A transcriptionally active pRb–E2F1–P/CAF signaling pathway is central to TGFβ-mediated apoptosis

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Transforming growth factor-β (TGFβ) modulates the expression of multiple apoptotic target genes; however, a common and central signaling pathway, acting downstream of TGFβ and leading to cell death, has yet to be uncovered. Here, we show that TGFβ-induced apoptosis in cancer cells requires the transcription factor E2F1 (E2 promoter-binding factor 1). Using the E2F1 knockout mouse model, we also found E2F1 to be required for TGFβ-mediated apoptosis in normal cells. Moreover, we found TGFβ to increase E2F1 protein stability, acting at the post-translational level. We further investigated the molecular mechanisms by which E2F1 contributes to TGFβ-mediated apoptosis and found that TGFβ treatment led to the formation of a transcriptionally active E2F1–pRb–P/CAF complex on multiple TGFβ pro-apoptotic target gene promoters, thereby activating their transcription. Together, our findings define a novel process of gene activation by the TGFβ–E2F1 signaling axis and highlight E2F1 as a central mediator of the TGFβ apoptotic program.

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Transforming growth factor-β (TGFβ) and its related family members are involved in the regulation of a wide range of fundamental cellular processes, including the regulation of growth, differentiation, and apoptosis.1 TGFβ, the prototype of the family, is a vital factor in the maintenance of homeostasis between cell growth and apoptosis. TGFβ exerts its tumor-suppressive effects by inhibiting cell-cycle progression, inducing apoptosis, and preventing immortalization through inhibition of telomerase activity. Loss or mutation of TGFβ signaling components is frequently observed in human cancer and further define a tumor-suppressive role for this growth factor.2

TGFβ ligands signal through serine/threonine kinase receptors that, once activated by ligand binding, recruit and phosphorlyate the canonical downstream mediators, Smad2 and Smad3. Once phosphorylated, Smad2 and Smad3 interact with Smad4 to then translocate to the nucleus where the Smad complex associates with diverse DNA-binding factors to regulate expression of target genes in a cell- and tissue-specific manner. These partner proteins, which act as co-activators or co-repressors, are differentially expressed in different cell types and are thus thought to provide a basis for tissue and cell type-specific functions for TGFβ ligands.3

TGFβ induces a number of apoptotic responses and its ability to do so varies greatly depending on the cell type.4 Understanding the basis of this variability requires elucidating the molecular mechanisms involved in regulating TGFβ-mediated apoptosis. TGFβ signaling activates caspases in various epithelial cell types5,6 and transcriptionally induces DAPK (death-associated protein kinase) in hepatoma cells.7 TGFβ also induces apoptosis by antagonizing PI3K (phosphatidylinositol 3-kinase)/Akt signaling activity through expression of the lipid phosphatase SHIP (SH2-domain-containing inositol-5-phosphatase) in hematopoietic cells.8 Transcriptional up-regulation of pro-apoptotic proteins such as Bax (Bcl-2-associated X protein) and down-regulation of pro-survival Bcl-2 (B-cell lymphoma 2) family members have also been implicated in TGFβ-mediated programmed cell death.9,10 However, these mechanisms are context and tissue-specific; a central mechanism acting downstream of TGFβ to induce apoptosis has not yet been described.

We previously demonstrated that the TGFβ inhibitory effect on telomerase activity and cell immortalization is dependent on both Smad3 and the transcription factor E2F1 (E2 promoter-binding factor 1), highlighting E2F1 as an important mediator of TGFβ tumor-suppressive effects.11 The E2F family of transcription factors is a group of DNA-binding proteins that are central regulators of cell-cycle progression. The transcriptional activity of E2F1–5 is regulated primarily via their association with members of the retinoblastoma family of pocket proteins, which include pRb (retinoblastoma tumor-suppressor protein)/p105, p107, and p130.12 E2F1, the founding member and best-characterized of the family, has a unique role compared with other E2Fs, showing characteristics of being both an oncogene and a tumor suppressor, as it is able to induce both cell-cycle progression and apoptosis. Though an increase in E2F1 activity has been reported in several types of
tumors supporting an oncogenic role for E2F1, transgenic mice overexpressing E2F1 display aberrant cell apoptosis. Furthermore, E2F1 knockout mice develop highly malignant tumors and show defects in thymocyte apoptosis, highlighting E2F1 as a potent tumor suppressor. The nature of this dichotomy is proposed to be based on the degree to which E2F1 is expressed in the context of the cell cycle and/or following DNA damage, and the notion that different threshold levels of E2F1 are required for differential transactivation of its target gene promoters, which may favor either survival or apoptosis. Interestingly, E2F1 mutants that are unable to promote cell-cycle progression retain their ability to induce programmed cell death, indicating that induction of the cell cycle and apoptosis are separable functions of E2F1. Given our previous findings that E2F1 is required for TGFβ-mediated inhibition of hTERT (human telomerase reverse transcriptase) and that TGFβ promotes increased E2F-DNA-binding activity in pre-apoptotic hepatoma cell nuclear extracts, we investigated whether E2F1 could also mediate another arm of the TGFβ tumor-suppressive response and regulate apoptosis.

We found TGFβ to regulate the transcription of a number of pro-apoptotic genes in an E2F1-dependent manner in cancer cell lines from various tissues. Using embryonic fibroblasts from the E2F1 knockout mouse model, we also found E2F1 to be required for TGFβ-mediated apoptosis in normal cells. Moreover, we found TGFβ to increase E2F1 protein stability, acting post-translationally. We further investigated the molecular mechanisms by which E2F1 contributes to TGFβ-mediated cell death and found that TGFβ could promote formation of a transcriptionally active E2F1-pRb-P/CAF (p300/CREB-binding protein-associated factor) complex on the promoters of TGFβ-targeted apoptotic genes to activate their transcription. Together, our results underline E2F1 as a central mediator of the TGFβ pro-apoptotic response and highlight the E2F1-pRb-P/CAF signaling pathway as a critical regulator of TGFβ-mediated cell death.

Results

TGFβ-mediated apoptosis is dependent on E2F1. We first examined the pro-apoptotic effect of TGFβ in various model systems, including two human hepatoma cell lines (HuH7 and HepG2), a human melanoma cell line (WM278), and a human keratinocyte cell line (HaCaT). Cells were stimulated or not with TGFβ as indicated and apoptosis was assessed using MTT or calcein-AM assays. Data are represented as mean ± S.D. Values were assessed as being significantly different at p < 0.05 as determined by ANOVA (Figure 1a and b). Expression of endogenous E2F1 in these cells was assessed by immunofluorescence and shown to increase following DNA damage, indicating that induction of the cell cycle and apoptosis is separable functions of E2F1. Together, our results underline E2F1 as a potent tumor suppressor. The nature of this tumor-suppressive response and regulate apoptosis.

E2F1 is required for TGFβ-mediated regulation of pro-apoptotic target genes. TGFβ signaling activates multiple pro-apoptotic pathways in cancer cell lines of various origins. Supporting an oncogenic role for E2F1, transgenic mice overexpressing E2F1 display aberrant cell apoptosis. Furthermore, E2F1 knockout mice develop highly malignant tumors and show defects in thymocyte apoptosis, highlighting E2F1 as a potent tumor suppressor. The nature of this dichotomy is proposed to be based on the degree to which E2F1 is expressed in the context of the cell cycle and/or following DNA damage, and the notion that different threshold levels of E2F1 are required for differential transactivation of its target gene promoters, which may favor either survival or apoptosis. Interestingly, E2F1 mutants that are unable to promote cell-cycle progression retain their ability to induce programmed cell death, indicating that induction of the cell cycle and apoptosis are separable functions of E2F1. Given our previous findings that E2F1 is required for TGFβ-mediated inhibition of hTERT (human telomerase reverse transcriptase) and that TGFβ promotes increased E2F-DNA-binding activity in pre-apoptotic hepatoma cell nuclear extracts, we investigated whether E2F1 could also mediate another arm of the TGFβ tumor-suppressive response and regulate apoptosis.

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Cell Viability (% of control)

HuH7 HepG2 WM278 HaCaT

Cell Viability (% of control)

0h 24h 48h 72h 0h 24h 48h 72h

Cell Viability (% of control)

0h 4h 8h 16h 24h

Cell Viability (% of control)

0h 24h 48h 72h

Cell Viability (% of control)

Apoptotic Cells (%) 7.2 ± 0.28 15.4 ± 1.63 9.5 ± 0.92 7.4 ± 0.21

Apoptotic Cells (%)

control siRNA E2F1 siRNA

control siRNA E2F1 siRNA

control siRNA E2F1 siRNA

control siRNA E2F1 siRNA
products were analyzed by DNA gel electrophoresis (100 pM) for 24 h. The mRNA levels for the indicated genes were measured as in (a). (c) HuH7 cells were pre-treated for 30 min with cycloheximide (10 μM) or vehicle and then stimulated with TGFβ (100 pM) for the indicated times. The mRNA levels for the indicated genes were analyzed by RT-PCR and the amplified products were analyzed by DNA gel electrophoresis.

Figure 2 E2F1 is required for TGFβ-mediated regulation of proapoptotic genes. (a) HuH7 cells were stimulated with TGFβ (100 pM) and mRNA levels for the indicated genes were measured by real-time qPCR. Results are normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and shown relative to levels observed in untreated cells (set to 1). Data are represented as mean ± S.D. (b) HuH7 cells were transiently transfected with siRNA against E2F1 or a control non-silencing siRNA and stimulated with TGFβ (100 pM) for 24 h. The mRNA levels for the indicated genes were measured as in (a). (c) HuH7 cells were pre-treated for 30 min with cycloheximide (10 μM) or vehicle and then stimulated with TGFβ (100 pM) for the indicated times. The mRNA levels for the indicated genes were analyzed by RT-PCR and the amplified products were analyzed by DNA gel electrophoresis.

translational inhibitor cycloheximide (CHX) and stimulated with TGFβ as indicated. Interestingly, CHX treatment of the cells completely impaired the induction of these genes by TGFβ (Figure 2c). As a control, the mRNA expression levels of a direct TGFβ target gene, Smad7, were also examined and, as expected, were not affected by CHX treatment. These results indicate that TGFβ regulation of expression of its downstream pro-apoptotic target genes is indirect and requires the induction of a TGFβ-responsive transcriptional activator.

TGFβ rapidly and transiently induces E2F1 protein expression levels. Having shown that TGFβ indirectly induces the expression of these pro-apoptotic target genes and that E2F1 is required for this process, we next sought to determine whether E2F1 expression itself was regulated by TGFβ. TGFβ treatment induced a time-dependent decrease in E2F1 mRNA levels in HaCaT cells (Figure 3a), in agreement with previous reports.28,29 Surprisingly, however, we found TGFβ to rapidly and transiently induce E2F1 protein expression levels in these cells (Figure 3b). We then examined the TGFβ effect on E2F1 protein expression levels in human epithelial cancer cell lines originating from different tissues (melanoma, hepatocarcinoma, and colon carcinoma) and, as shown in Figure 3c, E2F1 protein levels were strongly induced by TGFβ in all the cell lines tested. This effect was transient, however, as longer exposure to TGFβ resulted in a return to basal E2F1 protein levels. Interestingly, in all cases the increase in E2F1 expression in response to TGFβ was very rapid, suggesting that TGFβ induces post-translational protein stabilization of E2F1. To address this, we performed a CHX chase in HaCaT cells treated or not with TGFβ (Figure 3d). In the presence of CHX, untreated cells showed progressive diminished levels of E2F1 over time. Conversely, TGFβ treatment maintained E2F1 levels throughout the chase, indicating that TGFβ indeed prolongs E2F1 half-life, by stabilizing E2F1 protein levels post-translationally.

TGFβ pro-apoptotic effects are impaired in E2F1-null embryonic fibroblasts. Having shown that TGFβ-induced apoptosis in various epithelial cancer cell lines requires E2F1, we next examined the contribution of E2F1 downstream of TGFβ-mediated cell death in normal cells. For this,
Skin blotting indicated times. Total cell lysates were analyzed for E2F1 protein levels by western

HaCaT cells were stimulated with TGF\(\beta\) (100 pM) for the indicated times and phospho-

Western blot analysis of total E2F1 protein levels in TGF\(\beta\)-treated cells of various origins, as indicated. (d) Western blot analysis of total cell lysates were analyzed by western blotting. (a) RT-PCR followed by DNA gel electrophoresis and (b) western blot to measure E2F1 RNA and protein levels, respectively. (c) Analysis of total E2F1 protein levels in TGF\(\beta\)-treated and -untreated cells (set to 1). Data are represented as mean ± S.D. (*\(P<0.05\))

role for E2F1 downstream of TGF\(\beta\) in the mediation of apoptosis in a normal cell setting in addition to multiple cell lines of various cancer origins.

Figure 3  TGF\(\beta\) rapidly and transiently induces E2F1 protein expression levels. HaCaT cells were stimulated with TGF\(\beta\) (100 pM) for the indicated times and subjected to (a) RT-PCR followed by DNA gel electrophoresis and (b) western blotting to measure E2F1 RNA and protein levels, respectively. (c) Western blot analysis of total E2F1 protein levels in TGF\(\beta\)-treated cells of various origins, as indicated. (d) Cycloheximide (CHX) chase analysis in HaCaT cells to address the potential contribution of TGF\(\beta\) in E2F1 post-translational stabilization. Cells were incubated with CHX (50 \(\mu\)g/mL) and treated or not with TGF\(\beta\) (100 pM) for the indicated times. Total cell lysates were analyzed for E2F1 protein levels by western blotting.

we used mouse embryonic fibroblasts (MEFs) isolated from wild-type and E2F1-deficient mice. Importantly, both wild-type (E2F1 \(+/+\)) and E2F1-null (E2F1 \(-/-\)) MEFs respond equally to TGF\(\beta\) stimulation, as assessed by the induction of Smad phosphorylation (Figure 4a). The pro-apoptotic effect of TGF\(\beta\), however, greatly differed in these two cell types. Although cell viability of the wild-type E2F1 \(+/+\) MEFs was potently decreased in response to TGF\(\beta\); this effect was severely impaired in the E2F1 \(-/-\) MEFs (Figure 4b). Correspondingly, TGF\(\beta\)-induced expression of Caspase 7 and Smac/DIABLO was significantly reduced in the E2F1 \(-/-\) MEFs (Figure 4c). Together, these findings highlight a critical
established at the transcriptional level, as their overexpression significantly reduced TGF/β-induced Caspase 7 and Smac/DIABLO mRNA levels (Figure 5b). These results indicate that TGF/β requires not only proper E2F1 function (DNA binding and transactivation), but the ability of E2F1 to interact with pRb in order to successfully induce apoptosis. To further address this, we examined whether TGF/β could induce association between endogenous E2F1 and pRb using co-immunoprecipitation studies. As shown in Figure 5c, TGF/β treatment indeed promotes the association between E2F1 and pRb. Altogether, these results indicate that pRb-E2F binding is required for TGF/β to induce apoptosis and that this association is induced by TGF/β itself, strongly supporting the fact that the pRb-E2F1 protein complex has a role downstream of TGF/β-mediated cell signaling, leading to apoptosis.

TGF/β induces formation of a transcriptionally active complex between pRb/E2F1 and the acetyltransferase P/CAF onto pro-apoptotic gene promoters. Given the classical model of E2F regulation, which implies that E2F1 must be in its unbound form in order to activate transcription, this raised the question as to how E2F1 activates these pro-apoptotic genes in response to TGF/β while remaining in its seemingly transcriptionally repressive pRb-E2F complex. Thus, we assessed whether TGF/β could in fact recruit positive regulators of transcription to the pRb-E2F1 complex. As TGF/β may activate gene transcription through histone acetyltransferases, including p300/CBP (cAMP-response element-binding protein (CREB)-binding protein) and P/CAF (p300/CBP-associated factor),31 we screened for the presence of these histone acetyltransferases in E2F1 and pRb immunoprecipitates in untreated versus TGF/β-treated cells. Interestingly, as shown
in Figure 6a, we found that TGF/β strongly promotes the association of both E2F1 and pRb to the acetyltransferase P/CAF. Moreover, these complexes appear to be P/CAF specific as we could not detect any association between pRb-E2F1 and p300/CBP.

We then addressed whether P/CAF is required for the activation of E2F1-responsive pro-apoptotic genes and induction of apoptosis in response to TGF/β. Loss of P/CAF expression by RNA interference dramatically reduced the TGF/β pro-apoptotic effect in these cells (Figure 6b). Moreover, the TGF/β-induced expression levels of Caspase 7 and Smac/DIABLO were notably reduced when P/CAF expression was silenced by siRNA (Figure 6c). As caspases require post-translational activation to become catalytically active and

Figure 6 TGF/β induces formation of a transcriptionally active complex between pRb/E2F1 and the acetyltransferase P/CAF, onto pro-apoptotic gene promoters. (a) Untreated and TGF/β-treated HuH7 cells were subjected to immunoprecipitation (IP) with the specified antibodies followed by western blotting (WB) to assess levels of P/CAF or CBP/p300 and associated E2F1 and pRb. (b) and (c) HuH7 cells were transiently transfected with siRNA against P/CAF or a control non-silencing siRNA and treated with TGF/β (100 pM) for 24 h. Cell viability was assessed by (b) calcein-AM assay, and Caspase 7 and Smac/DIABLO mRNA levels were measured by (c) real-time qPCR analysis. Results are normalized to GAPDH and shown relative to levels observed in untreated cells (set to 1). Data are represented as mean ± S.D. (d) The efficiency of P/CAF knockdown by siRNA was verified by real-time qPCR. (e) HuH7 cells were transiently transfected with a control siRNA or siRNA again P/CAF (left panel) or E2F1 (right panel) and treated with TGF/β (100 pM) as indicated. Activation of Caspase 3/7 was measured by Caspase-Glo 3/7 assay (Promega). Data are represented as mean ± S.D. (f) HuH7 cells were untreated or treated with TGF/β (100 pM) for the indicated times, and the binding of E2F1, pRb, and P/CAF to the p73, Apaf1, Smac/DIABLO, and Caspase 7 gene promoters was determined by chromatin immunoprecipitation (ChIP)
mediate cell death,\textsuperscript{32} we investigated whether the loss of TGF-\(\beta\)-induced caspase expression due to P/CAF knockdown was followed by a decrease in caspase activity. As shown in Figure 6e (left panel), blocking P/CAF expression severely impaired TGF-\(\beta\)-mediated Caspase 3/7 activation. This effect was similar to what was observed when E2F1 expression was silenced (Figure 6e, right panel). By 48 h, loss of either P/CAF or E2F1 expression nearly completely abolished TGF-\(\beta\)-induced caspase activation. Collectively, these findings support a critical role for P/CAF downstream of TGF-\(\beta\)/E2F1-dependent activation of pro-apoptotic genes and the mediation of programmed cell death.

To then assess the functional relevance of the TGF-\(\beta\)/E2F1–P/CAF complex in regulating TGF-\(\beta\)/E2F1-responsive pro-apoptotic genes identified above. Interestingly, as shown in Figure 6f, TGF-\(\beta\) treatment markedly induced recruitment of all three partners (E2F1, pRb, and P/CAF) to the p73, Apaf1, Caspase 7, and Smac/DIABLO gene promoters, concurring with the TGF-\(\beta\)/mediated increase in the mRNA levels of these pro-apoptotic genes and activation of the apoptotic program. These results highlight the E2F1–pRb–P/CAF pathway as a major signaling axis leading to apoptosis downstream of TGF-\(\beta\) in normal and cancer cells.

Discussion

Although various apoptotic mediators and signaling pathways have been implicated in TGF-\(\beta\)-mediated apoptosis, most of these regulatory mechanisms appear to be cell type-dependent or tissue-specific.\textsuperscript{4} This study defines a novel process of gene activation by the TGF-\(\beta\)/E2F1 signaling axis, which regulates the intrinsic apoptosis pathway through recruitment of the E2F1–pRb–P/CAF pathway as a critical mediator of TGF-\(\beta\) apoptotic program in multiple target tissues.

We identified a number of key pro-apoptotic TGF-\(\beta\) target genes that trigger the intrinsic apoptosis pathway through the induction of E2F1. Although these genes are functionally interrelated, our results imply that TGF-\(\beta\) regulates the intrinsic apoptosis pathway at multiple levels, consistent with the strong pro-apoptotic effect of this growth factor in its target tissues. However, we do not exclude the possibility that induction of other targets (or pathways) might also contribute to E2F1-dependent TGF-\(\beta\)-mediated cell death. Importantly, these results are corroborated using the E2F1 knockout mouse model, demonstrating that the TGF-\(\beta\)/E2F1 signaling pathway mediates TGF-\(\beta\)-induced cell death not only in a diseased state but in a normal cell setting as well.

Although it is well-established that E2F1 activity is intimately controlled through association with pRb, the precise mechanisms of this regulation are somewhat contradictory. The prevailing view holds that the pRb–E2F1 complex acts as a repressor of E2F target genes.\textsuperscript{13} Accordingly, disruption of this pRb–E2F1 complex is required to release free E2F1 in order to induce transcription of its target genes. Paradoxically, pRb–E2F1 complexes were recently shown to transcriptionally activate pro-apoptotic genes in response to DNA damage through recruitment of a histone acetyltransferase to the pRb–E2F1 complex.\textsuperscript{33} Interestingly, our results also challenge this dogma, and support a non-classic transcriptionally active pRb–E2F1 regulatory complex, as we show here that the pRb–E2F1 complex can also recruit an acetyltransferase (P/CAF) to activate transcription of pro-apoptotic genes in response to TGF-\(\beta\). Indeed, analysis with dominant-negative E2F1 mutants revealed that, in fact, pRb binding to E2F1 is required for TGF-\(\beta\)-mediated apoptosis.

Our results also indicate that TGF-\(\beta\) rapidly increases E2F1 protein levels, acting at the post-translational level. Interestingly, several lines of evidence have demonstrated that the E2Fs are often regulated by post-translational modifications such as phosphorylation,\textsuperscript{34} acetylation,\textsuperscript{35} and by the ubiquitin–proteasome pathways.\textsuperscript{36} Binding of pRb to E2F1 protects E2F1 from ubiquitination and proteolytic degradation,\textsuperscript{35} thereby increasing its stability. As TGF-\(\beta\) maintains pRb in a hypophosphorylated form, causing E2F1 to remain bound to pRb and suppressing activation of E2F1-responsive cell-cycle regulatory genes,\textsuperscript{36} it is likely that the TGF-\(\beta\) effect on E2F1 protein levels is mediated through induction of pRb–E2F1 association, revealing a new level of E2F1 regulation.

Moreover, the association of P/CAF to E2F1 may also contribute to the increased stability of E2F1 protein levels in response to TGF-\(\beta\), as P/CAF also binds and acetylates E2F1, prolonging its half-life. In fact, E2F1 acetylation by P/CAF has three functional effects on E2F1 activity: increased protein–half life, DNA-binding ability, and activation potential.\textsuperscript{35} Thus, P/CAF binding to E2F1 in response to TGF-\(\beta\) may in fact have multiple functional consequences, affecting not only E2F1 stability but its transcriptional-activating capability as well.

Additional post-translational modifications of E2F1 and/or pRb may also contribute to the formation of the pro-apoptotic complex. Notably, pRb holds a second alternate E2F1-binding site that does not interfere with E2F1’s transactivation domain.\textsuperscript{39} It is interesting to consider, then, whether TGF-\(\beta\) could somehow induce pRb and E2F1 to assume this alternate conformation. If so, this conformation should also allow for recruitment of P/CAF, which we have demonstrated here to be required for TGF-\(\beta\) to activate E2F1-dependent pro-apoptotic target genes. The coordinated recruitment of E2F1, pRb, and P/CAF to these pro-apoptotic gene promoters suggests the potential formation of a transcriptionally active pRb–E2F1 complex, which mediates the regulation of TGF-\(\beta\)/pro-apoptotic targets. Taken together, these results strongly support a pro-apoptotic role for the E2F1 pathway downstream of TGF-\(\beta\) and provide a potential mechanism for the activation of E2F1-responsive pro-apoptotic genes in response to TGF-\(\beta\).

It is interesting to consider that TGF-\(\beta\)/tumor-suppressive effects might utilize the functional interplay among the E2F family members, which affects E2F activity. It is well-established that TGF-\(\beta\) prevents cell-cycle progression, causing G1 arrest, by up-regulating expression of Cdk (cyclin-dependent kinase) inhibitors and by inhibiting both cdc25a (cell division cycle 25 homolog A)\textsuperscript{40} and c-myc\textsuperscript{41} by means of Smad–E2F4/5-pocket protein repressor complexes. The rapid surge in E2F1 that we observe in response to TGF-\(\beta\) may thus effectively
It is interesting to note that the E2F family acts via distinct pathways to regulate specific genes, yet all toward a global action of tumor suppression. We can thus consider the E2F family as ‘super-mediators’ of TGFβ tumor-suppressive effects. A better understanding of the mechanisms by which both TGFβ/1 and E2F1 exert their tumor-suppressive roles may prove useful for the development of novel therapeutic strategies aimed at restoring the apoptotic or tumor-suppressive response of the E2Fs in human cancer.

**Materials and Methods**

**Cell culture and transfections.** HaCaT, HuH7, HepG2, Moser, and SKCO cell lines, as well as MEFs were cultured in DMEM (HyClone, Logan, UT, USA) and WM278 cells in RPMI-1640 (HyClone). Medium for all cells was supplemented with 10% fetal bovine serum (FBS) (HyClone) and 2 mM L-glutamine ( Gibco, Grand Island, NY, USA), and cells were grown at 37°C in 5% CO2 conditions. Before treatment, cells were serum-starved for 24 h and all stimulations were done in serum-free medium containing 100 μM TGFβ/1 (PeproTech, Rocky Hill, NJ, USA). Cells were transiently transfected with different siRNAs against E2F1 (Ambion, Foster City, CA, USA) or P/CAF (Sigma-Aldrich, St. Louis, MO, USA), or with wild-type and mutant E2F1 expression vectors using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions.

**Viability assays.** Cells were seeded in triplicate in 96-well plates, at 10 000 cells/100 μl in medium supplemented with 2% FBS, and in the presence or absence of 100 μM TGFβ/1. Mitochondrial viability was determined by MTT colorimetric assay. Briefly, following 4–24 h of TGFβ/1 treatment, cells were incubated with 1 mg/ml MTT solution (Sigma-Aldrich) in the culture media for 2 h. Formazan crystals were solubilized overnight in 50% dimethyl formamide, 20% SDS, pH 4.7, and the absorbance of each well was measured at 570 nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Alternatively, cell viability was determined by the fluorescent calcine-AM method. Briefly, following 4–24 h of TGFβ/1 treatment, original culture medium was replaced with serum-free medium containing 2 μg/ml calcine-AM (BD Biosciences, San Diego, CA, USA) for 60 min at 37°C. Cells were then washed twice with PBS and the fluorescence of each well was monitored from the bottom of the wells at excitation and emission wavelengths of 485 and 520 nm, respectively, using a FLUOstar Optima microplate reader (BMG Labtech, Ortenberg, Germany).

**RNA isolation and real-time quantitative PCR.** Total RNA was isolated from cell lines using TRIzol reagent (Invitrogen) and reverse transcribed using random hexamers and M-MLV Reverse Transcriptase (Invitrogen), as per the manufacturer’s instructions. Subsequently, real-time qPCR was carried out using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) in a RotorGene 6000 PCR detection system (Corbett Life Science, Montreal Biotech Inc., Kirkland, QC, Canada). The conditions for qPCR were as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 20 s. Primer sequences are listed in Table 1. Where indicated, some cDNAs were amplified for 30 cycles instead and amplified products were analyzed by DNA gel electrophoresis.

**Immunoblotting and immunoprecipitation.** Cells were lysed in cold RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA), containing 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin.
Lysates were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with the specified antibodies overnight at 4°C: anti-E2F1 (KH65, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-β-tubulin (Sigma-Aldrich), and anti-phospho-Smad3 (BioSource, Camarillo, CA, USA). Following primary antibody incubation, membranes were washed twice in TBST (50 mM Tris-HCl at pH 7.6, 200 mM NaCl, 0.05% Tween20) and incubated with secondary antibody coupled to horseradish peroxidase (Sigma-Aldrich) at 1: 10 000 dilution for 1 h at room temperature. Membranes were then washed three times in TBST for 15 min. Immunoreactivity was revealed by chemiluminescence and detected using an Alpha Innotech Fluorochem Imaging system (Packard Canberra, Montreal, QC, Canada). Immunoprecipitations were performed overnight at 4°C using antibodies against E2F1 (C-20, Santa Cruz Biotechnology), pRB (Cell Signaling, Danvers, MA, USA), P/CAF (Abcam, Cambridge, MA, USA), and CBP/p300 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-Smad3 (BioSource, Camarillo, CA, USA). Following primary antibody incubation, membranes were washed twice in TBST (50 mM Tris-HCl at pH 8.1), and LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.1), high-salt wash buffer (0.1% SDS, 10 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.1), and LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and twice in TE buffer. Complexes were then eluted twice in 150 μl of freshly made elution buffer (1% SDS, 0.1 M NaHCO3) by incubating at 65°C for 10 min. To reverse cross-linking, 0.2 M NaCl and 1 μl of 10 mg/ml RNaseA was added to each sample, and they were incubated at 65°C overnight. Following this, 5 mM EDTA and 2 μl of 10 mg/ml protease K was added, and samples were incubated for at 45°C for 2 h. DNA was recovered using the QIAquick spin columns (Qiagen, MD, USA) as per the manufacturer’s protocol and PCR analysis was performed using primers specific for the indicated promoters, as listed in Table 2.

Statistical analysis. Results are expressed as mean ± standard deviation of at least three independent experiments. Statistical differences were determined by two-tailed unpaired t-test. *P<0.05 was considered statistically significant.

Conflict of Interest
The authors declare no conflict of interest.

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