displayed in the merged image (Figure 11A-E). This demonstrated that the co-localization between these two proteins is consistent and independent of the initial pattern of subnuclear localization adopted by C/EBPα-EGFP in COS7 cells.

### 4.3 The Amino-Terminal Domain of TCERG1 is Required for Co-Localization with C/EBPα in COS7 Cells

Based on results showing that an intact amino-terminus of TCERG1 was required and sufficient to reverse C/EBPα-mediated growth arrest (see Figure 6 and section 4.1.2) and transcriptional activity (McFie *et al.*, 2006), it was questioned if this domain was important for co-localization with C/EBPα. If the subnuclear localization of C/EBPα is a consequence of its activity and the ability of TCERG1 to inhibit the activities requires co-localization with C/EBPα, then mutants with the ability to inhibit C/EBPα activity should also occupy the same nuclear domains as C/EBPα. Thus, loss of TCERG1 translocation in the presence of C/EBPα could provide evidence for the domain(s) essential for co-localization with C/EBPα.

First, LSCM co-localization studies were performed to assess the nuclear distribution of each mCherry-TCERG1 truncation mutant in the absence of C/EBPα-EGFP. With the exception of mCherry-TCERG1 32-668 and 641-1098, the other mutants, mCherry-TCERG1 32-1098, 32-668 and 281-1098, localized in a pattern reminiscent of splicing factor-rich nuclear speckles (Figure 12A). Although absent from the nucleolus, the spatial distribution of mCherry-TCERG1 32-668 was notably different than that of mCherry-TCERG1 32-1098. It appeared to be reticular with no particular pattern or roughly defined foci and was randomly distributed throughout the nucleus. Also, mCherry-TCERG1 641-1098 did not completely localize to the nucleus, as confirmed by both direct visualization (Figure 12A) and Western blot analysis (Figure 9), suggesting that the SV40 NLS was not sufficient for the complete and

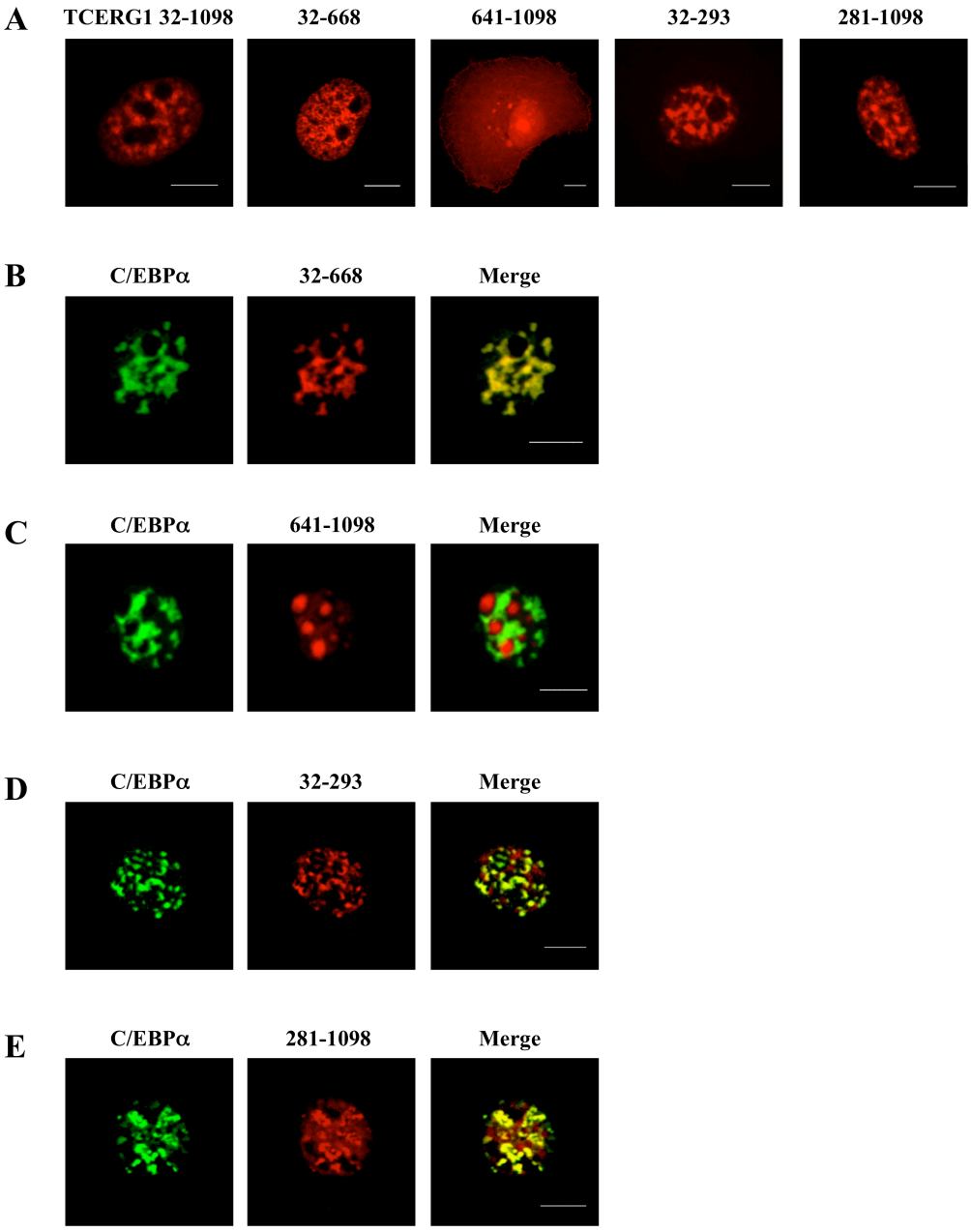


**Figure 12. Subnuclear Localization of TCERG1 Truncation Mutants When Co-Expressed with C/EBPα in COS7 Cells.**
Studies were performed in COS7 cells transiently transfected with vectors encoding fluorescent proteins, mCherry and EGFP, fused to TCERG1 (red) or C/EBPα (green), respectively. (A) Subnuclear distribution of each TCERG1 truncation mutant as indicated. (B-E) Co-expression of each TCERG1 truncation mutant with C/EBPα to examine their nuclear distrbution patterns. Each panel represents images of C/EBPα, TCERG1 mutants, and finally the merged image of the first two. Images were captured by Z-sectioning using an Olympus FV300 confocal microscope under 60x oil objective. A scale bar representing 10 μm based on the magnification is indicated in the bottom right of each image or panel where appropriate.

proper trafficking to the nucleus. Additionally, this mutant was localized within several distinguishable populations throughout the cell. mCherry-TCERG1 641-1098 mainly localized within the nucleus, both in an evenly dispersed pattern throughout the nucleoplasm and also in highly concentrated areas within the nucleolus or nucleoli of cells. Several bright globular foci in the cytoplasm were also present near to and adjacent to the nuclear periphery when this mutant was expressed. Regardless of the differences in subnuclear localization, and since mCherry-TCERG1 32-1098 was able to co-localize with C/EBPα independent of the initial nuclear residency of C/EBPα (see Figure 11), all mutants were examined for any pattern alterations in the presence of C/EBPα-EGFP. Much like with mCherry-TCERG1 32-1098, mCherry-TCERG1 32-668 completely co-localized with C/EBPα-EGFP despite its different localization pattern in the absence of C/EBPα-EGFP (Figure 12B). These results are intriguing because it demonstrates that localization of mCherry-TCERG1 32-668 to nuclear speckles was not required for nuclear translocation in the presence of C/EBPα-EGFP. Moreover, this led to the proposal that TCERG1 mutants with the ability to interact with or function to inhibit the activities of C/EBPα should also co-localize with C/EBPα, regardless of their initial subnuclear residence. This idea was confirmed by the results obtained for mCherry-TCERG1 641-1098, a mutant lacking the ability to reverse growth arrest (see Figure 6B), having no overlap with the distribution pattern of C/EBPα-EGFP (Figure 12C). Surprisingly, mCherry-TCERG1 mutants 32-293 and 281-1098, shown to have reduced ability to reverse growth arrest, also failed to completely co-localize with C/EBPα-EGFP (Figure 12D and E). However, partial co-localization indicated by areas of yellow in the merged image between these two mutants and C/EBPα-EGFP could be a result of limited interaction or decreased affinity to bind to C/EBPα, resulting from the deletion of critical regions required for co-localization and/or inhibitory activity toward C/EBPα. Furthermore, the lack of significant co-localization between C/EBPα-EGFP and either TCERG 32-293 or 281-1098 may result in the inability of the TCERG1 mutants to inhibit C/EBPα-EGFP-mediated growth arrest. This suggests that co-localization between TCERG1 and C/EBPα at sites occupied by C/EBPα is necessary to inhibit growth arrest. Collectively, an intact amino-terminal domain of TCERG1 is sufficient to inhibit C/EBPα activity and contains domains important for proper co-localization with C/EBPα.

## 4.4 Subnuclear Localization of TCERG1 and C/EBPα is Not Cell-Type Specific

In order to examine whether C/EBPα-mediated sequestration of TCERG1 to foci occupied by C/EBPα was cell-specific, co-localization experiments were repeated in HEK293T cells. Previous evidence supports that the amino terminal domain of TCERG1 contains functional motif(s) required for TCERG1-mediated inhibition of C/EBPα growth arrest and transcriptional activity in HEK293T cells (McFie *et al.*, 2006). To further support the hypothesis that the mechanism for TCERG1 inhibitory activity toward C/EBPα involved subnuclear sequestration, it was necessary to corroborate studies performed in COS7 cells by repeating these studies in a different cell line.

### 4.4.1 TCERG1 Redistributes to Subnuclear Structures Occupied by C/EBPα in HEK293T Cells

Co-localization studies in HEK293T cells were performed identically to those in COS7 cells. Upon the ectopic expression of EGFP or mCherry, each fluorescent protein was dispersed throughout the cell, similar to observations made in COS7 cells (compare Figure 10A and Figure 13A). The expression of C/EBPα-EGFP was noticeably different in HEK293T cells than in COS7 cells and was the only consistent pattern observed throughout the studies. More specifically, there were two non-nucleolar subpopulations of C/EBPα-EGFP observed within the nucleus: evenly dispersed throughout and highly concentrated within 5-10 distinct small foci (Figure 13A). Conversely, the subnuclear distribution of mCherry-TCERG1 32-1098 was similar to that observed in COS7 cells and strongly overlapped with SC35, indicative of splicing factor-rich nuclear speckle occupancy (Figure 13A and B).

Next, HEK293T cells were co-transfected with expression vectors for C/EBPα-EGFP and mCherry-TCERG1 32-1098 in order to examine the co-localization between the two proteins (Figure 13C). Ectopic expression of mCherry-TCERG1 32-1098 in the presence of C/EBPα-EGFP caused a complete co-localization of mCherry-TCERG1 32-1098 with C/EBPα-EGFP (Figure 13C). Next, mCherry-TCERG1 32-1098 was co-expressed with EGFP-Sp1 to show the alteration in TCERG1 localization was specifically influenced by C/EBPα-EGFP and not with other transcription factors. When expressed alone or in the presence of mCherry-TCERG1 32-1098, EGFP-Sp1 showed a dispersed pattern that was absent from the nucleoli

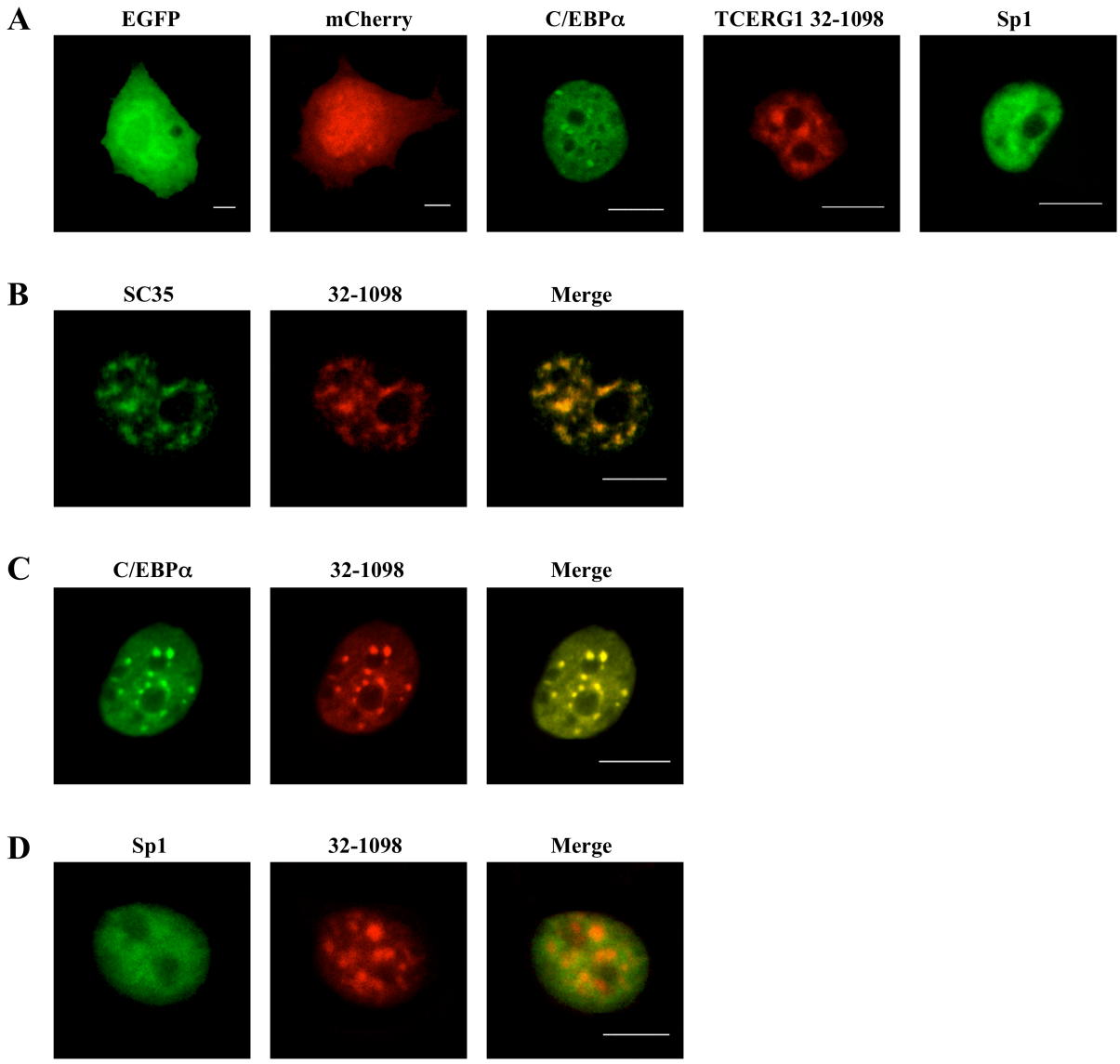


**Figure 13. C/EBPα Alters the Subnuclear Localization of TCERG1 in HEK293T Cells.** Transfected HEK293T cells expressing C/EBPα-EGFP and mCherry-TCERG1 32-1098 were prepared and assessed for the subnuclear distribution pattern of each protein. A scale bar representing 10 μm is indicated as appropriate based on magnification for each collection of images. (A) Cellular distribution pattern of enhanced green fluorescent protein and mCherry (red). Subnuclear localization of C/EBPα-EGFP, mCherry-TCERG1 32-1098, and EGFP-Sp1 have been included as controls. (B) Cells expressing mCherry-TCERG1 32-1098 were assessed for localization to splicing factor-rich nuclear speckles using endogenous SC35 as a marker protein detected by indirect immunostaining technique (anti-SC35, 1:1000; green). Areas of complete overlap between the proteins are indicated by a yellow colour in the merged image to the right. (C) Nuclear distribution of mCherry-TCERG1 32-1098 upon co-expression of C/EBPα. (D) Localization of mCherry-TCERG1 32-1098 upon the co-expression of transcription factor EGFP-Sp1, a previousy established negative control for TCERG1 activity (McFie *et al.*, 2006).

(Figure 13A and D). As observed in COS7 cells, there was virtually no overlap between the nuclear localization patterns of EGFP-Sp1 and mCherry-TCERG1 32-1098 in HEK293T cells (Figure 13D, merge panel). Thus, EGFP-Sp1 had no effect on the spatial distribution of mCherry-TCERG1 32-1098. Moreover, mCherry-TCERG1 32-1098 was unable to influence the subnuclear distribution of EGFP-Sp1, as expected since TCERG1 neither interacts with nor affects Sp1 transcriptional activity (McFie *et al.*, 2006). Therefore, the striking alteration in the subnuclear localization of TCERG1 by C/EBPα-EGFP was not of a general nature nor an artifact of fluorescent-fusion protein expression. The results obtained from the co-localization studies in HEK293T and COS7 cells reveal that subnuclear sequestration of TCERG1 to sites occupied by C/EBPα is specific but not cell-type dependent.

### 4.5 The Amino-Terminus of TCERG1 Contains Domain(s) Essential for Subnuclear Translocation by C/EBPα

To further validate the effects of C/EBPα on TCERG1 localization observed in COS7 cells, the next step was to assess the nuclear patterns observed upon co-expression of C/EBPα and various TCERG1 truncation mutants in HEK293T cells. If the effect of C/EBPα on TCERG1 subnuclear distribution is not cell-type specific and the mechanism by which this occurs is independent of the initial nuclear location of TCERG1, co-expression with C/EBPα, should yield similar results as that observed in COS7 cells. First, each mCherry-TCERG1 truncation mutant was expressed individually in order to evaluate its subnuclear distribution in HEK293T cells (Figure 14). With the exception of mCherry-TCERG1 641-1098 and 32-668 roughly defined large foci reminiscent of nuclear speckles were detected throughout the nucleoplasm but absent from the nucleolus. mCherry-TCERG1 641-1098 also had a significant cytoplasmic pool, suggesting that it did not properly partition to the nucleus and was partially retained in the cytosol. mCherry-TCERG1 32-668 had a more heterogeneous and irregularly dispersed appearance with no specific localization pattern apart from its exclusion from the nucleoli. The localization pattern for each mutant resembled those found in COS7 cells and therefore appeared consistent between cell types. Next, HEK293T cells were visually characterized following the co-expression of C/EBPα-EGFP and each mCherry-TCERG1

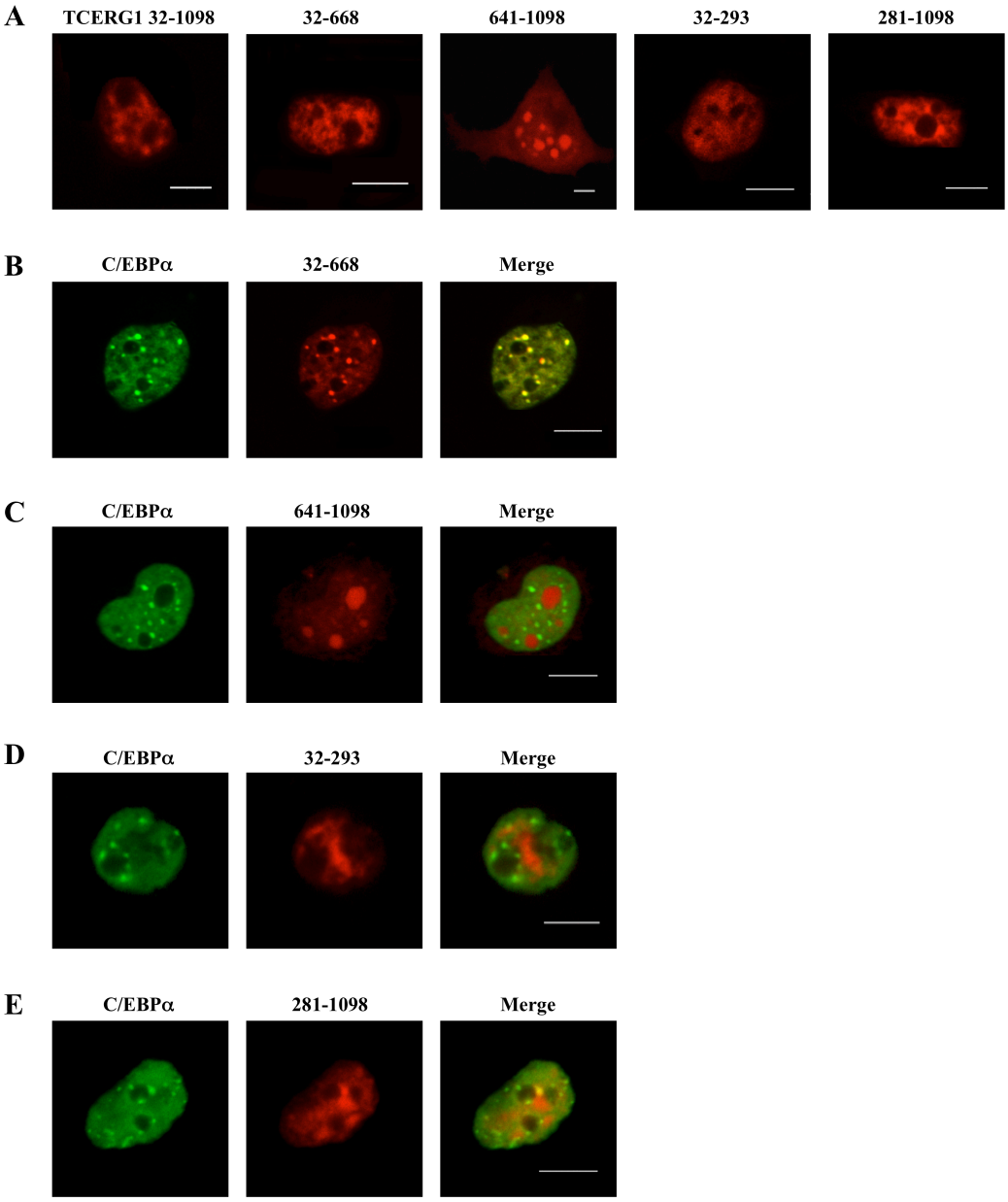


**Figure 14. Co-localization of TCERG1 Mutants and C/EBPα in HEK293T cells.**
HEK293T cells were co-transfected with mammalian expression vectors encoding fluorescent fusion proteins to C/EBPα (EGFP, green) and TCERG1 truncation mutants (mCherry, red). Cells were prepared and observed using LSCM. Images of HEK293T cell nuclei were collected as described in materials and methods. A scale bar of 10 μm is indicated as appropriate equalized to the magnification used. (A) Nuclear distribution of each mCherry-TCERG1 mutant in the absence of C/EBPα-EGFP. A significant cytosolic population is observed for mCherry-TCERG1 641-1098 in addition to nuclear localization. (B-E) Nuclear localization pattern upon co-expression of C/EBPα-EGFP and mCherry-TCERG1 truncation mutants. Areas that appear yellow in the merged image indicate an overlap between the localization patterns of each protein.

mutant. mCherry-TCERG1 32-668 was the only mutant that completely localized to areas occupied by C/EBPα-EGFP (Figure 14B) and virtually no overlap occurred between mCherry-TCERG1 641-1098 and C/EBPα-EGFP (Figure 14C), remarkably similar to results obtained in COS7 cells. More specifically, 641-1098 localized to areas within the nucleus completely devoid of C/EBPα-EGFP. Moreover, mCherry-TCERG1 truncation mutants 32-293 and 281-1098, which lack potentially important functional motifs, only partially overlapped with areas occupied by C/EBPα-EGFP (Figure 14D and E). Therefore, results from co-localization studies performed in HEK293T and COS7 cells reveal the incredible ability of TCERG1 to strongly associate with C/EBPα-occupied subnuclear domains and that specific regions within TCERG1 are important for this association.

### 4.6 TCERG1 Does Not Co-Localize with C/EBPβ

Although our laboratory has worked most extensively to investigate the effect of TCERG1 on C/EBPα activity, recent results showed that TCERG1 also has an inhibitory effect on the transcriptional activity of another C/EBP isoform, C/EBPβ (McFie *et al.*, 2006). Using reporter gene assays, McFie *et al.* (2006) demonstrated that TCERG1 inhibited the transactivation potential of C/EBPβ. Furthermore, TCERG1 inhibited the transactivation of C/EBPβ in the presence of transcriptional co-activation, p300. This suggests that TCERG1 has a dominant effect over p300-mediated activation of C/EBPβ transcriptional activity (McFie *et al.*, 2006). Based on these data, it is possible that TCERG1 associates with C/EBPβ and subnuclear localization may be involved in the inhibition of C/EBPβ activity by TCERG1. Because of the marked relocalization of TCERG1 to co-localize with C/EBPα, we sought to see if the same effect could be observed in the presence of C/EBPβ. First, EGFP-C/EBPβ was expressed alone in both COS7 and HEK293T cells to characterize its nuclear pattern in each cell line (Figure 15). The observations made of EGFP-C/EBPβ were indistinguishable from those made with C/EBPα-EGFP in that both proteins localized in a similar globular punctate pattern (compare Figure 10A with Figure 15A), while it was distributed in a more dispersed pattern with a small number of areas enriched with protein in HEK293T cells (compare Figure 13A with Figure 15C). Control experiments showing the expression of mCherry- TCERG1 32-

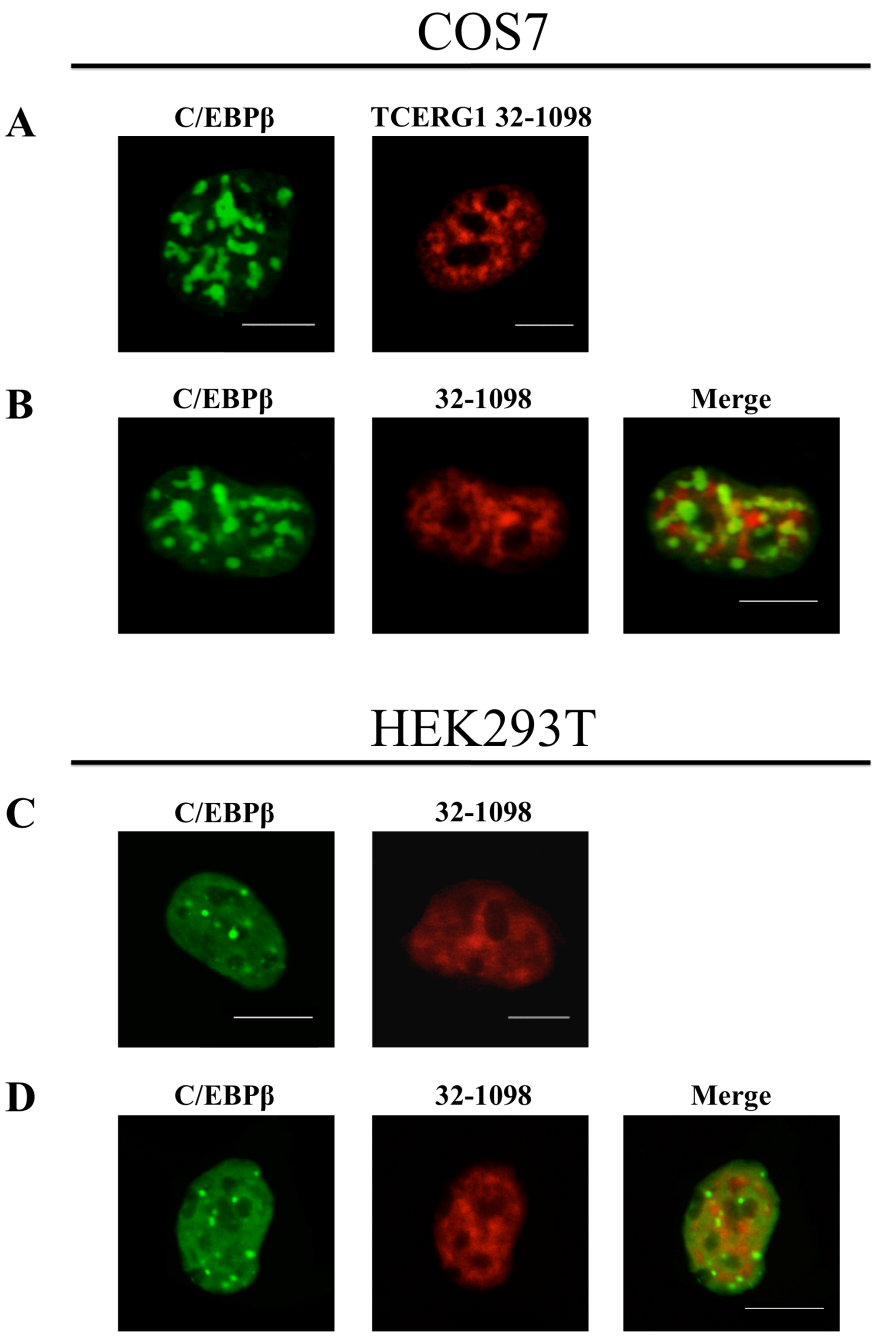


**Figure 15. C/EBPβ and TCERG1 Do Not Co-Localize in COS7 or HEK293T cells.**
COS7 and HEK293T cells were transfected with EGFP-C/EBPβ (green) and mCherry-TCERG1 32-1098 (red) and slides were prepared as described. A scale bar is shown where appropriate. (A-B) Nuclei of COS7 cells showing the nuclear localization pattern of EGFP-C/EBPβ and mCherry-TCERG1 32-1098 expressed individually (A) and together (B) in COS7 cells. A merged image is shown to display the overlap between EGFP-C/EBPβ and mCherry-TCERG1 32-1098. (C-D) Subnuclear distribution of EGFP-C/EBPβ and mCherry-TCERG1 32-1098 in the nuclei of HEK293T cells expressed separately (C) and together (D).

1098 displayed the typical nuclear speckle pattern characterized by the presence of large roughly shaped foci and a smaller dispersed population throughout the nucleoplasm. Additionally, all proteins expressed in these studies were absent from both nucleolar and cytoplasmic regions.

Next, COS7 and HEK293T cells were co-transfected with expression vectors encoding EGFP-C/EBPβ and mCherry-TCERG1 32-1098 and prepared for visual examination by LSCM (Figure 15B and D). Image analysis revealed that mCherry-TCERG1 32-1098 was retained in nuclear speckles and partially co-localized with regions occupied by EGFP-C/EBPβ in both cells lines, indicated by the lack of yellow colour generated in the merged image. These results indicate that EGFP-C/EBPβ had no influence on the nuclear distribution of mCherry-TCERG1 32-1098, in striking contrast to C/EBPα.

# 5. DISCUSSION

It was recently discovered that TCERG1 interacts with and co-represses C/EBPα activity (McFie *et al.*, 2006). Using various biochemical techniques, TCERG1 was shown to repress both the transcriptional and growth arrest activities of C/EBPα. Interestingly, it was found that C/EBPα mediated these activities through different domains, suggesting that TCERG1 may act in a global manner to repress all forms of C/EBPα activity. Results of the studies presented here further characterize TCERG1-mediated inhibition of C/EBPα growth arrest activity by investigating the domain(s) within TCERG1 that mediate this effect and provide evidence for a general mechanism by which this activity occurs. In addition, results from LSCM provide compelling evidence that links subnuclear localization with the inhibition of C/EBPα growth arrest activity by TCERG1. Collectively, these findings suggest this activity is mediated through the amino-terminal region of TCERG1 and possibly requires the additive effects of certain WW domains found within this domain. This work supports a model has been proposed for the TCERG1-dependent inhibition of C/EBPα-mediated activity involving a subnuclear retention mechanism.

## 5.1 The Amino-Terminal Domain of TCERG1 is Sufficient for Inhibiting C/EBPα–Mediated Growth Arrest of Cells

TCERG1 is a transcription elongation factor, shown to interact with both RNA polymerase II and splicing factor 1, suggesting that transcription and splicing events may be coupled (Carty *et al.*, 2000; Goldstrohm *et al.*, 2001). Recently, TCERG1 was identified as an interactor with C/EBPα using a yeast two-hybrid screening approach (McFie *et al.*, 2006). Using gene reporter assays, TCERG1 was also found to inhibit C/EBPα transcriptional activity. In addition, TCERG1 was found to inhibit the growth arrest function of C/EBPα in cultured cells. Finally, results using a TCERG1 truncation mutant containing only the carboxy-terminus indicate that this domain does not contain the region(s) important for mediating this activity. Collectively, these data suggested that motifs found in the amino-terminal domain of TCERG1 may be required for interaction and inhibition of C/EBPα-mediated transcriptional and growth

arrest activities (McFie *et al.*, 2006). However, it was unknown if the interaction and functional domains of TCERG1 lie in the same or different domain(s).

In order to map the region(s) within TCERG1 responsible for inhibiting C/EBPα growth arrest activity, several TCERG1 truncation mutants were constructed to examine the functional ability of different domains to reverse cells under growth arrest (Figure 6 and 7). Based on the data collected, the intact amino-terminal domain of TCERG1 was sufficient to reverse growth-arrested COS7 cells. This conclusion was supported by results obtained with several TCERG1 truncation mutants. First, a mutant lacking the entire amino-terminal region, TCERG1 641-1098, was unable to reverse C/EBPα-mediated growth arrest of COS7 cells, similar to results reported previously (McFie *et al.*, 2006). Second, TCERG1 32-668, consisting of the intact amino-terminal domain, was sufficient to inhibit the growth arrest activity of C/EBPα. Lastly, results from analysis of two additional TCERG1 mutants containing truncations in the amino-terminal domain, TCERG1 32-293 and 281-1098, had significantly reduced ability to reverse growth arrest of cells. Therefore, one could speculate that TCERG1 32-293 and 281-1098 are missing important functional motifs that are required to work together in order to achieve inhibition of C/EBPα activity. Analysis of the amino acid sequence for both truncation mutants revealed that each was missing different WW domains. Interestingly, results obtained from a study examining the involvement of different domains in TCERG1-mediated transcriptional repression show that WW1 and WW2 domains together are sufficient for repression of the α4-integrin promoter (Goldstrohm *et al.*, 2001). This conclusion was supported by data from reporter assays using TCERG1 mutants with different combinations of deletions or mutations of critical residues found within each WW domain. Additionally, data from Far-Western analyses identified pre-mRNA splicing factor SF1, which also functions as a transcriptional repressor (Zhang and Childs, 1998), as a TCERG1 WW-interacting protein. Moreover, WW1 and WW2 of TCERG1, but not WW3, was shown to specifically interact with SF1 by co-immunoprecipitation studies using TCERG1 mutants consisting of individual WW domains. These data were also confirmed by Far-Western analyses using probes constructed from WW 1-3 of TCERG1, of which the first two were found to interact with SF1 (Goldstrohm *et al.*, 2001). It has been well established that many WW-containing proteins require multiple weak interactions for full biological function and that these domains have different binding affinities (Macias *et al.*, 2002). Generally, these interactions function in a co-operative manner to

stabilize binding with its ligand, particularly when the protein or its ligand is large. Therefore, based on the evidence collected here, it is reasonable to speculate that TCERG1-mediated inhibition of growth-arrested cells by C/EBPα may occur as a result of interactions with C/EBPα via multiple WW domains in the amino-terminal region of TCERG1. Finally, WW-domains are characterized by the binding of short proline-rich motifs that have been categorized into four classes based on the minimal consensus sequences they bind: PPxY, PPLP, PPR, and phospho-S/TP, respectively, where "x" denotes any amino acid (Sudol *et al.*, 1995; Espanel and Sudol, 1999; Bedford *et al.*, 2000; Sudol and Hunter, 2000). In fact, C/EBPα has one characterized poly-proline motif (Nerlov and Ziff, 1994), and upon further inspection of the entire amino acid sequence of C/EBPα (Landschulz *et al.*, 1988a; Lincoln *et al.*, 1994; *Rattus norvegicus*: Accession number P05554), I have identified six other proline-enriched sequences that could serve as WW-ligand binding motifs (Figure 16). Thus, there are several regions within C/EBPα that could serve as TCERG1 WW-interacting sequences that could mediate interaction and subsequent inhibition of C/EBPα activity.

## 5.2 Characterization of TCERG1 and C/EBPα Spatial Distribution in COS7 and HEK293T Cells

Subnuclear compartments, or nuclear bodies, have been identified and classified based on function, pattern of distribution and their respective molecular components (Matera, 1999; Misteli, 2001; Wang *et al.*, 2002b). These subnuclear compartments can act as storage sites for specific factors and/or assembly stations for transcriptional, splicing, and recycling purposes. Several lines of evidence have demonstrated that splicing factors have the exceptional ability to translocate from specific areas and become concentrated at sites of active transcription (Fu and Maniatis, 1990; Bregman *et al.*, 1995; Misteli *et al.*, 1997; Melcak *et al.*, 2000; Mintz and Spector, 2000; Phair and Misteli, 2000; Carmo-Fonseca, 2002; Gorski *et al.*, 2006). Based on these data, it was hypothesized that a connection between subnuclear localization of TCERG1 and C/EBPα and the inhibition of C/EBPα-mediated activity.

To date, there has only been one study examining the subnuclear localization of TCERG1. These studies were conducted in HeLa cells and IMR90 human fibroblasts


**Figure 16. Putative TCERG1 WW-Binding Motifs in C/EBPα.**
WW domains bind to proline rich motifs classified by different ligand consensus sequences. Four classes of consensus sequences have been identified: PPxY, PPLP, [P]-PR and [phospho-S/T]P, respectively, where X denotes any amino acid (Espanel and Sudol, 1999; Bedford *et al.*, 2000; Sudol and Hunter, 2000). Seven putative proline-rich motifs (highlighted in grey) can be identified throughout the amino acid sequence of C/EBPα that may serve as TCERG1 WW-binding ligands. C/EBPα, *Rattus norvegicus*: Accession number P05554.

(Sanchez-Alvarez *et al.*, 2006). Therefore, in this study, it was necessary to characterize the spatial distribution of TCERG1 in both COS7 and HEK293T cells prior to further analysis. In studies performed by Sanchez-Alvarez *et al.* (2006), TCERG1 primarily localized within domains termed splicing factor-rich nuclear speckles (Hall *et al.*, 2006), designated by the many splicing factors that reside in these domains. In COS7 and HEK293T cells, TCERG1 32-1098 also localized within nuclear speckles, verified by immunofluorescent detection of SC35, a nuclear speckle marker (Fu and Maniatis, 1990). Collectively, localization studies performed in several cell lines suggest that TCERG1 strongly associates with splicing factor-rich nuclear speckles (Sanchez-Alvarez *et al.*, 2006) and thus, is an intrinsic property of TCERG1. Based on the results from several studies examining components of nuclear speckles (Mintz and Spector, 2000; Misteli, 2001; Carmo-Fonseca, 2002; Iborra and Cook, 2002; Gorski *et al.*, 2006; Hall *et al.*, 2006), these subnuclear structures likely serve as reservoirs from which

splicing factors, and possibly TCERG1, can be assembled into protein complexes or can be recruited to other domains found within the nucleus.

During the co-localization analysis of TCERG1 and C/EBPα, it was apparent that C/EBPα adopted several different subnuclear localization patterns. Upon reviewing the literature, various studies examining the nuclear localization pattern of several nuclear factors involved in chromatin remodeling or cell cycle regulation have revealed different patterns of occupancy determined by the stage of the cell cycle (Rountree *et al.*, 2000; Bozhenok *et al.*, 2002). However, the aforementioned studies used chemicals to synchronize cells, which were then released from certain stages of cellular arrest, allowing researchers to follow changes in protein localization throughout the cell cycle. In the studies presented here, cells were not subjected to chemicals to synchronize cells or cause cellular arrest. The possibility of synchronizing the cells by chemical means was explored but from repeated experiments it was concluded that the cells examined were not very robust and were sensitive to this type of treatment. Moreover, an interesting study examining the localization pattern of CBP in transiently transfected cells and untransfected cells expressing endogenous CBP revealed that different cell lines and conditions compartmentalize CBP into different nuclear patterns (LaMorte *et al.*, 1998). In certain cell lines, such as Chinese hamster ovary cells and COS-1 cells, CBP localizes in a dispersed reticular pattern and is found concentrated within promyelocytic leukemia nuclear bodies, whereas in HEK293, hepatocellular carcinoma-G2 and human epithelial cervical cancer cells, CBP localizes only in a dispersed pattern. Thus, it is possible that cells can adapt to the overexpression of a protein by dysregulating the localization of the protein, such as CBP to promyelocytic leukemia nuclear bodies. However, the primary localization pattern of C/EBPα in the majority of COS7 and HEK293T cells was consistent, suggesting that these sites of occupancy were the most stable.

Upon assessing the subnuclear distribution of C/EBPα in COS7 and HEK293T cells, the pattern of C/EBPα compartmentalization observed between these two cell types appeared noticeably different. Although several patterns of localization were visualized in COS7 cells, only one pattern was consistent in HEK293T cells. Previous studies examining the spatial distribution of C/EBPα have shown that C/EBPα localizes to peri-centromeric regions in mouse GHTF1-5 progenitor pituitary cells and 3T3-L1 adipocytes (Tang and Lane, 1999; Schaufele *et al.*, 2001, 2003; Zhang *et al.*, 2001; Liu *et al.*, 2002, 2007). A recent study provided strong

evidence to support that C/EBPα binds to mouse major α-satellite DNA repeats situated near peri-centromeric heterochromatin (Liu *et al.*, 2007). However, this pattern of localization has not been described in cell lines from other species. This could explain the apparent differences in the localization patterns of C/EBPα in the cell types examined in this study. In addition, various post-translational modifications may have cell-type specific responses that may affect the subnuclear localization of C/EBPα. In studies examining the localization of C/EBPα and C/EBPβ during 3T3-L1 pre-adipocyte differentiation, it has been observed that both isoforms adopt distinct patterns of subnuclear localization in a phosphorylation-dependent manner, a process which occurs within hours of their expression (Tang and Lane, 1999). Moreover, studies have shown that growth hormone affects the nuclear distribution of C/EBPβ, resulting from phosphorylation by mitogen-activated protein kinase. Therefore, it is feasible that in early stages of C/EBPα expression, certain post-translational events might be required for proper localization of C/EBPα within the nuclei of COS7 and HEK293T cells. Likewise, certain functional motifs found within C/EBPα may play a role in its subnuclear localization in a cell type-specific manner. Interestingly, it has been demonstrated that the $(QA)_{38}$ repeat domain of TCERG1 has been implicated in the nuclear restriction of TCERG1 in rat striatal cells (Arango *et al.*, 2006). However, this domain appears to play no such role in either COS7 or HEK293T cells based on the observation that TCERG1 281-1098, which lacks the QA repeat, exhibited proper nuclear restriction and distribution (see Figure 12A and 14A). Therefore, we cannot conclusively confirm that C/EBPα binds to peri-centromeric regions in cell lines of human and African green monkey origin and that localization of C/EBPα and -β to peri-centromeric regions may be restricted to murine-derived cell types. Further studies using heterochromatin markers such as heterochromatin protein 1-α and major centromere auto-antigen B or DNA-binding stains such as 4',6-diamidino-2-phenylindole or Hoechst stain are needed in order to show the heterochromatin-associated occupancy of C/EBPα in these cells types. Finally, it is important to note that the C/EBPα-EGFP fusion protein used in the localization studies reported in this thesis has been shown to retain transcriptional activity in GHFT1-5 cells (Schaufele *et al.*, 2001) as well as growth arrest activity reported here. This strongly suggests that the fusion protein compartmentalized appropriately, since an altered compartmentalization might be expected to alter its activity.

## 5.3 Link Between TCERG1-Mediated Inhibition of C/EBPα Growth Arrest Activity and Subnuclear Localization

Based on the data collected from growth arrest assays and co-localization studies, the intact amino-terminal domain of TCERG1 appears to be involved in both mediating an inhibitory response to growth arrest and is sufficient for co-localization with C/EBPα. This is supported by the evidence gathered from studies with TCERG1 32-668, which was able to reverse growth arrest to levels similar to 32-1098 and to completely co-localize with C/EBPα, despite its different initial pattern of localization.

During the co-localization studies between TCERG1 32-1098 and C/EBPα, it was intriguing to find that TCERG1 32-1098 completely co-localized with C/EBPα. This striking translocation of TCERG1 32-1098 to sites occupied by C/EBPα provided insight into a potential mechanism to explain how TCERG1 inhibits the activities of C/EBPα, leading us to consider the less-characterized alternative methods of inhibition involving subnuclear retention. The complete co-localization observed between TCERG1 and C/EBPα may be a result of direct interaction between these two proteins (McFie *et al.*, 2006) and this interaction alone could be responsible for the inhibition of C/EBPα activity. The formation of inactive TCERG1/C/EBPα complexes could invariably prevent the access of various co-factors involved in recruiting C/EBPα to functional compartments and/or activating C/EBPα activity. Furthermore, results from co-localization studies using TCERG1 truncation mutants suggest that the intact amino-terminal domain of TCERG1 is necessary to co-localize with C/EBPα within inactive nuclear storage compartments. This was further emphasized by the results obtained for TCERG1 641-1098, a mutant unable to interact with or function as an inhibitor of C/EBPα activity (McFie *et al.*, 2006). TCERG1 641-1098, whose amino-terminal domain was deleted, localized to areas within the COS7 and HEK293T cells distinct from C/EBPα (see Figure 12C and 14C). Therefore, the complete lack of inhibitory activity toward C/EBPα could be explained, in part, by the fact that this mutant no longer has the functional domains necessary to interact with or co-localize with C/EBPα. Finally, TCERG1 32-293 and 281-1098, both unable to significantly reverse growth arrest, also failed to co-localize completely with C/EBPα (Figure 6B, 12D, E and 14D, E). However, the partial co-localization of both with C/EBPα, observed by the faint regions of overlap, may be a result of limited interaction between TCERG1 and C/EBPα

contributed by amino-terminal functional motifs retained by each mutant. Research has shown that multiple weak interactions via aromatic amino acids found in the binding pockets of WW1 and WW2, but not WW3 are required for repression of the α4-integrin promoter (Goldstrohm *et al.*, 2001). Although that study focused on the effect of TCERG1 on transcriptional activity, these domains may be involved in mediating interactions with C/EBPα resulting in the inhibition of its anti-proliferative and transcriptional activity. Therefore, TCERG1 mutants missing either of these domains, such as the TCERG1 32-293 and 281-1098 mutants, which lack WW1 and WW2, respectively, may possess limited or unstable interactions with C/EBPα and thus reduced or limited ability to reverse growth arrest of cultured cells. However, the contribution of multiple interactions may not be limited to WW1 and WW2 since the $(QA)_{38}$ repeat was retained in several of the mutants studied. Although no biological role has been assigned to this unique repeat domain, it cannot be ruled out as a potential participant in TCERG1 inhibitory activity. Additionally, interactions with other nuclear factors may be required to assist in the translocation of TCERG1 to sites occupied by C/EBPα. Finally, the alteration in TCERG1 localization is likely a biologically relevant response since these observations could be demonstrated in different cell lines and occurs specifically in response to C/EBPα. Together, these data support the hypothesis that the intact amino-terminal domain of TCERG1 mediates the inhibition of C/EBPα activity using a general retention mechanism and may involve the additive effects of WW1 and WW2 of TCERG1.

### 5.4 TCERG1 Specifically Co-localizes with C/EBPα But Not C/EBPβ

It was recently shown that TCERG1 has the ability to inhibit the transcriptional activity of another C/EBP isoform, C/EBPβ (McFie *et al.*, 2006). Therefore, it was questioned if the mechanism by which this occurs may involve nuclear retention of C/EBPβ as well. Thus, co-localization studies were performed in COS7 and HEK293T cells and observed for alterations in TCERG1 subnuclear distribution upon the ectopic expression of C/EBPβ. Results indicated there was no relationship between inhibition of C/EBPβ and co-localization with TCERG1 in both cell types, as no apparent changes were detected in the compartmentalization of TCERG1. These findings were unexpected for a couple of reasons. First, previous studies suggested that

these two proteins interact and that TCERG1 inhibits the activity of C/EBPβ (McFie *et al.*, 2006). Secondly, C/EBPβ adopts a subnuclear localization pattern analogous to C/EBPα in the cell lines studied, therefore, one might expect TCERG1 and C/EBPβ to co-localize if TCERG1-mediated inhibition involves a retention mechanism. This marked difference between C/EBPα and C/EBPβ in relation to the localization of TCERG1 creates the possibility that TCERG1-mediated inhibition with respect to each isoform occurs by a different mechanism. Although the functions of C/EBP isoforms often overlap, different forms of regulation by TCERG1 may be induced in response to conditions marked by the expression of different C/EBP isoforms. Alternatively, in conditions where both C/EBP isoforms are expressed, C/EBPα-mediated translocation of TCERG1 to sites occupied by C/EBPα may allow TCERG1 to interact with and inhibit C/EBPβ in a C/EBPα-dependent manner. Identification of co-factors that may be directly or indirecty involved in the inhibition of C/EBPβ transcriptional activity by TCERG1 may provide insight into an alternative mechanism by which this down-regulation is achieved.

### 5.5 Model for the Mechanism By Which TCERG1 Inhibits the Activities of C/EBPα

The ordered nature of the nucleus is extremely complex and thus requires many levels of regulation and co-ordination between various nuclear factors in order for specific events to occur. Several possibilities exist for mechanisms that regulate the flux of nuclear body components to and away from intranuclear sites. These activities may be controlled by direct interaction, post-translational modification, altering the rate of mobility, and/or sequestration with or without the assistance of other nuclear factors (Carmo-Fonseca, 2002; Iborra and Cook, 2002; Corry and Underhill, 2005). Nuclear factors may also be negatively regulated by mechanisms involving the retention of proteins within certain subnuclear compartments, thereby altering access of co-factors or by preventing the ability of the protein to translocate to sites where it is functionally active. As such, mounting evidence suggests that TCERG1 inhibits the activities of C/EBPα through a subnuclear retention mechanism.

In recent years, our laboratory has provided compelling evidence for a new biological role for TCERG1 with respect to inhibition of the activities of a well-characterized transcription factor, C/EBPα (this study and McFie *et al*., 2006). What makes TCERG1 unique is that it

inhibits two very different activities of C/EBPα. Moreover, these activities are found within different domains of C/EBPα. Based on the studies presented here, I have proposed a model where TCERG1 inhibits all of the activities of C/EBPα by interacting with and retaining C/EBPα within inactive subnuclear storage sites (Figure 17). C/EBPα is consequently prohibited from redistributing to sites of active transcription or from interacting with other nuclear factors that may be involved in the translocation or activation of C/EBPα activity. Whether this inhibition is achieved by direct interaction between TCERG1 and C/EBPα or by an indirect mechanism involving other regulatory molecules recruited to or found near sites associated with C/EBPα remains unresolved. Therefore, it would be important to identify factors that may assist in the trafficking process responsible for the redistribution of TCERG1 to C/EBPα-containing regions. How is TCERG1 released from nuclear speckles allowing for its localization with C/EBPα? Does it result from random interactions between C/EBPα and free moving TCERG1 molecules not associated with nuclear speckles or is it a coordinated event in response to some type of signalling event? Several recent studies have confirmed the ability of nuclear factors to traverse the nucleus (Fu and Maniatis, 1990; Misteli *et al.*, 1997; Phair and Misteli, 2000; Gorski *et al.*, 2006; Kaiser *et al.*, 2008; Misteli, 2008b) However, it has not been well documented how this mobility is achieved beyond the influence of extracellular stimuli or as a result of post-translational modification. Preserving cells at various time points or using real-time live cell imaging to visualize protein movement should provide insight into the pathway TCERG1 travels to become co-localized with C/EBPα.

While the studies presented here focus on the growth arrest activity of C/EBPα, future studies could be expanded to incorporate the ability of TCERG1 to inhibit transcriptional activities of C/EBPα in the context of subnuclear localization. If TCERG1-mediated inhibition of C/EBPα transcriptional activity by means of a subnuclear retention mechanism can be verified, the discovery of this type of global regulation is of significant importance. To date, evidence for a retention mechanism resulting in the simultaneous inhibition of multiple activities of a transcription factor has yet to be described elsewhere. Thus, these findings present us with a unique opportunity to investigate the relationship between the regulation of transcription factor activity and subnuclear structure and, therefore, will be immensely beneficial to our understanding of all aspects of cellular regulation.

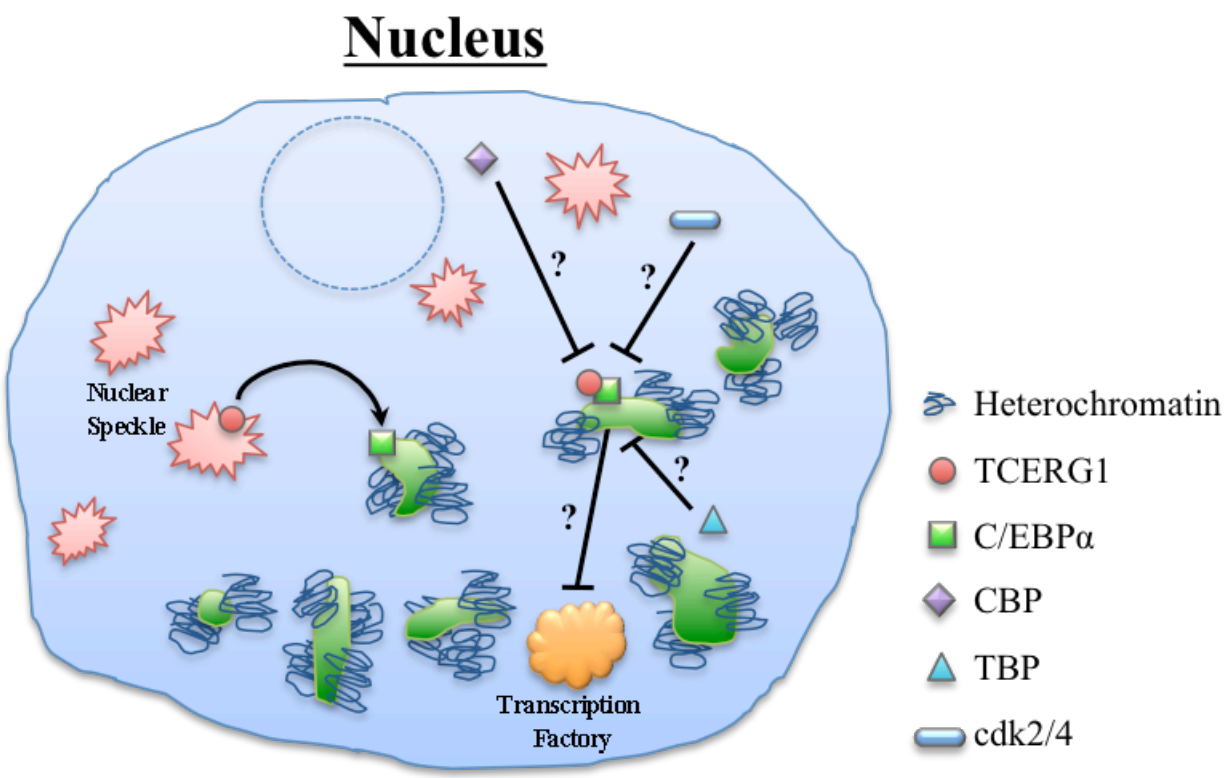


**Figure 17. Proposed Model for the Mechanism By Which TCERG1 Inhibits the Activities of C/EBPα.**

A simplified eukaryotic cell nucleus is shown containing nuclear components involved in TCERG1-mediated inhibition of C/EBPα activity. C/EBPα (green square) is concentrated in globular domains associated near inactive heterochromatin (Liu *et al.*, 2007). Co-factors, CBP (purple diamond) and TBP (aqua triangle), have been shown to associate with C/EBPα at these subnuclear domains by an unknown mechanism (Schaufele *et al.*, 2001). TCERG1 (red circle) is generally found within splicing factor-rich nuclear speckles (Sanchez-Alvarez *et al.*, 2006) but becomes associated with C/EBPα when co-expressed in cultured cells. The redistribution of TCERG1 to nuclear subcompartments occupied by C/EBPα results in the inhibition of C/EBPα-mediated activity. The association between TCERG1 and C/EBPα results in the inhibition of C/EBPα growth arrest activity by blocking interaction between C/EBPα and critical components of cell cycle progression, cdk2 and cdk4 (Wang *et al.*, 2001). The transcriptional activity of C/EBPα is downregulated by TCERG1 by preventing the formation of an active transcriptional complex between C/EBPα and transcriptional co-activators such as CBP and TBP. The resulting complex may be retained within inactive C/EBPα storage sites restricting its access to transcription factories, thereby providing an additional level of negative regulation. The validity of this model remains to be tested and requires further study to verify these hypotheses. Several factors such as cell state, signaling events and association and/or function with other proteins may be required for the redistribution of the nuclear factors discussed.

## 5.6 Future Directions

The collective results obtained from growth arrest and co-localization studies strongly implicate the amino-terminal region of TCERG1 as the key domain responsible for the inhibition of C/EBPα-mediated growth arrest activity. An intact amino-terminal domain of TCERG1 is also required for co-localization with C/EBPα. More specifically, WW1 and WW2 are the likely candidates involved in mediating these effects. If interaction between C/EBPα and TCERG1 via WW domains can be validated, it is probable that the inhibition mediated by TCERG1 results from its interaction with C/EBPα. Further studies are needed to examine the involvement of TCERG1 WW domains in mediating the inhibition of C/EBPα activity. TCERG1 mutants containing different combinations or entire deletions of individual WW domains could be generated and tested for their ability to interact with C/EBPα and restore the proliferative capacity of cell under growth arrest caused by C/EBPα. Additionally, the involvement of WW1 and WW2 could be addressed by mutating key residues within each WW domain required for ligand binding, which could then be assessed using various functional and protein interaction assays. Furthermore, examination is necessary to test each mutant in the context of TCERG1-mediated inhibition of C/EBPα transcriptional activity to determine if WW domains are involved. If indeed interaction between C/EBPα and the WW domains is a prerequisite for the inhibition of C/EBPα transcriptional activity, then these results in combination with those gathered from this study offer compelling evidence to support a model where TCERG1 exerts its inhibitory activity through a general mechanism involving subnuclear retention resulting from interactions provided by WW1 and WW2. Finally, although the data point toward the involvement of the first two WW domains in mediating the inhibitory activity of TCERG1, the potential role of the $(QA)_{38}$ repeat domain cannot be ignored. To date, no biological function has been given to this domain. Moreover, in studies presented by Goldstrohm *et al.* (2001), the impact of the $(QA)_{38}$ repeat on TCERG1-mediated repression of transcriptional activity was not addressed. Hence, this domain should be considered in future experiments to determine the requirements of the $(QA)_{38}$ repeat in TCERG1 inhibitory activity and/or interaction with C/EBPα. Finally, *in vitro* assays provide several limitations when assaying cells expressing C/EBPα, which is generally tightly controlled and limited to differentiated cell types. Additionally, the quantity of plasmid DNA expressing C/EBPα must

be low to prevent apoptosis (data not shown). Since all co-localization studies were performed in cell lines that do not express endogenous C/EBPα, additional studies should be performed in cell lines that can be induced to express endogenous C/EBPα, such as pre-adipocytes or stable cell lines with an on/off expression system. This would allow the examination of co-localization between TCERG1 and C/EBPα in situations where expression of C/EBPα can be controlled, mimicking a more natural environment. Moreover, it would be beneficial to repeat co-localization studies in each cell line with the detection of endogenous TCERG1 by immunofluorescence to substantiate the marked translocation of TCERG1 to C/EBPα-containing subnuclear compartments. Should results obtained from conditions examining the localization of endogenous protein be similar to those reported herein, then the data collected using fluorescent fusion proteins to TCERG1 and C/EBPα accurately reflects the physiological behaviour of the native protein.

Although extensive research has been able to identify numerous nuclear bodies (Matera, 1999; Misteli, 2001; Wang *et al.*, 2002b; Iborra *et al.*, 2003), none seem to fit the morphology or characterization of the sites occupied by C/EBPα in COS7 and HEK293T cells and at peri-centromeric regions in murine cell types. Thus, it would be of interest to determine the functional purpose of C/EBPα subnuclear localization, why it localizes to particular sites, and to find other factors that may be associated with these nuclear bodies. It has been demonstrated that C/EBPα localizes to peri-centromeric regions in a phosphorylation-dependent manner in 3T3-L1 pre-adipocytes (Tang and Lane, 1999). Thus, the role of phosphorylation in the localization of C/EBPα in COS7 and HEK293T cells could be examined by mutating key/susceptible phosphorylation sites within C/EBPα (reviewed in Khanna-Gupta, 2008) which can then be assessed for alterations in subnuclear compartmentalization. Studies in GHFT1-5 cells indicate that peri-centromeric localization of C/EBPα also requires the carboxy-terminal leucine zipper region, because when deleted, C/EBPα becomes dispersed throughout the nucleus (Liu *et al.*, 2002). Likewise, this study also demonstrates that a single amino acid substitution at position 296 from valine to alanine, causes C/EBPα to become dispersed in the nucleoplasm and no longer associate with peri-centromeric regions. Collectively, these findings provide a foundation from which one can explore the requirement of C/EBPα localization to peri-centromeric regions for TCERG1-mediated inhibition to occur. However, studies looking at the subnuclear localization of C/EBPα thus far have been limited to murine

cell types (Tang and Lane, 1999; Schaufele *et al.*, 2001, 2003; Wang *et al.*, 2001; Zhang *et al.*, 2001; Liu *et al.*, 2002; Day *et al.*, 2003; Enwright *et al.*, 2003; Demarco *et al.*, 2006). Therefore, one can only extrapolate so much when comparing to studies conducted in cell types of other species. Thus, the necessity for proper characterization of the specific subnuclear localization patterns of C/EBPα observed in COS7 and HEK293T cells is critical for understanding the relationship between function and subnuclear residency.

To date, there has only been one study showing the ability of C/EBPα to recruit transcriptional co-activators, CBP and TBP, to peri-centromeric regions in mouse progenitor pituitary GHTF1-5 cells (Schaufele *et al.*, 2001). Interestingly, our studies have suggested that TCERG1 does not interact with either of these co-factors (McFie *et al.*, 2006). It would be intriguing to investigate the co-localization of C/EBPα and CBP or TBP in the presence of TCERG1. Likewise, C/EBPα directly interacts with cdk2 and cdk4 to cause growth arrest (Wang *et al.*, 2001). Therefore, the localization of each with C/EBPα in the presence of TCERG1 could be examined to determine if cdk2 and cdk4 remain associated with C/EBPα. It can be anticipated that the interaction and co-localization between C/EBPα and TCERG1 would prevent proper binding of the aforementioned co-factors with C/EBPα, thus resulting in the inhibition of C/EBPα-mediated activity. Further insight into the inhibition of growth arrest by TCERG1 could also be gained by using a C/EBPα Ser193Ala mutant whose growth arrest activity was not only abolished but resulted in accelerated proliferation of hepatoma cells (Wang and Timchenko, 2005). This study also demonstrated that the interaction between C/EBPα and Rb results in cellular arrest, hence, co-localization between Rb and C/EBPα may be altered upon the overexpression of TCERG1 thereby releasing cells from growth arrest. However, the subnuclear distribution of C/EBPα with mutations affecting its activity must first be addressed before examining its co-localization with TCERG1.

Although there appears to be a link between TCERG1 inhibitory activity of C/EBPα-mediated growth arrest and subnuclear localization, these studies must be expanded to incorporate the inhibition of C/EBPα transcriptional activity. Does C/EBPα require transcriptional activity for redistribution of TCERG1 to sites occupied by C/EBPα? This could be tested by altering the transcriptional activity of C/EBPα by deleting important domains required for this activity or by mutating key amino acids within these domains. Alternatively, Liu *et al.* (2002) have shown that when EGFP is fused to the amino terminus of C/EBPα, it no

longer possesses transcriptional activity. Therefore, alterations in the localization of TCERG1 could be analyzed upon expression of the various C/EBPα mutants lacking transcriptional activity to determine if this activity is necessary. Moreover, a recent study presents data supporting the ability of pituitary-specific transcription factor Pit-1 to recruit C/EBPα to intranuclear sites occupied by Pit-1 (Enwright *et al.*, 2003). It would be interesting to examine the localization of TCERG1 in the context of GHFT1-5 pituitary cells expressing Pit-1. Would TCERG1 retain C/EBPα within peri-centromeric regions thereby inhibiting the activation of genes specifically co-regulated by Pit-1 and C/EBPα? Finally, identifying the TCERG1 interaction domain within C/EBPα would be pivotal in order to establish a definitive relationship between subnuclear compartmentalization and inhibition of C/EBPα-mediated activity through interactions with TCERG1 by performing co-localization studies where the interaction between TCERG1 and C/EBPα is abolished.

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