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Journal of Cellular Biochemistry 112:2317–2326 (2011)

Journal of Cellular Biochemistry

# TCERG1 Inhibits C/EBPα Through a Mechanism That Does Not Involve Sequestration of C/EBPα at Pericentromeric Heterochromatin

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## ABSTRACT

Transcriptional elongation regulator 1 (TCERG1) is a nuclear protein that participates in multiple events that include regulating the elongation of RNA polymerase II and coordinating transcription and pre-mRNA processing. More recently, we showed that TCERG1 is also a specific inhibitor of the transcription factor CCAAT enhancer binding protein α (C/EBPα). Interestingly, the inhibition of C/EBPα by TCERG1 is associated with the relocalization of TCERG1 from the nuclear speckle compartment to the pericentromeric regions where C/EBPα resides. In the present study, we examined additional aspects of C/EBPα-induced redistribution of TCERG1. Using several mutants of C/EBPα, we showed that C/EBPα does not need to be transcriptionally competent or have anti-proliferative activity to induce TCERG1 relocalization. Moreover, our results show that C/EBPα does not need to be localized to the pericentromeric region in order to relocalize TCERG1. This conclusion was illustrated through the use of a V296A mutant of C/EBPα, which is incapable of binding to the pericentromeric regions of heterochromatin and thus takes on a dispersed appearance in the nucleus. This mutant retained the ability to redistribute TCERG1, however in this case the redistribution was from the nuclear speckle pattern to the dispersed phenotype of C/EBPα V296A. Moreover, we showed that TCERG1 was still able to inhibit the activity of the V296A mutant. While we previously hypothesized that TCERG1 might inhibit C/EBPα by keeping it sequestered at the pericentromeric regions, our new findings indicate that TCERG1 can inhibit C/EBPα activity regardless of the latter's location in the nucleus. J. Cell. Biochem. 112: 2317–2326, 2011. 

**KEY WORDS:** CCAAT ENHANCER BINDING PROTEIN; TCERG1; SUBNUCLEAR COMPARTMENTALIZATION; NUCLEAR SPECKLES; CONFOCAL MICROSCOPY

It is now well accepted that the nucleus possesses a high degree of architecture [Schneider and Grosschedl, 2007]. Individual chromosomes are known to occupy distinct regions in this organelle that do not overlap with other chromosomes [Cremer et al., 2006]. Moreover, several different nuclear bodies exist that are distinguished by the nuclear proteins present in them and by the functions they perform. For example, nuclear speckles have been shown to concentrate several splicing factors, and to be a site where pre-mRNA processing occurs [Lamond and Spector, 2003]. Because nuclear bodies do not have membranes to define or organize them, the size, number, and subnuclear location of nuclear bodies is in constant flux. This heterogeneity is consistent with the high degree of mobility that nuclear proteins display, and may reflect the rapid exchange of molecules from the nuclear bodies that may function as storage depots or released into the nucleoplasm where they can be recruited for specific functions [Misteli, 2001].

An appreciation for the role that subnuclear localization plays in the regulation of gene expression and the activity of nuclear proteins is steadily increasing. In large part, this is due to a growing list of nuclear proteins that display subnuclear compartmentalization and to observations that their compartmentalization is often dynamic in nature. One of the best-studied examples of this is the transcription factor CCAAT enhancer binding protein α (C/EBPα). Regardless of the cell type studied, this protein is concentrated in pericentromeric regions of heterochromatin [Tang and Lane, 1999; Schaufele et al., 2001]. Interestingly, this region contains the major α-satellite repeat sequences, to which C/EBPα is able to bind in vitro [Liu et al., 2007], leading to speculation that the pericentromeric domains act as a storage depot for C/EBPα, from where it can then be released in order to be used elsewhere in the nucleus for its various functions. There is additional evidence to support this hypothesis. In the pituitary, C/EBPα and another transcription factor, Pit-1,

Grant sponsor: Hazel Constance Brooker Trust Fund; Grant sponsor: Department of Biochemistry and College of Graduate Studies & Research at the University of Saskatchewan.

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Received 25 November 2010; Accepted 11 April 2011 • DOI 10.1002/jcb.23154 • © 2011 Wiley-Liss, Inc.
Published online 18 April 2011 in Wiley Online Library (wileyonlinelibrary.com).

synergistically activate several gene promoters [Schaufele, 1996; Jacob and Stanley, 1999]. Using a pituitary progenitor cell line, Enwright et al. [2003] showed that C/EBPα, when expressed alone, was concentrated in pericentromeric heterochromatin as expected, whereas Pit-1 displayed a more dispersed appearance. However, when expressed simultaneously, C/EBPα underwent an altered subnuclear localization pattern that overlapped that of Pit-1. This co-localization of C/EBPα and Pit-1 correlated with their synergistic activation of the prolactin gene promoter. These data suggested that Pit-1 causes the release of C/EBPα from the α-satellite repeat regions in the pericentromeric regions, allowing C/EBPα to bind to and transactivate its target genes in the cell.

There is also evidence that C/EBPα recruits other transcription factors to the pericentromeric domain. These include one of its coactivators, CREB-binding protein (CBP), and the general factor TBP (TATA binding protein) [Schaufele et al., 2001]. While the regulatory consequences of this nucleoprotein assembly by C/EBPα are not known, the widespread role that CBP and TBP play in gene regulation suggest that entire programs of gene expression could be altered.

Recently, our laboratory identified yet another protein, transcriptional elongation regulator (TCERG1), that is specifically recruited to the pericentromeric regions by C/EBPα [Banman et al., 2010]. We initially identified TCERG1 in a two-hybrid screen as a protein that interacted with the transactivation domain of C/EBPα, and the physical interaction between these two proteins was confirmed by co-immunoprecipitation analysis. This same study also showed that TCERG1 inhibited C/EBPα activity [McFie et al., 2006]. In a follow-up study, we showed that TCERG1, which normally resides in nuclear speckles, is recruited by C/EBPα to the pericentromeric regions of the nucleus. Moreover, we showed that the relocalization of TCERG1 was required for its inhibitory activity towards C/EBPα [Banman et al., 2010]. This conclusion was based on studies using a panel of TCERG1 mutants, some of which showed a loss of inhibitory activity towards C/EBPα. Interestingly, these loss-of-function mutants also lost the ability to be relocalized by C/EBPα to the pericentromeric regions.

In the present study, we explore additional aspects of the relocalization of TCERG1 by C/EBPα. Our results indicate that this activity of C/EBPα is distinct from its other known activities. Moreover, we show that TCERG1 can be relocalized to other nuclear compartments as long as C/EBPα is in residence.

## MATERIALS AND METHODS

### PLASMIDS

The plasmids expressing the EGFP-C/EBPα and mCherry-TCERG1 (hereafter referred to as Ch-TCERG1) fusion proteins, HA-tagged TCERG1 and related mutants 32–668 and 641–1098, and the C/EBPα responsive reporter gene vector -68FX4-luc have been previously described [Banman et al., 2010]. The plasmid expressing GFP-C/EBPβ was a gift from C. Asselin (Université de Sherbrooke). The single amino acid substitution mutants of C/EBPα, S193A, and V296A, were generated in the plasmid expressing EGFP-C/EBPα using QuikChange™ site-directed mutagenesis (Stratagene). The EGFP-C/EBPα mutant containing three amino acid substitutions (Y67A, F77A, L78A) was generated by digesting pCMVα containing these mutations [Nerlov and Ziff, 1995] with *Bam*HI/*Pst*I, which released the coding region extending from amino acid 1 to 217 as well as some 5′ untranslated region. This fragment was then cloned into pEGFP-C/EBPα that had been cut with *Bgl*II/*Pst*I to generate pEGFP-C/EBPα-TM. All expression plasmids generated were verified by DNA sequencing.

### REPORTER GENE ASSAY

COS7 cells were subcultured into 60 mm plates to approximately 30% confluency. The next day, they were transfected with 0.1% Polyethylenimine "Max" (Polysciences Inc., Warrington, PA) at a ratio of 3:1 reagent to DNA with the total amount of DNA being 2.5 μg per plate. Cells were washed 4 hr after transfection, then cultured in DMEM containing 10% fetal calf serum (FBS) for an additional 48 hr. Cells were harvested and assayed for luciferase activity and protein concentration as previously described [McFie et al., 2006].

### TRANSFECTION, IMMUNOSTAINING, AND LASER-SCANNING CONFOCAL MICROSCOPY

COS7 cells were cultured in high-glucose DMEM supplemented with 10% FBS. On day 1, cells were sub-cultured to 20% confluency onto coverslips in 35 mm plates. The following day, cells were transfected as described above with 250 ng of EGFP-C/EBPα expression plasmid and/or 2.25 μg of Ch-TCERG1 expression plasmid per plate. Four hrs post-transfection, cells were washed once with PBS and then re-fed with medium containing 10% FBS. Fifty hours post-transfection (or as indicated in the figure legend), coverslips were washed twice with PBS and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After three 5 min washes in PBS, coverslips were mounted on slides using Prolong Gold Antifade reagent (Invitrogen).

In some instances, TCERG1 was detected by immunofluoresence as follows. Following fixation with paraformaldehyde, cells were washed twice with PBS for 5 min, then permeabilized with 0.15% Triton X-100 for 10 min. Cells were then washed twice with PBS for 5 min, followed by blocking with 3% bovine serum albumin for 15 min at room temperature. The BSA solution was replaced with anti-TCERG1 antibody (Bethyl Laboratories, 300A–360A) diluted 1:300 in blocking solution. After 1 hr at room temperature, cells were washed three times in PBS for 5 min, then incubated with either an Alexa Fluor® 594- or Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody diluted 1:1,000 for 45 min at room temperature. Coverslips were washed five times for 5 min with PBS, then mounted on slides as described above. Laser-scanning confocal microscopy was performed using an Olympus FV300 microscope housed in the Cell Signaling Laboratory, University of Saskatchewan.

## RESULTS

### C/EBPα DOES NOT REQUIRE ITS TRANSACTIVATION OR GROWTH ARREST ACTIVITY TO REDISTRIBUTE TCERG1

Initially, we examined whether the TCERG1 relocalization activity of C/EBPα was associated with either of its other two known

activities, namely its transactivation and growth arrest activities. Two transcriptionally inactive forms of C/EBPα were used in these studies. A triple amino acid substitution mutant (TM) of C/EBPα that has Y67, F77, and L78 mutated to alanines, was shown previously to be devoid of transactivation potential due to a reduction in its ability to interact with TBP and TFIIB [Nerlov and Ziff, 1995]. A second transcriptionally inactive form of C/EBPα was based on the study by Schaufele et al., who reported that when EGFP is fused to the amino terminus of C/EBPα, it results in a loss of C/EBPα activity [Liu et al., 2002]. This reversed fusion protein, termed EGFP-C/EBPα-RF, as well as EGFP-C/EBPα-TM were examined for their ability to relocalize Ch-TCERG1. As shown in Figure 1A, C/EBPα-RF localized to pericentromeric heterochromatin similar to wild-type C/EBPα. However, C/EBPα-TM consistently showed an altered pattern relative to wild-type protein, in that the compartments it occupied were less restricted. We also tested a third mutant, S193A, which is deficient in growth arrest activity. The serine mutated is required to be phosphorylated in order for C/EBPα to sequester cdk2 and cdk4 which is necessary for growth arrest activity [Wang and Timchenko, 2005]. This mutant also localized to pericentromeric chromatin similar to wild type (Fig. 1A). Also shown in Figure 1A is the typical nuclear speckle pattern of Ch-TCERG1 when expressed alone.

These three mutants were then co-expressed with Ch-TCERG1. In Figure 1B, the ability of C/EBPα-RF to relocalize Ch-TCERG1 is shown. When the two proteins were co-expressed, Ch-TCERG1 displayed an altered compartmentalization pattern that matched that of C/EBPα-RF as illustrated by the merged panel. Similar observations were made for C/EBPα-TM and C/EBPα-S193A (Fig. 1C,D, respectively). Of particular interest was the observation that despite C/EBPα-TM displaying an altered localization pattern relative to C/EBPα-RF or -S193A, it was still able to redistribute Ch-TCERG1 (Fig. 1C).

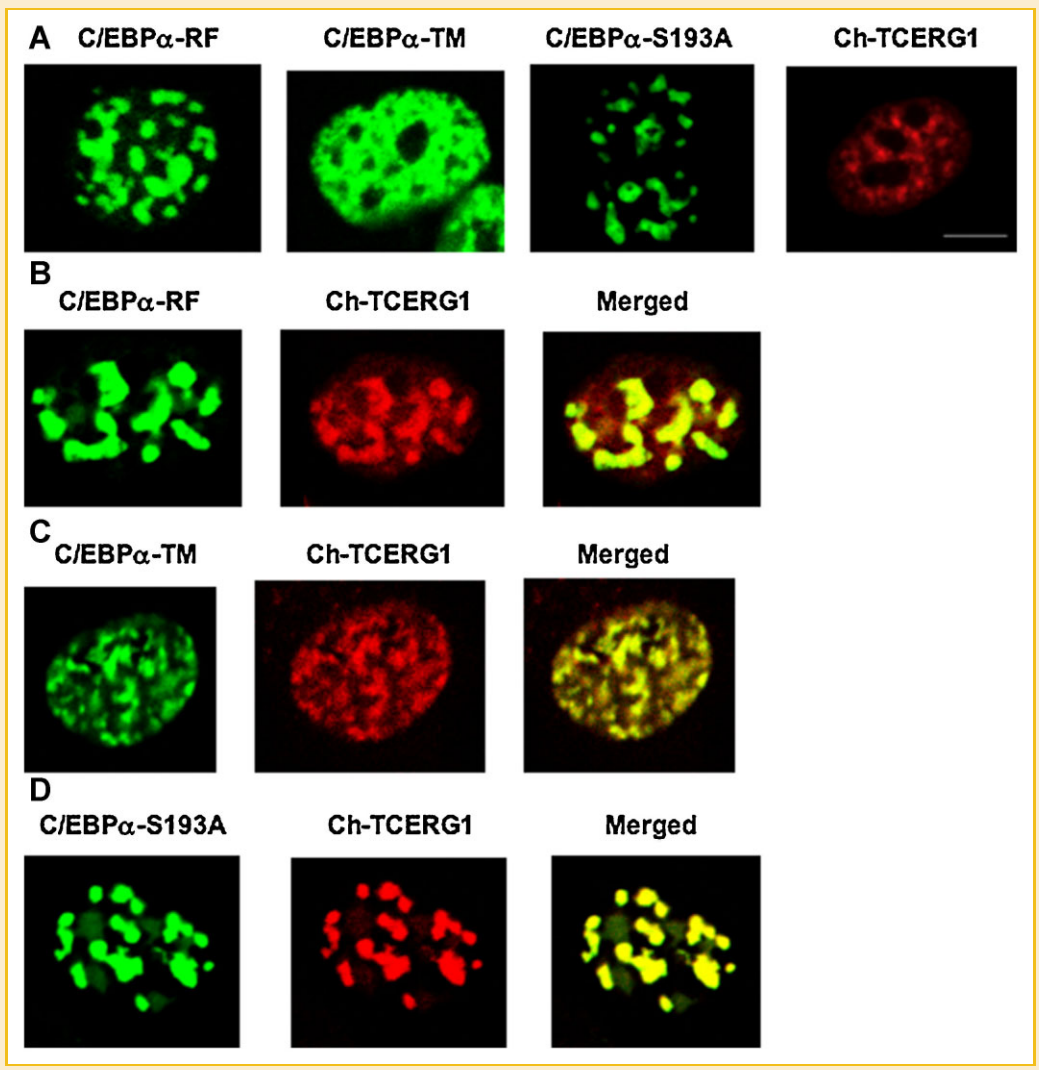


Fig. 1. C/EBPα does not need to be functionally active to relocalize TCERG1. A: COS7 cells were transfected with expression vectors for EGFP fusions of C/EBPα-RF, C/EBPα-TM, or C/EBPα-S193A, or with Ch-TCERG1. After 50 hr, cells were fixed and then analyzed by confocal microscopy. B–D: Cells were co-transfected with expression vectors for the indicated EGFP-C/EBPα fusion protein and Ch-TCERG1.

## C/EBPα CAN RELOCALIZE TCERG1 TO DIFFERENT NUCLEAR COMPARTMENTS

During the course of our study, we noted that approximately 75% of cells showed the typical pericentromeric compartmentalization of C/EBPα (data not shown). Another 10–15% showed a dispersed pattern which likely reflected cells in which insufficient time had elapsed to allow for pericentromeric localization of C/EBPα (data not shown). The remaining 10–15% of cells showed atypical patterns that fell into three categories. This is consistent with observations that during different phases of the cell cycle, proteins can undergo alteration of their subnuclear compartmentalization or the architecture of the compartment itself can change. We used these altered patterns to explore the issue of whether the relocalization of TCERG1 was destination-specific, i.e., could TCERG1 be relocalized to a region other than to pericentromeric regions. COS7 cells were co-transfected with plasmids for EGFP-C/EBPα and Ch-TCERG1, and then cells displaying the different C/EBPα compartmentalization patterns were examined for the localization pattern of TCERG1. Figure 2A shows the typical C/EBPα distribution pattern, as well as the relocalized Ch-TCERG1 that is entirely overlapping with C/EBPα. Figure 2B shows the dispersed pattern of C/EBPα that appeared in some cells. Interestingly, Ch-TCERG1 still co-localized with C/EBPα despite the significant change in compartmentalization pattern. Panels C and D show two other atypical C/EBPα localization patterns. In panel C, a punctate pattern is shown that consists of more numerous and smaller foci. Panel D shows a C/EBPα compartmentalization pattern that is partially dispersed with some non-uniform regions of accumulation including perinucleolar concentration. Regardless of the compartmentalization pattern, however, Ch-TCERG1 co-localized with C/EBPα as indicated by the merged panels (Fig. 2C,D).

While the data in Figure 2 suggest that TCERG1 can be relocalized to different cellular compartments, it could be argued that the different patterns arise simply from the pericentromeric regions adopting different patterns during different stages of the cell cycle. Thus, we examined this issue using a different approach. Schaufele's group identified a C/EBPα mutant, V296A, that lost its ability to bind to the α-satellite repeat sequence in the pericentromeric regions yet retained its ability to bind to consensus C/EBP binding sites as well as to C/EBP binding sites in gene promoters [Liu et al., 2007]. This mutant, therefore, does not show pericentromeric concentration but rather takes on a dispersed appearance. We confirmed that this mutant adopted the dispersed pattern in COS7 cells (Fig. 3A). When Ch-TCERG1 was co-expressed, it also took on a dispersed appearance and completely co-localized with the V296A C/EBPα mutant (Fig. 3B). This finding further supports our conclusion that C/EBPα can relocalize TCERG1 to different cellular compartments.

## TCERG1 INHIBITS C/EBPα V296A DESPITE ITS DISPERSED LOCALIZATION

We previously hypothesized that TCERG1 might inhibit C/EBPα activity by preventing the release of C/EBPα from the pericentromeric regions. This was based on several observations including the fact that both proteins can be co-immunoprecipitated, that TCERG1 is relocalized to pericentromeric regions by C/EBPα, and that Pit-1 appears to activate C/EBPα by causing its release from these regions. We thus reasoned that the V296A mutant of C/EBPα provided a tool to test this hypothesis further. Specifically, we predicted that since this mutant has reduced affinity for the repeat sequences in the pericentromeric regions and is dispersed in the nucleus, this mutant should be relatively insensitive to inhibition by TCERG1.

We tested this using a C/EBP responsive promoter in a luciferase reporter gene assay. Wild-type EGFP-C/EBPα was observed to stimulate promoter activity by threefold, and this promoter activation was significantly repressed by TCERG1 (Fig. 4A). The specificity of this inhibition was examined by using two mutants which we had examined previously for their ability to inhibit C/EBPα-dependent growth arrest [McFie et al., 2006]. The mutant possessing amino acids 32–668, which retains the ability to inhibit the anti-proliferative activity of C/EBPα, was also shown to inhibit EGFP-C/EBPα in the reporter gene assay shown in Figure 4A. Likewise, the mutant expressing amino acids 641–1098, which lacks inhibitory activity as assessed in a growth arrest assay, also failed to inhibit the ability of C/EBPα to activate a target gene promoter (Fig. 4A).

The response of the V296A mutant of EGFP-C/EBPα to TCERG1 was then examined. As shown in Figure 4B, V296A induced a stronger activation of the responsive promoter relative to wild-type C/EBPα, consistent with previous observations. However, similar to the wild-type protein, this mutant remained sensitive to inhibition by both full-length TCERG1 and the 32–668 mutant but not to the 641–1098 mutant (Fig. 4B).

## C/EBPα ALSO RELOCALIZES ENDOGENOUS TCERG1

Our analysis of TCERG1 localization to date utilized a mCherry fusion protein. While we previously established that Ch-TCERG1 localizes to nuclear speckles [Banman et al., 2010], it has not been established that endogenous TCERG1 also undergoes relocalization in response to C/EBPα. In order to assess this, we used immunofluorescence to track TCERG1. In Figure 5A, confocal images of COS7 cells that were transfected with Ch-TCERG1 or immunostained for TCERG1 (TCERG1 IF) are shown. Both Ch-TCERG1 and endogenous TCERG1 showed nucleolar exclusion as well as a dispersed pool of protein as well as a fraction that concentrates in irregular shaped domains. We then assessed how closely ectopically expressed Ch-TCERG1 overlaps with endogenous TCERG1 by immunostaining for TCERG1 in cells that had been transfected with Ch-TCERG1. The primary antibody used recognizes an epitope in TCERG1, thus the immunofluorescence analysis detected both endogenous TCERG1 as well as Ch-TCERG1. Based on our previous observation that Ch-TCERG1 displays two different compartmentalization patterns (data not shown), we assessed co-localization in cells displaying both of these patterns. In the top row of Figure 5B, the confocal image of a cell displaying the dispersed localization pattern of Ch-TCERG1 is shown. In this same cell, TCERG1 detected by immunofluorescence showed the same distribution pattern, completely overlapping with that of Ch-TCERG1 (merged panel). When a cell showing the more typical nuclear speckle pattern of Ch-TCERG1 was examined (Fig. 5B, bottom row), total cellular TCERG1 once again adopted

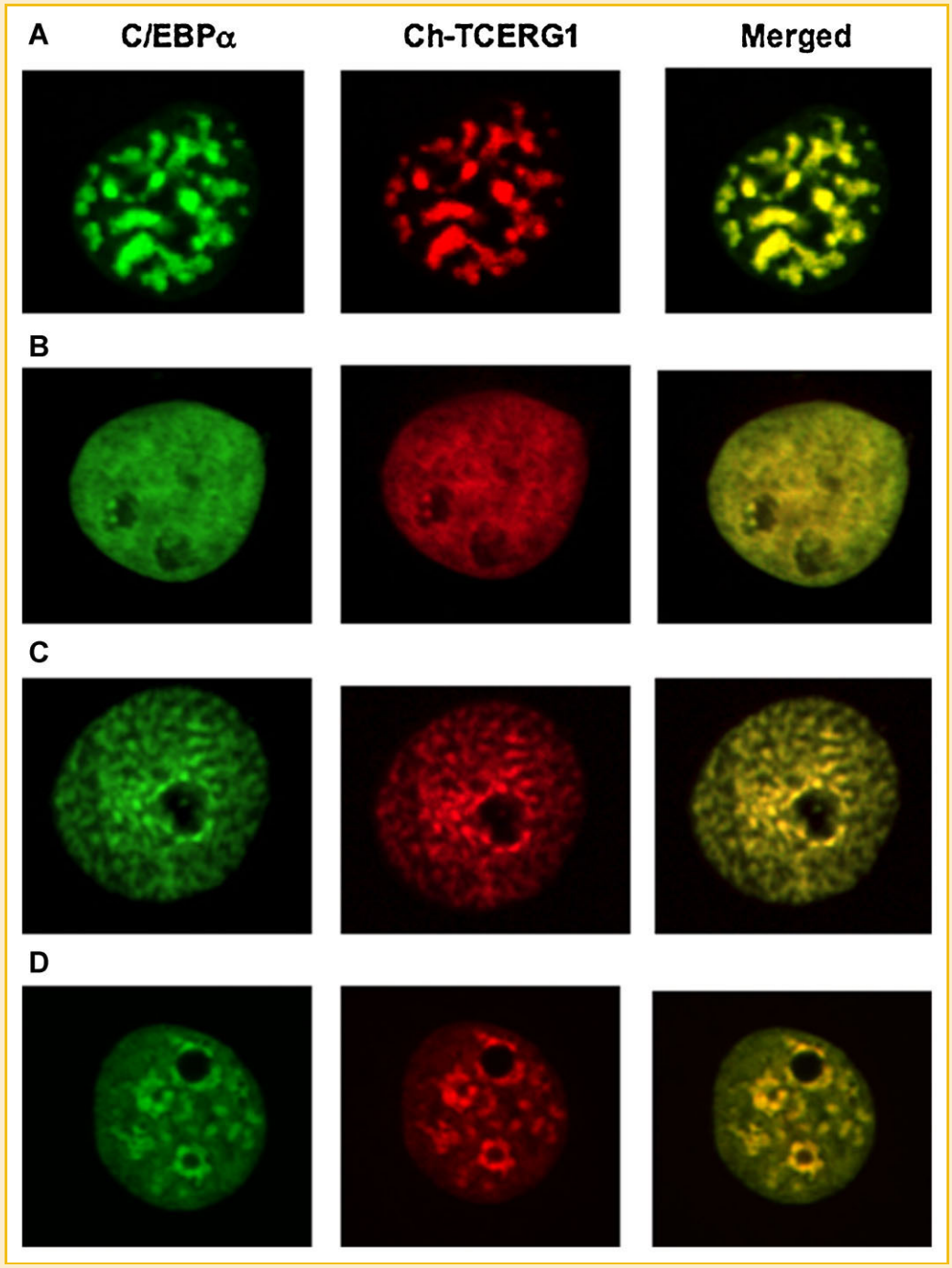


Fig. 2. C/EBPα relocalizes TCERG1 regardless of nuclear compartmentalization pattern. COS7 cells were co-transfected with expression vectors for EGFP-C/EBPα and Ch-TCERG1. After 50 hr, cells were fixed and analyzed by confocal microscopy. Panels A–D represent the four most common patterns of C/EBPα localization observed.

the identical compartmentalization. Despite the limitation of our immunofluorescence analysis, we can nonetheless conclude that there is no pool of endogenous TCERG1 that is occupying a different subnuclear compartment than Ch-TCERG1, since if that were the case, regions of green fluorescence on the merged panel would be apparent. Taken together, these data provide further evidence that the mCherry-TCERG1 fusion protein mimics the localization pattern of the endogenous protein.

We then examined whether endogenous TCERG1 could be relocalized in response to ectopic expression of EGFP-C/EBPα. As shown in Figure 5C, C/EBPα displayed its typical pericentromeric localization pattern, and endogenous TCERG1 was relocalized such that it adopted the same pattern as C/EBPα (merged panel). However, examination of the merged panel indicated that there was a population of TCERG1 that did not co-localize with C/EBPα but rather maintained its dispersed distribution pattern.

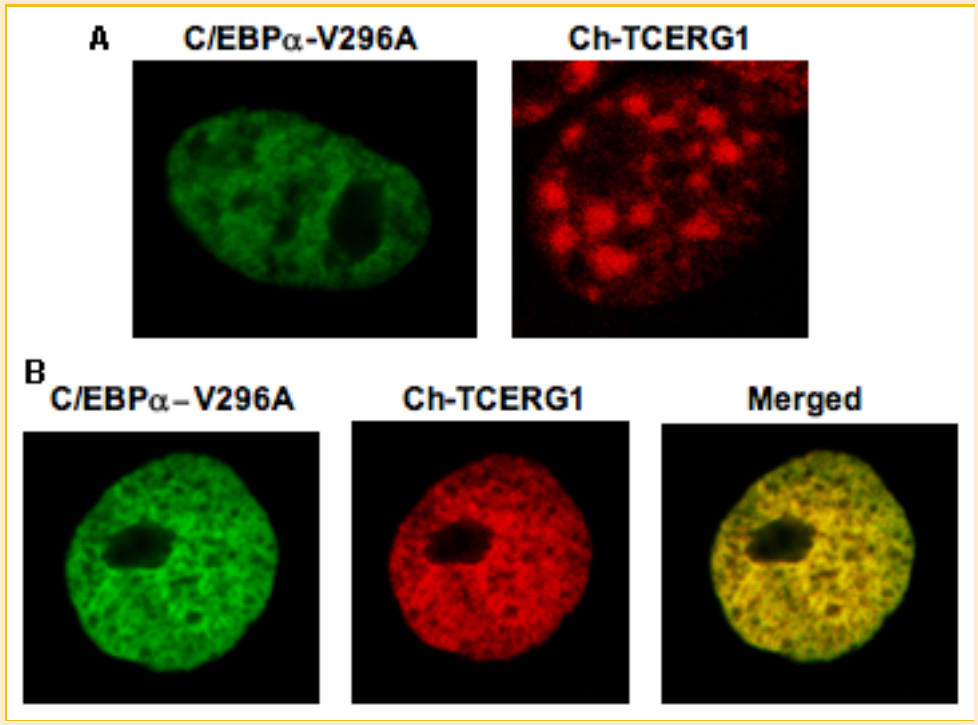


Fig. 3. C/EBPα does not need to be localized to pericentromeric heterochromatin in order to relocalize TCERG1. A: COS7 cells were co-transfected with an expression vector for either EGFP-C/EBPα-V296A or Ch-TCERG1 and then analyzed by confocal microscopy. B: Cells were co-transfected with expression vectors for EGFP-C/EBPα-V296A and Ch-TCERG1.

### TCERG1 CAN ALSO BE RELOCALIZED BY C/EBPβ

Previously we had shown that TCERG1 inhibits the transactivation activity of another C/EBP isoform, C/EBPβ [McFie et al., 2006]. Both α and β isoforms share almost identical DNA binding domains but possess disparate transactivation domains [Lekstrom-Himes and Xanthopoulos, 1998]. Based on these observations, we were interested in seeing whether C/EBPβ can also induce redistribution of TCERG1. As shown in Figure 6A, EGFP-C/EBPβ adopted a nuclear compartmentalization pattern similar to that displayed by C/EBPα, which is typical of pericentromeric localization. When EGFP-C/EBPβ was co-expressed with Ch-TCERG1, the two proteins adopted a pericentromeric localization pattern with near complete overlap (Fig. 6B). Thus, C/EBPβ also appears able to relocalize TCERG1 in the nucleus.

## DISCUSSION

C/EBPα has become a paradigm for studying how nuclear compartmentalization plays a role in the regulation of transcription factor activity. This transcription factor localizes to a very specific compartment, the pericentromeric region of heterochromatin, in a variety of cells [Tang and Lane, 1999; Schaufele et al., 2001; Banman et al., 2010]. This specific compartmentalization appears to be a result of C/EBPα binding to the α-satellite repeat sequences found in this region of the chromatin [Tang and Lane, 1999; Liu et al., 2007]. This conclusion is based on the observations that C/EBPα mutants that either lack the bZIP DNA binding domain or are deficient in binding to the repeat sequences do not show compartmentalization [Day et al., 2003; Liu et al., 2007]. It is also becoming clear from several studies that this compartmentalization plays an important role in regulating C/EBPα function. One illustration of this is the observation that Pit-1, which synergizes with C/EBPα to activate several pituitary-specific genes, induces the release of C/EBPα from the pericentromeric regions whereupon it co-localizes with Pit-1 [Enwright et al., 2003]. Moreover, this study showed that the redistribution of C/EBPα by Pit-1 is required for synergism. These and other data suggest that C/EBPα activity is regulated tightly by a storage-and-release mechanism [Corry and Underhill, 2005].

Previously, we reported on a nuclear protein called TCERG1 that we characterized as an inhibitor of C/EBPα [McFie et al., 2006]. Interestingly, upon C/EBPα expression, TCERG1 undergoes redistribution from the nuclear speckles to the pericentromeric region where it co-localizes with C/EBPα [Banman et al., 2010]. While we initially demonstrated this using a mCherry-TCERG1 fusion protein, in the present study we confirmed that endogenous TCERG1 also undergoes redistribution. Through the use of TCERG1 mutants, we were able to conclude that the redistribution of TCERG1 is associated with its ability to inhibit C/EBPα [Banman et al., 2010]. Coupled with the observation that one mode of C/EBPα activation is via its release from pericentromeric regions, we hypothesized that TCERG1 inhibits C/EBPα activity by blocking its release.

In the present study, one major issue we wanted to examine was whether the ability of C/EBPα to relocalize TCERG1 required either its transactivation activity or growth arrest activity. We used two

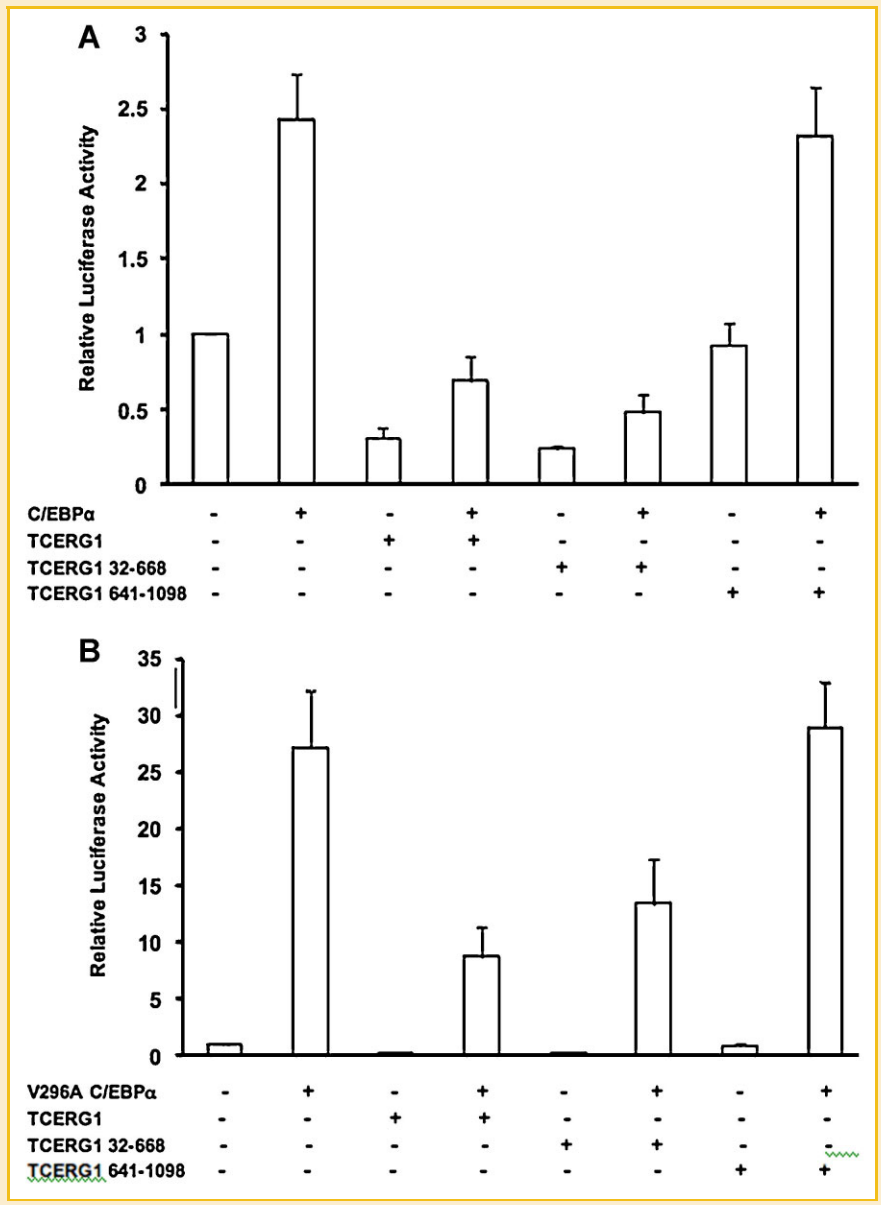


Fig. 4. The activity of the V296A mutant of C/EBPα is inhibited by TCERG1. COS7 cells were transfected with 250 ng of the -68FX4-luc reporter plasmid and where indicated, an expression plasmid (150 ng) for wt EGFP-C/EBPα (panel A) or V296A EGFP-C/EBPα (panel B) and an expression plasmid (1.5 μg) for TCERG1, TCERG1 32–668, or TCERG1 641–1098. Values shown are the mean ± SE of at least three experiments performed in duplicate, and are relative to the luciferase activity obtained with reporter gene only which was set at 1.0.

transcriptionally inactive mutants of C/EBPα as well as a growth arrest-deficient mutant, and found that all three redistributed TCERG1. These findings suggest that the relocalization activity of C/EBPα is a distinct function mediated by a unique domain. However, the location and nature of this domain is still a question mark. We originally cloned TCERG1 using a yeast two-hybrid screen, in which we used the N-terminal domain of C/EBPα (amino acids 6–217) as the interacting bait [McFie et al., 2006]. That observation, plus the fact that TCERG1 was able to inhibit the transactivation activity of a GAL4-C/EBPα hybrid containing the transactivation domain, suggests that the TCERG1 relocalization/interaction region lies within amino acids 6–217. One observation in the current study that may help focus our efforts to identify this domain is that C/EBPβ was also observed to be capable of relocalizing TCERG1. While the transactivation domains of the α and β isoforms are rather dissimilar, they do have a conserved bipartite domain that has been shown to bind TBP and TFIIB [Nerlov and Ziff, 1995]. Mutational analysis currently underway should allow us to assess the involvement of this domain and others in the redistribution of TCERG1.

The corresponding interaction domain in TCERG1 was suggested previously to lie in the amino terminal half of the protein which contains three WW domains [Banman et al., 2010], and the results of experiments testing several overlapping deletion mutants suggested that the interaction with C/EBPα is mediated by more than one

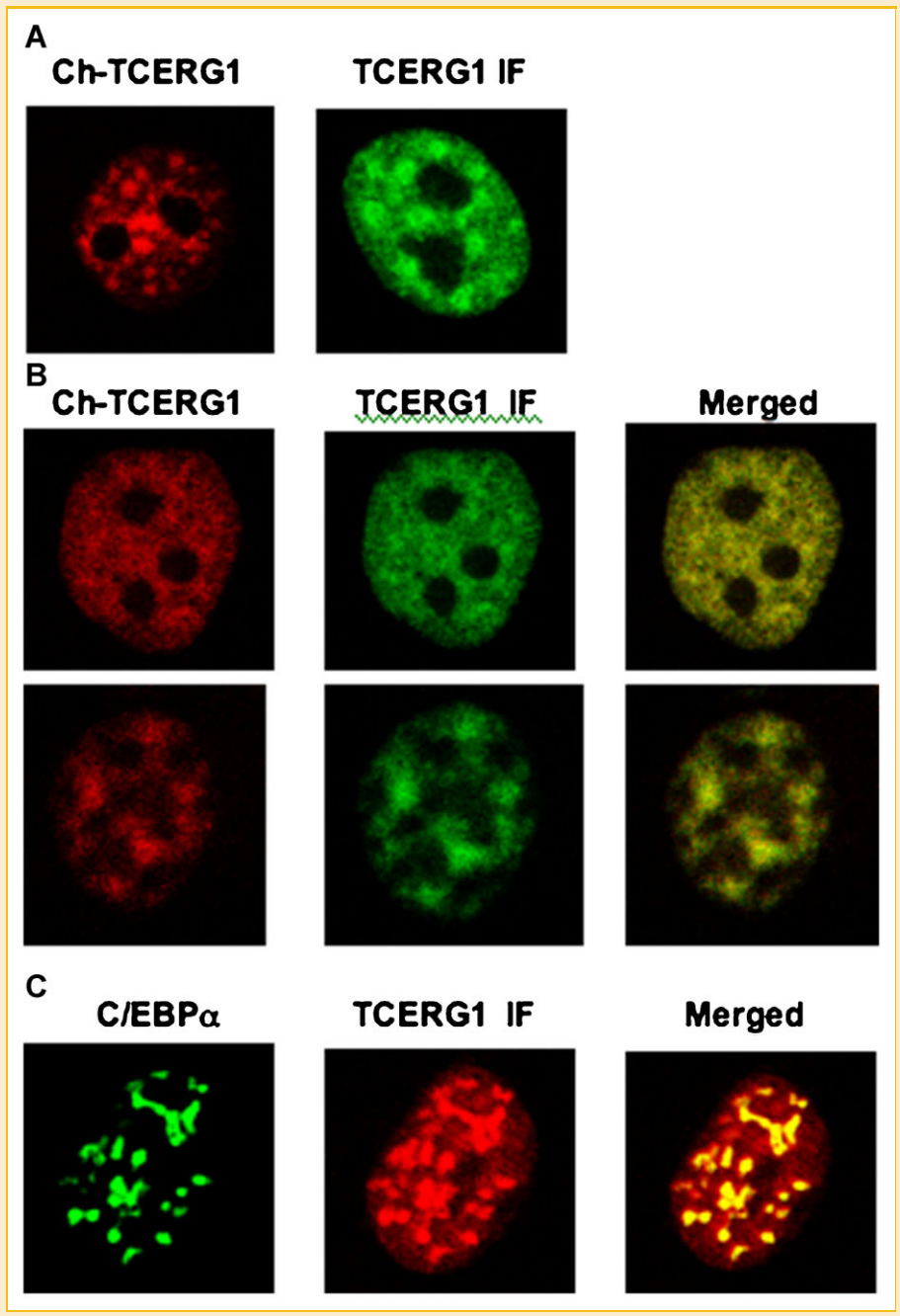


Fig. 5. Endogenous TCERG1 is also relocalized by C/EBPα. A: COS7 cells were either transfected with an expression vector for Ch-TCERG1, or immunostained for TCERG1 with an Alexa Fluor® 594-tagged secondary antibody. B: Cells were transfected with an expression vector for Ch-TCERG1 and then immunostained for TCERG1 with an Alexa Fluor® 488-tagged secondary antibody (green). Cells were then analyzed by confocal microscopy. The top row shows a cell displaying the dispersed compartmentalization pattern, while the bottom row is a cell displaying the speckle pattern. C: Cells were transfected with an expression vector for EGFP-C/EBPα, and then fixed after 50 hr followed by immunostaining for TCERG1 using an Alexa Fluor® 594-tagged secondary antibody (red). TCERG1 IF refers to TCERG1 detected by immunofluoresence.

domain. This finding is consistent with a number of studies showing that WW-containing proteins often bind to their target proteins through multiple weak interactions that usually involve more than one WW domain [Goldstrohm et al., 2001]. Indeed, WW domains are almost always present as clusters within their respective proteins. WW domains are also known to mediate interaction with proline-rich protein targets, which is a characteristic of C/EBPα [Ilsley et al., 2002]. Beyond this circumstantial evidence, there is no direct evidence to implicate the WW domains in the interaction with C/EBPα. Another unique domain in the amino terminal portion of TCERG1 that is a possible candidate for the interaction domain is the QA-repeat whose functional role remains unknown.

A major finding of the present study is that C/EBPα can relocalize TCERG1 to compartments other than the pericentromeric domains.

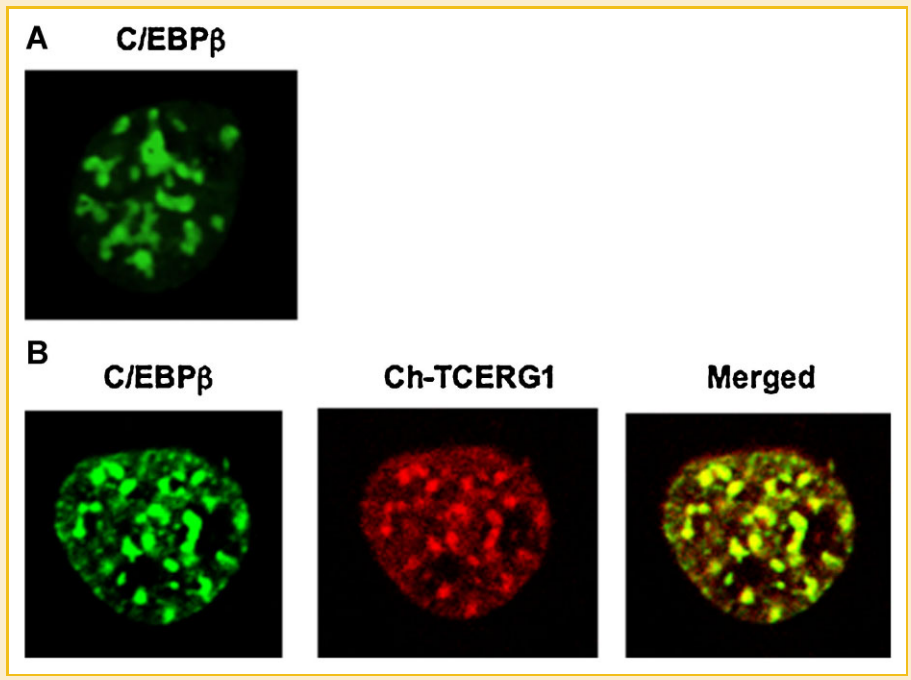


Fig. 6. TCERG1 is also relocalized by C/EBPα. A: COS7 cells were transfected with an expression vector EGFP-C/EBPα. After 50 hr, cells were fixed and analyzed by confocal microscopy. B: Cells were co-transfected with expression vectors for EGFP-C/EBPα and Ch-TCERG1.

While C/EBPα normally compartmentalizes to the pericentromeric regions of chromatin, we showed using the V296A mutant that TCERG1 is recruited to C/EBPα regardless of its subnuclear location. This suggests that the relocalizing activity is inherent to C/EBPα rather than requiring some other factor, such as another protein, that is present in pericentromeric heterochromatin. This is consistent with our previous observation that C/EBPα and TCERG1 could be co-immunoprecipitated from nuclear extracts, suggesting that the two proteins directly interact [McFie et al., 2006]. The overriding conclusion is that the primary determinant for TCERG1 localization is C/EBPα rather than a specific feature of a subcompartment.

Sanchez-Alvarez et al. [2006] first noted that endogenous TCERG1 (then referred to as CA150) was localized to nuclear speckles in HEK293 cells. However, they observed that there were two pools of TCERG1. One was diffusely present throughout the nucleoplasm, while a second was concentrated into speckles of irregular shape and size. In the present study using COS7 cells, we noted that a significant fraction of the cells showed a very similar compartmentalization pattern as observed by Sanchez-Alvarez et al. However, we also noted a second commonly present pattern where the majority of TCERG1 was dispersed throughout the nucleoplasm (Fig. 5B). The basis for these two distinct patterns is unclear. Even in cells where TCERG1 is predominantly concentrated at nuclear speckles, there is usually some fraction that is dispersed, suggesting that there may be some dynamic exchange of TCERG1 between these two pools. If so, then it is easy to imagine that in some cells, perhaps due to cells being in different stages of the cell cycle, the dynamics can change in either direction such that TCERG1 could either be predominantly in speckles or dispersed form. Conversely, the two distinct patterns could simply reflect the different architecture of nuclear speckles that has been observed during different phases of the cell cycle [Lamond and Spector, 2003]. Nuclear speckle organization can be affected by other factors or circumstances, such as when a cell undergoes generalized transcriptional inhibition [Lamond and Spector, 2003]. From the perspective of the present study, an interesting conclusion was that C/EBPα was able to induce TCERG1 relocalization regardless of which compartmentalization pattern TCERG1 displayed. The basis for this conclusion is that in cells in which C/EBPα and TCERG1 were co-expressed, all showed co-localization. Thus, it appears that TCERG1 doesn't need to be in nuclear speckles to be relocalized by C/EBPα, which is the same conclusion we made previously based on the behavior of several TCERG1 mutants [McFie et al., 2006]. These data, along with other evidence presented herein that shows that C/EBPα does not need to be localized to pericentromeric chromatin regions to cause relocalization of TCERG1, strongly suggest that the redistribution is driven by the two proteins themselves rather than mediated or modulated by nuclear architecture. Moreover, our finding that TCERG1 can inhibit C/EBPα activity regardless of whether it is localized to pericentromeric heterochromatin or is dispersed in the nucleoplasm suggests that the inhibition does not involve sequestering C/EBPα at inactive sites in the chromatin. Further analysis of the mechanism behind this inhibition awaits the identification of appropriate mutants of each protein.

The specific cellular functions for TCERG1 remain unclear. Shortly after the discovery of TCERG1, a study by Garcia-Blanco et al. suggested that it may function on some genes as a transcriptional elongation regulator [Sune and Garcia-Blanco, 1999]. Subsequent transcriptome analyses in HEK293 and HeLa cells following knockdown of TCERG1 identified a large number of

genes whose expression was affected [Pearson et al., 2008]. The altered expression of a number of these high confidence TCERG1 target genes was shown to be the result of altered mRNA processing. Of particular relevance to our work was the observation that of the genes whose expression was altered by mechanisms not involving altered mRNA processing, some of them are established or potential C/EBP-regulated genes. For example, the expression of the SOD2 gene, which was found to be upregulated as a result of TCERG1 knockdown, is regulated by C/EBP through a complex response unit which also involves NF-κB [Jones et al., 1997]. Another up-regulated gene that was identified, glutaminase, has two C/EBP binding sites in its promoter [Chung-Bok et al., 1997]. Indeed, Pearson et al. commented that their *en masse* gene expression analyses was not able to discriminate between a variety of possible mechanisms for the altered expression of genes resulting from TCERG1 knockdown [Pearson et al., 2008]. They commented further that one of these mechanisms included the potential for TCERG1 to modulate the activity of transcription factors, in a coregulator mode of function, which could lead to subsequent alterations in the expression of their target genes. Work from our lab, including that reported in the present study, suggest that C/EBPα and β may be examples of such regulators that are modulated by TCERG1 [McFie et al., 2006; Banman et al., 2010]. Precisely how TCERG1 inhibits C/EBP activity is the focus of ongoing studies in our laboratory.

## ACKNOWLEDGMENTS

We thank R. Day, M. Garcia-Blanco, and C. Asselin for plasmids, and D. Mousseau for use of the FV300 laser-scanning confocal microscope in the Cell Signalling Laboratory, University of Saskatchewan. This work was supported by the Hazel Constance Brooker Trust Fund. SLB was supported by a graduate scholarship from the Department of Biochemistry and College of Graduate Studies & Research at the University of Saskatchewan.

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