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Nuclear Redistribution of TCERG1 Is Required for Its Ability to Inhibit the Transcriptional and Anti-Proliferative Activities of C/EBPα

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ABSTRACT

Transcription elongation regulator 1 (TCERG1) is an inhibitor of transcriptional elongation, and interacts with transcription and splicing factors, suggesting that it assists in coupling and coordinating these two processes. Recently we showed that TCERG1 possesses an additional activity, that being to repress the transactivation and anti-proliferative activities of the transcription factor CCAAT/Enhancer Binding Protein α (C/EBPα). In the present study, we provide evidence that TCERG1 functions as an inhibitor of C/EBPα rather than a transcriptional co-repressor. This conclusion was based on reporter gene experiments demonstrating that TCERG1 was able to reverse not only C/EBPα-mediated transactivation of promoter activity, but also C/EBPα-mediated transrepression of a promoter which is inhibited by C/EBPα. These observations, along with our previous findings that TCERG1 inhibits cellular proliferation conferred by C/EBPα, support the relabeling of TCERG1 as an inhibitor C/EBPα. Using mutants of TCERG1, we showed that the inhibitory activity lies in the amino terminal region. Because C/EBPα and TCERG1 have been shown to occupy different subnuclear compartments, we examined whether nuclear relocalization of either protein was involved in the inhibition of C/EBPα by TCERG1. Using confocal microscopy, we showed that TCERG1 localizes to nuclear speckles in the absence of C/EBPα. However, when co-expressed with C/EBPα, TCERG1 localizes to pericentromeric sites where C/EBPα resides. Nuclear redistribution of TCERG1 is required for its inhibitory activity, since mutants that did not display nuclear relocalization also lacked C/EBPα-inhibitory activity. We propose that TCERG1 inhibits C/EBPα activity by keeping it retained in inactive, pericentromeric heterochromatin. J. Cell. Biochem. 109: 140–151, 2010. © 2009 Wiley-Liss, Inc.

KEY WORDS: CCAAT/ENHANCER BINDING PROTEIN; TCERG1; GROWTH ARREST; NUCLEAR REDISTRIBUTION; NUCLEAR SPECKLES

Transcription elongation regulator 1 (TCERG1), previously known as CA150, was originally characterized to inhibit RNA polymerase II (RNAPII) elongation on specific genes through direct binding to the carboxy terminal domain of the polymerase [Sune and Garcia-Blanco, 1999; Carty and Greenleaf, 2002]. Subsequently, non-targeted mass spectrometry studies showed that TCERG1 interacts with a large number of transcriptional and splicing proteins and is part of the spliceosome [Carty and Greenleaf, 2002; Lin et al., 2004]. These observations, along with other studies showing that TCERG1 is localized to speckles in the nucleus that are believed to act as storage and assembly sites for splicing factors, suggest that TCERG1 functions to couple and coordinate transcription and splicing processes [Sanchez-Alvarez et al., 2006].

In order for TCERG1 to coordinate these two processes, it would need to interact with several proteins simultaneously, which in turn would require it to possess a number of protein interaction motifs. This is in fact the case, with TCERG1 possessing three WW and six FF domains that are both well-established protein-protein interaction modules and often reside together in nuclear proteins [Sune et al., 1997]. WW domains are modules that bind to proline-rich domains of target proteins [Macias et al., 2002], and the three WW domains in TCERG1 allow for interaction with several splicing factors that possess proline-rich motifs [Goldstrohm et al., 2001; Lin et al., 2004]. FF domains usually appear in clusters of tandem repeats that allow multiple albeit weak interactions with their target protein [Bedford and Leder, 1999]. In the case of TCERG1, the six FF domains mediate the interaction with RNAPII [Sanchez-Alvarez et al., 2006]. TCERG1 also possesses a glutamine–alanine $(QA)_{38}$ repeat in the amino terminus domain, yet despite its unique feature it has yet to be assigned any critical role except to be

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required for nuclear localization in certain cell types [Arango et al., 2006].

Recently, our lab identified another, apparently unrelated function for TCERG1 that emerged from a yeast two-hybrid screen of a human liver cDNA library to search for potential co-regulators of the transcription factor C/EBPα. C/EBPα is a basic region-leucine zipper protein that is expressed at high levels in adipose, liver, and lung, and it transactivates a large number of genes involved in nutrient metabolism. Using an inactivated transactivation domain of C/EBPα as bait, we identified TCERG1 as a possible interactor [McFie et al., 2006]. The ability of TCERG1 to physically interact with C/EBPα was confirmed by co-immunoprecipitation, and we went on to show that TCERG1 inhibited the C/EBPα-dependent transactivation in reporter gene assays. Moreover, we demonstrated that TCERG1 could reverse C/EBPα-mediated anti-proliferation (herein referred to as growth arrest) of cells, which is an activity distinct from its transactivation function and mediated by a separate region of the protein as well. Thus, it appeared that through its direct interaction with C/EBPα, TCERG1 globally inhibited the activities of this transcription factor.

In the present study, we further characterized the C/EBPα-inhibitory activity of TCERG1 using reporter gene assays, and have concluded that rather than being a co-repressor, it instead acts as an inhibitor of C/EBPα. Moreover, using confocal microscopy, we provide evidence that nuclear relocalization of TCERG1 is involved in the mechanism whereby it exerts this inhibitory effect.

MATERIALS AND METHODS

CELL LINES

HepG2 cells were maintained in DMEM/F12 supplemented with 10% FBS, while COS7 and HEK293T cells were maintained in DMEM supplemented with 17 mM glucose and 10% FBS.

PLASMIDS

The pNF/0.75-luc vector, consisting of the rat HNF-6 promoter linked to the luciferase reporter gene [Rastegar et al., 2000], was a gift from F. Lemaigre (Université de Catholique Louvain). The −68FX4-luc reporter gene is a PEPCK promoter mutant containing four copies of the −355/−200 region of the promoter linked to the −68 to +76 minimal PEPCK promoter [Roesler et al., 1992]. The G5-SV40-luc reporter plasmid, consisting of five GAL4 binding sites linked to the 5′ end of the SV40 early promoter, which drives expression of the luciferase reporter gene [Yu et al., 2000], was a gift from W. Strätling (Universitäts-Krankenhaus Eppendorf). The expression plasmid for the GAL4-TCERG1 fusion protein was generated by cloning the coding region for TCERG1 (amino acids 32–1098) into the appropriate restriction sites in pM2 [Sadowski and Ptashne, 1989]. T7-tagged TCERG1 (originally referred to as T7-CA150) was a gift from M. Garcia-Blanco (Duke University) [Sune et al., 1997]. Hemagglutinin-tagged TCERG1 mutants were generated by cloning *Bgl*II/*Eco*RI fragments of the human TCERG1 coding region into pHA3 plasmid (which contains coding regions for three tandem hemagglutinin epitopes cloned into pRc-CMV, Invitrogen) that was a gift from D. Anderson (Saskatoon Cancer Centre). For TCERG1 mutants which lacked the endogenous nuclear localization signal (NLS) as a result of the cloning strategy, the coding region for the NLS was generated by annealing two synthetic oligonucleotides coding for the NLS, and then cloned into the *Bgl*II site in the pHA3 vector. The sequences of the two oligonucleotides were 5′-GATCCCCTAAGAAGAAGAGGAAAGTCA-3′ (coding strand) and 5′-GATCTGACTTTCCTCTTCTTCTTAGGG-3′ (non-coding). All mutants generated were sequenced to verify the accuracy of cloning and the integrity of the open reading frame. The expression plasmid for EGFP-C/EBPα, with the EGFP linked to the carboxy terminus of C/EBPα [Liu et al., 2002], was a gift from R. Day (University of Virginia). GFP-Sp1 expression plasmid was a gift from O. Rohr (Université de Strasbourg) [Marban et al., 2005], and GFP-C/EBPβ was a gift from C. Asselin (Université de Sherbrooke, Quebec) [Gheorghiu et al., 2001]. Cherry fluorescent protein fusions of TCERG1 FL, 281–1098, and 641–1098 were generated by cloning *Bgl*II/*Eco*RI fragments into mCherry-C1 (Clontech). Mutants 32–293 and 32–668 were cloned into mCherry-C1 as *Bgl*II/*Hin*dIII fragments. MSV-C/EBPα, an expression vector for C/EBPα, was described previously [Park et al., 1990]. RSV-βgal was used an internal transfection efficiency control and has been described previously [Roesler et al., 1993].

LUCIFERASE REPORTER GENE ASSAYS

On the day of transfection, HepG2 cells were sub-cultured into 60 mm plates to approximately 30% confluency and allowed to attach for several hours. Cells were then transfected with Lipofectamine supplemented with Plus reagent (Invitrogen). In all transfections, an expression plasmid for β-galactosidase was included to monitor transfection efficiency. After 40 h, cells were harvested and assayed for luciferase and β-galactosidase activities, and protein concentration as previously described [McFie et al., 2006].

GROWTH ARREST ASSAY

Growth arrest assays were performed in COS7 cells as previously described with some minor modifications [McFie et al., 2006]. Briefly, on day 1 cells were sub-cultured to 20% confluency. The next day, they were transfected with plasmids that expressed EGFP or EGFP-C/EBPα and where appropriate TCERG1 or its related mutants. Transfection was performed using 0.1% polyethylenimine linear (Polysciences, Inc., Warrington, PA) using a polyethylenimine to DNA ratio of 3:1. Cells were washed and fresh medium was added 4 h post-transfection. The next day (day 3), cells were sub-cultured to 50% in duplicate. On day 4, approximately 150 green fluorescing colonies were counted using an Olympus IX-70 inverted fluorescent microscope at 20X objective, and assessed for either being under growth arrest (single cell) or dividing (cluster of two or more cells). Images were captured using SPOT advanced software (Diagnostic Instruments, Sterling Heights, MI) coupled with a digital camera.

LASER-SCANNING CONFOCAL MICROSCOPY

COS7 cells were sub-cultured at 20% confluency in 60 mm plates, and were transfected using 0.1% polyethylenimine at a 3:1 reagent to DNA ratio the following day. One microgram of EGFP or GFP vectors and 10 μg mCherry vectors were used in all transfections. Cells were subsequently sub-cultured to 50% in duplicate 12–24 h

post-transfection in 6-well plates containing sterilized # 1 1/2 glass cover slips. Cells were maintained at 37°C/5% CO_2 for approximately 24 h before washing once with phosphate-buffered saline (PBS) and fixed with a 4% paraformaldehyde/PBS solution for 15 min. Cells were washed three times with PBS for 5 min. Cover slips were air-dried and mounted onto microscope slides using Prolong® Gold Antifade reagent plus DAPI (Invitrogen) and stored in the dark at room temperature for 24 h. For SC35 localization experiments, cells were permeabilized using 0.2% Triton X-100 for 5 min at room temperature following fixation, and then washed twice with PBS for 5 min. Cells were blocked for 15 min at room temperature with 1% bovine serum albumin in PBS, followed by the addition of primary monoclonal SC35 antibody (Abcam, ab11826) diluted to 1:1,000 using blocking solution and incubated at room temperature for 1 h. Cover slips were then washed three times with PBS for 5 min, then incubated with Alexa Fluor® 488 conjugated goat anti-mouse secondary antibody (Invitrogen) diluted to 1:200 in blocking solution for 45 min at room temperature. Cover slips were washed five times with PBS, followed by air drying and mounting as described above. Laser-scanning confocal microscopy was performed using an Olympus FV300 microscope (Cell Signalling Laboratory, University of Saskatchewan).

RESULTS

TCERG1 IS NOT A CO-REPRESSOR OF C/EBPα, BUT RATHER AN INHIBITOR

In a previous study, we identified TCERG1 as a nuclear protein that physically interacted with C/EBPα, and subsequently determined that it repressed C/EBPα-mediated transactivation [McFie et al., 2006]. We thus tentatively labeled it as a co-repressor. However, the finding that TCERG1 also reversed the growth arrest mediated by C/EBPα, which is a function distinct from its transactivation ability, called into question whether it functioned as a classical co-repressor [McFie et al., 2006]. We used two different approaches to address this issue. First, we examined whether TCERG1 could inhibit a strong promoter when it was recruited through a heterologous DNA-binding domain. Co-repressors typically either possess chromatin-modifying activities that repress gene expression, such as histone deacetylase activity or recruit proteins to promoters that possess these activities [Berger, 2001; Privalsky, 2004]. Therefore, tethering of a co-repressor via a GAL4 domain will inhibit promoter activity, such has been observed for the methyl-CpG-binding protein 2 and Small Unique Nuclear receptor CoRepressor [Zamir et al., 1997; Yu et al., 2000]. We used the SV40 early promoter since it is unresponsive to native TCERG1 [Sune and Garcia-Blanco, 1999]. Five GAL4 binding sites were ligated to the 5′ end of this promoter, and was then examined for its ability to be repressed by co-expression of a GAL4-TCERG1 fusion protein in a luciferase reporter gene assay in HepG2 cells. As shown in Figure 1A, the GAL4 DNA-binding domain had no effect on promoter activity, and the GAL4-TCERG1 fusion protein similarly showed no repressor activity. We also tested native TCERG1 for its effects on this promoter, and as expected based on a previous study [Sune and Garcia-Blanco, 1999], it provided no repression. These data indicated that, unlike classical co-repressors, TCERG1 has no intrinsic transcription repression activity. In order to further address this issue, we examined the effect of TCERG1 on two classes of C/EBPα-responsive promoters; those that are activated by C/EBPα and those that are inhibited. We hypothesized that if TCERG1 functioned as a co-repressor, over-expression of TCERG1 should reverse C/EBPα-mediated transactivation and enhance C/EBPα-mediated transrepression. We used the −68FX4 promoter as a strong C/EBPα-transactivated promoter, and the HNF6 gene promoter as a representative of genes that are repressed by C/EBPα [Rastegar et al., 2000]. As shown in Figure 1B, C/EBPα activated the −68FX4 promoter, and this transactivation was repressed by co-expression of TCERG1. Interestingly, the HNF6 promoter response to C/EBPα and TCERG1 was exactly the opposite (Fig. 1C). C/EBPα expression resulted in an inhibition of promoter activity, which was completely reversed by co-expression of TCERG1. These findings, together with our previous data showing that TCERG1 reverses C/EBPα-mediated growth arrest in cells, suggest that TCERG1 is more accurately classified as an inhibitor of C/EBPα rather than as a co-repressor.

THE C/EBPα INHIBITORY ACTIVITY LIES WITHIN RESIDUES 32–668 OF TCERG1

We next investigated what region of TCERG1 confers the C/EBPα inhibitory activity. Hemagglutinin-tagged deletion mutants were generated as shown in Figure 2. The endogenous nuclear localization region was deleted in mutants 641–1098 and 32–293, therefore we cloned the NLS into these expression vectors. All mutants were expressed at similar levels as determined by Western blot analysis (data not shown), and all showed almost exclusive localization to the nucleus, except for mutant 641–1098 where some cytosolic presence was seen (see confocal microscopy data in Fig. 5A). These mutants were expressed in COS7 cells and examined for their ability to inhibit C/EBPα-mediated growth arrest. Growth arrest was quantified by transfecting cells with either enhanced green fluorescent protein (EGFP) (control) or EGFP-C/EBPα, sub-culturing them into well-separated single cells, and then assessing on day 4 the percentage of green fluorescent colonies that consisted of a single cell (growth arrested) or of two or more cells (proliferating) (Fig. 3A). In these growth arrest studies, EGFP was used simply as a way to visualize transfected cells for assessment. Initially, we confirmed that neither full-length TCERG1 (TCERG1 FL) nor any of the mutants showed any effect on cell proliferation compared to control cells when tested in the absence of C/EBPα (Fig. 3B), with all experimental groups having between 65% and 73% of cells demonstrating proliferative capacity. These mutants were next examined for their ability to inhibit C/EBPα-dependent growth arrest. Control cells (in the absence of C/EBPα and TCERG1 expression) showed 65% clusters and 35% single cells 4 days after cells were sub-cultured (Fig. 3C). In the presence of C/EBPα expression, 85% of cells were present as single cells and thus under growth arrest. Co-expression of TCERG1 FL entirely reversed the growth arrest produced by C/EBPα, as did mutant 32–668, supporting with our hypothesis that the amino terminal domain contains the C/EBPα inhibitory domain. Consistent with our previous findings [McFie et al., 2006], the carboxy terminal mutant 641–1098 showed little if any inhibitory activity. Two additional

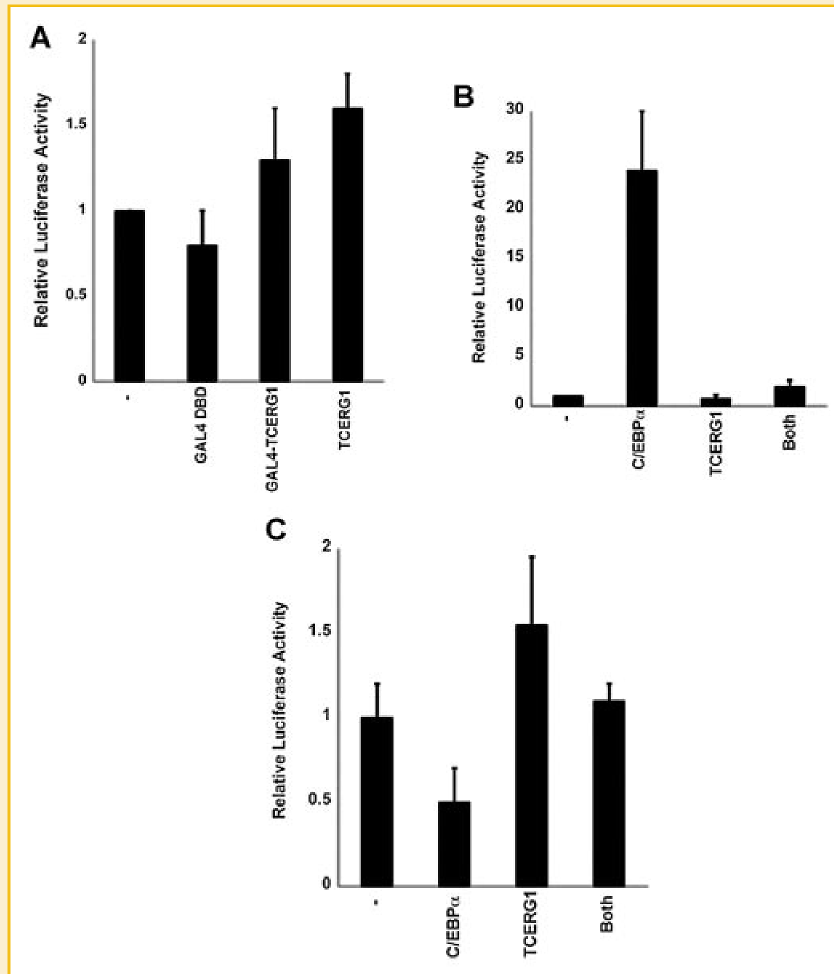

Fig. 1. TCERG1 inhibits C/EBPα but has no intrinsic repressor activity. A: HepG2 cells were transfected with 2 μg G5-SV40-luc reporter gene along with 5 μg of expression vectors for the GAL4 DNA binding domain (GAL4 DB), GAL4-TCERG1, or TCERG1. B: HepG2 cells were transfected with 1 μg −68FX4-luc reporter gene along with expression vectors for C/EBPα (2 μg) and/or TCERG1 (7 μg). C: HepG2 cells were transfected with 1 μg of pNF/0.75-luc along with expression vectors for C/EBPα (50 ng) and/or TCERG1 (1 μg). In all transfections, 1 μg of RSV-βgal was included as an internal control to monitor transfection efficiency. Values shown are the means ± SE of at least three experiments performed in triplicate, and are relative to the luciferase activity obtained with reporter gene only which was set at 1.0.

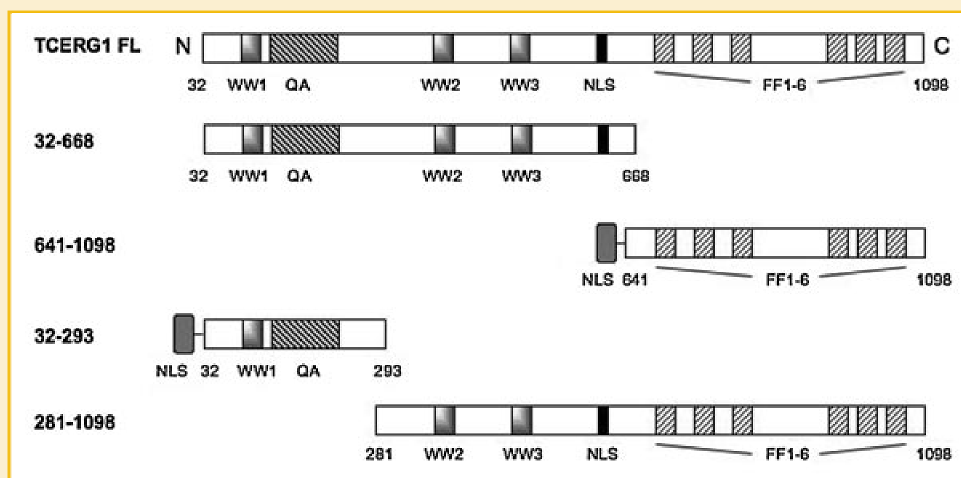

Fig. 2. Schematic of TCERG1 mutants used in this study. A schematic of the full-length TCERG1 (TCERG1 FL) along with the related mutants are shown, with the structural and functional domains highlighted. The mutants are named based on the amino acids of TCERG1 that are included in each mutant, which are shown underneath each mutant at the amino and carboxy ends. QA refers to the glutamine/alanine repeat; NLS refers to the nuclear localization signal.

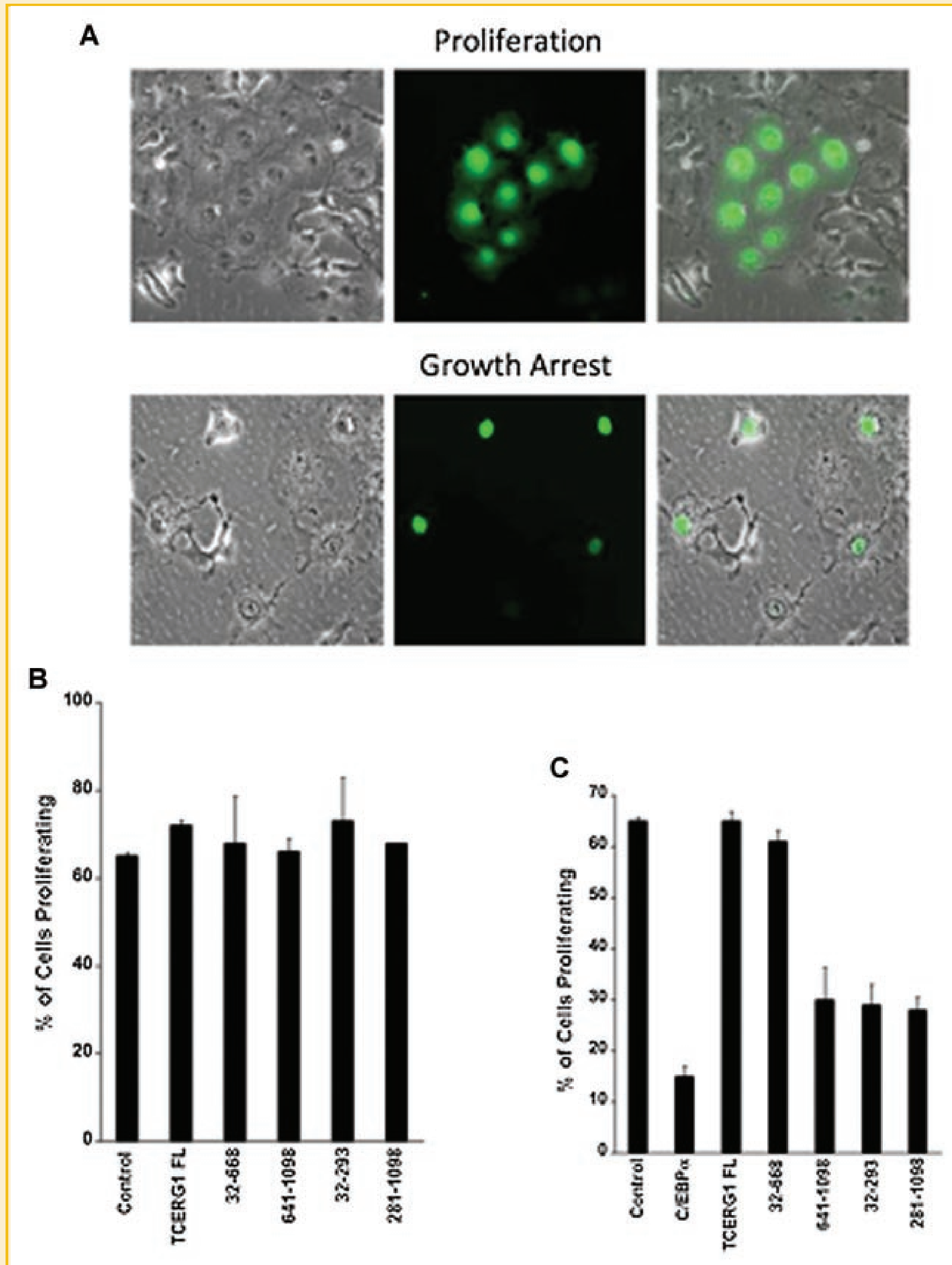

Fig. 3. The amino terminus of TCERG1 can reverse C/EBPα-induced growth arrest. A: An example of results from growth arrest assays, showing COS7 cells that are proliferating or under growth arrest. The left-hand panel shows a phase-contrast image; the middle panel shows the green fluorescence emitted by EGFP; and the right-hand panel shows the overlay of the two images. B: COS7 cells were transfected with an expression vector for EGFP (control) along with expression vectors for the TCERG1 mutants indicated, and assessed for proliferation or growth arrest as described in the Materials and Methods Section. Values shown are reported as being the percent of colonies appearing as clusters of two or more cells, evidence of proliferation. C: The same experiment as described in panel B was performed, except that the TCERG1 mutants were tested in the presence of EGFP-C/EBPα. Control indicates proliferation assessed when EGFP alone was expressed. The values shown are the means ± SE of at least three experiments performed in duplicate.

mutants were generated to further dissect the amino terminal domain. However, neither 32–293 nor 281–1098 showed significant ability to reverse C/EBPα-mediated growth arrest (Fig. 3C).

C/EBPα REDISTRIBUTES TCERG1 IN THE NUCLEUS IN COS7 CELLS

While both C/EBPα and TCERG1 localize to the nucleus, the specific subnuclear locations in which they reside differ. C/EBPα has been shown to localize to pericentromeric regions in a wide variety of cells and is used as a pericentromeric marker [Tang and Lane, 1999; Schaufele et al., 2001; Zhang et al., 2001], while TCERG1 has been shown to concentrate in nuclear speckles [Sanchez-Alvarez et al., 2006], which are compartments known to be rich in splicing factors [Lamond and Spector, 2003]. However, proteins are capable of moving quite rapidly in the nucleus, and as a result their subnuclear localization pattern can be dynamic in nature [Misteli, 2001]. Thus, we were interested in whether the inhibition of C/EBPα by TCERG1 involved relocalization of either protein.

EGFP-tagged C/EBPα and mCherry-tagged TCERG1 were used in confocal microscopy experiments in COS7 cells. The EGFP-C/EBPα expression vector that we used has the EGFP fused to the carboxy terminus of C/EBPα; this fusion protein was shown previously to retain its transactivation activity and to localize within the nucleus similarly to native C/EBPα [Schaufele et al., 2001]. As shown in Figure 4A, while EGFP alone showed no specific cellular localization pattern, EGFP-C/EBPα displayed nuclear-restricted expression, and the pattern was very similar to the pericentromeric heterochromatin staining pattern observed in COS7 by others [Weisbart et al., 2000; Warton et al., 2004]. To further confirm that C/EBPα is localizing to pericentromeric domains, we analyzed the compartmentation demonstrated by GFP-C/EBPβ since this isoform also shows pericentromeric localization [Tang and Lane, 1999]. As can be seen in Figure 4A, C/EBPβ showed a nuclear distribution pattern similar to that of C/EBPα.

mCherry alone localized diffusely throughout the cell, without any specific subcellular compartmentation observed (Fig. 4A). mCherry-tagged TCERG1 FL however, localized only to the nucleus and showed a discrete pattern reminiscent of nuclear speckles. We confirmed that the subnuclear compartment to which it localized were nuclear speckles by comparing it with the sites occupied by endogenous SC35, a splicing factor that has been shown to reside in nuclear speckles. As can be observed in the merged panel in Figure 4B, TCERG1 and SC35 entirely overlapped in their nuclear localization.

As a negative control for the co-localization experiments, we also examined GFP-tagged Sp1 based on our previous study where we showed that TCERG1 was unable to inhibit the transactivation produced by this transcription factor [McFie et al., 2006]. Sp1 showed primarily diffuse localization throughout the nucleus, although there were several foci where it was highly concentrated (Fig. 4A).

We next examined the localization patterns of C/EBPα and TCERG1 when they were co-expressed in COS7 cells (Fig. 4C). The subnuclear localization pattern of C/EBPα did not change, maintaining a pattern similar to that when it was expressed in the absence of TCERG1. However, the pattern for TCERG1 localization changed significantly to one resembling that of C/EBPα, which was confirmed by merging the two patterns (where yellow indicates regions of overlap). Strikingly, there were no areas of red fluorescence in the merged panel, indicating that all of the TCERG1 co-localized with C/EBPα. In contrast, when Sp1 and TCERG1 were co-expressed, TCERG1 maintained the nuclear speckle pattern observed when it was expressed alone (compare Fig. 4D and Fig. 4A). Merging of the Sp1 and TCERG1 signals indicated that there was little if any specific overlap in their localization within the nucleus, with both red and green fluorescence observable.

The TCERG1 mutants shown in Figure 2 were then examined for their nuclear localization pattern and their ability to co-localize with C/EBPα. Initially, mCherry-tagged TCERG1 mutants were analyzed in COS7 cells in the absence of C/EBPα (Fig. 5A). All of the mutants, except for 641–1098, showed primarily nuclear localization. When examined in the presence of C/EBPα expression, distinct differences between the mutants were observed. The only mutant that showed complete co-localization with C/EBPα was TCERG1 32–668 (Fig. 5B), and it appeared to adopt the typical pattern displayed by C/EBPα, that is, underwent nuclear redistribution. This observation was in contrast to the 641–1098 mutant, which showed little if any co-localization with C/EBPα (Fig. 5C), which is consistent with its functional inactivity as tested in growth arrest assays described above. The other two mutants, 32–293 and 281–1098, showed partial overlap with C/EBPα (Fig. 5D,E). However, neither mutant showed any significant change in its localization pattern in response to C/EBPα expression, but rather it appeared that their inherent localization patterns simply overlapped to some degree with that of C/EBPα (compare Fig. 5A with Fig. 5D and E, and data not shown). Nonetheless, the presence of discrete, red fluorescent foci in the merged panels of each of these two mutants indicates that they do not co-localize with C/EBPα with the same efficiency as full-length TCERG1 or the 32–668 mutant.

C/EBPα REDISTRIBUTES TCERG1 IN THE NUCLEUS OF HEK293 CELLS

The co-localization experiments were repeated in HEK293T cells to examine whether C/EBPα-induced redistribution of TCERG1 occurred in cell lines other than COS7. HEK293 cells were chosen since we had shown previously that TCERG1 inhibits the C/EBPα-dependent growth arrest in these cells [McFie et al., 2006]. As shown in Figure 6A, C/EBPα when expressed alone displayed a pattern typical of pericentromeric localization, with discrete nuclear foci present, and was similar to that of C/EBPβ. TCERG1 displayed a significantly different pattern. Figure 6B shows that TCERG1 localized to nuclear speckles since it overlapped with SC35. In cells where C/EBPα and TCERG1 were co-expressed, the pattern of TCERG1 changed to closely resemble that of C/EBPα, with complete overlap again being observed (Fig. 6C). Similar to what we observed in COS7 cells, Sp1 and TCERG1 displayed no overlap nor was there any change in TCERG1 distribution in the nucleus when co-expressed with Sp1 (Fig. 6D). The N-terminus and C-terminus domain mutants of TCERG1 were also examined. Figure 7A shows the nuclear distribution of TCERG1 FL and the two mutants when expressed alone. The 32–668 mutant had a distribution pattern similar to that of full-length protein (Fig. 7A). Moreover, its

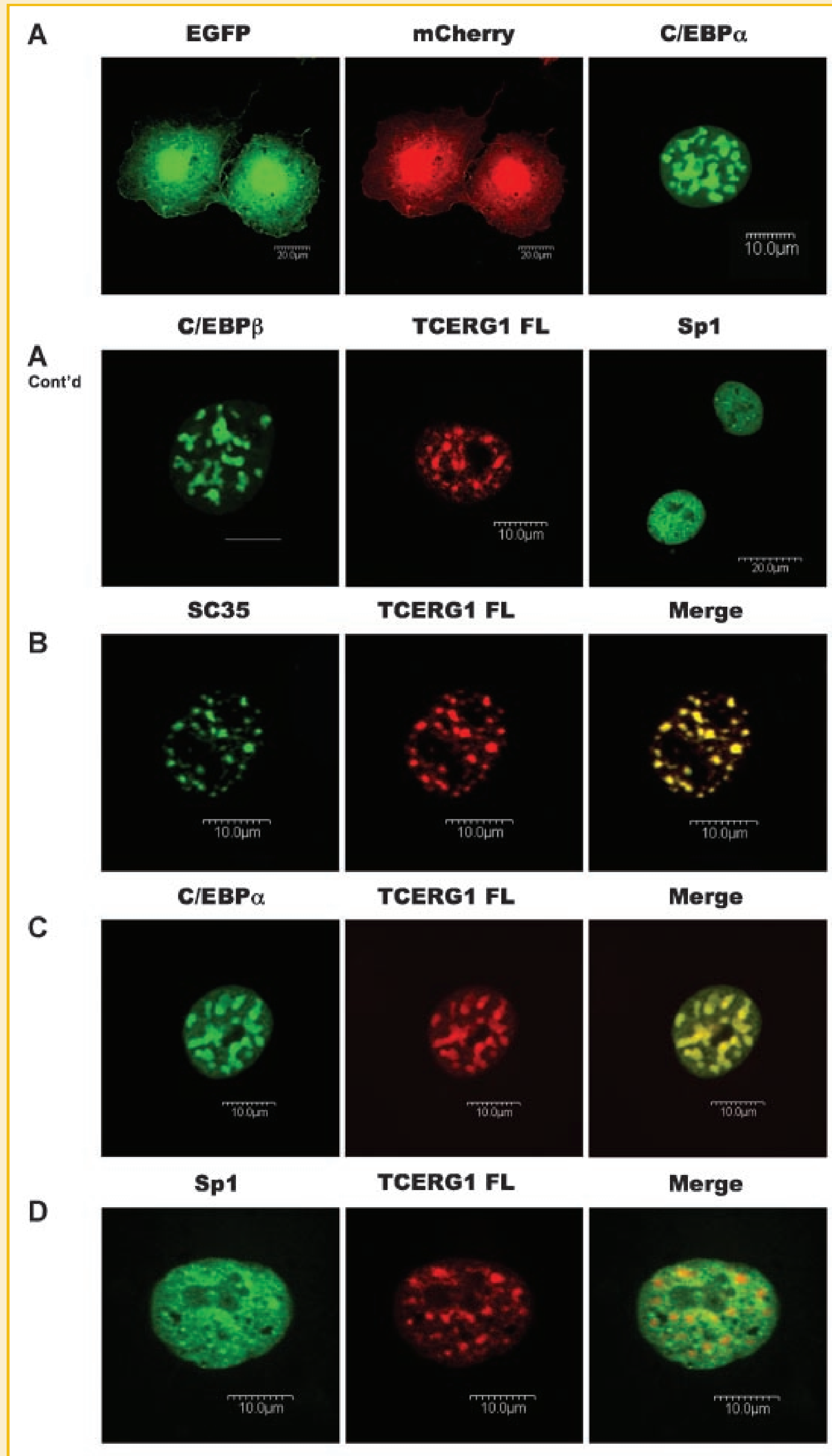

Fig. 4. TCERG1 redistributes itself to intranuclear sites occupied by C/EBPα. A: COS7 cells were transfected with expression vectors for EGFP, mCherry, EGFP-C/EBPα, GFP-C/EBPβ, mCherry-TCERG1 FL, or GFP-Sp1, and then analyzed by confocal laser-scanning microscopy. B: Cells were transfected with mCherry-TCERG1 FL. SC35 was detected using a primary antibody in conjunction with an Alexa Fluor® 488 conjugated secondary antibody. C: Cells were transfected with expression vectors for EGFP-C/EBPα and mCherry-TCERG1 FL. D: Cells were transfected with expression vectors for GFP-Sp1 and mCherry-TCERG1.

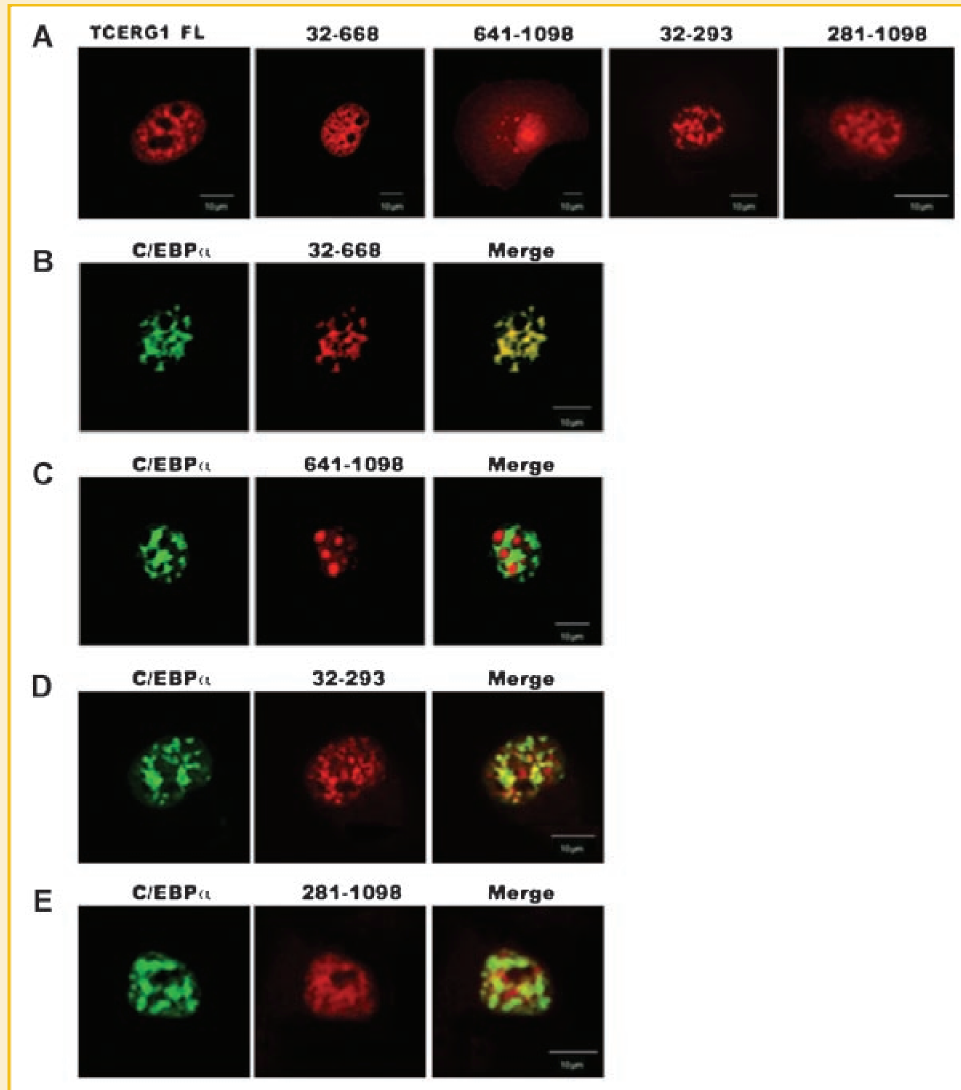

Fig. 5. The amino terminus of TCERG1 is sufficient for C/EBPα-induced intranuclear redistribution. A: COS7 cells were transfected with expression vectors for mCherry-TCERG1 FL or its related mutants, and then analyzed by confocal laser-scanning microscopy. B–E: Cells were transfected with an expression plasmid for the indicated mCherry-TCERG1 mutant along with EGFP-C/EBPα.

subnuclear localization pattern was significantly altered when co-expressed with C/EBPα, with complete co-localization observed (Fig. 7B). The carboxy terminal domain mutant of TCERG1, 641–1098, showed only partial nuclear localization when expressed alone (Fig. 7A), and the pattern within the nucleus was significantly altered relative to full-length, with one or more bright foci usually being present. In the presence of C/EBPα, its pattern remained unaltered and no overlap of localization with C/EBPα was observed (Fig. 7C).

DISCUSSION

TCERG1 was originally characterized as a gene-specific transcriptional elongation factor, and was shown to form a complex with the phosphorylated form of RNAPII as well as with other elongation factors. Subsequently, an expanded nuclear role for TCERG1 was suggested by the observations that it is involved in pre-mRNA splicing and able to interact with several components of the spliceosome. Thus, the emerging model for the biological role of TCERG1 is that it is a nuclear protein that couples the processes of transcriptional elongation and mRNA processing. In further support of this hypothesis is the observation that TCERG1 localizes to discrete nuclear compartments called nuclear speckles, which have been shown to be enriched in splicing factors and to serve as a reservoir of these proteins that are recruited to sites of active gene transcription.

Work from our laboratory has suggested that the biological role for TCERG1 may be even broader. In our efforts to identify cellular interactors with C/EBPα using a two-hybrid screen, TCERG1 was identified as a potential candidate [McFie et al., 2006]. Subsequent characterization indicated that not only did C/EBPα and TCERG1

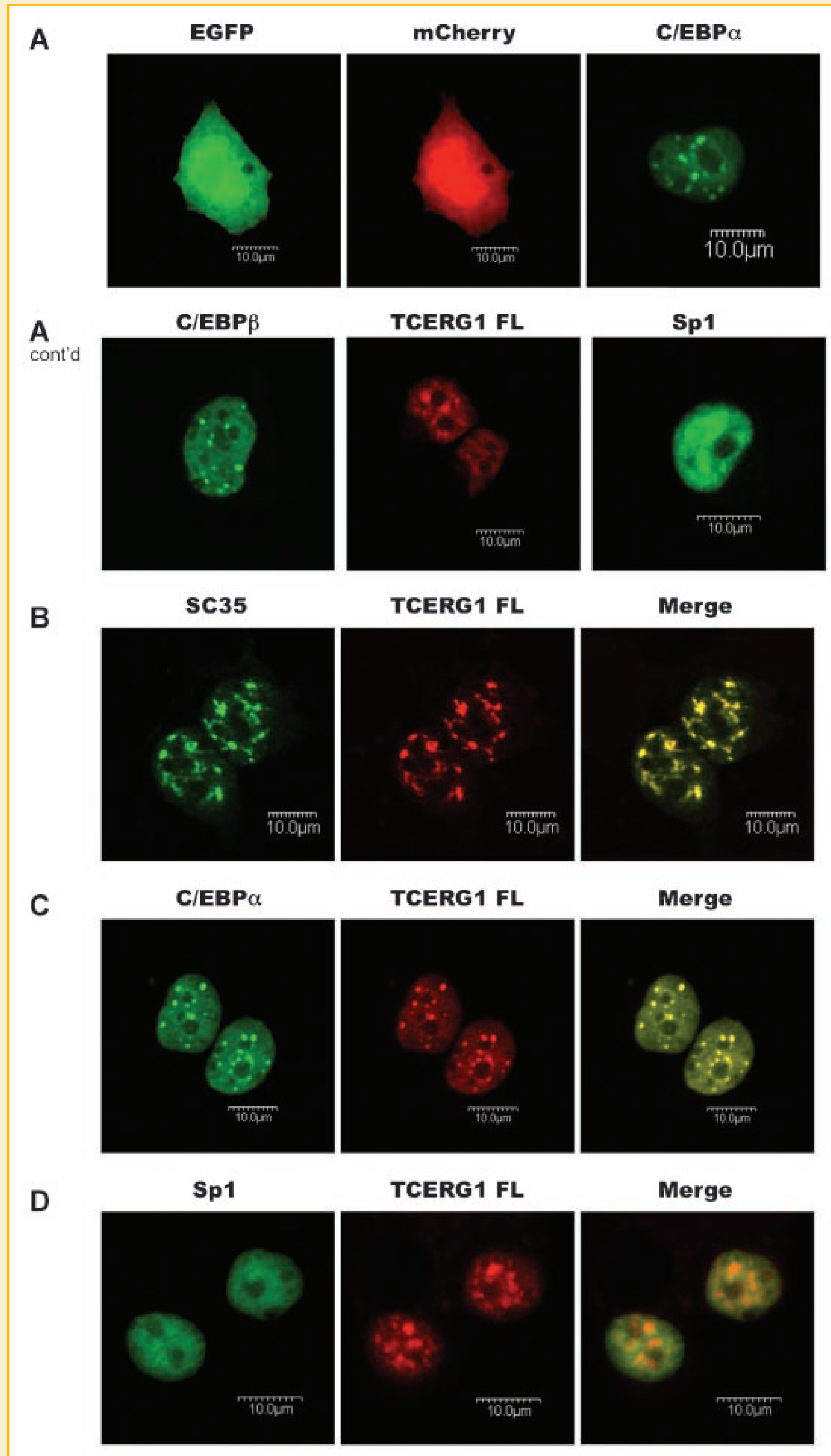

Fig. 6. C/EBPα induces TCERG1 redistribution in the nuclei of HEK293 cells. A: HEK293 cells were transfected with expression vectors for EGFP, mCherry, EGFP-C/EBPα, GFP-C/EBPβ, mCherry-TCERG1 FL, or GFP-Sp1, and then analyzed by confocal laser-scanning microscopy. B: Cells were transfected with mCherry-TCERG1 FL. SC35 was detected using a primary antibody in conjunction with an Alexa Fluor® 488 conjugated secondary antibody. C: Cells were transfected with expression vectors for EGFP-C/EBPα and mCherry-TCERG1 FL. D: Cells were transfected with expression vectors for GFP-Sp1 and mCherry-TCERG1.

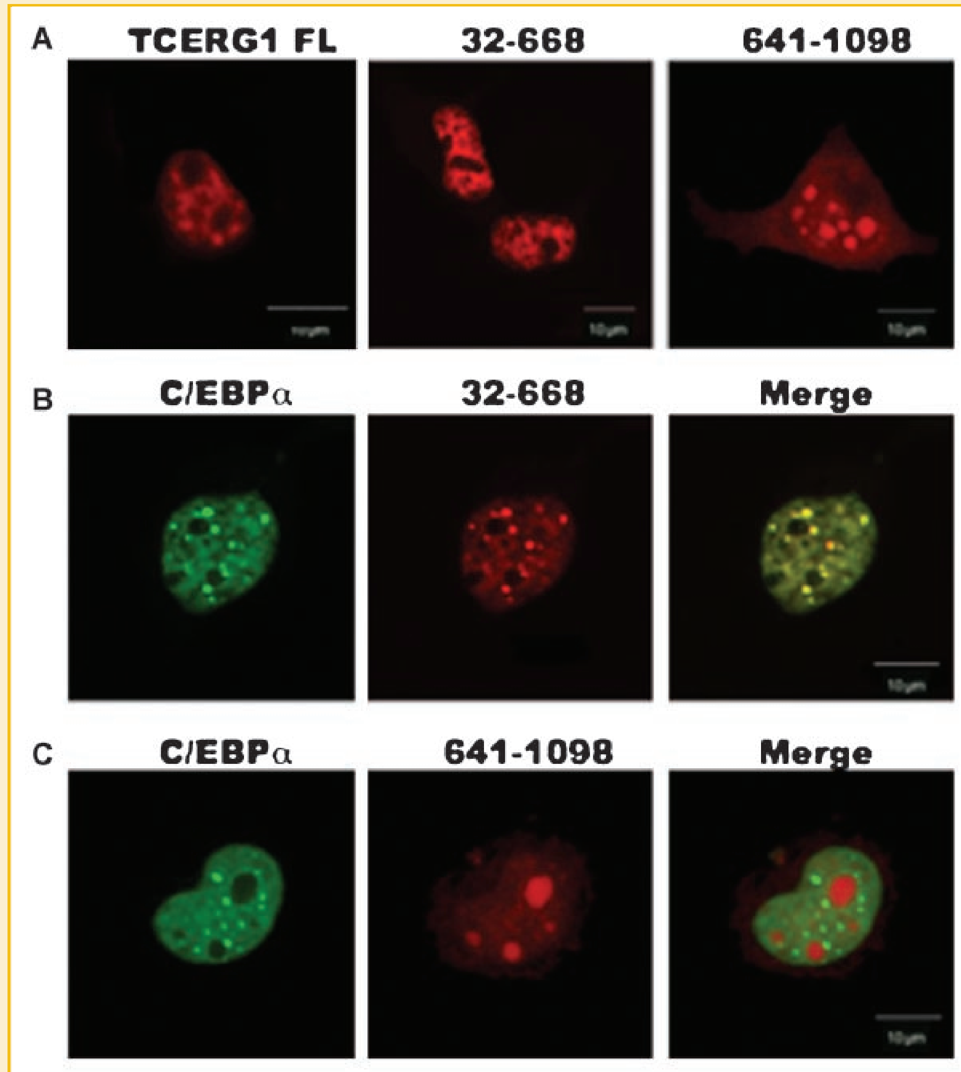

Fig. 7. The amino terminus of TCERG1 is sufficient for C/EBPα-induced intranuclear redistribution in HEK293T cells. A: HEK293T cells were transfected with expression vectors for mCherry-TCERG1 FL or its related mutants, and then analyzed by confocal laser-scanning microscopy. B,C: Cells were transfected with an expression plasmid for the indicated mCherry-TCERG1 mutant along with EGFP-C/EBPα.

physically interact in the cell, but that there was also a functional consequence to this interaction. Over-expression of TCERG1 inhibited C/EBPα-mediated transactivation of a reporter gene, and also reversed C/EBPα-dependent growth arrest of cells. This finding was intriguing for two reasons. First, since TCERG1 was able to inhibit two distinct activities of C/EBPα, that is, transactivation and growth arrest, it was clear that the mechanism where by this inhibition occurred could not be explained by the known functions for TCERG1. Secondly, since these two distinct activities of C/EBPα are mediated by separate domains yet TCERG1 is able to inhibit both of these activities suggested that TCERG1 inhibits C/EBPα through a global, general mechanism.

While we originally labeled TCERG1 as a co-repressor of C/EBPα, due to its ability to block C/EBPα-dependent transactivation, the ability of TCERG1 to reverse C/EBPα-mediated growth arrest did cast some doubt on the accuracy of this label. The findings of the present study have now led us to the conclusion that a more precise label for TCERG1 is that it is an inhibitor of C/EBPα. This relabeling is based on the observation that regardless of whether C/EBPα was examined as a mediator of transactivation, transrepression, or growth arrest, over-expression of TCERG1 was able to inhibit all three of these activities (Figs. 1B,C and 3C). Moreover, our results suggest that TCERG1 has no endogenous transcriptional repressor activity (Fig. 1A).

These observations ruled out certain mechanisms that might explain how TCERG1 inhibits the activity of C/EBPα, but left the question unanswered. An examination of the literature related to the biology of C/EBPα and how its activity is regulated suggested that nuclear compartmentation may be involved. Rather than being evenly dispersed throughout the nucleoplasm, C/EBPα and related isoforms localize to pericentromeric regions in heterochromatin. However, this localization pattern can be dynamic in nature.

Enwright et al. [2003] showed that co-expression of the transcription factor Pit-1 triggered movement of C/EBPα from the pericentromeric compartments to the subnuclear sites occupied by Pit-1. Pilipuk et al. [2003] provided evidence that subnuclear localization can be regulated by extracellular stimuli, by showing that C/EBPβ exhibits a diffuse distribution in the nuclei of preadipocytes, but rapidly moves to pericentromeric regions upon treatment with growth hormone. It has also been shown that C/EBPβ and C/EBPδ both move to pericentromeric domains concomitant with adipocyte differentiation [Tang and Lane, 1999]. While these studies highlight that C/EBP family members are capable of nuclear redistribution, there have also been studies suggesting that C/EBPs are able to recruit nuclear factors to their pericentromeric location [Schaufele et al., 2001]. Since C/EBPα and TCERG1 had been reported to occupy distinct nuclear compartments, pericentromeric regions, and nuclear speckles, respectively, we set out to examine whether redistribution of either protein occurred when they were co-expressed in cells.

It became evident from initial experiments that C/EBPα and TCERG1 adopted different nuclear distribution patterns in COS7 cells when expressed individually, and our experimentation further showed that as expected, they localized to pericentromeric and nuclear speckles, respectively. When they were co-expressed, it was evident that it was the distribution pattern of TCERG1, and not C/EBPα, that became altered. Moreover and perhaps most dramatically, the redistribution of TCERG1 was always observed to be complete, with no evidence of any TCERG1 (red foci) remaining in the nuclear speckles. The same total redistribution was observed with the N-terminal domain mutant TCERG1 32–668, despite the fact that this mutant displayed a different nuclear distribution pattern in COS7 cells compared with full-length TCERG1 in the absence of C/EBPα (Fig. 5). This finding indicates that C/EBPα-mediated redistribution of TCERG1 is independent of TCERG1's initial site of residence in the nucleus.

The ability of the N-terminus TCERG1 mutant to be re-localized by C/EBPα is also consistent with our hypothesis that it is through the N-terminal domain that TCERG1 physically interacts with C/EBPα. This hypothesis was initially based on the fact that the TCERG1 clone identified by our two-hybrid screen contained the coding region for amino acids 89–480, which indicated that this domain was sufficient for interaction with C/EBPα [McFie et al., 2006]. This was indirectly supported by other experiments in the same study showing that that carboxy terminal domain (amino acids 641–1098) was incapable of either interacting with C/EBPα in a two-hybrid assay, repressing the activity of C/EBPα, or inhibiting C/EBPα-mediated growth arrest. Furthermore, in the present study, we show that the C-terminal domain mutant (TCERG1 641–1098) was incapable of being re-localized by C/EBPα. It should also be noted that the N-terminal domain mutant, 32–668, retained full capacity to inhibit C/EBPα-mediated growth arrest (Fig. 3C). Thus, the cumulative evidence suggests a model whereby C/EBPα recruits TCERG1 from nuclear speckles, through direct interactions with the amino terminus of TCERG1, to pericentromeric regions, which leads to repression of C/EBPα activity. Furthermore, the present study allows the conclusion that only domains in the amino terminus of TCERG1 are required for this entire process to occur.

Precisely what sub-domains in the amino terminus of TCERG1 are responsible for the C/EBPα inhibitor activity is not yet known, but the WW1 and WW2 domains are likely candidates. WW domains are motifs known to mediate interactions with proteins that have proline-rich domains, which C/EBPα possesses [Landschulz et al., 1988]. Secondly, WW-domain proteins usually have multiple such domains that are engaged in the interaction with target proteins. The third piece of evidence suggesting that WW1 and WW2 are both required for interaction with C/EBPα comes from our mutational analyses presented in this study. Mutants that lacked WW2 (32–293) or WW1 (641–1098 and 281–1098) were shown in growth arrest assays to have lost most of their C/EBPα inhibitory activity, whereas mutant 32–668 that possesses both of these domains retained full C/EBPα inhibitory activity and co-localization ability. Moreover, co-localization experiments indicate that mutants 32–293 and 281–1098 only partially overlap with C/EBPα, and no evidence of C/EBPα-induced nuclear redistribution was evident. Interestingly, WW1 and WW2 have also been implicated in the transcriptional repression activity of TCERG1, and for its ability to interact with splicing factors [Goldstrohm et al., 2001]. For these reasons, one can reasonably speculate that both WW1 and WW2 domains are required for the C/EBPα inhibitory activity of TCERG1. However, a role for the QA repeat, which lies between the two WW domains, cannot be ruled out based on the mutants that we examined.

The finding that TCERG1 can be recruited to intranuclear sites occupied by C/EBPα suggests a mechanism whereby TCERG1 is able to inhibit C/EBPα activity. As mentioned earlier, C/EBPα concentrates at pericentromeric regions within heterochromatin, and this localization as been shown to require the DNA-binding activity of C/EBPα [Schaufele et al., 2001]. Interestingly, α-satellite repetitive DNA is abundant at pericentromeric regions [Joseph et al., 1989], and C/EBPα can bind to this repeat sequence [Liu et al., 2007]. This suggests that the pool of C/EBPα that collects in these pericentromeric regions may serve as a reservoir for this protein. We speculate that C/EBPα can then be recruited from this reservoir to gene promoters where it can regulate transcription or to other nuclear compartments where it can mediate growth arrest. Evidence to support this hypothesis comes from studies showing that the transcription factor Pit-1, which synergistically binds to and activates the prolactin and growth hormone gene promoters in conjunction with C/EBPα [Schaufele, 1996], is able to translocate C/EBPα away from its pericentromeric regions to sites where Pit-1 resides [Enwright et al., 2003]. Based on this scenario, a possible mechanism for the inhibitory action of TCERG1 would be that it blocks the translocation of C/EBPα, thereby keeping it retained in inactive heterochromatin regions. While this hypothesis remains to be tested, the findings of our study add to the growing number of examples that emphasize the importance of nuclear localization of proteins in the regulation of gene expression and other nuclear processes.

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