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

J Korah¹, N Falah¹, A Lacerte¹ and JJ Lebrun^{*1}

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

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Transforming growth factor- β (TGF β) 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 TGF β , the prototype of the family, is a vital factor in the maintenance of homeostasis between cell growth and apoptosis. TGF β exerts its tumor-suppressive effects by inhibiting cell-cycle progression, inducing apoptosis, and preventing immortalization through inhibition of telomerase activity. Loss or mutation of TGF β signaling components is frequently observed in human cancer and further define a tumor-suppressive role for this growth factor.²

TGF β ligands signal through serine/threonine kinase receptors that, once activated by ligand binding, recruit and phosphorylate 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 TGF β ligands.³

TGF β 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 TGF β -mediated apoptosis. TGF β signaling activates caspases in various epithelial cell types^{5,6} and transcriptionally

induces DAPK (death-associated protein kinase) in hepatoma cells.⁷ TGF β 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 TGF β -mediated programmed cell death.^{9,10} However, these mechanisms are context and tissue-specific; a central mechanism acting downstream of TGF β to induce apoptosis has not yet been described.

We previously demonstrated that the TGF β 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 TGF β 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

¹Division of Medical Oncology, Department of Medicine, McGill University Health Center, Montreal, Canada

^{*}Corresponding author: JJ Lebrun, Department of Medicine, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Quebec H3A 1A1, Canada. Tel: +514 843 1553; Fax: +514 982 0893; E-mail: JJ.Lebrun@mcgill.ca

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Abbreviations: CHX, cycloheximide; E2F, E2 promoter-binding factor; MEF, mouse embryonic fibroblast; P/CAF, p300/CREB-binding protein-associated factor; PI3K, Phosphatidylinositol 3-kinase; pRb, retinoblastoma tumor-suppressor protein; Smac/DIABLO, second mitochondrial-derived activator of caspase/direct IAP-binding protein with low pI; TGF β , transforming growth factor- β

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tumors^{13,14} supporting an oncogenic role for E2F1, transgenic mice overexpressing E2F1 display aberrant cell apoptosis.¹⁵ Furthermore, E2F1 knockout mice develop highly malignant tumors and show defects in thymocyte apoptosis, highlighting E2F1 as a potent tumor suppressor.¹⁶ 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.¹⁷ 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 E2F1.¹⁸ Given our previous findings that E2F1 is required for TGF β -mediated inhibition of hTERT (human telomerase reverse transcriptase)¹¹ and that TGF β promotes increased E2F-DNA-binding activity in pre-apoptotic hepatoma cell nuclear extracts,¹⁹ we investigated whether E2F1 could also mediate another arm of the TGF β tumor-suppressive response and regulate apoptosis.

We found TGF β 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 TGF β -mediated apoptosis in normal cells. Moreover, we found TGF β to increase E2F1 protein stability, acting post-translationally. We further investigated the molecular mechanisms by which E2F1 contributes to TGF β -mediated cell death and found that TGF β could promote formation of a transcriptionally active E2F1-pRb-P/CAF (p300/CREB-binding protein-associated factor) complex onto the promoters of TGF β -targeted apoptotic genes to activate their transcription. Together, our results underline E2F1 as a central mediator of the TGF β pro-apoptotic response and highlight the E2F1-pRb-P/CAF signaling pathway as a critical regulator of TGF β -mediated cell death.

Results

TGF β -mediated apoptosis is dependent on E2F1. We first examined the pro-apoptotic effect of TGF β 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 TGF β as indicated and apoptosis was assessed using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) cell viability assay as well as calcein-AM (calcein-acetoxymethyl ester) assay, a more sensitive assay for early apoptosis detection.²⁰ All cell lines tested were strongly growth inhibited by TGF β treatment in a time-dependent manner (Figures 1a and b). To address the

contribution of E2F1 in mediating this TGF β response, we used RNA interference to reduce the expression of endogenous E2F1. Interestingly, we found that the effect of TGF β on cell viability (Figure 1c) and early apoptosis (Figure 1d) in all the cell lines tested was almost completely prevented when E2F1 expression was silenced, indicating that E2F1 is required for mediating the TGF β pro-apoptotic response in multiple cell lines of various origins.

To further investigate the role of E2F1 in TGF β -mediated apoptosis, we performed fluorescence-activated cell sorting (FACS) following AnnexinV and propidium iodide staining. Although TGF β treatment markedly increased the number of apoptotic cells in control siRNA-transfected HuH7 cells (Figure 1f, left panels), E2F1 knockdown completely abolished this effect (Figure 1f, right panels), consistent with cell viability and calcein-AM results. Fluorescence imaging following AnnexinV staining further confirmed these findings (Figure 1g). Taken together, these results indicate that TGF β has a strong pro-apoptotic function in various cell lines and that these effects require the transcription factor E2F1.

E2F1 is required for TGF β -mediated regulation of pro-apoptotic target genes. TGF β signaling activates multiple pro-apoptotic genes and pathways in a cell- and tissue-specific manner.⁴ Independently of TGF β , the E2F pathway is also involved in multiple distinct apoptotic mechanisms. In varying cell types and tissues, E2F1 alone has been shown to activate numerous pro-apoptotic genes, including *Apaf1* (apoptotic protease activating factor 1), *p14ARF*, *p73*, *Caspase 3*, *Caspase 7*, *Caspase 8*, *Chk2* (checkpoint kinase 2), *Ask-1* (apoptosis signal-regulating kinase 1), and *Smac/DIABLO* (second mitochondrial-derived activator of caspase/direct IAP-binding protein with low pI).^{21–27}

To assess whether TGF β and E2F1 share any common downstream apoptotic targets, we examined the regulation of representative E2F1-responsive pro-apoptotic genes in TGF β -treated human hepatoma HuH7 cells, which express both functional p53 and pRb. As shown in Figure 2a, TGF β potentially induced mRNA expression of *Apaf1*, *Caspase 3*, *Caspase 7*, *p73*, and *Smac/DIABLO*, suggesting that TGF β induces apoptosis in HuH7 cells by the intrinsic mitochondrial pathway. Importantly, this analysis also revealed *Smac/DIABLO* as a novel TGF β target. Loss of E2F1 expression markedly impaired the TGF β -mediated induction of each of these target genes (Figure 2b), indicating that E2F1 is required for TGF β -mediated regulation of its pro-apoptotic downstream target genes. Moreover, these data provide a novel pathway by which TGF β regulates these genes and reveals E2F1 as a widespread co-transducer of TGF β -induced activation of the intrinsic mitochondrial pathway.

To then examine whether these pro-apoptotic genes are direct targets of TGF β , cells were treated or not with the

Figure 1 TGF β -mediated apoptosis is dependent on E2F1. (a and b) The specified cell lines were untreated or treated with TGF β (100 pM) for the indicated times and assessed for cell viability by (a) MTT and (b) calcein-AM assays. Data are represented as mean $\pm$ S.D. (c, d) Cells were transiently transfected with two different siRNAs against E2F1 or a control non-silencing siRNA and assessed by (c) MTT and (d) calcein-AM assays. (e) The efficiency of E2F1 knockdown by siRNA was verified by immunoblotting with an E2F1 specific antibody. (f and g) Activation of the apoptotic program by TGF β was assessed by AnnexinV staining followed by (f) FACS or (g) fluorescence microscopy, in HuH7 cells transiently transfected with a control, non-targeting siRNA, or E2F1 siRNA. In (f), values represent the percentage of early and late apoptotic cells and represent the mean $\pm$ S.D. (h) Expression of endogenous E2F1 in these cells was assessed by immunofluorescence

Figure 2 E2F1 is required for TGF β -mediated regulation of proapoptotic genes. (a) HuH7 cells were stimulated with TGF β (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 $\pm$ S.D. (b) HuH7 cells were transiently transfected with siRNA against E2F1 or a control non-silencing siRNA and stimulated with TGF β (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 μ M) or vehicle and then stimulated with TGF β (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 TGF β as indicated. Interestingly, CHX treatment of the cells completely impaired the induction of these genes by TGF β (Figure 2c). As a control, the mRNA expression levels of a direct TGF β target gene, *Smad7*, were also examined and, as expected, were not affected by CHX treatment. These results indicate that TGF β regulation of expression of its downstream pro-apoptotic target genes is indirect and requires the induction of a TGF β -responsive transcriptional activator.

TGF β rapidly and transiently induces E2F1 protein expression levels. Having shown that TGF β 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 TGF β . TGF β 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 TGF β to rapidly and transiently induce E2F1 protein expression levels in these cells (Figure 3b). We then examined the TGF β 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 TGF β in all the cell lines tested. This effect was transient, however, as longer exposure to TGF β resulted in a return to basal E2F1 protein levels. Interestingly, in all cases the increase in E2F1 expression in response to TGF β was very rapid, suggesting that TGF β induces post-translational protein stabilization of E2F1. To address this, we performed a CHX chase in HaCaT cells treated or not with TGF β (Figure 3d). In the presence of CHX, untreated cells showed progressive diminished levels of E2F1 over time. Conversely, TGF β treatment maintained E2F1 levels throughout the chase, indicating that TGF β indeed prolongs E2F1 half-life, by stabilizing E2F1 protein levels post-translationally.

TGF β pro-apoptotic effects are impaired in E2F1-null embryonic fibroblasts. Having shown that TGF β -induced apoptosis in various epithelial cancer cell lines requires E2F1, we next examined the contribution of E2F1 downstream of TGF β -mediated cell death in normal cells. For this,

Figure 3 TGF β rapidly and transiently induces E2F1 protein expression levels. HaCaT cells were stimulated with TGF β (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 β -treated cells of various origins, as indicated. (d) Cycloheximide (CHX) chase analysis in HaCaT cells to address the potential contribution of TGF β in E2F1 post-translational stabilization. Cells were incubated with CHX (50 μ g/mL) and treated or not with TGF β (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 β stimulation, as assessed by the induction of Smad phosphorylation (Figure 4a). The pro-apoptotic effect of TGF β , however, greatly differed in these two cell types. Although cell viability of the wild-type E2F1^{+/+} MEFs was potently decreased in response to TGF β , this effect was severely impaired in the E2F1^{-/-} MEFs (Figure 4b). Correspondingly, TGF β -induced expression of *Caspase 7* and *Smac/DIABLO* was significantly reduced in the E2F1^{-/-} MEFs (Figure 4c). Together, these findings highlight a critical

Figure 4 TGF β pro-apoptotic effects are impaired in E2F1-null embryonic fibroblasts. (a) Wild-type (WT) and E2F1^{-/-} mouse embryonic fibroblasts (MEFs) were untreated or treated with TGF β (100 pM) for the indicated times and phospho-Smad3 levels of total cell lysates were analyzed by western blotting. (b) WT and E2F1^{-/-} MEFs were stimulated or not with TGF β (100 pM) for 24 h and cell viability assessed by calcein-AM assay. (c) *Caspase 7* and *Smac/DIABLO* mRNA levels in TGF β -treated WT and E2F1^{-/-} MEFs were measured by 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 $\pm$ S.D. (* P < 0.05)

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

E2F1 DNA-binding, transactivation, and pRb-interaction are required for TGF β -mediated apoptosis. To further understand the molecular mechanisms underlying the role of E2F1 in the induction of programmed cell death by TGF β , we next addressed the contribution of E2F1's principal regulator, pRb. For this, we used dominant-negative E2F1 mutant forms to alter E2F1 function and/or binding to pRb. Importantly, the DNA-binding-deficient mutant, E2F1 (E132), and the transactivation-defective mutant, E2F1 (1–374), are both reportedly unable to activate transcription, whereas the E2F1 Y411C mutant, which has lost its ability to interact with pRb, retains similar transcriptional-activating potential as its wild-type E2F1.³⁰ Interestingly, transient overexpression of each of these mutants drastically impeded the effect of TGF β on cell viability in HuH7 cells (Figure 5a). The antagonistic effects of these E2F1 mutants were further

Figure 5 E2F1 DNA-binding, transactivation, and pRb-interaction are required for TGF β -mediated apoptosis. HuH7 cells transiently transfected with empty vector or mutant E2F1 expression constructs as indicated were untreated or treated with 100 pM TGF β for 24 h. (a) Cell viability was assessed by calcein-AM assay, with bars representing means $\pm$ S.D. (b) *Caspase 7* and *Smac/DIABLO* mRNA levels were measured by real-time qPCR analysis. Results are normalized to *GAPDH* and show the mean $\pm$ S.D., expressed as relative to levels observed in untreated cells (set to 1). (c) HuH7 cells untreated or treated with TGF β (100 pM) were subjected to immunoprecipitation (IP) with the specified antibodies followed by western blotting (WB) to assess levels of associated E2F1 and pRb

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 $\pm$ 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 against 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 $\pm$ 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,³² we investigated whether the loss of TGF β -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 β -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 β -induced caspase activation. Collectively, these findings support a critical role for P/CAF downstream of TGF β in the E2F1-dependent activation of pro-apoptotic genes and the mediation of programmed cell death.

To then assess the functional relevance of the TGF β -induced pRb-E2F1-P/CAF complex in regulating TGF β transcriptional responses, we performed chromatin immunoprecipitation assays to determine whether this complex is recruited to the pro-apoptotic target gene promoters in response to TGF β . We examined the promoters of the TGF β - and E2F1-responsive pro-apoptotic genes identified above. Interestingly, as shown in Figure 6f, TGF β 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 β -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 β in normal and cancer cells.

Discussion

Although various apoptotic mediators and signaling pathways have been implicated in TGF β -mediated apoptosis, most of these regulatory mechanisms appear to be cell type-dependent or tissue-specific.⁴ This study defines a novel process of gene activation by the TGF β -E2F1 signaling axis, and highlights the pRb-E2F1-P/CAF pathway as a wide-ranging and critical mediator of the TGF β apoptotic program in multiple target tissues.

We identified a number of key pro-apoptotic TGF β target genes that trigger the intrinsic apoptosis pathway through the induction of E2F1. Although these genes are functionally interrelated, our results imply that TGF β 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 β -mediated cell death. Importantly, these results are corroborated using the E2F1 knockout mouse model, demonstrating that the TGF β -E2F1 signaling pathway mediates TGF β -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.¹² 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.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 β . Indeed, analysis with dominant-negative E2F1 mutants revealed that, in fact, pRb binding to E2F1 is required for TGF β -mediated apoptosis.

Our results also indicate that TGF β 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,³⁴ acetylation,³⁵ and by the ubiquitin-proteasome pathways.³⁶ Binding of pRb to E2F1 protects E2F1 from ubiquitination and proteolytic degradation,³⁷ thereby increasing its stability. As TGF β maintains pRb in a hypophosphorylated form, causing E2F1 to remain bound to pRb and suppressing activation of E2F1-responsive cell-cycle regulatory genes,³⁸ it is likely that the TGF β 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 β , 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.³⁵ Thus, P/CAF binding to E2F1 in response to TGF β 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-specific binding site that does not interfere with E2F1's transactivation domain.³⁹ It is interesting to consider, then, whether TGF β 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 β 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 β pro-apoptotic targets. Taken together, these results strongly support a pro-apoptotic role for the E2F1 pathway downstream of TGF β and provide a potential mechanism for the activation of E2F1-responsive pro-apoptotic genes in response to TGF β .

It is interesting to consider that TGF β tumor-suppressive effects might utilize the functional interplay among the E2F family members, which affects E2F activity. It is well-established that TGF β 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)⁴⁰ and c-myc⁴¹ by means of Smad-E2F4/5-pocket protein repressor complexes. The rapid surge in E2F1 that we observe in response to TGF β may thus effectively

initiate the TGF β apoptotic program, without affecting cell cycle, as TGF β maintains transcriptional repression of factors required for S-phase entry through the other E2F family members. Moreover, E2F4, in complex with pRb or p107, is capable of binding to E2F-binding sites on the E2F1 promoter, leading to its repression after 4 h of TGF β treatment.²⁸ Thus, it is conceivable that TGF β treatment leads to increased levels of E2F1, triggering the activation of pro-apoptotic genes. Subsequently, in addition to directly inhibiting cell-cycle regulatory genes, E2F4 may repress E2F1 levels following longer stimulation with TGF β , further preventing cell-cycle progression.

The present work delineates a novel process of gene activation by the TGF β -E2F1 signaling axis and supports a role for the E2F family as potent co-transducers of TGF β signals. Combined with previous studies from our lab and others, these findings highlight the crucial role for the E2F family in regulating TGF β tumor-suppressive effects and we propose the following model of E2F tumor-suppressive action downstream of TGF β (Figure 7):

- (1) TGF β induces E2F4/5 recruitment into classical repressive pRb-E2F-HDAC (histone deacetylase) complexes, which target key cell-cycle regulators, such as *cdc25a*⁴⁰ and *c-myc*,^{41,42} preventing cell-cycle entry.
- (2) TGF β also induces E2F1 recruitment into repressive E2F-HDAC complexes, inhibiting hTERT expression and suppressing immortalization, as we have previously demonstrated.¹¹
- (3) The current study demonstrates that TGF β can also recruit E2F1 into transcriptionally active pRb-E2F1-P/CAF complexes, increasing the expression of multiple pro-apoptotic target genes and inducing programmed cell death.

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 TGF β tumor-suppressive effects. A better understanding of the mechanisms by which both TGF β 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 pM TGF β 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 μ l in medium supplemented with 2% FBS, and in the presence or absence of 100 pM TGF β . Mitochondrial viability was determined by MTT colorimetric assay. Briefly, following 24–72 h of TGF β 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 calcein-AM method. Briefly, following 4–24 h of TGF β treatment, original culture medium was replaced with serum-free medium containing 2 μ g/ml calcein-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 μ g/ml aprotinin, 2 μ g/ml leupeptin, and 1 μ g/ml pepstatin.

Figure 7 The pRb/E2F signaling pathway mediates three distinct arms of TGF β tumor-suppressive effects. See text for details

Table 1 PCR primer sequences

Apaf1 forward	5'-CTCTCATTGCTGATGTCGC-3'
Apaf1 reverse	5'-TCGAAATACCATGTTTGGTCA-3'
TAp73 forward	5'-CATGGAGACGAGGACGTA-3'
TAp73 reverse	5'-CTGTAACCCCTGGGAGGTGA-3'
Caspase3 forward	5'-AGCGAATCAATGGACTCTGG-3'
Caspase3 reverse	5'-CGGCCTCCACTGGTATTTTA-3'
Caspase7 forward	5'-GCAGTGGGATTGTGCTTCT-3'
Caspase7 reverse	5'-CCCTAAAGTGGGCTGTCAAA-3'
Smac/DIABLO forward	5'-AATGTGATTCTGGCGGTTA-3'
Smac/DIABLO reverse	5'-AGCTGGAACCACTTGGATG-3'
GAPDH forward	5'-GCCTCAAGATCATCAGCAATGCCT-3'
GAPDH reverse	5'-TGTGGTCATGAGTCCTTCCACGAT-3'
Smad7 forward	5'-TCCTGCTGTGCAAAGTTTC-3'
Smad7 reverse	5'-CAGGCTCCAGAAGAAGTTGG-3'

Lysates were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with the specified antibodies overnight at 4 °C: anti-E2F1 (KH95, 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 in TBST four times 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). Protein A-sepharose (Amersham Biosciences, Uppsala, Sweden) was added for 2 h at 4 °C, and beads were then washed four times with cold lysis buffer. The immunoprecipitates were eluted with 2 $\times$ SDS Laemmli sample buffer, boiled for 5 min, and subjected to immunoblotting.

Annexin-V apoptotic assays. Apoptotic cells were analyzed using an Annexin V apoptosis detection kit (Santa Cruz Biotechnology). Following TGF β treatment, cells were collected by trypsinization, pelleted by centrifugation, washed with PBS, and each sample was incubated with 0.5 μ g Annexin V-FITC and 10 μ l propidium iodide (50 μ g/ml) in the supplied incubation buffer for 15 min. Cells were then analyzed using FACS in an Accuri C6 flow cytometer (BD Biosciences). For fluorescence microscopy, cells were plated on glass coverslips at 80% confluence. Following TGF β treatment, cells were washed with PBS and subjected to Annexin V-FITC staining for 15 min as described above. Stained coverslips were mounted onto slides with SlowFade Gold Antifade with DAPI (Invitrogen), and immediately examined.

Immunofluorescence. Cells plated on glass coverslips were fixed with 4% paraformaldehyde, permeabilized in PBS containing 0.1% Triton X-100 for 3 min, washed with PBS, and blocked with 2% bovine serum albumin (BSA) for 30 min. Cells were then incubated with anti-E2F1 antibody (Santa Cruz Biotechnology) for 1 h, washed with PBS, and incubated with AlexaFluor568 goat anti-mouse IgG secondary antibody (Invitrogen) for 1 h. After a final wash, stained coverslips were mounted with SlowFade Gold Antifade with DAPI (Invitrogen) and examined using a Zeiss LSM-510 Meta Axiovert confocal microscope (Carl Zeiss, Thornwood, NY, USA).

Caspase activity. Cells were plated in triplicate in 96-well dishes, at 10 000 cells/100 μ l in medium supplemented with 2% FBS, and in the presence or absence of 100 pM TGF β . Caspase 3/7 activity was measured using the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, following TGF β treatment, cells were incubated with Caspase-Glo reagent (Promega) for 1.5 h at room temperature, and the luminescence of each sample was measured using a luminometer (EG & G Berthold, Bad Wildbad, Germany).

CHX chase. Cells were seeded in 60-mm² plates and grown to 85% confluence. Following overnight serum-starvation, the cells were incubated, in the presence or absence of 100 pM TGF β , with 50 μ g/ml CHX (Sigma-Aldrich) for the indicated times and analyzed by immunoblotting.

Table 2 ChIP primer sequences

Apaf1 forward	5'-GCCCCGACTTCTTCCGGCTCTTCA-3'
Apaf1 reverse	5'-GAGCTGGCAGCTGAAAGACTC-3'
TAp73 forward	5'-TGAGCCATGAAGATGTGCGAG-3'
TAp73 reverse	5'-GCTGCTTATGGTCTGATGCTTATGG-3'
Caspase7 forward	5'-TTTGGGCACCTGGAGCGCG-3'
Caspase7 reverse	5'-AAGAGCCCAAGCGACCCGT-3'
Smac/DIABLO forward	5'-TTCCCTTCAAGCCCTGGCCCGAAC-3'
Smac/DIABLO reverse	5'-ACGCCCCACCCAAGGAAGCAGTC-3'

Chromatin immunoprecipitation. Protein complexes were cross-linked to DNA by adding formaldehyde directly to tissue culture medium to a final concentration of 1%. Crosslinking was allowed to proceed for 10 min at room temperature and was then stopped by the addition of glycine to a final concentration of 0.125 M. Cross-linked cells were harvested, washed with PBS, pelleted by centrifugation at 2000 r.p.m. for 5 min at 4 °C, and lysed in nuclear lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)), supplemented with 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 2 μ g/ml pepstatin, for 10 min on ice. The resulting chromatin solution was sonicated for five pulses of 20 s to generate 300–2000 bp DNA fragments. After centrifugation at 14 000 r.p.m. for 10 min at 4 °C, the supernatant was immunocleared by incubation with protein A-sepharose beads for 2 h at 4 °C. Immunocleared chromatin was immunoprecipitated overnight with 5 μ g of the indicated antibodies. Antibody–protein–DNA complexes were then isolated by immunoprecipitation with 40 μ l protein A-sepharose beads (Amersham) for 2 h with rotation at 4 °C. Beads were washed consecutively for 10 min each with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 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 μ l 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 μ l 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 μ l of 10 mg/ml proteinase 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 $\pm$ 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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