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Regulation of c-MYC transcriptional activity by transforming growth factor-beta 1-stimulated clone 22

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c-MYC stimulates cell proliferation through the suppression of cyclin-dependent kinase (CDK) inhibitors including P15 (*CDKN2B*) and P21 (*CDKN1A*). It also activates E-box-mediated transcription of various target genes including telomerase reverse transcriptase (*TERT*) that is involved in cellular immortality and tumorigenesis. Transforming growth factor-beta 1 (TGF-β1)-stimulated clone 22 (TSC-22/*TSC22D1*) encodes a highly conserved leucine zipper protein that is induced by various stimuli, including TGF-β. TSC-22 inhibits cell growth in mammalian cells and in *Xenopus* embryos. However, underlying mechanisms of growth inhibition by TSC-22 remain unclear. Here, we show that TSC-22 physically interacts with c-MYC to inhibit the recruitment of c-MYC on the *P15* (*CDKN2B*) and *P21* (*CDKN1A*) promoters, effectively inhibiting c-MYC-mediated suppression of *P15* (*CDKN2B*) and also *P21* (*CDKN1A*) promoter activities. In contrast, TSC-22 enhances c-MYC-mediated activation of the *TERT* promoter. Additionally, the expression of TSC-22 in embryonic stem cells inhibits cell growth without affecting its pluripotency-related gene expression. These results indicate that TSC-22 differentially regulates c-MYC-mediated transcriptional activity to regulate cell proliferation.

KEYWORDS
c-MYC, cyclin-dependent kinase inhibitor, growth inhibition, transcriptional regulation, TSC-22

1 | INTRODUCTION

MYC is a proto-oncogene that encodes a basic helix-loop-helix leucine zipper (bHLH-LZ) transcriptional factor named c-MYC. Overexpression of c-MYC and MYC family proteins has been detected in numerous human cancers, including Burkitt's lymphoma (c-MYC), neuroblastoma (N-MYC), and small cell lung cancer (L-MYC).[1-3] *MYC* is an early response gene that is activated in response to mitogenic signals[4] and has a short half-life with tight transcriptional, post-transcriptional, and post-translational controls.[4-6]

c-MYC dimerizes with MAX, and the c-MYC-MAX heterodimer can activate various genes by directly binding to a specific DNA sequence, termed E-box (5′-CACGTG-3′).[7] E-boxes are found in the promoters of a large group of c-MYC-induced genes that also include protein-coding genes (eg telomerase reverse transcriptase

Abbreviations: bHLH-LZ, basic helix-loop-helix leucine zipper; CDK, cyclin-dependent kinase; ID2, inhibitor of DNA binding 2; LIF, leukemia inhibitory factor; TERT, telomerase reverse transcriptase; TSC-22, transforming growth factor-β1-stimulated clone 22.

Zheng and Suzuki contributed equally to this work.

[*TERT*], cyclin D2 [*CCND2*], and ribosomal RNA genes).[8-10] In contrast, c-MYC can repress various genes, including cyclin-dependent kinase inhibitors such as *P15* (*CDKN2B*) and *P21* (*CDKN1A*),[11,12] through its interaction with the zinc finger protein MIZ-1 at the initiator elements of their promoters.[11,13] c-MYC-mediated transcriptional activation or repression of the target genes influences many biological processes, including the promotion of cell proliferation, immortalization, inhibition of terminal differentiation, and induction of apoptosis.

Transforming growth factor-beta 1 (TGF-β1)-stimulated clone 22 (TSC-22/*TSC22D1*) was first identified as a target gene of TGF-β1 in mouse osteosarcoma cells.[14] TSC-22 and TSC-22 family members (KIAA0669/*TSC22D2*, GILZ/*TSC22D3*, and THG-1/*TSC22D4*) have a conserved TSC-box and a leucine zipper domain.[15] These proteins form homo- or heterodimers with other family members. During mouse embryogenesis, TSC-22 is expressed at the site of epithelial-mesenchymal interaction[16] and has transcriptional repressor activity when fused to a heterologous DNA-binding domain.[15] The expression of TSC-22 in cultured cells or in *Xenopus* embryo inhibits cellular proliferation.[17,18] However, the mechanism of growth inhibition by TSC-22 has not been determined. During our trial to elucidate the mechanism of TSC-22, we found that TSC-22 bound to c-MYC.

In the present study, we investigated the regulation of c-MYC transcriptional activity by TSC-22 and showed the mechanism of growth inhibition by TSC-22.

2 | MATERIALS AND METHODS

2.1 | Cell culture

HEK293T cells and HaCaT cells were obtained from the ATCC and Dr N.E. Fusenig, respectively. These were cultured in DMEM (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% FBS, penicillin G (100 U/mL), and streptomycin sulfate (0.1 mg/mL; Wako Pure Chemical Industries, Ltd, Osaka, Japan). HaCaT cells stably expressing FLAG-TSC-22 were maintained in culture medium supplemented with 1 μg/mL puromycin (Sigma). MGZ5 ES cells were maintained on feeder-free, gelatin-coated plates in leukemia inhibitory factor (LIF)-supplemented medium as described previously.[19]

2.2 | DNA constructs

c-MYC cDNA was provided by Drs B. Blackwood and R.N. Eisenman. Expression constructs pcDNA3-c-MYC and pcDNA3-FLAG-inhibitor of DNA binding 2 (ID2) were described previously.[20,21] Dr M. Eilers provided cDNA for MIZ-1, which we cloned into pcDEF3. TSC-22 cDNA from HaCaT cells was cloned into both pcDNA3 and pCAGIP (for transfection into ES cells) vectors. c-MYC, TSC-22 deletion and 4LA (L77A, L84A, L91A, and L98A) mutants were generated using PCR. The *P21* promoter WWP-luc,[22] *P15*-luc,[23] and pGL3-EBox2-luc[8] were provided by Drs B. Vogelstein, X.F. Wang, and R. Dalla-Favera, respectively. These constructs were transfected into cells using the FuGENE6 reagent (Promega, Madison, WI, USA) according to the manufacturer's recommendations.

2.3 | Immunoprecipitation and immunoblotting

For immunoprecipitation, cells were solubilized in a lysis buffer containing Tris-HCl (20 mmol/L, pH 7.5), NaCl (150 mmol/L), Nonidet P-40 (1%), Trasylol (1.5%), and PMSF (1 mmol/L). After clearing by centrifugation, total cell lysates or immunoprecipitates obtained using the indicated antibodies were subjected to SDS-PAGE. Proteins were electrotransferred onto PVDF membranes (Millipore, Burlington, MA, USA) and subjected to immunoblotting. We used anti-c-MYC (9E10; Santa Cruz Technology, Santa Cruz, CA, USA), FLAG (M2; Sigma), TSC-22 (Abnova, Taipei, Taiwan), and α-TUBULIN (Millipore, Burlington, MA, USA) antibodies as primary antibodies for immunoblotting. Reacted antibodies were detected using an enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, UK). For reblotting, we stripped the membranes according to the manufacturer's protocol.

2.4 | Cell proliferation assay

HaCaT cells and their derivatives were seeded into 12-well plates at a density of 1×10^4 cells per well and cultured for the indicated time periods. Number of cells was counted using a hematocytometer. MGZ5 ES cells were transfected with pCAGIP-empty, FLAG-TSC-22, and FLAG-ID2. After 24 hours, cells were trypsinized and equal numbers of cells were seeded on gelatin-coated 6-cm plates and cultured for 6 days in a feeder-free and LIF-supplemented medium. After photographing the colonies under a phase-contrast microscope (Olympus, Tokyo, Japan), cells were stained with crystal violet and colony numbers were measured with image analysis software (Image J; NIH, Bethesda, MD, USA).

2.5 | Luciferase assay

We transfected the cells using FuGENE6 transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Luciferase activity in the cell lysates was determined by a luciferase reporter assay system (Promega, Madison, WI, USA) using a luminometer (AutoLumat LB953; EG & G Berthold, Bad Wildbad, Germany). Luciferase activities were normalized to β-galactosidase activity of cotransfected CH110 (GE Healthcare).

2.6 | Chromatin immunoprecipitation

ChIP was carried out as described previously,[24] with some modifications. Cells were treated with 1% formaldehyde at 37°C for 20 minutes and washed twice with PBS. The cells were resuspended in 3 mL TE buffer supplemented with PMSF (1 mmol/L) and sonicated. Soluble chromatin was collected by centrifugation for 10 minutes at 16 000 × *g* in a microfuge and adjusted to 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, and 140 mmol/L NaCl. Immunoprecipitation reactions containing 1 mL chromatin solution, 25 μL

protein A-Sepharose beads, and 1 μg control IgG or anti-c-MYC antibody (N262; Santa Cruz) were incubated with end-over-end rotation overnight at 4°C. The immunoprecipitates were washed sequentially four times with RIPA buffer containing NaCl (0.3 mol/L), once with RIPA buffer containing no NaCl, and once with TE. DNA was then eluted with elution buffer (10 mmol/L DTT, 1% SDS, and 0.1 mol/L $NaHCO_3$). Following reverse-cross-linking at 65°C for 6 hours, DNA was treated with proteinase K and purified using a PCR purification kit (Qiagen, Hilden, Germany). DNA was eluted into 20 μL (immunoprecipitates) and 50 μL (input) of elution buffer, and 1 μL of this solution was used for PCR analysis using the PCR primers listed in Table S1.

2.7 | Reverse transcription-PCR

Total RNA was isolated using ISOGEN II (Nippon Gene, Tokyo, Japan). RT was carried out using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA) and PCR was done using Ex Taq polymerase (TaKaRa Bio Inc., Shiga, Japan). PCR primers are listed in Table S2.

2.8 | Immunofluorescence

Immunofluorescence in HaCaT cells stably expressing TSC-22 was carried out using anti-TSC-22 and anti-c-MYC (N262; Santa Cruz) primary antibodies followed by incubation with Alexa 488-labeled goat anti-mouse IgG and Texas Red-labeled goat anti-rabbit IgG secondaries (Molecular Probes, Eugene, OR, USA). Nuclei were stained with Hoechst 33342 (Sigma). Intracellular localization of TSC-22 and c-MYC was observed using a fluorescence microscope (Axiovert 200; Carl Zeiss AG, Oberkochen, Germany).

2.9 | Statistical analysis

Statistical analyses of the data was carried out with the *t* test using a statistics function in Microsoft Excel (Microsoft, Redmond, WA, USA) or Prism 5 (Graphpad Software, La jolla, CA, USA). Probability values <.05 were considered significant and indicated as $*P < .05$, $**P < .01$.

3 | RESULTS

3.1 | TSC-22 overexpressing cells form smaller colonies

To investigate the function of TSC-22 in cell proliferation, we generated HaCaT human keratinocyte cells stably expressing TSC-22 (Figure 1A). HaCaT cells expressing TSC-22 (clone #11 and #17) exhibited slower proliferation than Mock-transfected cells (Figure 1B). We also examined the effects of TSC-22 on the colony formation of mouse ES (MGZ5) cells. MGZ5 cells were transfected with TSC-22 or ID2 overexpression plasmids and the transfected cells were selected using puromycin for 6 days (Figure S1A). We used ID2 as a reference because ID2 suppresses ES cell differentiation and sustains self-renewal,[25] and also regulates MYC-mediated tumorigenesis.[26] Using this experimental procedure, we can assess clonal propagation of ES cells only if they maintain their pluripotency.[27] Compared with Mock- or ID2-transfected cells, TSC-22-transfected cells formed smaller colonies but kept an undifferentiated morphology (Figure 1C,D). However, the total number of colonies in Mock-, TSC-22-, and ID2-transfected cells was similar (Figure 1E). The expression of c-MYC protein and undifferentiated markers (*Nanog*, *Oct4*, and *Rex1* mRNA)

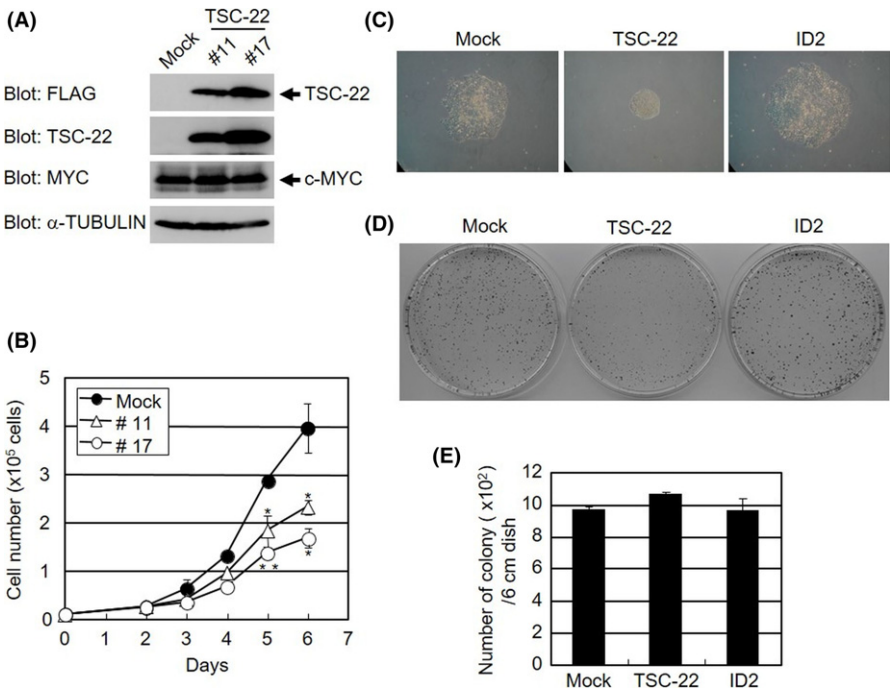

FIGURE 1 TSC-22 inhibits cell proliferation. A, Immunoblot analysis showing expression of TSC-22, c-MYC, and α-TUBULIN in FLAG-TSC-22-expressing HaCaT cells (clones #11 and #17), as indicated. B, Cell growth of Mock and FLAG-TSC-22-expressing HaCaT cells (clones #11 and #17). Mean ± SD, n = 3. $*P < .05$, $**P < .01$ (on days 5 and 6). C-E, Mouse ES cells (MGZ5 cells) were transfected with Mock, FLAG-TSC-22, and FLAG-ID2 expression plasmids, as indicated, transferred to 6-cm dishes and cultured for 6 d in the presence of puromycin. C, Morphology of colonies transfected with Mock, FLAG-TSC-22, and FLAG-ID2 cDNAs, as indicated. D, Whole view of the colonies in 6-cm dishes stained with crystal violet. E, Number of colonies. Mean ± SD. n = 3

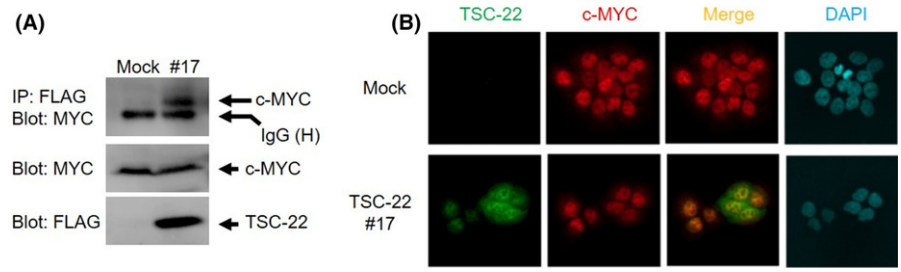

FIGURE 2 TSC-22 interacts with c-MYC in HaCaT cells. A, Interaction of TSC-22 and c-MYC in Mock- and FLAG-TSC-22-expressing HaCaT cells (clone #17). Cell lysates were immunoprecipitated with anti-FLAG antibody (IP), followed by immunoblotting using antibodies as indicated (Blot). IgG (H), immunoglobulin heavy chain used for IP. B, Localization of TSC-22 in FLAG-TSC-22-expressing HaCaT cells (clone #17). Cells were cultured on coverslips and stained with anti-TSC-22 and c-MYC antibodies. Nuclei were counterstained with DAPI

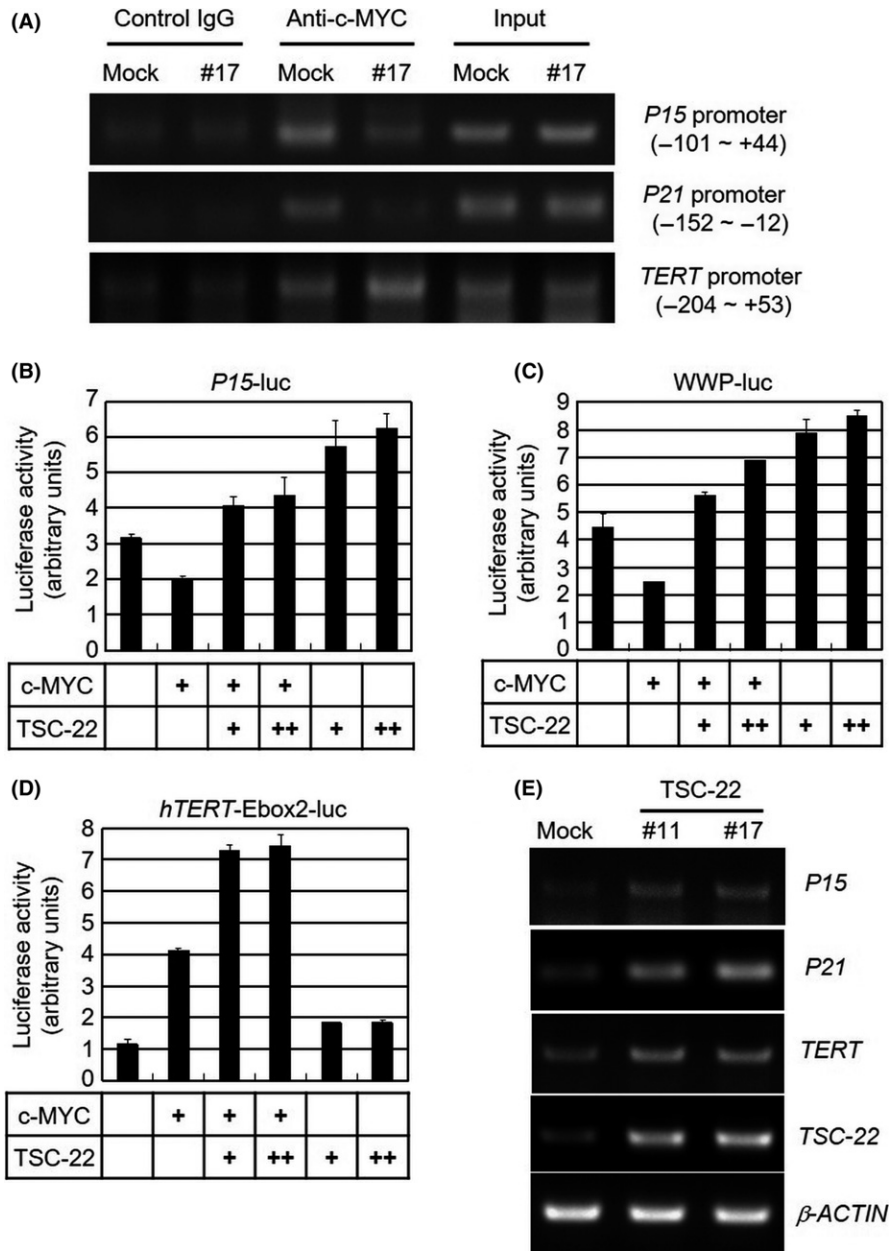

FIGURE 3 Effects of TSC-22 on c-MYC-mediated transcriptional activation and repression. A, ChIP analysis of c-MYC binding to the *P15*, *P21*, and *TERT* promoters in Mock- and FLAG-TSC-22-expressing HaCaT cells (clone #17). Control IgG was used as a negative control. B-D, 293T cells were transfected with the luciferase reporter constructs; (B) *P15* (*CDKN2B*)-luc, (C) WWP (*CDKN1A*)-luc, and (D) *hTERT* E-box2-luc, along with various combinations of c-MYC and TSC-22 expression plasmids, and luciferase activity was measured. Mean ± SD. n = 3. E, Expression of *P15*, *P21*, and *TERT* mRNA in Mock- and FLAG-TSC-22-expressing HaCaT cells (clone #11 and #17). β-actin was used as a loading control

was similarly maintained in Mock-, TSC-22-, and ID2-transfected cells (Figure S1B). As colony numbers and morphology were similar across groups, these data lead to the conclusion that TSC-22 specifically affects proliferation and not other differentiation parameters.

3.2 | TSC-22 interacts with c-MYC

Next, we verified the interaction between transfected TSC-22 and endogenous c-MYC. Immunoprecipitation analysis clearly showed the interaction between FLAG-TSC-22 and c-MYC (Figure 2A) and, furthermore, TSC-22, which is distributed mainly in nuclei, co-localized with c-MYC in the nucleus (Figure 2B).

3.3 | TSC-22 cancels c-MYC-mediated suppression of *P15* (*CDKN2B*) and *P21* (*CDKN1A*) and enhances c-MYC-mediated activation of *TERT*

c-MYC is known to stimulate cell growth through transcriptional downregulation of CDK inhibitors, including P15 and P21.[11,12] We therefore investigated the binding of c-MYC to the *P15* (*CDKN2B*, −101 to +44), *P21* (*CDKN1A*, −152 to −12), and *TERT* (−204 to +53) promoters using chromatin immunoprecipitation. Recruitment of c-MYC was reduced on the *P15* and *P21* promoters but enhanced on the *TERT* promoter in TSC-22-transfected HaCaT cells (Figure 3A) and, as shown in Figure 3B,C, c-MYC suppressed the transcriptional activity of the *P15* and *P21* promoters (*P15*-luc and WWP-luc,

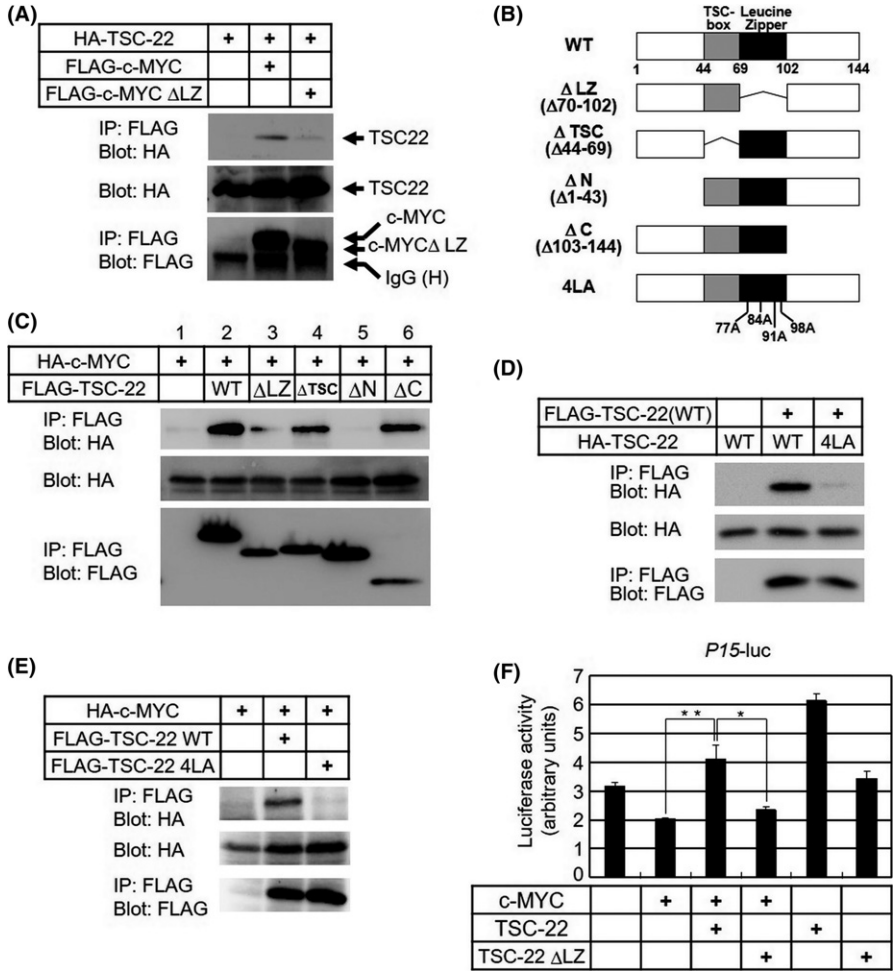

FIGURE 4 Domains required for TSC-22 and c-MYC interaction. A,C,D,E, 293T cells were transfected with expression plasmids, as indicated. Cell lysates were immunoprecipitated with anti-FLAG antibody (IP), followed by immunoblotting using antibodies as indicated (Blot). A, Leucine zipper domain (LZ) of c-MYC is required for TSC-22 and c-MYC interaction. IgG (H), IgG heavy chain used for IP. B, Schematic representation of the structures of TSC-22 wild-type (WT), deletion mutants of leucine zipper domain (ΔLZ), TGF-β1 stimulated clone 22 box (ΔTSC), N-terminal part (ΔN), C-terminal part (ΔC), and four leucine-to-alanine mutations in the LZ domain (4LA). C, Interaction of c-MYC and WT or deletion mutants of TSC-22. N-terminal part and LZ domain of TSC-22 are required for TSC-22-c-MYC interaction. D, Homodimer formation of FLAG-TSC-22 WT and HA-TSC-22 WT or HA-TSC-22 4LA, as indicated. E, Heterodimer formation of HA-c-MYC and FLAG-TSC-22 WT or FLAG-TSC-22 4LA, as indicated. F, *P15*-luc activity suppressive effects of c-MYC are canceled by TSC-22 WT but not by TSC-22 ΔLZ. 293T cells were transfected with the luciferase construct *P15* (*CDKN2B*)-luc along with various combinations of the indicated expression plasmids, and luciferase activity was measured. Mean ± SD. n = 3. $^{*}P < .05$, $^{**}P < .01$

respectively). Importantly, TSC-22 canceled c-MYC-mediated suppression of P15 and P21 in an expression level-dependent way. Furthermore, TSC-22 stimulated *P15* and *P21* promoter activity in the absence of c-MYC transfection, suggesting that TSC-22 inhibits endogenous c-MYC activity. Next, we investigated the effect of TSC-22 on c-MYC-mediated promoter activation by using the *TERT* promoter luciferase construct (pGL3-EBox2-luc), which contains two E-boxes that are activated by c-MYC.[8] As shown in Figure 3D, TSC-22 stimulated c-MYC-mediated activation of the *TERT* promoter but showed no activity in the absence of c-MYC coexpression. Corresponding to these reporter results, the amount of endogenous mRNA of P15, P21, and *TERT* was increased in HaCaT cells stably expressing TSC-22 (Figure 3E).

3.4 | LZ and N-terminal domains are required for c-MYC and TSC-22 interaction

The c-MYC family proteins possess the bHLH-LZ domain at their C-terminal.[28] We examined the bHLH-LZ domain of c-MYC for its role in the interaction with TSC-22. As shown in Figure 4A, c-MYC ΔLZ, without the LZ domain, did not interact with TSC-22. Furthermore, we constructed TSC-22 mutants (Figure 4B) and examined their interactions with c-MYC, finding that TSC-22 ΔN, without the 43 N-terminal amino acids, completely lost interaction with c-MYC (Figure 4C, lane 5) and the interaction between TSC-22 ΔLZ and c-MYC was significantly reduced (Figure 4C, lane 3). As TSC-22 family members are known to form homo- or heterodimers through their LZ domains, we then constructed TSC-22 4LA, which has four leucine-to-alanine mutations in the LZ domain (L77A, L84A, L91A and L98A), and examined the role in homodimer formation and interaction with c-MYC. As shown in Figure 4D,E, TSC-22 4LA lost homodimer formation and heterodimer formation with c-MYC. These results suggested that the N-terminal domain (amino acids: 1-43) and LZ domain of TSC-22 are involved in the interaction with c-MYC. Finally, we examined the role of interaction between c-MYC and TSC-22 for the regulation of *P15* promoter activity. As shown in Figure 4F, TSC-22 ΔLZ was unable to inhibit c-MYC-mediated suppression of the *P15* promoter activity. These results indicated that the interaction between TSC-22 and c-MYC is essential for the inhibition of c-MYC-mediated suppression of the *P15* promoter activity.

3.5 | TSC-22 enhances c-MYC-MAX but suppresses c-MYC-MIZ-1 heterodimer formation

Reports have shown c-MYC to activate or repress various genes through interaction with MAX and MIZ-1, respectively.[8-10,12,13] We examined the interaction of these proteins with TSC-22 by immunoprecipitation. As shown in Figure 5A, TSC-22 bound to c-MYC, but not to MAX and MIZ-1. We next examined the effect of TSC-22 in c-MYC-MAX and c-MYC-MIZ-1 heterodimer formation. As shown in Figure 5B,C, the interaction between c-MYC and MAX was enhanced in the presence of TSC-22 (Figure 5B, lanes 2 and 3). However, the interaction between c-MYC and TSC-22 was reduced

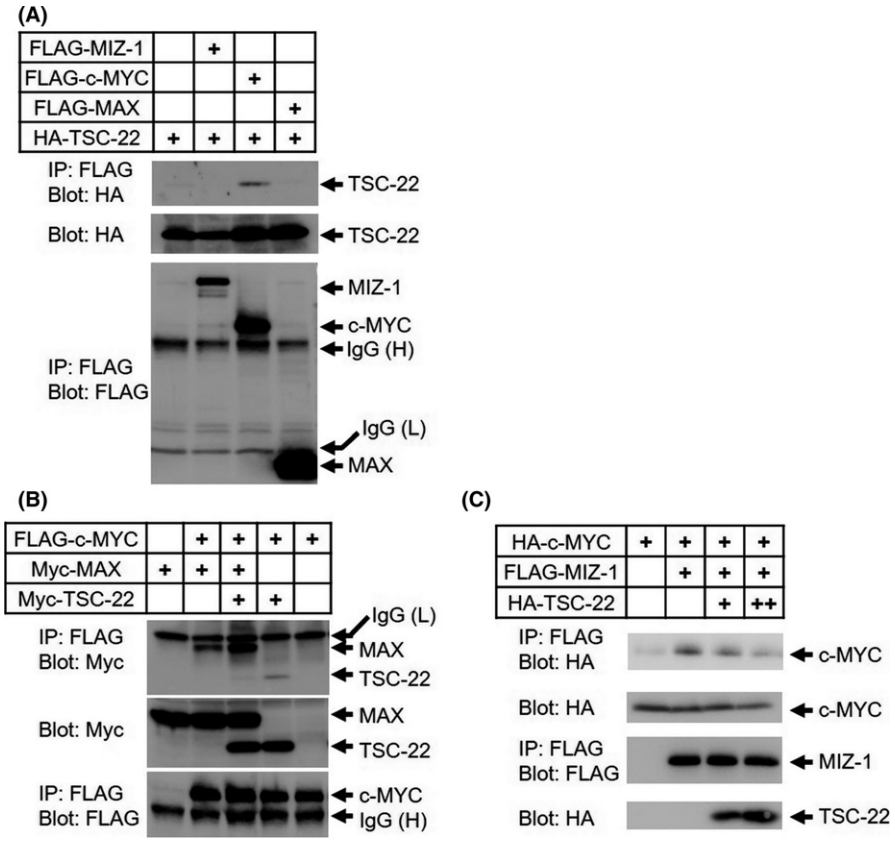

FIGURE 5 Effects of TSC-22 on c-MYC-MAX and c-MYC-MIZ-1 heterodimer formation. A-C, 293T cells were transfected with expression plasmids, as indicated. Cell lysates were immunoprecipitated with anti-FLAG antibody (IP), followed by immunoblotting using antibodies as indicated (Blot). IgG (H) and IgG (L), IgG heavy chain and light chain used for IP, respectively. A, Interaction of TSC-22 with c-MYC but not with MIZ-1 and MAX. B, TSC-22 enhances c-MYC-MAX interaction and MAX competes with TSC-22 for c-MYC binding. C, TSC-22 suppresses c-MYC-MIZ-1 interaction

in the presence of MAX (Figure 5B, lanes 3 and 4). In contrast, TSC-22 interaction to c-MYC suppressed c-MYC-MIZ-1 interaction (Figure 5C, lanes 3 and 4). These results suggested that TSC-22 binds to c-MYC and enhances the c-MYC-MAX heterodimer formation after only transient involvement, but suppresses the c-MYC-MIZ-1 heterodimer formation.

4 | DISCUSSION

In the present study, we showed that TSC-22 regulates transcriptional activity of c-MYC by interaction with the c-MYC LZ domain and differentially affecting c-MYC-MAX and c-MYC-MIZ-1 heterodimer formation. Correspondingly, TSC-22 inhibited c-MYC recruitment to the *P15* and *P21* promoters (Figure 3A) and canceled c-MYC-mediated suppression of *P15* and *P21* (Figure 3B,C). In contrast, TSC-22 enhanced c-MYC recruitment to the *TERT* promoter and promoted c-MYC-mediated transcriptional activation of the *TERT* promoter (Figure 3A,D).

The X-ray structure of the MYC-MAX-DNA (E-box) complex shows that the MYC-MAX heterodimer dimerizes to form a large heterotetramer, which allows MYC to upregulate expression of the target genes even if their promoters have widely separated E-boxes.[29] We observed that TSC-22 enhances MYC-MAX dimer formation (Figure 5B), raising the possibility that TSC-22 enhances the c-MYC-induced activation of *TERT* expression by enhancing MYC-MAX interaction to the E-box (Figure 3A,D,E). In contrast, the formation of MYC-MIZ1 on the *P15* and *P21* promoters may be impaired by the suppression of MYC-MIZ-1 heterodimer formation (Figures 3A and 5C). Further global genomic analyses of c-MYC-MAX and c-MYC-MIZ-1 target genes would help to understand the likely mechanism of action and further functions of TSC-22.

Several studies have reported downregulation of TSC-22 in tumors, including those found in the salivary gland, brain, and prostate.[18,30,31] Furthermore, *TSC22D1* is hypermethylated and silenced in T-cell- or natural killer-large granular lymphocyte leukemia, and Tsc-22-deficient mice are susceptible to tumorigenesis in a carcinogen-induced mouse liver-tumor model by the ablation of a RAF interacting Tsc-22 that negatively regulates RAS-mediated transformation.[32,33] Our current results indicated further function of TSC-22 as a tumor suppressor protein through binding to c-MYC. Our current findings provide a novel TGF-β-induced-TSC-22-mediated regulatory network to the canonical c-MYC function.[33]

We verified the effect of Tsc-22 on the clonal propagation of mouse ES cells. As shown in Figures 1C,D and S1B, Tsc-22 inhibited ES cell colony growth without affecting pluripotency-related gene expression. A growing amount of evidence suggests that the dormancy of tissue stem cells plays a critical role not only in stem cell maintenance but also in the properties of cancer stem cells, including resistance to therapy and cancer relapse.[34] In the mouse leukemia model described earlier, the recovery of TSC-22 expression increased the survival of mice with leukemia.[32] Furthermore, targeted disruption of *Tsc22d1* in mice enhanced the proliferation and in vivo repopulation efficiency of hematopoietic precursor cells.[32] The results of the current study provide novel insights into the potential roles of TSC-22 in stem cell dormancy through the modification of c-MYC-mediated transcriptional regulation to enhance *P15*, *P21* and *TERT* expression. Further studies are therefore needed to understand the regulation of stem cell dormancy by TSC-22.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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