Role of the pRb/E2F Pathway in TGFβ-Mediated Tumour Suppression

Juliana Korah

Department of Experimental Surgery
McGill University
Montreal, Canada
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Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.

- Marie Curie

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Abstract

Tumour formation is characterized by a series of well-defined events that occur in virtually all human cancers, including the ability of cells to attain immortalization, sustain proliferation and evade apoptosis. These cell growth and cytostatic processes are normally regulated by various growth factors that act in concert to maintain proper cellular homeostasis. As a result, deregulation of these growth factor signalling pathways leads to uncontrolled cell growth and tumour formation. In particular, transforming growth factor-β (TGFβ) exerts a central role in preventing tumour formation in virtually all cell types and tissues. TGFβ tumour suppressive effects are mainly illustrated by its ability to inhibit cell growth, induce cell death and prevent cell immortalization. TGFβ-mediated prevention of cell immortalization relies on inhibition of telomerase activity. While expression of hTERT, the protein component of telomerase, is increased in most cancer cells, studies from our laboratory revealed that TGFβ efficiently represses hTERT gene expression in both normal and cancer cells through multiple signalling pathways. We further found that the inhibition of hTERT by TGFβ requires the synthesis of an intermediate molecule that we identified as the transcription factor E2F1, and showed that interfering with E2F1 activity impedes the TGFβ inhibitory effect on telomerase activity. The E2F family of transcription factors plays a central role in regulating cell-cycle progression. Deregulation of these factors is a common event in most human cancers. Interestingly, E2F1 has been shown to have the ability to induce both cell cycle progression and apoptosis, though the mechanisms of E2F-mediated apoptosis have not been fully elucidated. TGFβ itself is a potent pro-apoptotic factor, as it modulates the expression of multiple apoptotic genes in various tissues. However, a common and central signalling pathway, acting downstream of TGFβ and leading to cell death, had yet to be uncovered. Interestingly, recent work from our laboratory highlighted E2F1 as a central factor downstream of TGFβ-induced apoptosis in cancer cells. Using the E2F1 knockout mouse model, we found E2F1 to be required for TGFβ-mediated apoptosis in normal cells as well. 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 pro-apoptotic target gene promoters, thereby activating their transcription. These findings define a novel process of gene activation by the TGFβ-E2F1 signalling axis, and uncover the pRb/E2F1 pathway as a wide-ranging and critical mediator of the TGFβ apoptotic programme in multiple target tissues. We further determined that TGFβ induces pRb/E2F1-dependent transcriptional activation of several autophagy-related genes, potentially leading to autophagic cell death. Together, our studies support a role for the pRb/E2F pathway as a potent co-transducer of TGFβ signalling and highlight the pivotal role for pRb/E2F in mediating TGFβ tumour-suppressive effects.

Résumé

La formation de tumeurs est caractérisée par une série d'évènements bien définis qui sont communs à tous les cancers, notamment l'habileté des cellules à atteindre l'immortalisation, maintenir la prolifération, et éviter l'apoptose. Ces procédés cytostatiques et de croissance cellulaire sont normalement régulés par divers facteurs de croissance qui agissent de concert pour maintenir l'homéostasie cellulaire. De ce fait, un dérèglement dans la signalisation de ces facteurs mène à une croissance incontrôlée et à la formation de tumeurs. Plus particulièrement, le facteur de croissance β (TGFβ) exerce un rôle central en agissant comme suppresseur de tumeurs dans quasiment tous les types cellulaires et tissus. Les effets en tant que suppresseur de tumeurs du TGFβ sont illustrés par son habileté à inhiber la croissance cellulaire, induire la mort cellulaire, et prévenir l'immortalisation cellulaire. La capacité du TGFβ à prévenir l'immortalisation cellulaire s'appuie sur le fait qu'il inhibe l'activité de la télomérase. Bien que l'expression de hTERT, la protéine faisant partie du complexe de la télomérase, soit accrue dans la plupart des cellules cancéreuses, des études de notre laboratoire ont révélé que le TGFβ peut réprimer l'expression du gène codant pour hTERT dans les cellules autant normales que cancéreuses à travers des voies de signalisation multiples. Nous avons de plus trouvé que l'inhibition de hTERT par le TGFβ requiert la synthèse d'une molécule intermédiaire que nous avons identifié comme étant le facteur de transcription E2F1, et avons démontré que d'interférer avec l'activité du E2F1 empêche les effets inhibiteurs du TGFβ sur l'activité de la télomérase. La famille des facteurs de transcription E2F joue un rôle central dans la régulation de la progression du cycle cellulaire. La dérégulation de ces facteurs est un évènement commun dans la plupart des cancers. Il a été démontré que le E2F1 possède l'habileté d'induire autant la progression du cycle cellulaire que l'apoptose, cependant, le mécanisme par lequel le E2F1 induit l'apoptose n'est pas complètement élucidé. Le TGFβ est lui-même un facteur pro-apoptotique puissant, car il module l'expression de plusieurs

gènes apoptotiques dans une variété de tissus. Cependant, une voie de signalisation commune et centrale, agissant en amont du TGFβ et amenant à la mort cellulaire, reste toujours à démontrer. Des travaux récents de notre laboratoire soulignent le E2F1 comme étant un facteur central en amont de l'apoptose médiée par le TGFβ dans les cellules cancéreuses. En utilisant un modèle de souris où le E2F1 a été inhibé, nous avons pu déterminer que le E2F1 est requis pour l'apoptose médiée par le TGFβ également dans les cellules normales. Nous avons investigué plus en profondeur les mécanismes moléculaires par lesquels le E2F1 contribue à l'apoptose médiée par le TGFβ et avons trouvé qu'un traitement par le TGFβ mène à la formation du complexe transcriptionellement actif E2F1-pRb-P/CAF sur le promoteur de plusieurs gènes pro-apoptotiques pour activer leur transcription. Ces découvertes définissent un nouveau procédé d'activation des gènes par l'axe de signalisation TGFβ-E2F1, et a permis d'élucider la voie pRb/E2F1 comme un médiateur critique et à grande portée du programme apoptotique du TGFβ et ce, dans plusieurs tissus cibles. Nous avons de plus déterminé que le TGFβ induit l'activation de la transcription de gènes autophagiques par le complexe pRb/E2F1. Collectivement, nos études supportent un rôle pour le complexe pRb/E2F1 dans la voie de signalisation du TGFβ et dans ses effets en tant que suppresseur de tumeurs.

Acknowledgements

When you spend so much time in one place, countless people come and go, many of which leave their mark in one way or another. I would like to take this opportunity to thank those who have been an important part of my graduate school experience, both in and out of the lab.

Firstly, I would like to thank my supervisor, Dr. Jean-Jacques Lebrun, for his guidance throughout my PhD and for allowing me to develop on my own as an independent researcher. Your passion for science is inspiring and your patience with students (especially a stubborn lot like us) has been much appreciated over the years. I will surely carry all that I have learned as your student with me throughout my future scientific endeavours.

I would also like to thank my Supervisory Research Committee: Dr. John Mort, Dr. Suhad Ali, and Dr. Mario Chevrette, for their support and insightful discussions about my research throughout my studies. Special thanks to Dr. Ali for allowing me to collaborate with her lab and for the helpful discussions regarding my future goals and career.

To my fellow lab members, both past and present, many thanks for all your help and support throughout the years. To my former lab members, particularly Annie Lacerte, thank you for mentoring me through the early steps of grad school. To my current (and some more recently 'retired') lab members, it has been a pleasure working with you and I am so grateful for your friendship, particularly:

Nadège Fils-Aimé – my cheerleader, sister from another mister, and dear friend. I couldn't imagine working at the lab without your cheery presence (and singing!) and your constant support, both personal and professional, not to mention countless midnight motivational texts! I wish you every happiness and success and look forward to seeing what incredible things you will accomplish.

Meiou Dai – dear Mama Meiou, who takes such good care of us all, and is certainly our lab go-to girl. I still want to be you when I grow up. Thank you for all your help over the years, for your friendship and support. Oh the stories, the laughs, the tears (!), and the memories we have shared, I look forward to the many more that are to come.

Jimin Guo, Amal Al-Odaini, Thomas Sontag, and Manuel Villatoro – thank you for your friendship, kindness, good laughs, and memorable times. You have all been such great lab members, and even greater friends.

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To my beautiful and brilliant sisters, Nadine and Caroline, who manage to balance professionalism and grace with such ease and inspire me to work hard and to reach further in everything I do (and to do so stylishly). I cannot thank you enough for your continuous support and motivation. You are both an inspiration and your love and encouragement means the world to me.

To my Mum, for guiding me to become the person I am today. From day one of kindergarten to my final days of grad school (a lifetime of school!), you have always believed in me and helped me believe in myself. It has been a long and sometimes arduous journey, but your faith in me and trust in my goals has helped me to achieve them. Thank you for your unequivocal love and support, always.

Many thanks to Karin & Stan Balchunas for your continued encouragement and confidence in me and my endeavours. To my extended family, the Knopps, for always asking how my cells were doing and whether I had cured cancer yet – thank you for keeping me motivated and reminding me to keep sight of the 'bigger picture'. I am ever so grateful for your constant support.

To my darling husband, Ryan, I am profoundly indebted to you. We met during my first year of grad school and you have been my beacon of strength throughout these last seven (*seven!*) years. You have kept me grounded and your enthusiastic encouragement (not to mention weekly motivations to complete my thesis and apply for jobs already!) has helped me push through this final year. I adore you and am so very excited to start the next chapter in our life together.

Finally, to the greatest teachers I have ever had, my Babçia and Dziadzia. I try to lead my life by your example, as you have taught me the most important things that I will ever need to know. I know you both would have been so proud of me. I love and miss you and dedicate this thesis to you.

Preface & Contribution of Authors

The current thesis is presented in a manuscript-based format. The contents of the thesis in its entirety were written by myself, and revised by my supervisor, Dr. Jean-Jacques Lebrun. This thesis comprises five chapters, as follows:

Chapter 1

The first chapter is an introduction and review of the literature, giving a broad overview on TGFβ signalling and the role of TGFβ in cancer, with a focus on the tumour suppressive effects of TGFβ.

Chapter 2

The second chapter addresses the role of TGFβ in cell immortalization. It contains original published work demonstrating that the TGFβ inhibitory effect on telomerase activity and cell immortalization is dependent on both Smad and non-Smad signalling, and the transcription factor E2F1, highlighting E2F1 as an important mediator of TGFβ tumour suppressive responses. For this study, I performed all experiments for Figure 5, and repeated all experiments in Figure 1 and Figure 2, Figure 3b, Figure 4e, 4f, and 4g. Annie Lacerte, who is co-first author of this publication, designed and performed the initial experiments for Figure 1 and Figure 2, and conducted all experiments for Figure 3a, Figure 4a-d. Mélanie Roy repeated some of the experiments in Figures 1 and 4. The first draft of the manuscript was written by Annie Lacerte, and the final version was revised by myself and Dr. Jean-Jacques Lebrun. Dr. Xiang-Jiao Yang kindly provided the HDAC expression vectors used in Figure 1 and Dr. Serge Lemay provided helpful advice for the study design. This study was published as follows:

Lacerte A[*], **Korah J**[*], Roy M, Yang XJ, Lemay S, and Lebrun JJ. Transforming Growth Factor-β inhibits telomerase through SMAD3 and E2F transcription factors, *Cellular Signalling* (2008) 20: 50–59.

[*] These authors contributed equally to this work

Chapter 3

The third chapter comprises a published study describing a central mechanism by which TGFβ induces apoptosis in both normal and cancer cells of various origins. This study demonstrates that TGFβ increases E2F1 expression post-translationally, further leading to the formation and binding of a transcriptionally active E2F1-pRb-P/CAF complex on multiple TGFβ pro-apoptotic target gene promoters, thereby activating their transcription and highlighting E2F1 as a novel and key mediator of the TGFβ apoptotic programme. I designed and performed all of the experiments and prepared the manuscript, which was revised by Dr. Jean-Jacques Lebrun. Nisrine Falah repeated some of the real-time qPCR experiments in Figure 2a. Annie Lacerte performed some of the initial experiments in Figure 3a and 3b. This study was published as follows:

Korah J, Falah N, Lacerte A, and Lebrun JJ. A transcriptionally active pRb-E2F1-P/CAF signalling pathway is central to TGFβ-mediated apoptosis, *Cell Death and Disease* (2012) 3, e407.

The original published versions of the manuscripts in Chapter 2 and Chapter 3 have been included in the *Appendix* at the end of this thesis.

Chapter 4

The fourth chapter includes an original study in which I investigated the molecular mechanisms of an alternative form of programmed cell death, and uncovered a novel process of TGFβ-mediated autophagy. This study shows that TGFβ induces autophagy at least partially through the pRb/E2F1 pathway and transcriptional activation of autophagy-related genes, further underlining the central relevance of the pRb/E2F1 pathway downstream of TGFβ signalling. For this study, I designed and performed all experiments and wrote the manuscript, which is currently in preparation for submission:

Korah J, and Lebrun JJ. The pRb/E2F1 pathway mediates TGFβ-induced autophagy, (*in preparation*).

Chapter 5

The fifth and final chapter provides a general discussion of the findings from these studies and a brief reflection on their potential clinical implications. Based on my publication in *Cell Death and Disease* described in Chapter 3, I was recently invited to write a book chapter on the role of TGFβ signalling in tumour suppression. Parts of Chapter 5 were reproduced, with permission, from this book chapter:

Korah J, and Lebrun JJ. Role of the pRb/E2F pathway in TGFβ-mediated tumour suppression. Book Chapter in 'Oncology – Theory & Practice'. *iConcept Press* (2013).

In addition to the publications described above, I have also collaborated in and co-authored the following articles during my doctoral studies:

Garic D, Humbert L, Fils-Aimé N, **Korah J**, Zarbafian Y, Lebrun JJ, and Ali S. Development of buffers for fast semi-dry transfer of proteins, *Analytical Biochemistry* (2013) 441(2):182–4.

Guo J, **Korah J**, Rajadurai CV, Dai M, , Fils-Aimé N, Park M, and Lebrun JJ. Breast Cancer Anti-Estrogen Resistance-3 (BCAR3) inhibits TGFbeta/Smad signaling and associates with favorable breast cancer disease outcomes, *Oncogene* (submitted, 2013).

Benyoucef Y, Shams A, Liu F, **Korah J**, Guo J, Garic S, Dai M, Yasruel Z, Lebrun JJ, and Ali S. Prolactin signaling in human breast cancer reveals a suppressive role and a novel pathway linking Nudt21 and Paraspeckles to carcinogenesis, *Cancer Research* (submitted, 2013).

Al-Odaini AA, Dai M, Yun W, **Korah J**, Ali S, and Lebrun JJ. KiSS1 gene as a novel mediator of TGFβ pro-invasive effects in triple negative breast cancer. (*in preparation)*.

Abbreviations

ALK	*activin receptor-like kinases*
Apaf-1	*apoptotic protease activating factor 1*
ARTS	*apoptosis-related protein in the TGFβ signalling pathway*
Ask-1	*apoptosis signal-regulating kinase-1*
ATG	*autophagy-related gene*
Bad	*Bcl-2-associated death promoter protein*
Bax	*Bcl-2-associated X protein*
Bid	*BH3 interacting domain death agonist*
Bcl-2	*B-cell lymphoma 2*
bHLH	*basic-helix-loop-helix*
BMP	*bone morphogenetic protein*
BNIP3	*Bcl-2/adenovirus E1B 19kD -interacting protein 3*
BSA	*bovine serum albumin*
Calcein-AM	*calcein-acetoxymethyl ester*
CBP	*CREB-binding protein*
cdc25A	*cell division cycle 24 homolog A*
CDK	*cyclin-dependent kinase*
CDKI	*cyclin-dependent kinases inhibitor*
Chk2	*checkpoint kinase 2*
ChIP	*chromatin immunoprecipitation*
CHX	*cycloheximide*
Co-Smad	*common Smad*
CREB	*cAMP-response element-binding protein*
DAPK	*death-associated protein kinase*
DMEM	*Dulbecco's modified Eagle's medium*
DMSO	*dimethyl sulfoxide*
DRAM	*damage regulated autophagy modulator*
E2F	*E2 promoter-binding factor*
ERK	*extracellular signal regulated kinase*
EMT	*epithelial to mesenchymal transition*
FACS	*fluorescence-activated cell sorting*
FADD	*Fas-associated protein with death domain*
FasL	*Fas ligand*
FBS	*fetal bovine serum*
G1 phase	*Gap 1 phase*
G2 phase	*Gap 2 phase*
GABARAP	*gamma-aminobutyric acid receptor-associated protein*
GAPDH	*glyceraldehyde-3-phosphate dehydrogenase*
GRK2	*G protein-coupled receptor kinase 2*
HAT	*histone acetyltransferase*
HDAC	*histone deacetylase*
hTER	*telomerase RNA template*
hTERT	*human telomerase reverse transcriptase*
IAP	*inhibitor of apoptosis*
Id	*Inhibitor of Differentiation/DNA binding*
IFN-γ	*interferon gamma*
I-Smads	*inhibitory Smads*
JNK	*c-Jun N-terminal kinase*
LAP	*latency-associated protein*
LTBP	*latent TGFβ-binding protein*

M phase	*Mitosis phase*
MAP1LC3	*microtubule-associated protein 1 light chain 3*
MAPK	*mitogen-activated protein kinase*
MEF	*mouse embryonic fibroblast*
MH	*MAD homology*
MIS	*Müllerian inhibitory substance*
MMP	*Matrix metalloproteinase*
MOMP	*mitochondrial outer membrane permeabilization*
mTOR	*mammalian target of rapamycin*
MTT	*methylthiazolyldiphenyltetrazolium bromide*
NF-κB	*nuclear factor κB*
NLS	*nuclear localization signal*
p15	*p15Ink4b*
p21	*p21CIP1, p21CIP1/WAF1*
PARP	*poly(ADP-ribose) polymerase*
PBS	*phosphate buffer saline*
P/CAF	*p300/CREB binding protein-associated factor*
PCD	*programmed cell death*
PI	*propidium iodide*
PI3K	*phosphatidylinositol 3-kinase*
PIK3C3	*phosphatidylinositol 3-kinase, catalytic subunit type 3*
pRb	*retinoblastoma tumour suppressor protein*
PTEN	*phosphatase and tensin homolog*
RPMI	*Roswell Park memorial institute medium*
R-Smad	*receptor-regulated Smad*
RT-PCR	*reverse transcription polymerase chain reaction*
S phase	*Synthesis phase*
SAPK	*stress-activated protein kinase*
SBE	*Smad binding element*
SHIP	*SH2-domain-containing inositol-5-phosphatase*
siRNA	*small interfering RNA*
Smac/ DIABLO	*second mitochondrial-derived activator of caspase/direct IAP-binding protein with low pI*
TAK1	*TGFβ-activated kinase 1*
TβRI	*TGFβ type I receptor*
TβRII	*TGFβ type II receptor*
TGFβ	*Transforming growth factor-beta*
TIEG1	*TGFβ-inducible early response gene*
TNF	*tumour necrosis factor*
TRAF6	*TNF receptor associated factor 6*
TRAIL	*TNF-related apoptosis-inducing ligand*
TSA	*trichostatin A*
ULK	*Unc-51-Like Kinase*
VPS34	*vacuolar sorting protein 34*

Chapter 1 | General Introduction and Literature Review

1.1 TGFβ Signalling Pathway

The transforming growth factor-β (TGFβ) superfamily comprises widespread and evolutionarily conserved polypeptide growth factors that are involved in the regulation of a multitude of diverse fundamental physiological processes, including cell growth, embryonic development, adult tissue homeostasis, immune regulation, tissue remodeling and repair, and disease pathogenesis [1-7]. At the cellular level, TGFβ regulates cell proliferation, differentiation, migration, immortalization, and cell death [2,3,5].

TGFβ1, the founding member of the TGFβ superfamily, was characterized in the early 1980s following its isolation from human platelets [8], human placenta [9], and bovine kidneys [10]. At the time, it was termed transforming growth factor for its ability to elicit transformation of normal fibroblasts, as demonstrated by their ability to grow in soft agar in an anchorage-independent manner [11]. Soon after its discovery, TGFβ was found to also act as an inhibitor of cell proliferation, thus establishing a dual role of TGFβ in cell-growth control. Since then, it has become evident that TGFβ ligands and their receptors are expressed in virtually all tissues and affect a wide range of cellular processes during embryogenesis and throughout adulthood.

1.1.1 TGFβ ligands

Since the initial discovery of TGFβ1, a steadily growing number of structurally and functionally related members have been identified. The TGFβ superfamily comprises well over 40 members, and these include isoforms of TGFβ (1 to 3), activins (A and B), bone morphogenic proteins (BMPs 1 to 20), growth and differentiation factors (GDFs), inhibins (A and B), nodal, lefty, Müllerian inhibiting substance (MIS), and others [12,13]. Based on their sequence similarity and the specific signalling pathways that they activate, ligands of the TGFβ

superfamily are generally divided into two subfamilies: the TGFβ/Activin/Inhibin/Nodal subfamily, and the BMP/GDF/MIS subfamily. Sequence homology between these subfamilies is approximately 30-50%, while for members within a given subfamily, sequence homology increases to 60-80% [14]. While each ligand presents distinct features of action, they all share common structural features as well as common machinery to transmit intracellular signals.

Structurally, all TGFβ superfamily members are disulfide-bound homodimer or heterodimer complexes that contain a characteristic and conserved spaced pattern of 7-9 cysteine residues which plays an important role in their tertiary structure. Each monomer contains 6-8 cysteine residues that form intramolecular disulfide bonds to form a tight structure known as the cysteine knot, and an additional cysteine residue involved in the inter-subunit disulfide bond linking two monomers to form a dimer. This results in a highly stable protein with a "butterfly-shape" structure [15].

Three distinct TGFβ isoforms (TGFβ-1, -2, -3), each encoded by a different gene, have been identified and characterized. Though these isoforms share approximately 70% sequence homology, TGFβ1 has been the most extensively characterized and most widely studied [13,14] and will hereinafter be referred to simply as TGFβ.

1.1.2 TGFβ receptors

TGFβ family ligands signal through serine/threonine kinase receptors. In contrast to the large number of TGFβ ligands, there are in fact relatively few receptors, which have been structurally and functionally divided into two subgroups, namely type I and type II receptors. At present, 7 type I receptors and 5 type II receptors have been identified in mammals [14]. Both types of receptors consist of an N-terminal extracellular ligand-binding domain, a single transmembrane-spanning

domain, and a cytoplasmic serine/threonine kinase domain. Prior to ligand binding, these receptors present as homodimers at the cell membrane [16].

Many of these receptors were cloned by different groups concurrently and, as a consequence, most type I receptors have several names. Originally termed activin receptor-like kinases (ALKs), the receptors were renamed accordingly once their physiological ligands were determined. For instance, the type I TGFβ receptor, which was originally known as ALK5, is now more commonly referred to as TβRI. On their own, the type I receptors have a relatively low affinity for their ligands, and typically associate with ligand-bound type II receptors or bind ligand cooperatively with type II receptors [17]. A defining feature of type I receptors, not contained within type II receptors, is the presence of a glycine-serine-rich juxtamembrane domain termed the GS box. Upon ligand binding, the type II receptor, which has constitutive kinase activity, phosphorylates the GS box, thereby activating the type I receptor kinase. The ligand-bound type II receptor phosphorylates serine and threonine residues within a characteristic TTSGSGSG sequence of the type I receptor's GS box, which immediately precedes its kinase domain. Phosphorylation within this region of the type I receptor is required for signalling through these receptors [18].

Remarkably, though TGFβ family ligands are numerous, they utilize a relatively small number of receptor combinations to mediate their highly diverse signalling effects. This is accomplished through finely tuned developmental ligand and receptor expression patterns [19]. Moreover, a single ligand is able to activate various typeI/typeII receptor complexes, thus determining pathway specificity and eliciting distinct downstream responses. For instance, TGFβ complexed to its type II receptor not only recruits its main type I receptor (TβRI/ALK5), but can also recruit other type I receptors, namely ALK1 or ALK2, resulting in distinct signalling [20]. These ligand-receptor complex variations are outlined in Figure 1.1.

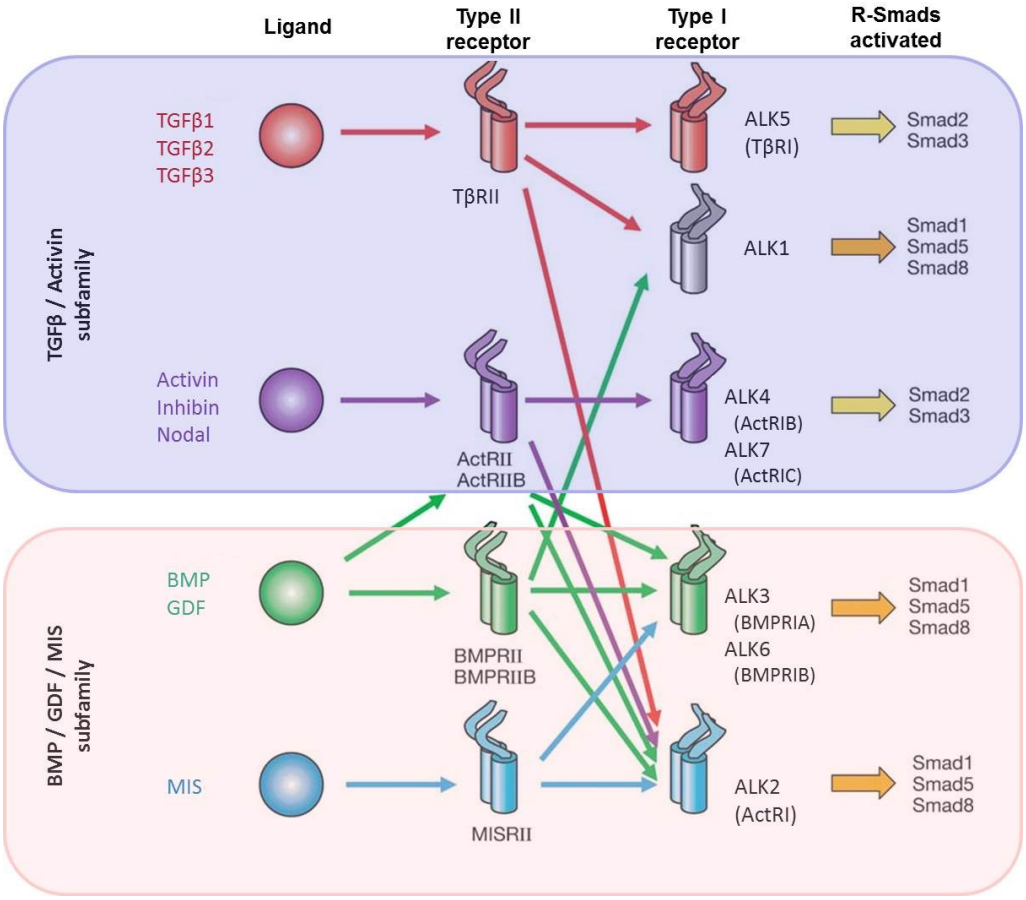

Figure 1.1: Signalling specificity of TGFβ superfamily members.
(Adapted from *Feng and Derynck, 2005; Akhurst and Hata, 2012*) [19,21].

1.1.3 TGFβ activation

TGFβ is synthesized and secreted as an inactive precursor molecule, the TGFβ large latent complex (LLC), comprising a propeptide or latency-associated peptide (LAP), the latent TGFβ-binding protein (LTBP), and the mature TGFβ homodimer [22]. LAPs bind to TGFβ dimers with high affinity, inhibiting ligand binding to its receptor by masking the receptor-interacting epitopes of TGFβ [23],

and retaining TGFβ in an inactive form. TGFβ activation requires its dissociation from the LLC. Multiple regulatory processes have been implicated in disrupting LAP-TGFβ association, including enzymatic proteolysis by furins [24], plasmin [25], and matrix metalloproteinases [26], as well as acid-, alkali-, and heat-induced proteolysis [22,27].

1.1.4 TGFβ signalling – canonical Smad signalling

Once activated, TGFβ ligands bind to the extracellular domain of the constitutively autophosphorylated type II TGFβ receptor (TβRII). This ligand-TβRII complex triggers recruitment and association of the type I receptor (TβRI) into the complex. The dimeric TGFβ ligand associates with pairs of type II receptors and type I receptors, to form a heterotetrameric complex [3]. Within this complex, the type II receptor transphosphorylates the GS domain of the type I receptor, activating its kinase activity and promoting recruitment and phosphorylation of the canonical downstream mediators, the receptor-regulated Smad proteins (R-Smads), Smad2 and Smad3. Once phosphorylated, R-Smads are released from the receptor complex and interact with their common mediator Smad (Co-Smad), Smad4, to form heterodimers (1 R-Smad/1 Co-Smad) or heterotrimers (2 R-Smads/ 1 Co-Smad). These Smad complexes then translocate to the nucleus where they associate with diverse DNA binding factors to regulate expression of target genes in a cell- and tissue-specific manner [2]. 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 [19,28].

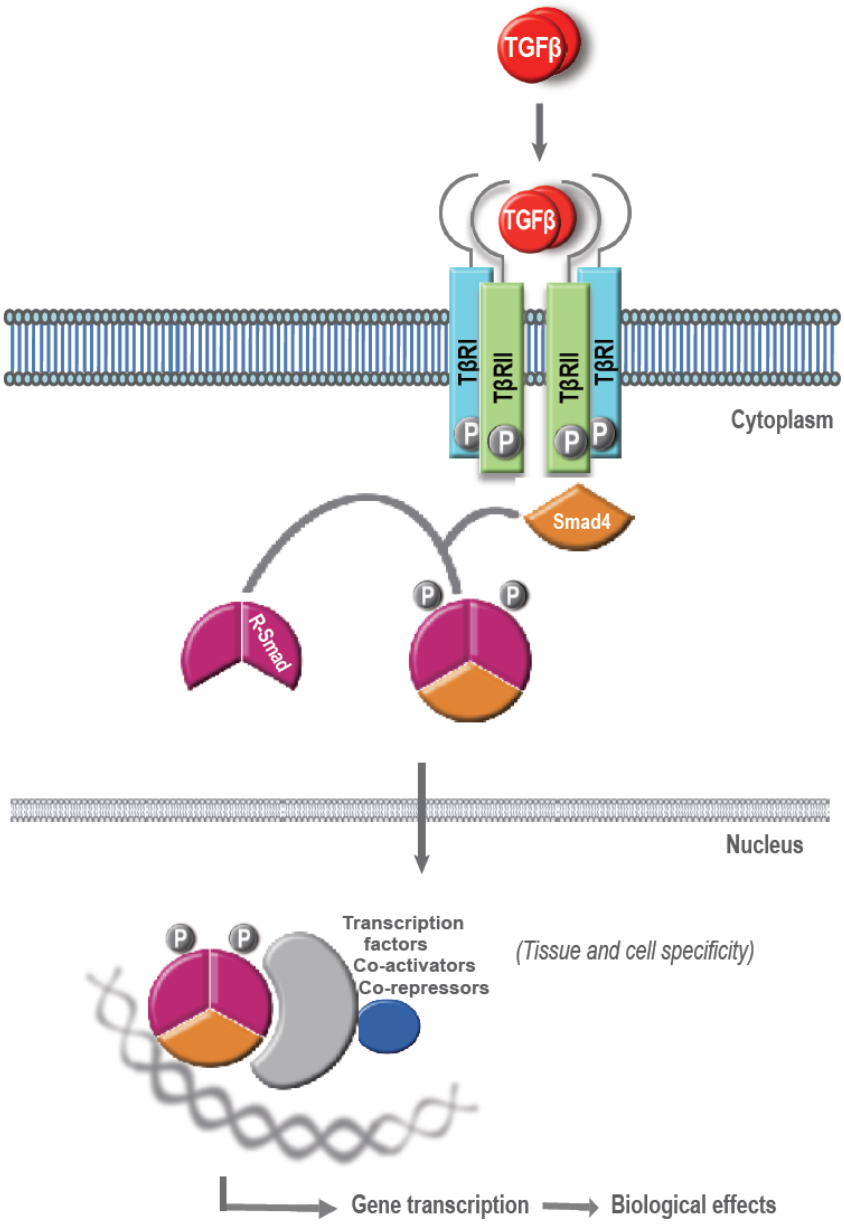

Figure 1.2: TGFβ signalling through the canonical Smad pathway.
(Adapted from *Lebrun, 2012*) [29].

1.1.5 The Smads

The fundamental intracellular effector molecules of TGFβ signalling are the Smad family of proteins. The mammalian term Smad is a portmanteau of the two founding members of the family, the *Caenorhabditis elegans* protein SMA (so called because animals carrying *sma* gene mutations exhibit a small body size) and the *Drosophila melanogaster* protein MAD (Mothers Against Decapentaplegic) [19,30]. At present, eight mammalian Smad proteins have been identified and, based on their structural and functional properties, classified into three distinct subgroups: receptor-regulated Smads (R-Smads), common-mediator Smads (Co-Smads), and the inhibitory Smads (I-Smads) [31]. Altogether, the Smad proteins range in size from 42 to 60 kDa and are composed of two highly conserved domains, an N-terminal Mad Homology 1 (MH1) domain and a C-terminal Mad Homology 2 (MH2) domain [3]. The MH1 domain is highly conserved between the R-Smads and Co-Smads and elicits sequence-specific DNA binding activity, may mediate nuclear import, and negatively regulates the MH2 domain. The MH2 domain is highly conserved among all Smads and is involved in mediating specific receptor interaction, formation of Smad complexes, and interaction with DNA-binding co-factors and transcriptional regulators. These domains form globular structures and are tethered by a less conserved proline-rich linker region that contains multiple phosphorylation sites involved in mediating crosstalk with other signalling pathways [32,33] and a PY motif that mediates interactions with Smad-ubiquitination-regulatory-factors (Smurfs) and is a primary site for negative regulation of TGFβ signalling.

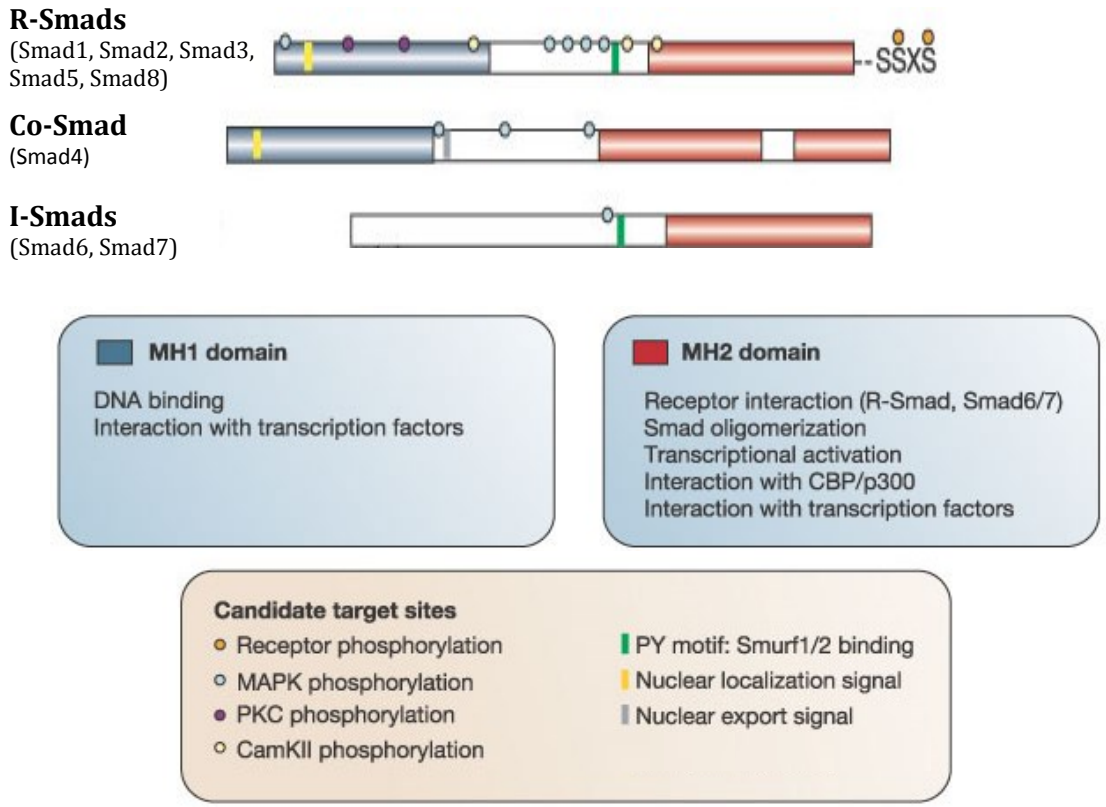

Figure 1.3: Structure of the Smads and functional properties of the various structural domains.

(Adapted from *Derynck and Zhang, 2003*) [34].

Receptor-regulated Smads

The R-Smad subgroup contains five members (Smad1, Smad2, Smad3, Smad5, and Smad8) and are the only Smad proteins that are directly phosphorylated by the type I receptor kinase. While Smad2 and Smad3 are downstream effectors for signalling by TGFβ/activin/nodal ligands, Smad1, Smad5 and Smad8 mediate signalling by BMPs and MIS. The activated type I receptor phosphorylates two serine residues within a conserved C-terminal SSXS motif located in the MH2 domain of R-Smads only. R-Smad phosphorylation promotes their dissociation

from type I receptors, association with Co-Smads, and heterocomplex translocation and accumulation in the nucleus, where the complexes bind to various co-activators or co-repressors to regulate target gene expression. The MH1 domain of R-Smads contains a characteristic β-hairpin structure that mediates specific R-Smad binding to the DNA sequence CAGAC, termed the Smad Binding Element (SBE), as well as some GC-rich promoter elements, but the Smads themselves have relatively poor DNA binding ability [32,35,36]. This β-hairpin loop displays high sequence homology among the R-Smads, suggesting that they all bind to a similar sequence. However, the most common spliced form of Smad2 contains a unique 30-residue insert within its MH1 domain which disrupts the conformation of the β-hairpin and prevents Smad2 DNA binding [32]. While phosphorylation of the MH2 domain by the type I receptor leads to activation of the R-Smads, phosphorylation of the linker region by various intracellular kinases, such as mitogen-activated protein kinase (MAPK) [37,38], protein kinase C (PKC) [39], calcium-calmodulin-dependent protein kinase II (CamKII) [40], and G protein-coupled receptor kinase 2 (GRK2) [41], inhibits Smad signalling. As such, the linker region acts as a primary site for negative regulation of Smad signalling.

Common-mediator Smads

Thusfar, the only identified mammalian Co-Smad is Smad4, which acts as a partner for all R-Smads. Though structurally similar to R-Smads, Smad4 lacks the C-terminal SSXS phosphorylation motif in its MH2 domain and is therefore not phosphorylated by the type I receptor kinase [42]. The MH1 domain of Smad4 contains a nuclear localization signal (NLS) and its linker region contains a nuclear export signal (NES), both of which are constitutively active, allowing for continuous Smad nucleocytoplasmic shuttling [43]. Additionally, Smad4 contains a Smad activation domain (SAD) within its linker region. This domain overlaps both the linker and the MH2 domain has been shown to be essential for mediating interactions with various transcription factors and transcriptional activation [44].

I-Smads

The I-Smads, Smad6 and Smad7, are structurally and functionally divergent Smads involved in inhibiting or modulating TGFβ and BMP signalling. The N-terminal domain of I-Smads shares little similarity with the MH1 domain of the other Smads, and does not bind to DNA [31]. Smad7 overexpression inhibits both TGFβ and BMP signalling through Smads2/3 and Smad1, respectively [45], while Smad6 preferentially inhibits BMP signalling [46]. The I-Smads inhibit signalling at multiple levels. Though these Smads contain a conserved MH2 domain, similar to Smad4, they also lack the C-terminal SSXS motif. As such, it has been proposed that I-Smad association with the type I receptor is more stable than that of the R-Smads, allowing for competitive interference with R-Smad recruitment, thus preventing R-Smad phosphorylation and activation [45,46]. Moreover, Smad6 competes with Smad4 to prevent active Smad1/Smad4 heterocomplex formation and favour formation of signalling inactive Smad1/Smad6 heterocomplexes [46] to further antagonize BMP signalling. Additionally, I-Smads contain a PY motif that mediates interactions with Smurfs. Both Smad6 and Smad7 can interact directly with Smurf E3 ubiquitin ligases that target Smads as well as Smad-associated receptors for proteasomal degradation, thus terminating signalling [45,46]**.** Incidentally, TGFβ signalling induces Smad7 expression, providing a TGFβ-induced negative feedback loop.

1.1.6 Smad-interacting partners

Though the SBE DNA sequence CAGAC appears in approximately every 1024 bp of the human genome [47], the affinity of the Smads for their DNA binding site is relatively low [32]. As such, multiple Smad binding sites and additional transcription factor binding to DNA adjacent to the SBE are required for high-affinity binding to ensure sufficient Smad-mediated transcriptional activation [28]. Smads directly interact with a wide range of transcription factors, which may be functionally expressed in different cell types or tissues, thus providing another

basis for tissue- and cell type-specific functions of TGFβ ligands [48-50]. Moreover, Smad association with various transcriptional co-activators or co-repressors enables TGFβ ligands to positively or negatively regulate gene transcription [31,48]. These Smad binding partners contribute to target gene specificity, pathway specificity, cell-type and tissue specificity, and specific TGFβ-mediated transcriptional effects.

Smad2, Smad3, and Smad4 interact with a vast array of DNA-binding transcription factors, including forkhead, homeodomain, Runx, and zinc-finger protein families, basic helix–loop–helix (bHLH) proteins (E2F4/5, Max, TFE3, and MyoD, for instance) as well as several bHLH-Zip family members (ATF2, ATF3, C/EBPβ, c-Jun, JunB), among others [51-55]. The first identified Smad-interacting transcription factor was the forkhead transcription factor FoxH1/FAST1 that binds cooperatively with the Smad2/Smad4 complex to an activin responsive element within the Mix.2 gene promoter. In response to activin, FoxH1 associates with the MH2 domain of Smad2 and, upon nuclear translocation with Smad4 complexing, Smad4 and FoxH1 bind to the SBE and neighbouring DNA, respectively, on the target gene promoter [49,56]. Additionally, TGFβ-mediated growth inhibition depends on the binding of another forkhead transcription factor, FoxO, to the Smad3/Smad4 complex to transcriptionally induce expression of the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ [57]. The zinc-finger transcription factor Sp1 has also been implicated in Smad3/4-mediated activation of $p21^{CIP1/WAF1}$ expression in response to TGFβ [58]. Moreover, TGFβ-activated Smad3/Smad4 complexes have been shown to functionally cooperate with the bHLH-Zip transcription factor TFE3, which binds to DNA through an E-box element, and activates TGFβ-induced transcription by binding to adjacent sites in the PAI-1 promoter [59].

Conversely, Smad-interacting partners may also be involved in TGFβ-mediated transcriptional repression of target genes. For instance, C/EBPβ interacts with TGFβ-induced Smad3/Smad4 complexes, which represses C/EBP transcriptional

activity at C/EBP binding sites and thus inhibits transcription from the leptin gene promoter, leading to TGFβ-mediated inhibition of adipogenesis [51]. Moreover, TGFβ-mediated repression of Id1 (Inhibitor of differentiation/DNA binding 1) relies on the functional cooperation between Smad3 and ATF3 (activating transcription factor 3). Interestingly, ATF3 itself is transcriptionally induced by TGFβ, and then associates with the active Smad3/Smad4 complex at the Id1 promoter to inhibit Id1 expression [60].

1.1.7 Smad transcriptional co-activators and co-repressors

In addition to cooperating with transcription factors, the Smads may also recruit transcriptional co-activators or co-repressors to further regulate target gene expression. TGFβ-activated R-Smad/Smad4 complexes have been shown to recruit a number of co-activators, including p300 and CBP (CREB-binding protein) [61-63], P/CAF (p300/CBP-associated factor) [64], MSG1 (melanocyte-specific gene 1) [65], and SMIF (Smad4-interacting factor) [66] to enhance their DNA binding and activate TGFβ-mediated transcriptional regulation. p300/CBP and P/CAF are histone acetyltransferases, suggesting that their regulatory role in TGFβ-induced transcriptional activation of target gene promoters involves chromatin remodeling.

Conversely, Smads also bind to transcriptional co-repressors such as TGIF (TG-interacting factor) [67], Ski (Sloan-Kettering Institute proto-oncogene) [68,69] and SnoN [70] (Ski-related novel gene), and recruit chromatin condensing histone deacetylases (HDACs) to target gene promoters, repressing their transcription. In some cases, binding to these co-repressors interferes with the ability of the Smads to associate with co-activators. For instance, Ski and SnoN bind to the same region of the MH2 domain of Smad4 required for complex formation with R-Smads, thus competitively preventing formation of an active Smad transcriptional complex [71]. Moreover, Ski further mediates transcriptional repression of TGFβ

target genes through the recruitment of the nuclear transcriptional co-repressor (N-CoR) and HDACs to TGFβ responsive promoters [65]. In osteoblasts, TGFβ transcriptional repression of osteocalcin relies on Smad3 binding to the transcription factor Runx2 and recruitment of HDAC4/5 to this transcriptional complex at the osteocalcin promoter [72].

1.1.8 TGFβ signalling – non-canonical pathways

Alternatively, TGFβ activates other intracellular signalling pathways independently of the Smads. These non-canonical mediators of TGFβ signalling include mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K), and Rho-like GTPases [73]. MAPK family members include the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), and the stress-activated c-Jun N-terminal kinases (JNKs) and p38 kinase, and are mainly involved in cell proliferation, differentiation, cell movement, and cell death. In response to TGFβ, the activated TβRI recruits and phosphorylates ShcA adaptor proteins on serine and tyrosine residues, which induces ShcA association with growth factor receptor binding protein 2 (Grb2) and GTP-exchange factor Sos [79]. The resulting ShcA/Grb2/Sos complex then sequentially activates its downstream mediators Ras, Raf, MEK1/MEK2 and ERK1/ERK2, leading to the induction of TGFβ-mediated epithelial-to-mesenchymal transition (EMT) [74-76]. TGFβ also signals through the stress-activated kinases JNK and p38. TGFβ induces binding of TNF receptor associated factor 6 (TRAF6) to the activated TGFβ receptor complex, and subsequent recruitment and activation of TGFβ-activated kinase 1 (TAK1), a MAP kinase kinase kinase that mediates the activation of downstream JNK and p38 [77-79]. Moreover, TGFβ-activated JNK and p38 can also enhance Smad signalling by directly phosphorylating Smad3 [80] or the transcription factors ATF2 and c-Jun, which bind cooperatively with active Smad complexes at defined TGFβ target gene promoters [55,81,82]. This functional crosstalk between pathways mediates a number of TGFβ responses, including induction of apoptosis

and EMT [76,81,83-85]. Additionally, TGFβ activates PI3K and Akt, through indirect binding of PI3K to the active TGFβ receptor complex [73], resulting in the mediation of translational responses through mTOR/S6K that are involved in regulating cell growth inhibition [86] and induction of EMT [87]. Finally, TGFβ has also been shown to signal through the Rho-like GTPase pathway, by activating or stabilizing RhoA, Cdc42, and Rac, which, in turn, have roles in numerous TGFβ responses, such as cytoskeleton reorganization, cell motility, and invasion [88,89]. These signalling pathways are outlined in Figure 1.4.

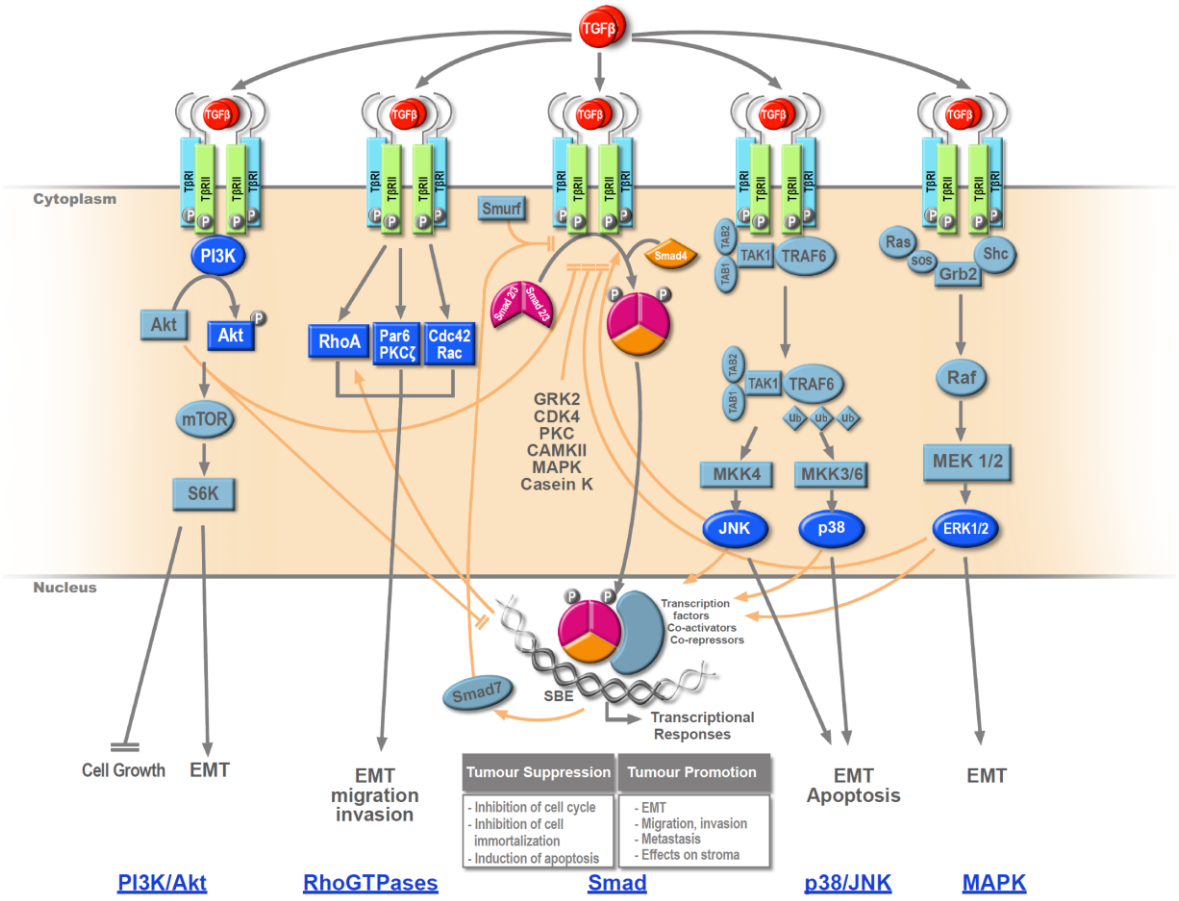

Figure 1.4: Non-canonical TGFβ signalling pathways.

(Adapted from *Lebrun, 2012*) [29].

1.2 TGFβ and Cancer

1.2.1 Dual role of TGFβ signalling in tumourigenesis

TGFβ is a vital factor in the maintenance of cellular homeostasis through regulation of a diverse set of cellular processes, including cell growth and proliferation, differentiation, and cell death in various cell types and tissues. The importance of this regulatory role is illustrated by the variety of human diseases in which deregulation of TGFβ signal transduction pathways has been implicated. Indeed, reduced TGFβ signalling leads to hyperproliferative disorders and tumour development, as well as inflammatory and autoimmune diseases. Conversely, increased TGFβ signalling has been implicated in immunosuppression and tumour metastasis [6,7,90].

Intriguingly, while TGFβ acts as a tumour suppressor in normal cells and early carcinoma, its protective effects are often lost during cancer progression. Concurrently, its tumour promoting and pro-invasive responses prevail, leading to further tumour growth and metastasis [91-93] (Figure 1.5). Though widely studied, the precise mechanistic basis for this dichotomous function of TGFβ in human cancers remains unclear.

TGFβ exerts its main tumour suppressive effects by inhibiting cell cycle progression, preventing immortalization through inhibition of telomerase activity, and inducing apoptosis. These are outlined in Figure 1.6 and will be discussed in depth below. Nonetheless, many types of tumour cells have acquired the capacity to circumvent the tumour suppressive activity of TGFβ.

In some cases, tumour cells manage to evade the suppressive effects of TGFβ by acquiring inactivating mutations in core signalling components of the TGFβ pathway (discussed below). Alternatively, in other tumours, the TGFβ signalling pathway itself is unaffected, but cells acquire downstream alterations to

selectively disable the tumour suppressive arm of the pathway. In the latter approach, tumour cells may utilize the remaining intact TGFβ regulatory functions to their advantage, generating added potential for tumour progression. In these tumours, TGFβ becomes an oncogenic factor by promoting epithelial-to-mesenchymal transition (EMT), cell migration and invasion. Meanwhile, TGFβ also affects the tumour stroma to promote angiogenesis and suppress immunosurveillance [29,91,94].

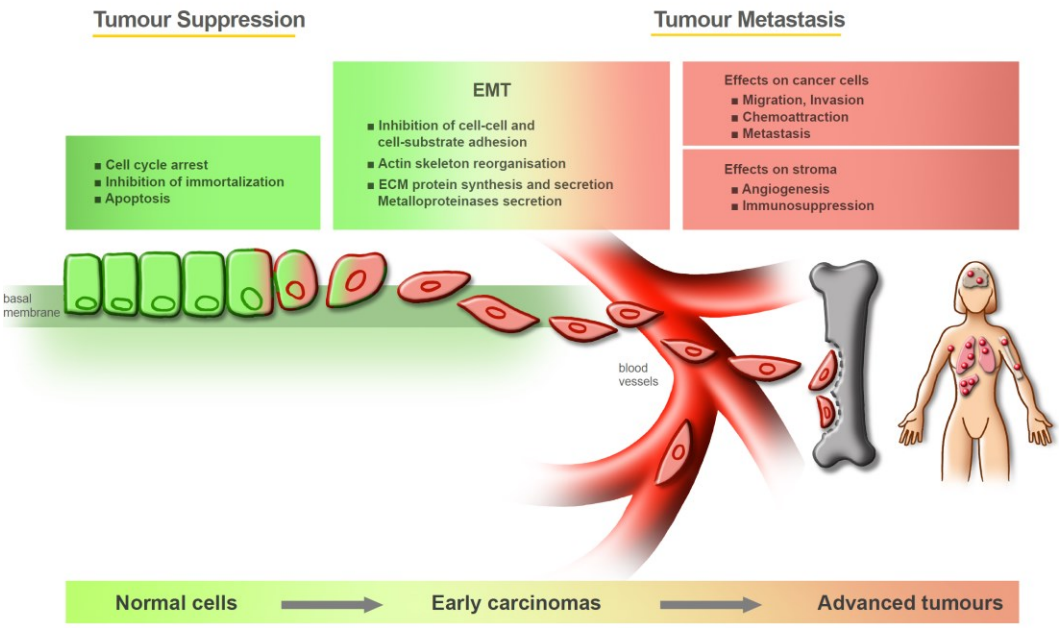

Figure 1.5: Dual role of TGFβ in cancer
(Adapted from *Lebrun, 2012*) [29].

The role of TGFβ signalling as a tumour suppressor pathway is evidenced by the presence of various inactivating mutations in genes encoding TGFβ signalling components in human cancer. These alterations, which occur in human cancers of

various origin, disrupt the tumour suppressive functions of TGFβ, thus favouring tumour development. Indeed, virtually all tumours of epithelial origin, which comprise the vast majority of all human cancers, become resistant to the tumour suppressive effects of TGFβ. This may occur due to defects in TGFβ receptors or Smads, a decrease in cell surface expression of TGFβ receptors, elevated expression of TGFβ itself, or an increase in expression of inhibitory Smads [91,93,95].

1.2.1.1 TGFβ receptor mutations and alterations

Mutations in the gene that encodes the TβRII are frequently observed in colon [96-98], gastric [97,99,100], head-and-neck [101], and ovarian cancers [102] and often occur as insertions or deletions in the kinase domain of TβRII, resulting in truncated or inactivated forms of the receptor. These mutations have also been reported in pancreatic, lung, liver, breast, biliary tract, and brain (glioma) tumours [103-107]. Though they occur less frequently, inactivating mutations in TβRI have also been observed, most notably in ovarian [108,109], breast [110], pancreatic [107], head-and-neck [111] carcinomas, as well as T-cell lymphomas [112], generally occurring as frameshift and missense mutations. Interestingly, mutations in TβRI have not been found to be accompanied by TβRII mutations [109].

Moreover, reduced TGFβ receptor expression or availability at the cell surface has also been reported in tumour cells, allowing them to become resistant to the growth inhibitory effect of TGFβ [113,114]. Transcriptional silencing of the TGFβ receptor genes may result either from promoter hypermethylation, defective expression of the transcription factors that regulate their expression, or mutations within the TGFβ receptor promoter that disrupt transcription factor binding [133].

1.2.1.2 Smad mutations

Similarly, mutations in the genes that encode the Smads have been detected in several cancers. Most notably, Smad4 was initially characterized as a tumour suppressor gene that was homozygously deleted in 50% of pancreatic carcinomas [115]. Since then, Smad4 mutations have been reported, albeit less frequently, in various other tumour types, including colorectal, gastric, hepatocellular, breast, lung, bladder, kidney, biliary tract, prostate, cervical and ovarian tumours [19,95,116-119]. Smad2 gene mutations have also been observed in some cases of lung, liver, colorectal, ovarian, and cervical cancers [109,116,120,121]. Though Smad3 mutations have not been identified, tumour-associated defects in Smad3 expression have been reported in some gastric cancers and certain types of leukemia [122,123]. Tumour-associated mutations in Smad4 and Smad2 occur most frequently as inactivating missense or nonsense point mutations in the MH2 domain, interfering with Smad phosphorylation and heteromeric complex formation, and inactivating Smad transcriptional activity [116,120,121,124,125]. Mutations in the MH1 domain of Smad4 impair its DNA-binding ability [126,127]. Additionally, many MH1 and MH2 domain point mutations or C-terminal truncations confer decreased Smad2 and Smad4 protein stability through proteasomal degradation [127-129].

Alternatively, increased expression of Smad transcriptional repressors may also provide a mechanism by which tumour cells impair TGFβ responsiveness and develop resistance to its tumour suppressive effects. For instance, elevated expression of two Smad repressors, Ski and SnoN, have been reported in melanoma [130,131], leading to decreased TGFβ/Smad signalling. Similarly, enhanced levels of the inhibitory Smad family member, Smad7, as observed in several human tumour types, including pancreatic [132], endometrial [133], thyroid follicular [134], and esophageal squamous cell carcinoma [135], may also impair TGFβ responsiveness. Evidently, tumour cells have developed numerous approaches to evade the growth inhibitory response of TGFβ.

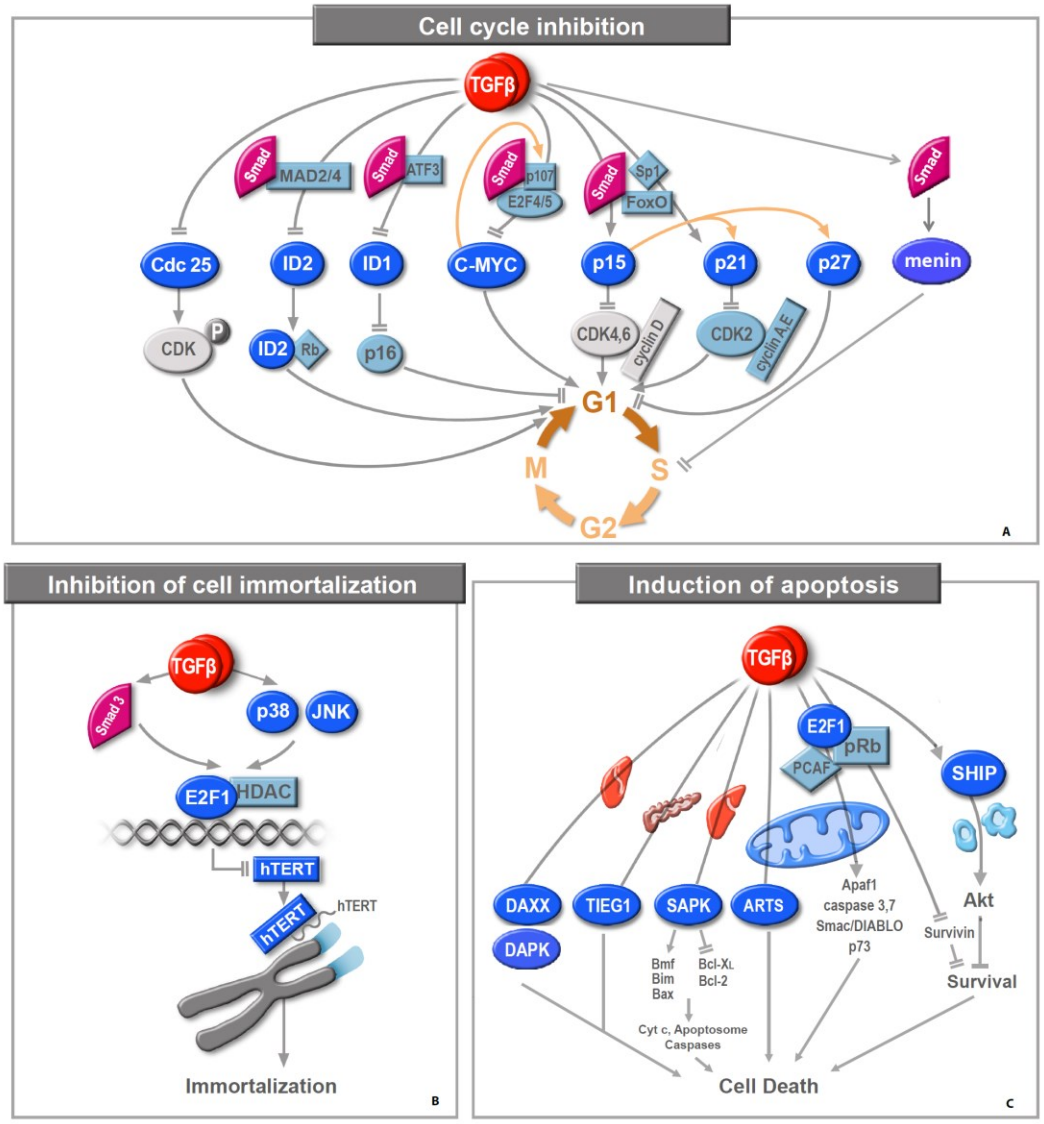

Figure 1.6: TGFβ tumour suppressive effects

(Adapted from *Lebrun, 2012*) [29].

1.2.2 TGFβ tumour suppressive effects: Cell Cycle Inhibition

1.2.2.1 Overview on cell cycle regulation

The cell cycle is a series of molecular events that take place in a cell leading to its replication and division. In eukaryotes, the cell cycle comprises four distinct phases: G1 (Gap1) phase, during which the cell grows in size; S phase (synthesis), during which DNA replication occurs; G2 (Gap 2) phase, during which cell growth continues and proteins are synthesized in preparation for the final M phase (mitosis), during which the duplicated chromosomes are distributed and the cell divides into two daughter cells [136,137]. Two main checkpoints, the G1/S checkpoint and the G2/M checkpoint, are used by the cell to monitor and regulate the progress of the cell cycle and to ensure that damaged or incomplete DNA is not passed on to daughter cells. The transition from G_1 to S phase is a rate-limiting step in the cell cycle and is also known as restriction point [156,157].

Cell cycle progression is tightly regulated by cyclin-dependent kinases (Cdks), which associate with their regulatory cyclins. These cyclin-Cdk complexes phosphorylate members of the retinoblastoma (Rb) protein family, which release E2F transcription factors that mediate the transcription of numerous cell cycle regulatory genes, allowing for cell cycle progression from G_1 to S phase [138,139].

1.2.2.2 The E2F family of transcription factors

The E2F family of transcription factors is a group of DNA-binding proteins that regulate the transcription of a multitude of genes involved in numerous cell functions, including cell cycle regulation, DNA replication, chromatin assembly and condensation, and DNA repair [139-141]. In mammals, the E2F family comprises eight genes, which encode nine major protein species, as shown in Figure 1.7. Though all family members contain a DNA-binding domain, the transcriptional

activity of E2F1 through 5 is regulated primarily via their association with members of the retinoblastoma family of pocket proteins, which include the retinoblastoma tumour suppressor protein, pRb/p105, and its homologs p107 and p130 [139]. Additionally, E2F1 through 6 contain a dimerization domain required for their association with members of the dimerization-partner (DP) family, which enables them to bind DNA and function as transcriptional regulators. While E2F7 and E2F8 do not interact with pocket proteins or DP proteins, they are able to bind DNA as homodimers or as E2F7–E2F8 heterodimers [142-147]. Classically, E2F family members have been grouped as either transcriptional activators (E2F1, E2F2, E2F3a) or transcriptional repressors (E2F3b, E2F4 through 8). More recently, however, this classification has been reconsidered, given that DNA microarray studies reveal that activation of the 'activator' E2Fs leads to repression of nearly as many genes as they activate [148].

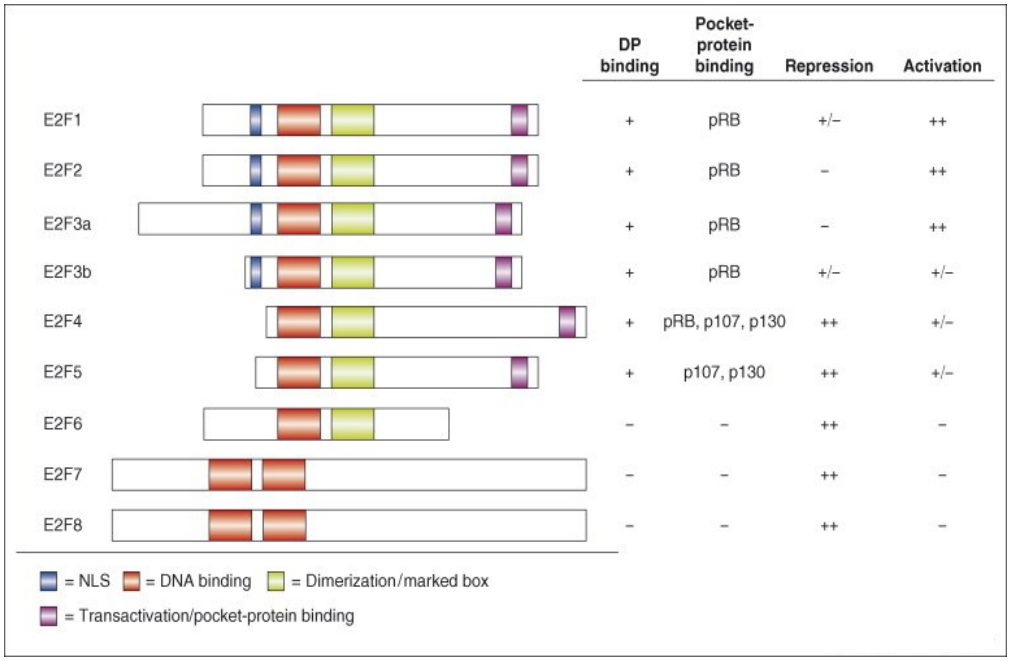

Figure 1.7: The E2F family of transcription factors

(Adapted from *Iaquinta and Lees, 2007*) [149].

Most classic E2F target genes are transcriptionally regulated by the cyclical repression or activation by different E2F members, depending on the phase of the cell cycle. In G_0/G_1, it is well established that E2F4 and E2F5, in complex with Rb pocket proteins and histone deacetylaces (HDACs), bind to and actively repress E2F-responsive gene promoters. Concurrently, 'activating' E2Fs are bound by pRb, thus inhibiting their potential to activate transcription. Upon mitogenic signalling, cyclin-cdk complexes phosphorylate the pocket proteins, disrupting pocket protein–E2F complexes. Consequently, E2F4 and E2F5 are exported from the nucleus, thus allowing E2F1, E2F2, and E2F3 to bind to and activate the promoters of E2F-responsive cell cycle genes, driving cell cycle progression [141].

Generally, pocket protein binding to E2F inhibits its transcriptional activity by binding to residues within the transactivation domain of E2F, thus impairing the ability of E2F to recruit the transcriptional machinery. Moreover, the pRb–E2F complex may also recruit chromatin modifiers and remodeling factors to E2F-responsive promoters. These transcriptional co-repressors include histone deacetylases, histone methyltransferases and DNA methyltransferases [148]

E2F1, the founding member and best-characterized of the family, has a unique role compared to other E2Fs, showing characteristics of being both an oncogene and a tumour 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 tumours [150,151] supporting an oncogenic role for E2F1, transgenic mice overexpressing E2F1 display aberrant cell apoptosis [152]. Furthermore, E2F1 knockout mice develop highly malignant tumours and show defects in thymocyte apoptosis, highlighting E2F1 as a potent tumour suppressor [153]. 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 [154].

Moreover, posttranslational modifications of E2F1 in response to DNA damage have been shown to direct E2F1 from cell cycle progression to apoptotic E2F target genes, resulting in apoptotic induction [155,156]. 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 [157].

1.2.2.3 TGFβ-mediated cell cycle arrest

TGFβ induces cell cycle arrest in G1 through transcriptional induction of the Cdk inhibitors $p15^{INK4B}$ [158] and $p21^{CIP1/WAF1}$ [159]. This regulation is Smad-dependent and requires Smad association with the transcription factors FoxO [57] and Sp1 [160,161].

The antiproliferative effect of TGFβ also relies on transcriptional inhibition of growth-promoting factors, such as c-myc [162], cdc25A [163], and Id transcription factors [164-166]. While c-myc is a well-established activator of cell growth and proliferation [167,168], it also directly inhibits $p15^{INK4B}$ [169] and $p21^{CIP1/WAF1}$ [170]. Thus, inhibition of c-myc expression by TGFβ not only relieves its proliferative role but also further contributes to the induction of the Cdk inhibitors and cell cycle arrest [171]. The Cdk tyrosine phosphatase cdc25A normally dephosphorylates inhibitory sites on Cdk4 and Cdk6, resulting in their activation. Transcriptional repression of cdc25A by TGFβ allows for sustained phosphorylation and inactivation of these Cdks, thus preventing cell cycle progression [172]. TGFβ inhibits both c-myc and cdc25A by recruiting Smad-E2F4/5-pRb family member repressor complexes to these gene promoters [162,163]. Members of the Id (Inhibitor of Differentiation/DNA binding) family of transcription factors prevent differentiation, promote cell proliferation through interaction with pRb, and have been implicated in promoting tumourigenesis [165,173]. Id1 has also been shown to delay cellular senescence in primary mammalian cells through repression of the cell cycle regulatory protein

p16^{INK4a} [174]. TGFβ transcriptionally induces activating transcription factor 3 (ATF3), which associates with Smad3 to inhibit Id1 expression [164]. Id2 overexpression results from transcriptional activation by c-myc [165], thus c-myc down-regulation by TGFβ contributes to inhibition of Id2 expression. Repression of these transcription factors by TGFβ largely contributes to this growth factor's anti-proliferative effect.

Moreover, previous studies from our laboratory demonstrated that TGFβ induces expression of the tumour suppressor menin in pituitary adenoma cells, leading to G1 arrest. We further found that menin interacts with Smad3, and that inactivating menin expression blocks TGFβ signalling and antagonizes TGFβ-mediated cell growth inhibition [4,175,176].

1.2.3 TGFβ tumour suppressive effects: Inhibition of Immortalization

1.2.3.1 Overview on cell immortalization

Most normal human cells are only able to replicate a limited number of times due to the progressive shortening of the ends of the chromosomes (telomeres) with each cell division, as DNA polymerases fail to fully replicate the genetic material. Consequently, the length of the telomeres shortens to a critical point, triggering cell senescence or cell death to avoid genomic instability and loss of important chromosomal DNA [177]. Conversely, cancer cells are not limited by such a fixed number of replication cycles but instead achieve immortalization. This is due to the constitutive activity of telomerase, an enzyme that adds telomeric DNA repeats at the ends of newly duplicated telomeres, thereby preserving their length throughout successive replication cycles and protecting chromosomes from degradation [178].

1.2.3.2 Telomeres and telomerase

Telomeres are specialized structures composed of repeated DNA sequences at the ends of linear chromosomes. Human telomeres are composed of a variable number of tandem repeats of the hexanucleotide sequence, TTAGGG, that are bound by specific proteins, and are therefore of variable length [179]. In humans, this telomeric sequence extends from 2 to 50 kb, with an average length of 10-15 kb [178]. Telomeres and their associated proteins are involved in stabilizing the ends of chromosomes by forming a cap structure that protects the chromosome ends from recombination, end-to-end fusion, and recognition as damaged DNA [177,180]. Telomeres are also characterized by a single-stranded 3'-end overhang which folds back and anneals with the double-stranded telomeric DNA repeat to form a loop structure called the T-loop [177,181]. Telomeric DNA associates with numerous proteins, including the telomere repeat binding factors 1 and 2 (TRF1 and TRF2), forming large protecting complexes [182].

Replication of chromosome ends poses a particular problem – known as the 'end replication problem' – in that conventional DNA polymerase is unable to fully replicate the 3' end of the lagging strands of linear DNA. Consequently, telomeres in most cells shorten by 50-200 nucleotides with each cell division. When telomeres become too short, they trigger either growth arrest or apoptosis to avoid genomic instability and loss of important chromosomal DNA [177]. As such, normal mammalian somatic cells divide a limited number of times, with the maximum number being referred to as the Hayflick limit [183]. It has been suggested, then, that this telomere shortening acts as a 'molecular clock' that monitors the number of cellular divisions and limits life span [184]. At the Hayflick limit, one or more critically shortened telomeres trigger a permanent growth arrest known as replicative senescence, or mortality stage 1 (M1) (Figure 1.8). The M1 mechanism causes a growth arrest mediated by critical cell cycle checkpoint genes such as p53, p16 and pRb. If the actions of these tumour suppressor genes are blocked, either by mutation or by binding of viral oncoproteins, cells can

escape replicative senescence and continue to divide and suffer further telomere loss until they reach a second proliferative block known as *crisis*, or mortality stage 2 (M2). M2 is characterized by massive cell death triggered by critically short and dysfunctional telomeres [185,186]. Rare survivor cells that escape crisis are able to maintain telomere length, in most cases by upregulation or reactivation of the enzyme telomerase, which is able to repair and maintain telomere length. This then leads to unlimited proliferative capacity, that is, cellular immortalization [177].

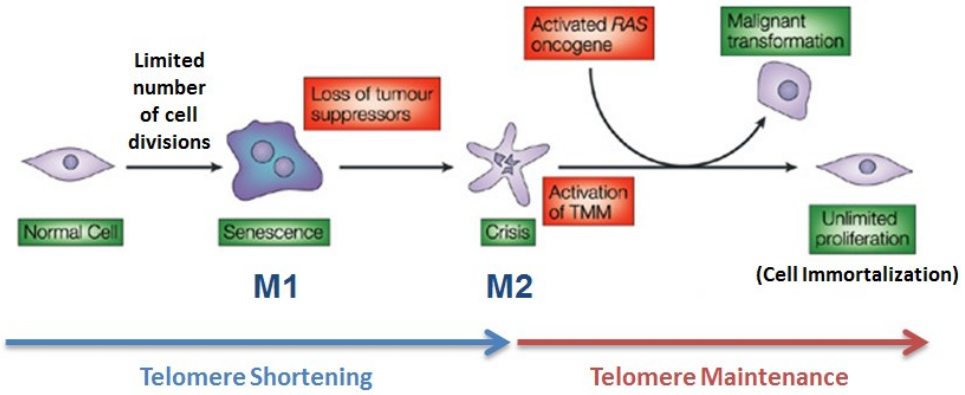

Figure 1.8: Telomerase bypasses senescence (M1) and crisis (M2) by activating a telomere maintenance mechanism (TMM), leading to cell immortalization. (Adapted from *Neumann and Reddel, 2002; Shay, 2005*) [182,187]

Human telomerase is a specialized reverse transcriptase complex composed of both an RNA component (hTR) that provides the template for the addition of new telomeric repeats, and a catalytic protein subunit known as human telomerase reverse transcriptase (hTERT). Although hTR is highly expressed in virtually all mammalian cells, the expression of hTERT is restricted to cells that exhibit telomerase activity, indicating that hTERT expression is the rate-limiting component of the telomerase enzyme [177,180]. Most somatic cells have little or no

telomerase activity due to strong repression of hTERT. Exceptions include cells with high proliferative potential, such as basal cells of the epidermis, embryonic cells, germ cells, activated lymphocytes, intestinal crypt cells, and certain stem cells, which require telomerase activity for proliferation and long-term viability [188]. In contrast, the expression of hTERT is elevated in 85-90% of cancer cells [182,189]. This is by far the most commonly observed abnormality acquired by tumour cells and is used as a diagnostic marker for cancer. Reactivation of telomerase activity is mainly due to the loss of repression of the hTERT gene in cancer cells.

1.2.3.3 Regulation of telomerase activity

Though telomerase activity is regulated at various levels, such as mRNA splicing, hTR and hTERT modifications, and assembly and accessibility of the telomerase ribonucleoprotein on the telomeres, transcriptional control of the hTERT gene is considered the key event in the activation of telomerase activity observed in cancer cells [188,190]. Indeed, several studies have demonstrated that the transcriptional regulation of hTERT expression is the rate-limiting step in the activation of telomerase activity in most cells, including cancer cells [194,197,191].

The hTERT gene was first cloned in 1999 [192], and since then a great number of studies have been conducted in attempt to understand how the hTERT gene is transcriptionally regulated in both normal and cancer cells. Sequence analysis of the hTERT promoter has revealed hundreds of putative binding sites for various transcription factors, suggesting that the hTERT gene is under multiple levels of regulation. Indeed, several transcription factors have been identified in regulating hTERT expression, some as transcriptional activators, others as transcriptional repressors. This coincides with the fact that hTERT is transcriptionally repressed in many normal cells and is reactivated or upregulated during immortalization. Some of these regulators will be discussed here.

Transcriptional Activators of hTERT:

The hTERT core promoter region contains two E-boxes containing DNA binding elements for c-myc/Mad/Max transcription factors. c-myc/Max heterodimers bind to these E-boxes, activating hTERT gene expression. In fact, the expression of c-myc seems to parallel hTERT expression, in that it is elevated in highly proliferative cells and immortal cells and downregulated during differentiation. Overexpression of c-myc has been shown to induce hTERT expression and telomerase activity in normal human mammary epithelial cells and primary fibroblasts. However, though c-myc-induced activation of hTERT expression is important in human cells, it is insufficient to account for the transforming activity of c-myc [177].

The Sp1 transcription factor binds to GC-boxes of promoters to activate a large number of genes. Sp1 interacts with numerous components of the general transcription machinery to help initiate transcription of TATA-less promoters [193]. The hTERT promoter, which is a TATA-less promoter, contains several GC-boxes that are potential binding elements for Sp1. Mutation of all GC-boxes abolishes hTERT promoter activity, thus demonstrating that Sp1 is absolutely required for hTERT promoter activity [194]. Though the exact mechanism remains unclear, Sp1 has been shown to cooperate with c-myc to activate hTERT transcription in a cell type-specific manner, suggesting the involvement of other transcription factors in this regulation.

The hTERT promoter also contains two potential estrogen response elements. Numerous studies have shown that estrogen activates telomerase through direct transcriptional regulation of hTERT expression in hormone-sensitive tissues. Tamoxifen, an anti-estrogen drug commonly used as adjuvant therapy to treat breast cancer, has been shown to reduce telomerase activity in breast cancer cell lines [195]. The antagonistic effect of tamoxifen on estrogen-induced telomerase

activity is consistent with its inhibitory effect on activation of the hTERT promoter by estrogen. Progesterone and androgens have also been implicated in regulating telomerase by targeting the hTERT promoter [196,197]. The finding that various steroid hormones directly regulate telomerase may provide further insight into the molecular mechanisms of tumour formation in hormone-dependent tissues as well as clinical management of hormone-dependent cancers.

Transcriptional Repressors of hTERT:

Mad1 counteracts the effect of c-myc on hTERT expression. Both c-myc and Mad proteins can dimerize with the ubiquitously expressed Max protein [168]. c-myc/Max heterodimers bound to E-boxes activate gene expression, while Mad/Max heterodimers compete for binding to E-boxes and repress transcription. This switch from c-myc/Max to Mad1/Max results in repression of hTERT transcription, thus inhibiting telomerase activity [198].

Since telomerase is upregulated in most human cancers and highly proliferative somatic cells and downregulated with cell cycle exit and differentiation, this suggests that cell cycle regulators such as p53 may also be involved in the regulation of telomerase. Indeed, p53 inhibits telomerase activity through the transcriptional repression of hTERT [199]. This inhibition occurs within hours after induction of p53, that is, before cell cycle arrest or apoptosis take place. The transcriptional repression of hTERT by p53 may therefore be independent of cell cycle arrest or apoptosis [177]. The critical cell cycle regulators pRb and E2F1 have also been implicated in the inhibition of telomerase [200,201]. Aside from its well established role in promoting cell proliferation, E2F1 has been found to also function as a tumour suppressor by repressing hTERT [202]. Upon overexpression of E2F1, telomerase activity is repressed in human cells. Interestingly, there are a number of E2F1 consensus DNA binding motifs in the core hTERT promoter. Moreover, several studies have established that HDACs are involved in the

transcriptional repression of hTERT [191,203]. It has been demonstrated that that the assembly of complexes formed of E2F, pocket proteins (potentially pRb) and HDACs regulate hTERT gene expression in normal human fibroblasts. Disruption of either protein in the E2F/pocket proteins/HDAC complexes de-represses hTERT gene expression [204], indicating that each of these is a key regulator of hTERT expression in human cells. This is supported by the fact that deregulation of the pRb/E2F pathway is common in the majority of cancers.

1.2.3.4 TGFβ-mediated inhibition of telomerase activity

TGFβ also inhibits telomerase activity in various cell lines, however the precise mechanisms by which it represses hTERT expression remain unclear. Several factors have been implicated in the inhibition of telomerase by TGFβ. For instance, TGFβ has been shown to suppress human and rat TERT expression indirectly by inhibiting c-myc expression [205,206]. However, another study in MCF7 breast cancer cells suggested that hTERT expression is not repressed by the inhibition of c-myc but rather by direct action of Smad3 on the hTERT promoter in response to TGFβ [207]. In this proposed pathway, TGFβ mediates Smad3 binding to c-myc and inactivates its expression, while c-myc recruits Smad3 to the hTERT promoter resulting in transcriptional repression of the hTERT gene [207]. Another study revealed a role for the Smad interacting protein-1 (SIP1) in TGFβ-mediated hTERT repression [208]. The different mechanisms observed in different cell systems may in fact reflect cell type-specific effects rather than a central mechanism.

As presented in Chapter 2 of this thesis, studies from our laboratory demonstrate that repression of telomerase by TGFβ is mediated not only through the Smad pathway but also requires the Erk1/2 and p38 kinase pathways, as well as histone deacetylase activity. Moreover, we found that the inhibitory effect of TGFβ on hTERT expression is dependent on the transcription factor E2F1, highlighting E2F1 as an important mediator of TGFβ tumour suppressive effects [209].

1.2.3.5 Telomerase and Cancer

It has been proposed that cellular senescence may have evolved, in part, to protect long-lived organisms such as humans against the early development of cancer [210]. Consequently, upregulation of telomerase in order to bypass senescence may be critical for continuous tumour cell growth. Contrary to normal cells, tumour cells exhibit no net loss of average telomere length with cell division, strongly suggesting that telomere stability may be required for cells to escape from replicative senescence and proliferate indefinitely [189]. Cell immortalization may result from gene mutation(s) in the telomerase repression pathway. Reactivation of telomerase activity may therefore be a rate-limiting step required for the continuing proliferation of advanced cancers.

However, carcinogenesis is a multi-step process in which a normal cell undergoes immortalization and then oncogenesis to become a fully transformed malignant cell. Even after immortalization is achieved, the cells are not yet oncogenic; additional genetic alterations are required for malignant transformation [211,212]. Though hTERT overexpression does not cause tumour formation, per se, it does significantly increase a cell's lifespan [210] and this immortality comes at a price. As immortal cells replicate their DNA more often, they thus have an increased chance of accumulating damaging mutations favouring transformation. This also helps to explain why cancer is primarily a disease of an aging population.

There is mounting evidence that telomere-associated events are indeed relevant to carcinogenesis. Numerous studies have demonstrated that ectopic expression of telomerase in telomerase-null, mortal human cells stabilizes telomeres and promotes immortalization, which is crucial for cell transformation [210,213,214]. Others have shown that the conversion of human fibroblasts or epithelial cells to transformed cancer cells by an activated oncogene (such as Ras) is facilitated by hTERT expression and requires immortalization [215]. Furthermore, inhibiting telomerase activity in immortal human cancer cell lines leads to apoptosis or

senescence [216,217]. Again, it is important to note that though immortality is necessary, it is not sufficient for malignant transformation.

Interestingly, advanced cancer cells are usually characterized by short telomeres. This is likely due to the fact that they proliferate for an extended period of time before reactivating telomerase activity [178]. In normal cells, progressive telomere shortening would eventually initiate a DNA damage response [218], thus limiting cell proliferation and promoting apoptosis. Conversely, in cancer cells with deficient DNA damage-induced checkpoints (such as p53-deficient cancer cells), shortened telomeres could actually contribute to the genomic instability usually observed in cancer cells [219], thereby further promoting mutations within the cancer cells. Selection for cells with reactivated telomerase activity would then be favoured, promoting indefinite cell proliferation [178].

Analysis of telomerase knockout mice has been instrumental in studying the roles of telomeres and telomerase in tumour formation. In p53 and telomerase double knockout mice, tumour onset is significantly accelerated [220], demonstrating that p53 is also an important mediator of the cellular response to short telomeres [221]. In contrast, tumourigenesis is reduced in mice that are simultaneously deficient in both telomerase and tumour suppressor genes other than p53, such as *p19ARF*, *p16*, and *Apc* [222-224], suggesting that short telomeres suppress tumour formation even in the absence of critical tumour-suppressor pathways.

Interestingly, several studies have shown that telomerase has additional functions not related to net telomere lengthening that enhance survival and proliferation. Mice studies appear to be a useful model to examine the impact of telomerase activation on cell proliferation since mice have relatively long telomeres (25-40 kb) and so the role of telomerase in lengthening short telomeres is less critical. These studies demonstrate that during tumourigenesis in mice, telomerase activity increases even in the presence of sufficiently long telomeres [225,226]. Moreover, first generation telomerase knockout mice were shown to be significantly less

susceptible to tumour development than their wild-type counterparts when exposed to chemical carcinogens. In contrast, transgenic mice overexpressing *mTERT* are twice as likely to develop epidermal tumours upon chemical carcinogenesis [227]. Additionally, mice with constitutive mTERT expression are more susceptible to developing both induced and spontaneous tumours as they age, compared to wild-type controls, independently of telomere length maintenance [227,228]. These studies strongly suggest that telomerase expression may cooperate with oncogenic factors, and more frequently with age, to promote tumourigenesis in mice.

Interestingly, some cancer cells and immortal human cell lines that lack telomerase activity are still able to maintain or elongate their telomeres by a telomerase-independent mechanism known as alternative lengthening of telomeres, or ALT [178] . This process involves homologous recombination to construct and maintain telomeres and is observed in approximately 10-15% of cancers or cancer cell lines. It has been proposed that tumours presenting ALT-phenotypes, or so-called telomerase-negative tumours, have potentially higher chromosomal instability than telomerase-positive tumours [229,230].

1.2.4 TGFβ tumour suppressive effects: Induction of Apoptosis

1.2.4.1 Overview on apoptosis

Programmed cell death by apoptosis is a fundamental mechanism for regulating cell number and tissue homeostasis [231]. The apoptotic programme is tightly controlled through the action of numerous effectors and complex pathways. Deregulation of these pathways may lead to various pathological conditions, such as developmental defects, autoimmune disorders, neurodegeneration, or cancer [232]. In mammals, cells undergo apoptosis through two major pathways, namely the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway. In

both pathways, the main effectors are the caspases (*c*ysteine-*asp*artic acid prote*ases*), which are activated and cleave specific cellular substrates, leading to a number of biological and morphological changes that are characteristic of apoptosis: cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation, and ultimately engulfment by macrophages or neighboring cells [233,234].

Briefly, the extrinsic pathway is mediated by the stimulation of specific death receptors upon binding of their ligands, such as FasL, tumour necrosis factor (TNF), or TNF-related apoptosis-inducing ligand (TRAIL). These receptors are characterized by an intracellular domain called the death domain. Death ligand stimulation results in oligomerization of the receptors and recruitment of the adaptor protein Fas-associated death domain (FADD), which in turn recruits procaspases-8 and -10, to form the death-inducing signalling complex (DISC) [235,236]. Once activated at the DISC, these caspases promote cell death either by activating effector caspases (caspases-3, -6, and -7) which function as downsteam effectors of the cell death programme, or by cleaving the Bcl-2 family member Bid, thus triggering mitochondrial-mediated apoptosis [237,238].

The intrinsic pathway is mediated by diverse apoptotic stimuli, including DNA damaging agents, growth factor deprivation, hypoxia, or oxidative stress. The key event of this pathway involves mitochondrial outer membrane permeabilization (MOMP) [239]. Mitochondrial integrity is regulated by several proteins from the Bcl-2 family, which may be pro-apoptotic (including Bax, Bak, Bok, Bid, Bad, Bim, Bmf, Bcl-X_S, Noxa, and PUMA) or anti-apoptotic (including Bcl-2 proper, Bcl-X_L, and Mcl-1) [240,241]. Upon initiation of apoptotic signalling, Bax and/or Bak associate with the mitochondrial membrane to form the mitochondrial apoptosis-induced channel (MAC), allowing for the release of cytochrome *c* and other pro-apoptotic factors from the mitochondria, and initiatiang a caspase cascade [242]. Anti-apoptotic Bcl-2 proteins can disrupt this interaction, thus preventing mitochondria permeabilization. Once released from the mitochondria, cytosolic

cytochrome *c* binds to apoptosis protease-activating factor 1 (Apaf-1) and procaspase-9, generating a multi-protein complex called the apoptosome. Within the apoptosome, caspase-9 is activated, which in turn activates effector caspases, promoting the execution of apoptosis [243].

In order to proliferate without restraint, tumour cells not only need to bypass cell-cycle checkpoints, but also evade cell death pathways. Indeed, acquired resistance to apoptosis is a hallmark of tumour development in virtually all types of cancer [244]. Tumour cells can acquire resistance to apoptosis by various means, but perhaps most commonly by loss of one of the principle apoptotic regulators, p53. Indeed, more than half of all types of human cancers exhibit mutated or lost p53 gene expression [245]. Cancer cells can additionally compromise the activity of p53 by overexpressing inhibitors or suppressing activators of p53. Moreover, tumour cells may also evade apoptosis by the overactivation of anti-apoptotic factors, such as Bcl-2, or by the downregulation of pro-apoptotic factors, such as Bax and Bak [246]. Defects in the apoptotic pathways may not only enable proliferation of neoplastic cells, but also lead to their escape from immunosurveillance. Moreover, as many cancer therapies act primarily by inducing apoptosis, tumour cell inactivation of the apoptotic pathways also influences the efficacy of therapeutic treatments.

1.2.4.2 TGFβ-mediated apoptosis

TGFβ induces a number of apoptotic responses and its ability to do so varies greatly depending on the tissue or cell type [5]. Understanding the basis of this variability requires elucidating the molecular mechanisms involved in regulating TGFβ-mediated cell death. Several apoptotic regulators have been implicated in mediating TGFβ apoptotic responses. In hepatocarcinomas, TGFβ transcriptionally induces the death-associated protein kinase (DAPK), which

promotes cell death in a Smad-dependent manner by modulating the activation potential of the mitochondrial membrane, potentially contributing to cytochrome *c* release and caspase activation[247]. In pancreatic epithelial cells, TGFβ induces the zinc finger transcription factor TGFβ-inducible early-response gene (TIEG1), leading to cell death [248]. Moreover, TGFβ promotes delocalization of the mitochondrial septin-like protein ARTS (apoptosis-related protein in TGFβ signalling pathway) from the mitochondrion to the nucleus where it binds to and inactivates inhibitors of apoptosis (IAPs), resulting in activation of caspases and the apoptotic programme [249]. Previous work from our lab showed that TGFβ can also induce apoptosis by antagonizing PI3K/Akt signalling activity through Smad-mediated transcriptional induction of the lipid phosphatase SHIP (SH2-domain-containing inositol-5-phosphatase) in haematopoietic cells. Increased SHIP expression impedes the phosphorylation and activation of Akt, a major pro-survival kinase, resulting in cell death in both B and T lymphocytes [250].

TGFβ also antagonizes survival signalling by inhibiting expression of survivin, a member of the mammalian IAPs, through the association of Smad3 with Akt, leading to programmed cell death in colon cancer [251-253]. In normal cells, survivin expression is confined to the G_2/M phase of the cell cycle and is required for regulating mitosis [254]. Conversely, survivin expression is highly overexpressed in numerous cancers and has been associated with inhibition of various apoptotic pathways, thus contributing to tumour maintenance and progression [255-261]. In fact, antagonizing survivin expression or activity induces spontaneous apoptosis, enhances apoptosis induced by chemotherapeutic agents, and/or inhibits tumour growth [262,263]. In prostate cancer, it has been proposed that de-regulated TGFβ-survivin signalling may contribute to tumour progression, as survivin expression positively correlates with tumour stage or loss of expression of the TGFβ receptors [264,265]. In prostate epithelial cells, transcriptional repression of survivin by TGFβ is Smad2- and Smad3-dependent and involves recruitment of a pRb/E2F4 repressive complex to the survivin promoter [266].

Moreover, the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signalling pathway also plays a critical role in mediating TGFβ apoptotic responses, through Smad interaction with the activator protein 1 (AP1) [267,268]. TGFβ causes both Smad- and SAPK/p38-dependent transcriptional induction of the pro-apoptotic Bcl-2 family members Bmf and Bim, which in turn activate Bax, leading to mitochondrial release of cytochrome *c* and activation of the apoptosome, resulting in caspase-dependent apoptosis in hepatocytes and B-lymphocytes [269,270]. Conversely, TGFβ inhibits expression of the anti-apoptotic factors Bcl-X_L and Bcl-2 in various cell types [271-274].

Each of these signalling events eventually couples the TGFβ signalling pathway to the apoptotic machinery, leading to changes in expression, localization, and activation of various apoptotic effectors [275]. The TGFβ apoptotic response in normal and tumour cells is multifaceted, incorporating both pro-apoptotic and pro-survival pathways and an array of cytoplasmic and nuclear effectors. The net decision of whether TGFβ will promote apoptosis or favor survival likely depends on additional signalling inputs that the cell receives. Though numerous TGFβ apoptotic mediators and pathways have been defined, these regulatory mechanisms have been mostly cell type- and tissue-specific [5]. Recently, we investigated the molecular mechanisms of TGFβ-induced apoptosis and uncovered a novel and wide-ranging mechanism of TGFβ-mediated cell death involving the pRb/E2F pathway, which will be described in Chapter 3.

1.2.4.3 Alternative cell death mechanism: autophagy

TGFβ has also been implicated in inducing another form of cell death, that of autophagy. Autophagy is an evolutionarily conserved process by which a cell self-digests its own components through a lysosomal degradative pathway in response to various stress conditions, such as nutrient deprivation, growth factor depletion, or hypoxia. Briefly, the general mechanism of autophagy involves the formation of a double membrane-bound vesicle called an autophagosome that envelops and

sequesters a targeted region of the cell. The autophagosome then fuses with a lysosome, forming an autolysosome, in which the sequestered contents are degraded by lysosomal hydrolases. The resulting degradation products can then be recycled for different purposes, such as new protein synthesis, energy production, and gluconeogenesis [276]. As such, autophagy is often considered a pro-survival mechanism, protecting cells and maintaining homeostasis under poor nutrient conditions or cell stress [277]. However, mounting evidence indicates that autophagy plays a role in several vital biological processes, including cell death. In fact, autophagy resulting in total destruction of the cell is actually considered to be a form of programmed cell death (PCD type II). Cell death can be elicited in a number of ways that are morphologically distinct from classical apoptosis. Indeed, three types of programmed cell death were identified in the early 1970s, based on the role of lysosomes inside the cell [278]. PCD Type II, or autophagic cell death, is morphologically distinct from PCD Type I (apoptotic cell death) by the presence of autophagic vacuoles within the dying cells, the absence of phagocyte recruitment, and, in some cases, by caspase independence [278,279]. How autophagy affects cell death depends on the type and context of the cell. Moreover, autophagy can also contribute to apoptosis, as several autophagy-related proteins have been shown to induce cell death and to engage the apoptotic pathway. In effect, there is extensive molecular crosstalk between autophagy-related and apoptosis-related proteins [280-283].

Autophagy is regulated by a large number of genes which function collaboratively in the formation and enclosure of the autophagosome. Some of the key autophagy-related genes (ATGs) include ATG6 (also known as Beclin-1), which is involved in the early stages of autophagosome formation; ATG5, which participates in ubiquitin-like conjugation systems that are essential for elongation of the autophagosome membrane; and ATG8 (also called microtubule-associated protein 1 light chain 3, MAP1LC3 or simply LC3), which is a ubiquitin-like protein required for autophagosome membrane expansion and closure. Importantly, LC3 is converted from its soluble cytosolic form (LC3-I) to the

membrane-bound, autophagosome-associated form (LC3-II) and this lipidation of LC3 is characteristic to autophagy [283-285].

In addition to normal cell growth and homeostasis, autophagy has been implicated to play a protective role in preventing the progression of a number of human diseases, including muscular disorders, some types of neurodegeneration, and cancer [277,279,280,286]. Autophagy in fact plays a dual role in cancer, demonstrating evidence of both a tumour-promoting and a tumour-suppressive role in a context-dependent manner. While many studies support a role for autophagy in maintaining tumour cell survival in response to metabolic stress or hypoxia and thus promoting the growth of solid tumours [287-290], an increasing number of reports suggest that there is a complex interplay between autophagy and cell death, indicating a tumour-suppressive role for this process [291-294].

Autophagy was initially recognized as a potential tumour suppressive mechanism based on the mono-allelic deletion of Beclin-1 in human tumours [295] as well as murine studies demonstrating that autophagy-defective Beclin-1-heterozygous mice are prone to tumourigenesis [296,297]. Since then, a number of key autophagy regulators have been found to be mutated or lost in various cancers and mice deficient for these genes are prone to tumour development (summarized in Table 1).

In addition to these mutations, other evidence suggesting that autophagy serves an anti-cancer role is derived from its regulation, in that tumour suppressors (such as p53, pRb, and PTEN) have been shown to induce autophagy, while oncogenes (such as PI3K, Akt, and Bcl-2) can inhibit autophagy [298-301] .

Protein	Alteration	Cancer type
Atg2B	Frameshift mutation	Gastric cancer
		Colorectal cancer
Atg5	Frameshift mutation	Gastric cancer
		Colorectal cancer
Atg9b	Frameshift mutation	Gastric cancer
		Colorectal cancer
Beclin-1	Mono-allelic deletion	Breast cancer
		Ovarian cancer
		Prostate cancer
	CpG hypermethylation	Breast cancer
	Loss-of-heterozygosity	Breast cancer
	Missense mutations	Gastric cancer
		Colorectal cancer
		Breast cancer
FIP200	Compound heterozygous deletions	Breast cancer
	Loss-of-heterozyosity	
UVRAG	Mono-allelic deletion	Colon cancer
	Frameshift mutation	Colon cancer
		Gastric cancer

Table 1: Atg gene mutations found in a number of distinct cancer types. **(**Wirawan *et al*., 2012) [302].

1.2.4.4 TGFβ-mediated autophagy

While TGFβ has been implicated in both sides of the 'autophagic coin', a recent study by Kiyono and colleagues demonstrated that activation of autophagy may in fact contribute to TGFβ-mediated tumour suppressive effects. They found that TGFβ induces accumulation of autophagosomes and the lipidation of LC3 and enhances the degradation of long-lived proteins. Moreover, they showed that induction of autophagy relies on both Smad-dependent and Smad-independent signalling and proceeds via transcriptional activation of a number of autophagic genes in hepatocellular carcinoma cells, potentiating the tumour-suppressive

effects of TGFβ in these cells [303]. In addition to cancer cells, TGFβ has been shown to induce autophagy in mammary and renal epithelial cells as well as mesangial cells [304-306]. These studies provide emerging evidence for a novel TGFβ-mediated tumour suppressive pathway, though the precise mechanisms and therapeutic implications of which remain to be fully elucidated. We recently assessed the contribution of the pRb/E2F pathway to autophagy activation by TGFβ and discovered that this pathway is also involved in the transcriptional activation of numerous autophagic genes and induction of autophagy in response to TGFβ. This study will be presented in Chapter 4.

Chapter 2 | Transforming Growth Factor-β inhibits telomerase activity through Smad3 and E2F Transcription factors

Annie Lacerte, Juliana Korah, Mélanie Roy, Xiang-Jiao Yang, Serge Lemay, and Jean-Jacques Lebrun

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[*] These authors contributed equally to this work

2.1 Preface

The TGFβ signalling pathway exerts one of its tumour suppressive effects through inhibition of cell immortalization in both normal and cancer cells. Most normal human cells are only capable of replicating a limited number of times due to the progressive shortening of telomeric ends with each cell division, as DNA polymerases fail to fully replicate the genetic material. In contrast, cancer cells are not limited in their number of replication cycles, and are consequently immortalized, due to the constitutive activity of telomerase. TGFβ has been shown to inhibit telomerase activity in various cell types, but the precise mechanisms by which it represses hTERT expression remain unclear. The different mechanisms observed in different cell systems may in fact reflect cell type-specific effects rather than a central mechanism. In this chapter, we investigated the mechanisms of hTERT repression by TGFβ and defined key modulators involved in this TGFβ tumour suppressive effect.

2.2 Abstract

Cancer arises from multiple genetic changes within the cell, among which constitutive telomerase activity and attainment of immortality are central. Expression of hTERT, the protein component of telomerase, is increased in most cancer cells. Transforming growth factor-β (TGFβ), a potent tumour suppressor, has been reported to regulate hTERT expression. We found that TGFβ represses hTERT expression in normal and cancer cells and that this effect is mediated through Smad3 but also requires Erk1/2, p38 kinase and histone deacetylase activity. Furthermore, we identified four critical E2F transcription factor binding sites within the hTERT gene promoter that confer the TGFβ response. Finally, using the E2F-1 knockout model, we showed that loss of E2F-1 abolishes TGFβ inhibition of telomerase expression. These findings highlight the prominent role of TGFβ in regulating telomerase expression and identify Smad3 and E2F-1 as critical mediators of TGFβ effects in both normal and cancer cells.

2.3 Introduction

In humans, tumour formation and progression are characterized by several hallmarks [244]. Cancer cells acquire the ability to become resistant to growth arrest signals, to proliferate in the absence of growth factors and to benefit from increased vascularisation. They also evade apoptosis and escape the immune system, become invasive and attain immortalization. In normal human somatic cells, cell division occurs a limited number of times as the length of the ends of chromosomes (telomeres) shortens with each cell division, leading to senescence and cell death. Cancer cells are not limited by such a fixed number of replication but are instead immortalized. This is due to constitutive telomerase activity, which adds telomeric DNA repeats at the ends of newly duplicated telomeres, thereby preserving their length throughout successive replication cycles and protecting chromosomes from degradation [178].

The telomerase enzyme contains an RNA component, the human telomerase RNA template or hTER and a protein component, the human telomerase reverse transcriptase or hTERT [307]. The hTERT protein is a key determinant of telomerase activity, as its expression is normally suppressed in somatic cells [188]. However, ninety percent of human cancers show increased expression of telomerase, a process that leads to cell immortalization [189]. This is by far the most commonly observed abnormality acquired by tumour cells and is used as a diagnosis marker for cancer [308]. Reactivation of telomerase activity is mainly due to the loss of repression of the hTERT gene in cancer cells. Although telomerase activity is regulated at various levels, such as mRNA splicing and accessibility of the telomeres, the transcriptional control of the hTERT gene is a key event in the increased telomerase activity observed in cancer cells [190].

Growth factors from the TGFβ family have a profound impact on cell homeostasis and act as tumour suppressors, through regulation of cell growth arrest and apoptosis. Interestingly, TGFβ has also been shown to regulate telomerase activity, through repression of the hTERT gene [205,309]. TGFβ 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, co-activators and/or co-repressors to regulate expression or repression of the target genes in a cell and tissue specific manner [310].

TGFβ inhibits telomerase activity in various cell lines, however, the precise mechanisms by which it represses hTERT expression remain unclear. Several factors have been shown to be involved downstream of TGFβ-mediated inhibition of telomerase activity. It has been proposed that TGFβ decreases human and rat TERT expression indirectly through inhibition of c-myc expression [205,206]. However, a recent study in MCF-7 breast cancer cells suggested that the TGFβ inhibitory effect on hTERT was not mediated through inhibition of c-myc

expression, but rather involved a direct interaction of Smad3 and c-myc on the hTERT promoter, leading to inhibition of c-myc transcriptional activity [207]. Another study suggested a role for the Smad interacting protein-1 (SIP1) in TGFβ-mediated hTERT repression [208]. Thus, the different mechanisms observed using different cell systems may reflect cell specific effects rather than a central mechanism.

In this paper, we show that TGFβ inhibitory effects on hTERT expression are mediated not only through the Smad pathway but also require the Erk1/2 and p38 kinase pathways, as well as histone deacetylase activity. Using truncation and deletion mutant forms of the hTERT promoter construct, we showed that TGFβ repressed hTERT gene promoter activity through the −252 to the +3 region, proximal to the start site. Interestingly, we found that several binding sites for E2F family members were critical for TGFβ-mediated inhibition of the hTERT gene promoter. We further demonstrated that interfering with E2F activity resulted in complete reversal of TGFβ-mediated hTERT inhibition, thus highlighting the E2F transcription factors as central mediators of the TGFβ inhibitory effects on telomerase activity. Finally, using the E2F-1 knockout mice model, we found that TGFβ-mediated inhibition of hTERT expression is reversed in the E2F-1 null mutant cells, thus highlighting E2F-1 itself as critical to TGFβ-mediated repression of telomerase activity. A better understanding of the mechanisms regulating hTERT gene expression in normal and cancer cells may prove useful for the development of cancer therapy.

2.4 Materials & Methods

Plasmid constructions

hTERT-2k GFP reporter construct was digested with BamHI and KpnI to separate the hTERT promoter insert (−1934 to +78, ATG as +1, GenBank sequence gi: 4210970) from the pGFP vector. This hTERT promoter insert was ligated into pGL3-basic vector cut with BglII and KpnI. The resulting hTERT (−1934)-lux

reporter construct was confirmed by sequencing. Sequential deletion mutants of hTERT promoter reporter were done using Erase-a-Base System (Promega, Madison, WI, USA) according to manufacturer instructions. All constructs were confirmed by sequencing.

RT-PCR

For reverse transcription reactions, total RNA was prepared from cells treated or not with TGFβ, using Trizol reagent (Invitrogen) according to the manufacturer's protocol. When used, inhibitors were added to the starvation media 30 min prior to starting the time course. cDNA synthesis was carried out using Superscript First Strand Synthesis System for RT-PCR (Invitrogen) using random primers and 5 µg of total RNA. Primers sequences used for the PCR reactions of the different human genes were as follows: hTERT (LT5: 5′-CGGAAGAGTGTCTGGAGCAA-3′; LT6: 5′-GGATGAAGCGGAGTCTGGA-3′); GAPDH (sense: 5′-ACCACCATGGAGAAGGCTGG-3′; antisense: 5′-CTCAGTGTAGCCCAGGATGC-3′); E2F1 (sense: 5′-TGCAGAGCAGATGGTTATGG-3′; antisense: 5′-ATCTGTGGTGAGGGATGAGG-3′); Densitometry analysis was performed using Alpha Innotech Corporation (San Leandro, CA) Fluorochem 8000 software version 3.04. The linear amplification range of each PCR was tested on the adjusted cDNA. The conditions were chosen so that none of the RNA analyzed reached a plateau at the end of the amplification protocol, i.e. they were in the exponential phase of amplification.

Cell Culture

HaCaT,HuH7,MCF-7,MEFs and CHO cells were cultured in DMEM (Hyclone Laboratories Inc., Logan, UT) in the presence of 10% fetal bovine serum (FBS) (Hyclone) and 2 mM L-glutamine. All stimulations were done in serum-free media containing 100pM TGFβ (Peprotech), for the periods of time indicated in the figures.

siRNA transfections

Smad2 and Smad3 siRNAs were purchased from Ambion and introduced into HaCaT cells by reverse transfection using Lipofectamine 2000 reagent (Invitrogen). Briefly, 10nM of each siRNA was mixed with Lipofectamine 2000 reagent and Optimem media (Invitrogen) directly into 60 mm^2 plates. 7.5×10^5 HaCaTcells were then added to each transfection mix and incubated 72 hours at 37°C in a 5% CO_2 incubator. Cells were then trypsinized and fed into 6-well plates for 16 hours. siRNA transfected cells were then incubated in serum-free media 30 minutes before adding TGFβ for the periods of time indicated in the figures. siRNA-Smad2-#1 (sense GGUCUCAUCAAUUAAAGCA, antisense: UGCUUUAAUUGAUGAGACC), siRNA-Smad2-#2 (sense GGUAAUGUAUCAUGAUCCA, antisense: UGGAUCAUGAUACAUUACC), siRNA-Smad3-#1 (sense GCACAUAAUAACUUGGACC, antisense: GGUCCAAGUUAUUAUGUGC), siRNA-Smad3-#2 (sense GGCCCAGUGCAUAUGCAAU, antisense: AUUGCAUAUGCACUGGGCC).

Immunoblotting

Cells were starved overnight in the absence of serum before being stimulated by TGFβ or activin. Cells were then harvested in RIPA buffer supplemented with 100mM sodium vanadate, 1mMphenylmethylsulphonyl fluoride (PMSF), 10μg/ml aprotinin, 10 μg/ml leupeptin and 2 μg/ml pepstatin. Whole-cell lysates were separated on a polyacrylamide gel, transferred to nitrocellulose and incubated with the indicated specific antibody overnight at 4 °C: [anti-hTERT (Calbiochem); anti-β-tubulin, anti-flag (Sigma); anti-phospho-Erk, anti-Erk, anti-phospho-p38, anti-p38 (Cell Signalling Technology), anti-phospho-Smad3 (BioSource); anti-Smad4 and anti-Smad2/3 (SantaCruz)]. After the primary antibody incubation, membranes were washed twice in TBST (50mMTris–Cl at pH 7.6, 200mMNaCl, 0.05%Tween 20), and incubated with the proper secondary antibody coupled to horseradish peroxidase (Sigma) at 1:10,000 dilution) for 1 hour at room temperature. Then, membranes were washed four times for 15 minutes in TBST. Immunoreactivity was normalized by chemiluminescence (ECL

reagent, Roche) according to the manufacturer's instructions and revealed using an Alpha Innotech Fluorochem Imaging system (Packard Canberra, Montreal, Quebec, Canada). Densitometry analysis was performed using Fluorochem 8000 software (Alpha Innotech, San Leandro, CA) that allows quantitative analysis of chemiluminescence under non-saturating conditions.

Luciferase Assays

HaCaT cells were transfected using Lipofectamine 2000 (Invitrogen) with 0.5 µg of luciferase reporter construct, 0.5 µg of β-galactosidase (pCMV-lacZ) expression vector and 0.1 µg to 0.8 µg of the different Smad expression vectors. For siRNA transfection, 10 nM of each Smad siRNAs were added to the transfection. The next day, cells were stimulated or not with TGFβ (100 pM) in starvation media. When inhibitors were used, they were added 30 min prior to TGFβ treatment. All experiments were repeated independently six times and the luciferase activity normalized to β-galactosidase values.

Statistical analysis

Results are expressed as mean±standard deviation. Differences were assessed by one-way ANOVA or the unpaired t test, where appropriate. $p<0.05$ was considered significant.

2.5 Results

2.5.1 TGFβ family members repress hTERT gene expression in a HDAC-dependent manner

To study the effect of TGFβ on *hTERT* expression, we used human epithelial cancer cell lines originating from different tissues (skin, breast and liver), as well as CHO cells. We first analyzed the effect of TGFβ on *hTERT* gene promoter

activity. For this, 2kb (-1978 to +73) of the *hTERT* gene promoter region was cloned in front of the luciferase gene. The resulting hTERT-lux construct was then transfected in the different cell lines, and the cells were stimulated or not with TGFβ. As shown in Figure 2.1a, TGFβ significantly decreased *hTERT* gene promoter activity in all cell lines tested to various extents. The strongest effect was observed in HaCaT cells (76% ± 8% inhibition), while more modest in HuH7 cells (32% ± 13% inhibition) and CHO cells (46% ± 24% inhibition) to weak in MCF-7 cells (24% ± 4% inhibition). We then examined if the decrease of *hTERT* gene promoter activity translated into reduced hTERT mRNA and protein levels. As illustrated in Figure 2.1b, TGFβ treatment of HaCaT cells resulted in a strong and rapid decrease of hTERT mRNA levels (left panels) followed by a significant decrease in hTERT protein level (right panels). Together, our results indicate that TGFβ acts as a potent inhibitor of hTERT expression in epithelial cancer cells.

Previous reports indicated that histone deacetylases (HDACs) repressed telomerase activity [190]. To assess whether inhibition of hTERT expression by TGFβ requires HDAC activity, we examined the effect of Trichostatin A (TSA), a class I and II HDAC inhibitor, on *hTERT* promoter activity. As shown in Figure 2.1c, increasing concentrations of TSA fully reversed the inhibitory effect of TGFβ on hTERT promoter activity and endogenous hTERT mRNA level. To further elucidate whether both class I and II HDAC proteins were involved in *hTERT* regulation, we overexpressed class I (HDAC1) and class II (HDAC4, HDAC5) cDNAs in HaCaT cells and analyzed their effect on *hTERT* gene promoter activity in the presence or the absence of TGFβ. Interestingly, while class I HDAC1 did not have any significant effect on hTERT promoter activity (Figure 2.1d), the two class II HDAC4 and HDAC5 significantly repressed hTERT gene promoter activity.

2.5.2 The Smad, Erk and p38 MAPK pathways are required for TGFβ-mediated hTERT inhibition

The receptor regulated (R-Smads) Smad2 and Smad3 are central to TGFβ signalling [34]. Blocking TGFβ receptor signalling using Smad7, a potent inhibitor that restrains Smad2/3 phosphorylation by the TGFβ type I receptor [311] and further targets the receptor complex to degradation [312,313] completely reversed the TGFβ inhibition of *hTERT* (Figure 2.2a). This demonstrated that functional TGFβ receptors and proper Smad2/3 signalling are required for inhibition of hTERT expression. To further address the relative contribution of Smad2 and Smad3, we used specific siRNAs (2 sets for each Smad) to efficiently and selectively block their expression. As shown in Figure 2.2b (upper panel), transfection of specific human Smad2 or Smad3 siRNAs led to potent inhibition of their relative expression. Importantly, the siRNAs were highly specific since Smad2 siRNAs did not affect the expression of the Smad3 protein and vice versa. Interestingly, blocking expression of Smad3, but not Smad2 led to a partial but significant reversal of the TGFβ inhibitory effect of *hTERT* gene promoter activity (Figure 2.2b, middle and lower panels), indicating that the TGFβ inhibitory effect on *hTERT* promoter activity is specifically mediated through Smad3 and is independent of Smad2. This is consistent with a recent study showing that TGFβ-mediated inhibition of *hTERT* is Smad3-dependent [207]. The partial reversal observed with the Smad3 siRNA also suggested that the Smad pathway is required but not sufficient for TGFβ to inhibit *hTERT* expression and suggested the requirement of additional pathways. TGFβ signals through activation of the canonical Smad pathway, but has also been reported to use other intracellular signalling cascades such as the p38 and Erk MAP kinases [34]. To determine whether these pathways were activated by TGFβ, HaCaT cells were stimulated for different periods of time with TGFβ and the levels of Erk1/2 and p38 phosphorylation examined using specific phospho-Erk1/2 or phospho-p38 antibodies. As shown in Figure 2.2c, both Erk1/2 and p38 kinases were strongly activated in response to TGFβ and these effects were specifically blocked when

cells were treated with either a specific MEK1/2 inhibitor (PD98059) or two different specific p38 kinase inhibitors (PD169316 and SB202190).

To address the contribution of these two pathways in TGFβ-mediated regulation of the *hTERT* promoter, HaCaT cells were transfected with hTERT-lux and stimulated or not with TGFβ in the presence or absence of increasing concentrations of the specific inhibitors. As shown in Figure 2.2d (upper panel), TGFβ-mediated inhibition of the *hTERT* promoter was significantly reversed when cells were pretreated with the MEK1/2 (PD98059) or with the two p38 (PD169316 and SB202190) inhibitors, while not affected in cells treated with a non-functional analog of the PD169316 inhibitor (SB202474). We then determined if this effect on the hTERT promoter was also observed at the mRNA level. Pre-treatment of HaCaT cells with PD98059 or PD169316 resulted in lower hTERT mRNA basal levels, as compared to cells treated with DMSO alone. However, the TGFβ effect on hTERT mRNA repression observed in cells treated with DMSO appeared to be blocked in cells treated with the two inhibitors (Figure 2.2d, lower panel), further suggesting a role for Erk and p38 kinases in regulating hTERT inhibition by TGFβ.

2.5.3 The hTERT core promoter region is required for TGFβ inhibition

To further identify the hTERT gene promoter elements which confer the TGFβ response, progressive deletion mutants of the *hTERT* promoter were generated and assessed for their TGFβ responsiveness by luciferase assays. As shown in Figure 2.3a, the results clearly indicate that the critical regulatory region for TGFβ-mediated inhibition of *hTERT* promoter activity was located between nucleotides –252 and +3. Interestingly, this 255 bp sequence of the *hTERT* promoter corresponds to the previously reported minimal promoter sequence necessary for its activity [190]. An internal deletion of this important regulatory region (–252 to +3 bp) was then introduced in the full length hTERT (-1934) gene promoter construct. As shown in Figure 2.3b, this deletion mutant (hTERT Δ-252,

+3-lux) construct lost its ability to respond to TGFβ. Interestingly, deletion of either half of the 255 bp region of the promoter (mutants hTERT Δ-252, -116-lux and Δ-116, +3-lux) did not reverse the TGFβ effect, indicating that both regions play a role in TGFβ-mediated inhibition of the *hTERT* promoter and suggesting the presence of redundant binding sites within the core region of the *hTERT* promoter (Figure 2.3b).

2.5.4 E2F DNA binding elements are involved in TGFβ-mediated inhibition of the hTERT promoter

The –252 to +3 *hTERT* promoter region contains two E-box DNA binding sites and five GC-boxes. E-box DNA elements of the *hTERT* promoter are recognized by the Myc/Mad/Max transcription factor family. In many cases, c-myc expression parallels *hTERT* expression, in that both are increased in highly dividing cells and down regulated during differentiation. On the other hand, Mad overexpression results in decreased *hTERT* promoter activity [314]. Both c-myc and Mad protein expression levels are known to be controlled by TGFβ signalling [315-317]. For this reason and as it was previously suggested that TGFβ inhibits hTERT through a decrease of c-myc protein [318], we evaluated the importance of each E-box in the TGFβ-mediated decrease of *hTERT* promoter activity by specific point mutations resulting in the disruption of DNA binding [319]. Mutation of either or both E-box sites did not reverse the TGFβ inhibition of the promoter activity (Figure 2.3a). The GC-boxes are DNA binding elements for the Sp1 transcription factor family. Sp1 transcription factors are known to cooperate with Smad proteins to regulate expression of several target genes [320]. However, mutations of the GC-box sites within the hTERT promoter, alone or in combination, did not affect TGFβ-mediated decrease of hTERT promoter activity, also ruling out the involvement of these sites in the TGFβ response (data not shown).

The –252 to +3 region also contains four E2F DNA binding elements and the dynamic assembly of the E2F/pocket protein/ HDAC complex has been

suggested to play a role in the regulation of hTERT [204]. Thus, we evaluated the potential role of the E2F DNA binding sites in TGFβ-induced *hTERT* repression by mutational analysis. Interestingly, while single, double and triple mutations of the E2F binding sites had little or no effect (Figure 2.4b, c and d, respectively), removal of all four E2F DNA sites (4XE2F mutant) completely reversed the TGFβ inhibitory response (Figure 2.4e), suggesting that TGFβ inhibitory effect on *hTERT* promoter activity is mediated through several E2F binding sites located throughout the 255 bp core promoter region.

To then investigate whether E2F transcription factors are required for TGFβ to inhibit *hTERT* expression, we used two dominant negative forms of E2F. We first used E2F-1(1-374), which only contains the DNA binding domain. Overexpression of this mutant was previously shown to act as a dominant negative by displacing endogenous E2F-complexes from E2F DNA binding sites [321]. Transcriptional activity of E2F family members is regulated by interactions with pocket proteins (Rb, p107, p130) that recruit HDAC proteins to repress target genes [139,322]. Thus, we also used a mutated form of E2F-1 (Y411C) which is unable to bind pocket proteins [321]. Interestingly, overexpression of increasing amounts of either dominant negative E2Fs significantly reversed TGFβ-mediated inhibition of the *hTERT* promoter (Figure 2.4f, 4g). Altogether, our results support the hypothesis that TGFβ inhibits telomerase activity through binding of an E2F/repressor complex, within the proximal region of the *hTERT* promoter.

2.5.5 TGFβ-mediated repression of hTERT is lost in embryonic fibroblasts E2F-1 null mutant mice

To further define the role and contribution of E2F downstream of TGFβ in normal cells, we used mouse embryonic fibroblasts (MEFs) isolated from the E2F-1 knockout mice [153]. Wild type (+/+) or E2F-1 null mutant (-/-) MEFs were stimulated or not with TGFβ and the level of Smad phosphorylation assessed by Western blot. As shown in Figure 2.5a, the wild type and E2F-1 (-/-) MEFs

responded to TGFβ. To next analyze the contribution of E2F-1 to TGFβ-mediated inhibition of *mTERT*, MEFs from wild type and E2F-1 null mice were stimulated with TGFβ for different periods of time and mTERT mRNA and protein levels were analyzed by RT-PCR and Western blot respectively. As shown in Figure 2.5b and c, while TGFβ potently inhibited both mTERT mRNA and protein levels in wild type cells, this effect was lost in the E2F-1 knockout cells, further highlighting the critical role played by E2F-1 in TGFβ-mediated repression of *mTERT* expression.

2.6 Discussion

In this study, we show that TGFβ inhibits hTERT expression in both normal and cancer cells. Our results indicate that the TGFβ effects on *hTERT* repression are mediated through the canonical Smad pathway but also require the activation of p38 and Erk kinases. Activation of these three pathways is necessary to decrease *hTERT* expression in response to TGFβ. We also found that E2F and HDAC activity are necessary for the mediation of the TGFβ inhibitory effects on *hTERT* expression. We further identified four critical E2F binding sites, within the proximal region of the core *hTERT* promoter, that confer the TGFβ response. Finally, using the E2F-1 (-/-) MEFs we show that the loss of E2F-1 abolishes the TGFβ inhibitory effect on TERT expression in normal mouse embryonic fibroblasts. Together, our results highlight the prominent role played by TGFβ in regulating telomerase activity and place E2F-1 center stage in the mediation of these effects in both normal and cancer cells.

Replicative senescence is a telomere–dependent mechanism that defines a limited number of successive cell divisions in somatic cells [187]. All dividing cells exhibit a progressive shortening of their telomeres due to the lack of hTERT expression observed in most human somatic cells [187]. Critically shortened telomeres then lead

to permanent growth arrest or senescence. Our results indicate that TGFβ signalling plays a major role in suppressing hTERT expression and as most normal human cell types respond to TGFβ this suggests that this growth factor provides a protective barrier against abnormal *hTERT* expression, thereby contributing to replicative senescence in normal somatic cells.

Our data indicate that Smad3 but not Smad2 is important for *hTERT* gene regulation by TGFβ. This is in agreement with previous observations by Li *et al*. highlighting *hTERT* as a Smad3-specific target gene [207]. Smad3 is essential but not sufficient for TGFβ to repress *hTERT* gene expression, which also requires both the Erk and p38 kinase pathways. In other cell systems, such crosstalk between these three pathways have been described to be important for the activation of the aggrecan gene [323] and the collagenase-3 gene [168], downstream of TGFβ. These results further strengthen the current paradigm that, in addition to the canonical Smad pathway, TGFβ signals through different cascades in a cell type dependent manner [168].

Previous studies investigating the role of E2F-1 in *hTERT* gene regulation have generated some controversial results. While some studies suggested that E2F-1 was required for telomerase activity in mouse and human cancer cells [200], others showed that E2F-1 induced repression of the hTERT gene [202,204,324]. It has also been proposed that E2F-1 exerts opposing regulatory roles in hTERT gene expression, by repressing hTERT in cancer cells, while activating the hTERT gene in normal somatic cells [325]. Our results indicate that the transcription factor E2F-1 plays a central role in regulating telomerase activity and that E2F-1 effects, at least downstream of TGFβ signalling, clearly lead to *hTERT* repression in normal and cancer cells.

A previous report, using the breast cancer cell line MCF7, suggested that SIP1, a TGFβ downstream effector, plays a role in regulating the *hTERT* promoter [326]. In another study, also using MCF7 cells, a Smad binding element (SBE) located

between –262 and –259 of the hTERT gene promoter was shown to be involved in TGFβ inhibition of the *hTERT* promoter [207]. Our results, however, indicate that this particular SBE is not critical for TGFβ-mediated *hTERT* repression in human keratinocyte (HaCaT) cells. As seen in Figure 2.1, the TGFβ effect on *hTERT* repression in MCF7 is much weaker that that observed in other cell types, thus it is conceivable that the TGFβ effects in that particular cell line may differ from those observed in other cell types. Downregulation of c-myc has also been previously suggested as a mechanism by which TGFβ could regulate *hTERT*. However, our results indicate otherwise, at least in the cell lines used in our studies. Indeed, TGFβ still potently repressed transcription of the *hTERT* gene promoter when both c-myc binding sites are removed. This is consistent with a recent study in MCF-7 cells showing that TGFβ negatively regulates telomerase activity via Smad3 interactions with c-myc and the *TERT* gene promoter, independent of c-myc downregulation [207]. However, in light of our results, it is unlikely that a Smad3/c-myc interaction plays a role in hTERT repression in human keratinocytes. This suggests that TGFβ utilizes distinct mechanisms to repress telomerase activity in a cell specific manner.

Recent studies have suggested a role for HDACs in hTERT gene repression in normal cells. Trichostatin A treatment results in increased telomerase activity [170,190,202,324] and HDAC complexes are shown to be recruited to the hTERT promoter via uncharacterized factors; Sp1 and/or Rb/E2F being potential candidates. Furthermore, a recent and elegant study has demonstrated that the assembly of complexes made up of E2F, pocket proteins and HDAC regulates hTERT gene expression in normal human fibroblasts [204]. A role for E2F in regulating hTERT activity was previously suggested, as E2F overexpression in human cells led to telomerase repression [202]. Another study demonstrated that endogenous p53 represses hTERT expression through a p21- and E2F/Rb-dependent pathway [324]. p53-induced p21 expression leads to decreased pRb phosphorylation and induces the recruitment of E2F family members and histone deacetylases to form complexes that inhibit transcription [324]. These data are

complementary to our results and combined, these studies highlight E2F and HDAC proteins as central mediators of TGFβ-induced telomerase repression.

TGFβ is a very potent negative regulator of the cell cycle, which can activate the expression of the cyclin-dependent kinase inhibitors p15 and p21, and repress Id proteins and c-myc in most cell types. Repression of c-myc by TGFβ has been extensively characterized. It involves interactions between Smad3, E2F4/5, the co-repressor p107 and HDAC activity [162]. Moreover, repression of cdc25A also involves E2F and HDAC [163]. It is interesting to note that in addition to c-myc and cdc25A, two critical cell cycle regulators, TGFβ also modulates hTERT expression through cooperation between the Smad proteins and E2F transcription factors. Thus these transcription factors appear to be critical regulators of TGFβ cytostatic responses.

By inhibiting the cell cycle, inducing apoptosis and preventing immortalization through the inhibition of telomerase activity, TGFβ exerts strong tumour suppressive effects. TGFβ-mediated inhibition of telomerase activity is of profound impact for this growth factor's tumour suppressive role. While hTERT involvement in cell immortalization is well characterized, recent studies indicated that telomerase possesses additional functions that are not related to net telomere length. In fact, increased telomerase expression enhances tumour formation even in the presence of very long telomeres in mice [327-330]. Overexpression of hTERT in human epithelial or neural cultured cells induces resistance to pro-apoptotic or anti-proliferative signals, including TGFβ [331]. In fibroblast cells, overexpression of hTERT with H-Ras produced tumours in nude mice, while a defective form of hTERT, which is unable to lengthen telomeres, is still able to cooperate with H-Ras to induce tumour formation. This suggests that the hTERT effect on tumour progression includes non-telomere function [332]. A recent study using microarrays demonstrated that telomerase is able to stimulate proliferation of epithelial cells by controlling expression of genes involved in cell proliferation [333]. Thus, the TGFβ inhibitory effect on hTERT activity not only leads to repression of

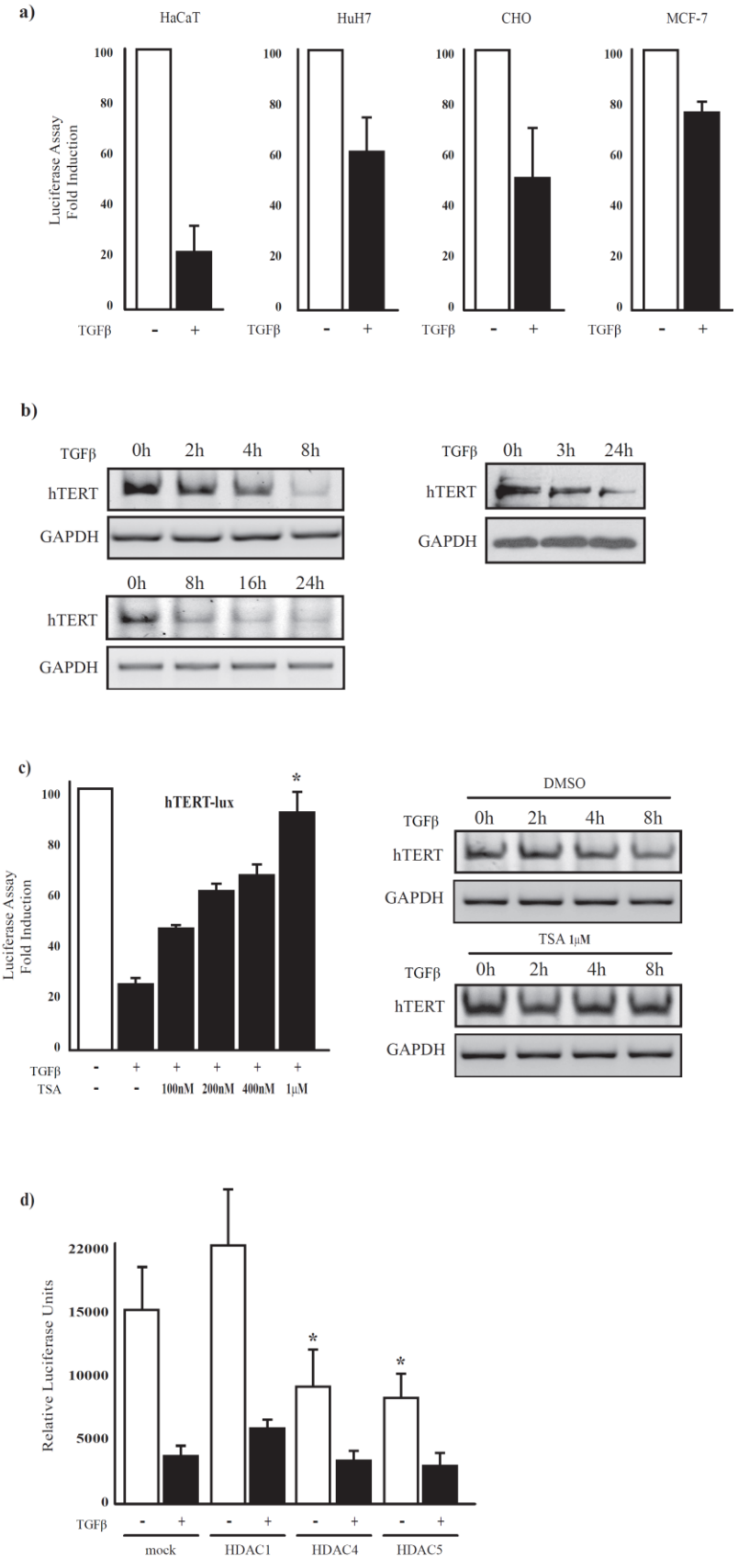
immortalization, but might also represent an important component of the cytostatic program induced by this growth factor to inhibit cell proliferation.

Deregulation of E2F-1 function is common in most human cancers and interestingly, like TGFβ, E2F-1 plays a dual role in cancer, acting as both a tumour suppressor and a tumour promoting agent. The oncogenic properties of E2F-1 and its regulatory role in the transition of the cell cycle from G1 to S phase, in order to activate genes required for DNA synthesis and cell cycle control are well characterized [334]. However, mice studies have revealed that E2F-1 could also act as a tumour suppressor gene. E2F-1 knockout mice exhibit apoptosis defects in thymocytes and develop highly malignant tumours [153,335], whereas transgenic mice expressing E2F-1 display aberrant cell apoptosis [336]. Thus, it is conceivable that, in addition to its inhibitory role on telomerase activity, E2F-1 may mediate some of the TGFβ pro-apoptotic responses. This role of E2F-1 in cell death and tumour suppression raises an interesting prospect as to its potential use in targeted therapy for human cancer.

2.7 Acknowledgements

hTERT-2k GFP reporter construct was kindly provided by Dr. Shiaw-Yih Lin (MD Anderson Cancer Center, the University of Texas). Dr. Kristian Helin (European Institute of Oncology) generously provided E2F-1 (1-374) and E2F-1 (Y411C) expression vectors. Finally, we are greatly thankful to Dr. Lili Yamasaki for providing us with the wild type and E2F-1 knockout MEFs. JJL is Research Scientist of the National Cancer Institute of Canada (NCIC) supported with funds provided by the Canadian Cancer Society. This work was supported by grants from the NCIC (015257 to JJL) and CIHR (to JJL)

2.8 Figures for Chapter 2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HDAC1
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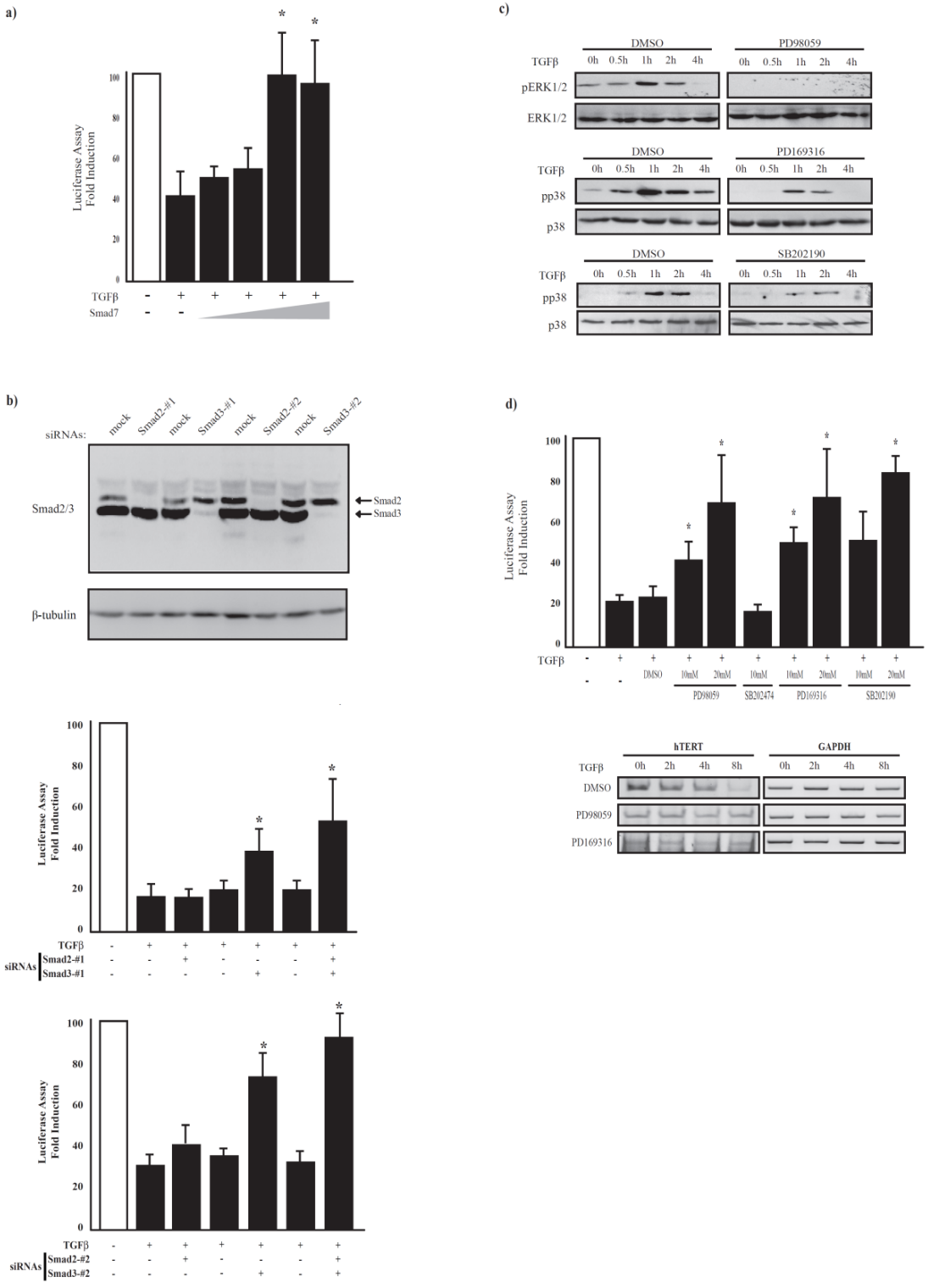
Figure 2.1: TGFβ inhibits hTERT expression in a HDAC dependent manner.

a) Cells were transfected with the hTERT-lux reporter, stimulated with TGFβ and assessed for luciferase. b) RT-PCR (left) and Western blot (right) analysis of hTERT mRNA and protein levels in HaCaT cells stimulated with TGFβ. c) hTERT (−1934)-lux transfected HaCaT cells were pre-treated with the indicated concentrations of TSA and stimulated with TGFβ before being assessed for luciferase (left) and hTERT mRNA levels by RT-PCR (right). d) HaCaT cells were transfected with hTERT (−1934)-lux and HDAC1, HDAC4 and HDAC5 expression vectors, as indicated, before being assessed for luciferase.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SB202190
hTERT
GAPDH
PD98059
PD169316

Figure 2.2: Figure 2.2 TGFβ requires the Smad, Erk and p38 MAPK pathways to inhibit hTERT promoter activity.

a) HaCaT cells transfected with hTERT-lux and increasing amounts of Smad7 cDNA (0.1 to 0.8 μg) were stimulated or not with TGFβ before being assessed for luciferase. b) HaCaT cells were transfected with two different sets of siRNAs against human Smad2 or Smad3 and Smad2 and 3 protein levels were analyzed by Western blot using an anti-Smad2/3 antibody (upper panel). HaCaT cells were then transfected with hTERT-lux and the different Smad2/3 siRNAs, as indicated, stimulated with TGFβ and assessed for luciferase (middle and lower panels). c) HaCaT cells were treated with PD98059, PD169316, or SB202190 as indicated, stimulated with TGFβ and protein phosphorylation levels were monitored by anti-phospho-Erk and anti-phospho-p38 by Western blot. Equal protein levels were verified by immunoblotting with anti-Erk1/2 and anti-p38. d) HaCaT cells were transfected with hTERT-lux with or without the different MAPK inhibitors, at the indicated concentrations for 30 min, stimulated or not with TGFβ for 16 h and assessed for luciferase (upper panel). Following treatment of HaCaT cells with MAPK inhibitors, hTERT and GAPDH mRNA levels were measured by RT-PCR (lower panel).

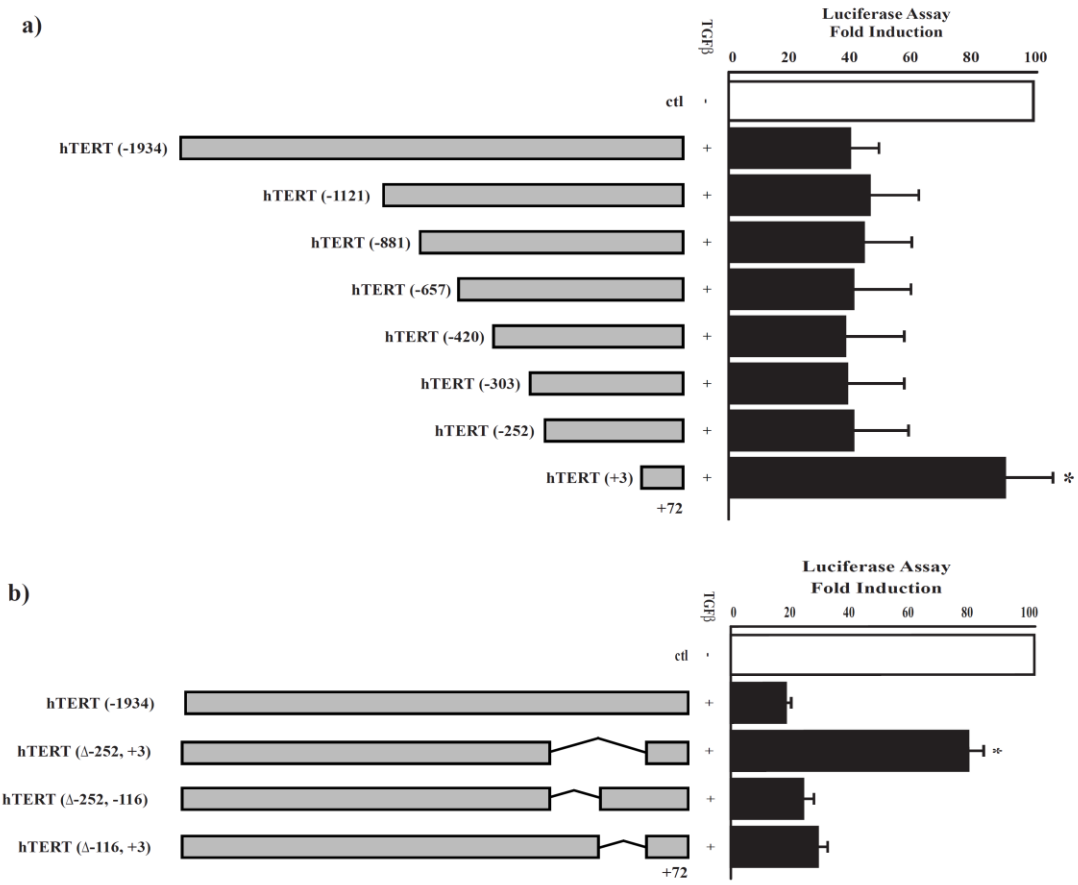

Figure 2.3: The −252 to +3 region of the hTERT gene promoter is required for TGFβ-mediated inhibition of hTERT.

Progressive deletion mutants a) or internal deletion mutants b) of the hTERT gene promoter were transfected in HaCaT cells and assessed for luciferase in response to TGFβ.

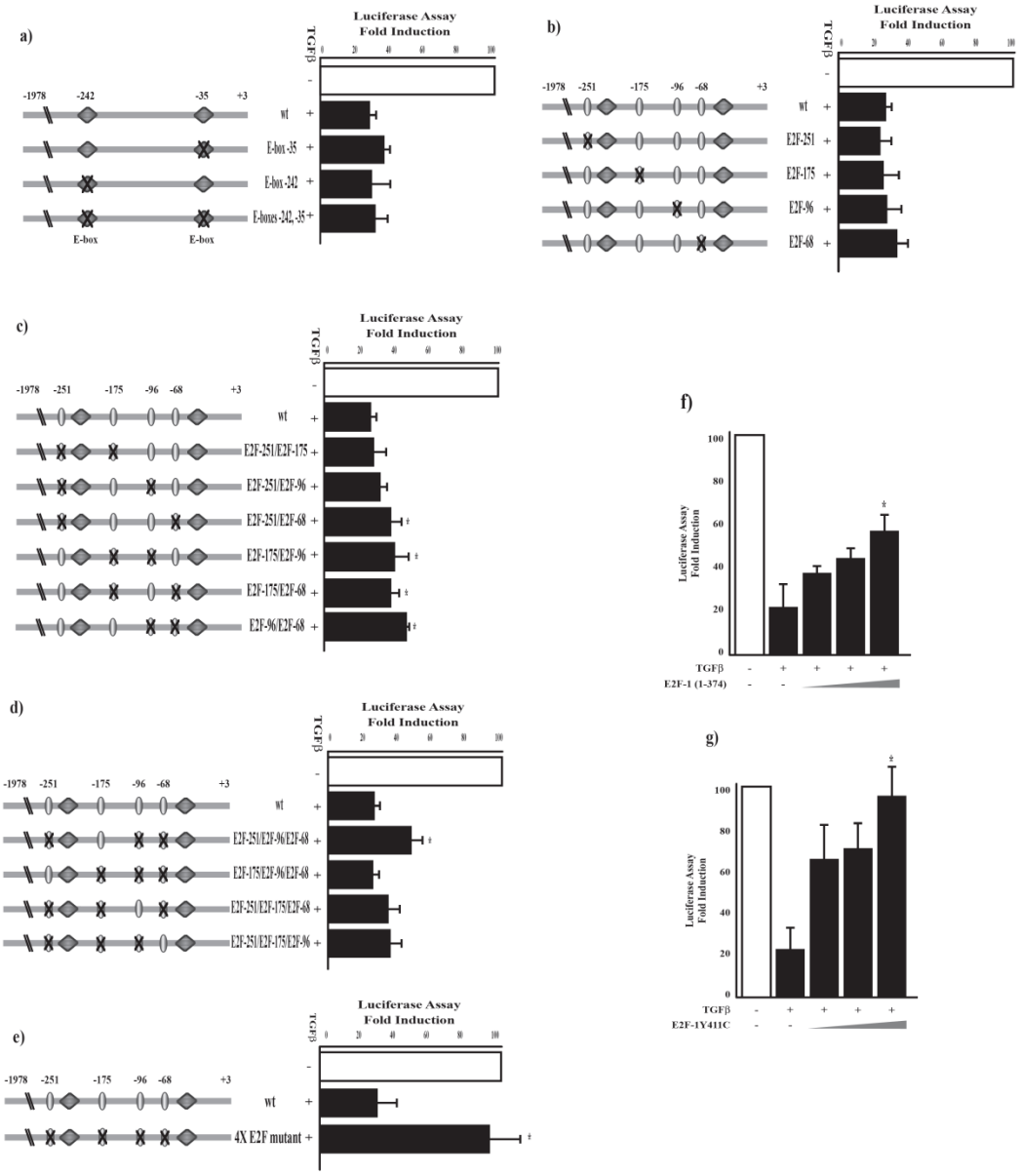

Figure 2.4: E2F is required for TGFβ-mediated inhibition of hTERT.

E-box mutants (a) and single (b), double (c), triple (d) or quadruple (e) E2F binding site mutants of the hTERT (−1934)-lux construct were transfected in HaCaT cells as indicated and assessed for luciferase in response to TGFβ. f, g) Dominant negative forms of E2F, f) (E2F-1 (1–374) and g) E2F-1 (Y411C) were transfected in HaCaT cells and luciferase activity assessed in response to TGFβ.

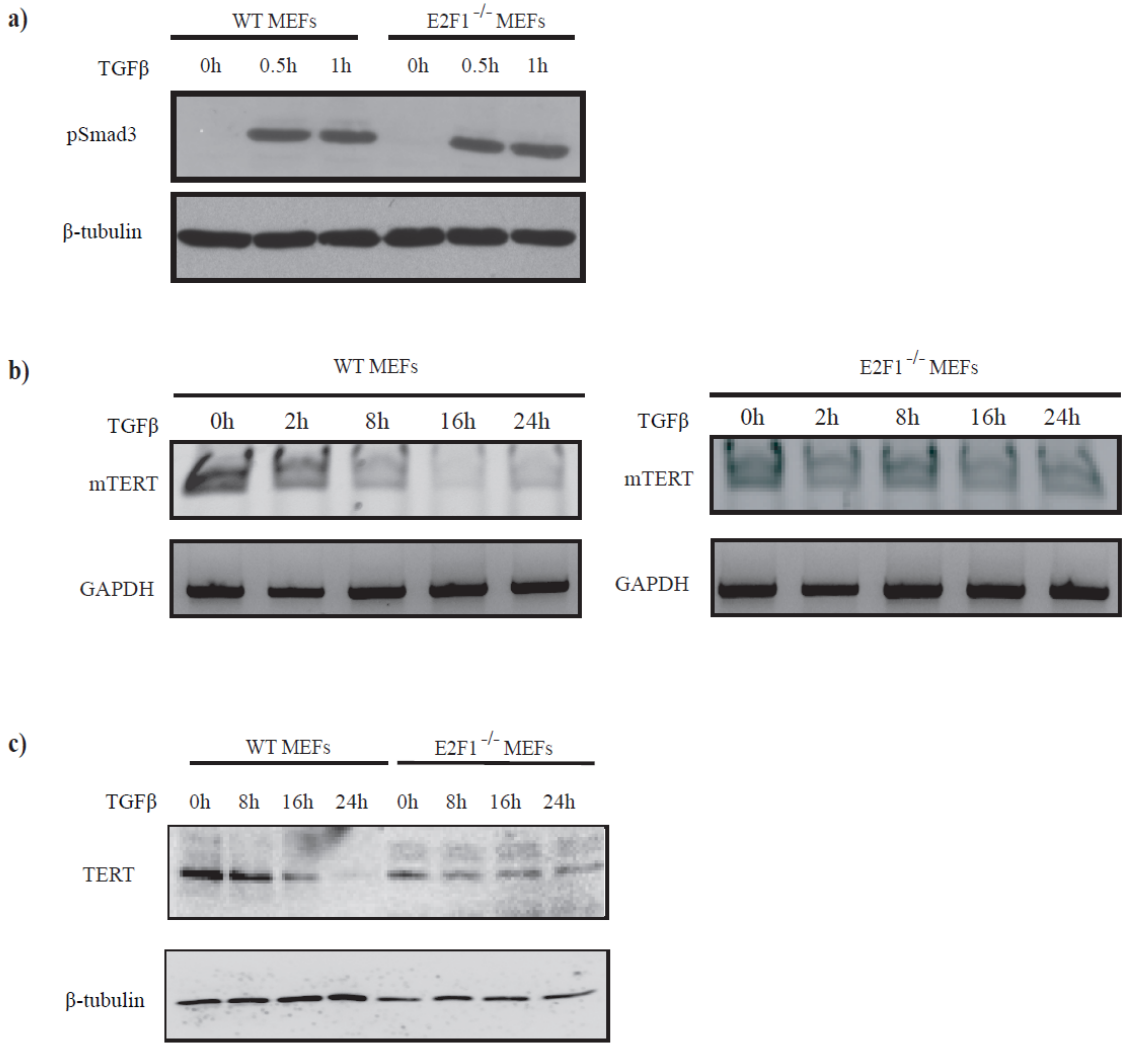

Figure 2.5: TGFβ-mediated repression of mTERT is lost in the E2F-1 null mutant mice.

Wild type and E2F-1 (−/−)MEFs were stimulated or not with TGFβ for the indicated times and phospho-Smad3 (a), TERT mRNA (b) and TERT protein (c) levels were assessed by Western blotting and RT-PCR.

Chapter 3 | A transcriptionally active pRb-E2F1-P/CAF signalling pathway is central to TGFβ-mediated apoptosis

Juliana Korah, Nisrine Falah, Annie Lacerte, and Jean-Jacques Lebrun

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3.1 Preface

The results from our study in Chapter 2 prompted us to investigate whether E2F1 could also be involved in mediating another arm of the TGFβ tumour suppressive response and regulate programmed cell death.

While various apoptotic mediators and signalling pathways have been implicated in TGFβ-mediated apoptosis, these defined regulatory mechanisms have been largely cell type- and tissue-specific. A better understanding of the specific regulatory mechanisms responsible for TGFβ-mediated apoptosis would provide clearer insight into the basis of this variability and, potentially, allow for the eventual integration of these various observations into a comprehensive pathway or global TGFβ apoptotic programme. In the study described here we assessed the role of the pRb/E2F1 pathway in mediating the TGFβ apoptotic programme in multiple target tissues and cell types.

3.2 Abstract

Transforming growth factor-β (TGFβ) modulates the expression of multiple apoptotic target genes, however, a common and central signalling 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. 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 the TGFβ pro-apoptotic target gene promoters, thereby activating their transcription. Together, our findings define a novel process of gene activation by the TGFβ-E2F1 signalling axis and highlight E2F1 as a central mediator of the TGFβ apoptotic program.

3.3 Introduction

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 tumour suppressive effects by inhibiting cell cycle progression, inducing apoptosis and preventing immortalization through inhibition of telomerase activity. Loss or mutation of TGFβ signalling components is frequently observed in human cancer and further define a tumour suppressive role for this growth factor [337].

TGFβ 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 TGFβ ligands [28].

TGFβ induces a number of apoptotic responses and its ability to do so varies greatly depending on the cell type [5]. Understanding the basis of this variability requires elucidating the molecular mechanisms involved in regulating TGFβ-mediated apoptosis. TGFβ signalling activates caspases in various epithelial cell types [269,338] and transcriptionally induces the death-associated protein kinase (DAPK) in hepatoma cells [247]. TGFβ also induces apoptosis by antagonizing PI3K/Akt signalling activity through expression of the lipid phosphatase SHIP (SH2-domain-containing inositol-5-phosphatase) in haematopoietic cells [250]. Transcriptional up-regulation of pro-apoptotic proteins such as Bax and down-regulation of pro-survival Bcl-2 family members have also been implicated in TGFβ-mediated programmed cell death [339,340]. 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, highlighting E2F1 as an important mediator of TGFβ tumour suppressive effects [209]. 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 through 5 is regulated primarily via their association with members of the retinoblastoma family of pocket proteins, which include the retinoblastoma tumour suppressor protein, pRb/p105, p107 and p130

[139]. E2F1, the founding member and best-characterized of the family, has a unique role compared to other E2Fs, showing characteristics of being both an oncogene and a tumour 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 tumours [150,151] supporting an oncogenic role for E2F1, transgenic mice overexpressing E2F1 display aberrant cell apoptosis [152]. Furthermore, E2F1 knockout mice develop highly malignant tumours and show defects in thymocyte apoptosis, highlighting E2F1 as a potent tumour suppressor [153]. 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 [154]. 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 [157]. Given our previous findings that E2F1 is required for TGFβ-mediated inhibition of hTERT [209] and that TGFβ promotes increased E2F-DNA-binding activity in pre-apoptotic hepatoma cell nuclear extracts [341], we investigated whether E2F1 could also mediate another arm of the TGFβ tumour 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 complex onto 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 signalling pathway as a critical regulator of TGFβ-mediated cell death.

3.4 Materials & Methods

Cell Culture & Transfections

HaCaT, HuH7, HepG2, Moser and SKCO cell lines, as well as mouse embryonic fibroblasts (MEFs) were cultured in DMEM (HyClone), and WM278 cells in RPMI-1640 (HyClone). Medium for all cells was supplemented with 10% fetal bovine serum (FBS) (HyClone) and 2mM L-glutamine (GIBCO) and cells were grown at 37°C in 5% CO_2 conditions. Prior to treatment, cells were serum-starved for 24 hours and all stimulations were done in serum-free medium containing 100pM TGFβ1 (Peprotech). Cells were transiently transfected with different siRNAs against E2F1 (Ambion) or P/CAF (Sigma-Aldrich), or with wild-type and mutant E2F1 expression vectors using Lipofectamine^{TM} 2000 reagent (Invitrogen), 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 pM TGFβ. Mitochondrial viability was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, following 24 to 72 hours of TGFβ treatment, cells were incubated with 1 mg/ml MTT solution (Sigma) in the culture media for 2 hours. 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 Bio-Tek microplate reader. Alternatively, cell viability was determined by the fluorescent calcein acetoxymethyl ester (calcein-AM) method. Briefly, following 4 to 24 hours of TGFβ treatment, original culture medium was replaced with serum-free medium containing 2 ug/ml calcein-AM (BD Biosciences) 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.

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 (BioRad) in a RotorGene 6000 PCR detection system (Corbett Life Science). Conditions for qPCR were as follows: 95°C for 30sec, 40 cycles of 95°C for 5sec and 60°C for 20sec. Primer sequences are listed in Table 2. Where indicated, some cDNAs were amplified for 30 cycles instead and amplified products were analyzed by DNA gel electrophoresis.

Immunoblotting & Immunoprecipitation

Cells were lysed in cold RIPA buffer (50mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA), containing 1 mM sodium orthovanadate, 1mM 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 (KH95, Santa Cruz Biotechnology), anti-β-tubulin (Sigma), anti-phospho-Smad3 (BioSource). Following primary antibody incubation, membranes were washed twice in TBST (50mM Tris-HCl at pH 7.6, 200mM NaCl, 0.05% Tween20), and incubated with secondary antibody coupled to horseradish peroxidase (Sigma) at 1:10,000 dilution for 1h 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). Immunoprecipitations were performed overnight at 4°C using antibodies against E2F1 (C-20, Santa Cruz Biotechnology), pRb (Cell Signalling), P/CAF (Abcam) and CBP/p300 (Santa

Cruz Biotechnology). Protein A-sepharose (Amersham Biosciences) was added for 2 hours at 4°C and beads were then washed four times with cold lysis buffer. The immunoprecipitates were eluted with 2xSDS 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 TGFβ treatment, cells were collected by trypsinization, pelleted by centrifugation, washed with PBS and each sample was incubated with 0.5 µg Annexin V-FITC and 10 µl Propidium Iodide (50 µg/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 TGFβ treatment, cells were washed with PBS and subjected to Annexin V-FITC staining for 15 minutes 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 hour, washed with PBS, and incubated with AlexaFluor568 goat anti-mouse IgG secondary antibody (Invitrogen) for 1 hour. 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.

Caspase Activity

Cells were plated in triplicate in 96-well dishes, at 10,000 cells/100 µl in medium supplemented with 2% FBS and in the presence or absence of 100 pM TGFβ.

Caspase 3/7 activity was measured using the Caspase-Glo® 3/7 Assay (Promega) according to the manufacturer's instructions. Briefly, following TGFβ treatment, cells were incubated with Caspase-Glo® reagent for 1.5 hours at room temperature, and the luminescence of each sample was measured using an EG & G Berthold luminometer.

Cycloheximide Chase

Cells were seeded in 60-mm^2 plates and grown to 85% confluence. Following overnight serum-starvation, the cells were incubated, in the presence or absence of 100 pM TGFβ, with 50 μg/ml cycloheximide (Sigma) for the indicated times, and analyzed by immunoblotting.

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 rpm 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 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin, for 10 min on ice. The resulting chromatin solution was sonicated for five pulses of 20 seconds to generate 300–2000 bp DNA fragments. After centrifugation at 14,000 rpm for 10min at 4°C, the supernatant was immunocleared by incubation with protein A-sepharose beads for 2 hours at 4°C. Immunocleared chromatin was immunoprecipitated overnight with 5 μg of the indicated antibodies. Antibody-protein-DNA complexes were then isolated by immunoprecipitation with 40 μl protein A-sepharose beads (Amersham) for 2 hours 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, 1mM EDTA, 10mM 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 1µl of 10 mg/ml RNaseA was added each sample, and they were incubated at 65°C overnight. Following this, 5mM EDTA and 2 µl of 10 mg/ml proteinase K was added, and samples were incubated for at 45°C for 2h. DNA was recovered using the QIAquick spin columns (Qiagen, Maryland, USA) as per the manufacturer's protocol and PCR analysis was performed using primers specific for the indicated promoters, as listed in Table 3.

Statistical Analysis

Results are expressed as mean ± standard deviation of at least 3 independent experiments. Statistical differences were determined by two-tailed unpaired *t*-test. $p < 0.05$ was considered statistically significant.

Table 2: PCR Primer Sequences

Apaf1 forward	5'-CTCTCATTTGCTGATGTCGC-3'
Apaf1 reverse	5'-TCGAAATACCATGTTTGGTCA-3'
TAp73 forward	5'-CATGGAGACGAGGACACGTA-3'
TAp73 reverse	5'-CTGTAACCCTTGGGAGGTGA-3'
Caspase3 forward	5'-AGCGAATCAATGGACTCTGG-3'
Caspase3 reverse	5'-CGGCCTCCACTGGTATTTTA-3'
Caspase7 forward	5'-GCAGTGGGATTTGTGCTTCT-3'
Caspase7 reverse	5'-CCCTAAAGTGGGCTGTCAAA-3'
Smac/DIABLO forward	5'-AATGTGATTCCTGGCGGTTA-3'
Smac/DIABLO reverse	5'-AGCTGGAAACCACTTGGATG-3'
GAPDH forward	5'-GCCTCAAGATCATCAGCAATGCCT-3'
GAPDH reverse	5'-TGTGGTCATGAGTCCTTCCACGAT-3'
Smad7 forward	5'-TCCTGCTGTGCAAAGTGTTC-3'
Smad7 reverse	5'-CAGGCTCCAGAAGAAGTTGG-3'

Table 3: 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'-TTTGGGCACTTGGAGCGCG-3'
Caspase7 reverse	5'-AAGAGCCCAAAGCGACCCGT-3'
Smac/DIABLO forward	5'-TTCCCTTCAAGCCCTGGCCCGAAC-3'
Smac/DIABLO reverse	5'-ACGCCCCCACCCAAGGAAGCAGTC-3'

3.5 Results

3.5.1 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 a cell viability assay (MTT) as well as calcein-AM assay, a more sensitive assay for early apoptosis detection [342]. All cell lines tested were strongly growth-inhibited by TGFβ treatment in a time-dependent manner (Figure 3.1A and B). To address the contribution of E2F1 in mediating this TGFβ response, we used RNA interference to reduce the expression of endogenous E2F1. Interestingly, we found that the effect of TGFβ on cell viability (Figure 3.1C) and early apoptosis (Figure 3.1D) in all cell lines tested was almost completely prevented when E2F1 expression was silenced, indicating that E2F1 is required for mediating the TGFβ pro-apoptotic response in multiple cell lines of various origins.

To further investigate the role of E2F1 in TGFβ-mediated apoptosis we performed fluorescence-activated cell sorting (FACS) following AnnexinV and PI staining. While TGFβ treatment markedly increased the number of apoptotic cells in control siRNA-transfected HuH7 cells (Figure 3.1F, left panels), E2F1

knockdown completely abolished this effect (Figure 3.1F, right panels), consistent with cell viability and calcein-AM results. Fluorescence imaging following AnnexinV staining further confirmed these findings (Figure 3.1G). Taken together, these results indicate that TGFβ has a strong pro-apoptotic function in various cell lines and that these effects require the transcription factor E2F1.

3.5.2 E2F1 is required for TGFβ-mediated regulation of pro-apoptotic target genes

TGFβ signalling activates multiple pro-apoptotic genes and pathways in a cell- and tissue-specific manner [5]. Independently of TGFβ, 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 *Apaf-1*(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) [343-349].

To assess whether TGFβ and E2F1 share any common downstream apoptotic targets, we examined the regulation of representative E2F1-responsive pro-apoptotic genes in TGFβ-treated human hepatoma HuH7 cells, which express both functional p53 and pRb. As shown in Figure 3.2A, TGFβ potently induced mRNA expression of *Apaf1*, *Caspase 3*, *Caspase 7*, *p73*, and *Smac/DIABLO*, suggesting that TGFβ induces apoptosis in HuH7 cells by the intrinsic mitochondrial pathway. Importantly, this analysis also revealed *Smac/DIABLO* as a novel TGFβ target. Loss of E2F1 expression markedly impaired the TGFβ-mediated induction of each of these target genes (Figure 3.2B), indicating that E2F1 is required for TGFβ-mediated regulation of its pro-apoptotic downstream target genes. Moreover, these data provide a novel pathway by which TGFβ regulates these genes and reveals E2F1 as a widespread co-transducer of TGFβ-induced activation of the intrinsic mitochondrial pathway.

To then examine whether these pro-apoptotic genes were direct targets of TGFβ, cells were treated or not with the 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 3.2C). 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.

3.5.3 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 3.3A), in agreement with previous reports [350,351]. Surprisingly, however, we found TGFβ to rapidly and transiently induce E2F1 protein expression levels in these cells (Figure 3.3B). 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 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 cycloheximide chase in HaCaT cells treated or not with TGFβ (Figure 3.3D). In the presence of cycloheximide, untreated cells showed progressive diminished levels of E2F1 over time. Conversely, TGFβ treatment maintained E2F1 levels throughout the chase, indicating that TGFβ is indeed able to prolong E2F1 half-life, by stabilizing E2F1 protein levels post-translationally.

3.5.4 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, 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 TGFβ stimulation, as assessed by the induction of Smad phosphorylation (Figure 3.4A). The pro-apoptotic effect of TGFβ, however, greatly differed in these two cell types. While cell viability of the wild-type $E2F1^{+/+}$ MEFs was potently decreased in response to TGFβ, this effect was severely impaired in the $E2F1^{-/-}$ MEFs (Fig. 4B). Correspondingly, TGFβ-induced expression of *Caspase 7* and *Smac/DIABLO* was significantly reduced in the $E2F1^{-/-}$ MEFs (Figure 3.4C). Together, these findings highlight a critical role for E2F1 downstream of TGFβ in the mediation of apoptosis in a normal cell setting in addition to multiple cell lines of various cancer origins.

3.5.5 E2F1 DNA-binding, transactivation and pRb-interaction are required for TGFβ-mediated apoptosis

To further understand the molecular mechanisms underlying the role of E2F1 in the induction of programmed cell death by TGFβ, 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 [352]. Interestingly, transient overexpression of each of these mutants drastically impeded the effect of TGFβ on cell viability in HuH7 cells (Figure 3.5A). The antagonistic effects of these E2F1 mutants were further established at the transcriptional level, as their overexpression significantly reduced TGFβ-

induced *Caspase 7* and *Smac/DIABLO* mRNA levels (Figure 3.5B). These results indicate that TGFβ 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 TGFβ could induce association between endogenous E2F1 and pRb using co-immunoprecipitation studies. As shown in Figure 3.5C, TGFβ treatment indeed promotes the association between E2F1 and pRb. Altogether, these results indicate that pRb–E2F binding is required for TGFβ to induce apoptosis and that this association is induced by TGFβ itself, strongly supporting that the pRb–E2F1 protein complex plays a role downstream of TGFβ-mediated cell signalling, leading to apoptosis.

3.5.6 TGFβ 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 TGFβ while remaining in its seemingly transcriptionally repressive pRb–E2F1 complex. Thus, we assessed whether TGFβ could in fact recruit positive regulators of transcription to the pRb–E2F1 complex. As TGFβ may activate gene transcription through histone acetyltransferases (HATs), including p300/CBP (CREB-binding protein) and p300/CBP-associated factor (P/CAF) [64], we screened for the presence of these HATs in E2F1 and pRb immunoprecipitates in untreated versus TGFβ-treated cells. Interestingly, as shown in Figure 3.6A, we found that TGFβ 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 TGFβ.

Loss of P/CAF expression by RNA interference dramatically reduced the TGFβ pro-apoptotic effect in these cells (Figure 3.6B). Moreover, the TGFβ-induced expression levels of *Caspase 7* and *Smac/DIABLO* were notably reduced when P/CAF expression was silenced by siRNA (Figure 3.6C). As caspases require post-translational activation to become catalytically active and mediate cell death [353], we investigated whether the loss of TGFβ-induced caspase expression due to P/CAF knockdown was followed by a decrease in caspase activity. As shown in Figure 3.6E (left panel), blocking P/CAF expression severely impaired TGFβ-mediated Caspase 3/7 activation. This effect was similar to what was observed when E2F1 expression was silenced (Figure 3.6E, right panel). By 48 hours, loss of either P/CAF or E2F1 expression nearly completely abolished TGFβ-induced caspase activation. Collectively, these findings support a critical role for P/CAF downstream of TGFβ in the E2F1-dependent activation of pro-apoptotic genes and the mediation of programmed cell death.

To then assess the functional relevance of the TGFβ-induced pRb–E2F1–P/CAF complex in regulating TGFβ transcriptional responses, we performed chromatin immunoprecipitation (ChIP) assays to determine whether this complex is recruited to the pro-apoptotic target gene promoters in response to TGFβ. We examined the promoters of the TGFβ- and E2F1-responsive pro-apoptotic genes identified above. Interestingly, as shown in Figure 3.6F, TGFβ 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 TGFβ-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 signalling axis leading to apoptosis downstream of TGFβ in normal and cancer cells.

3.6 Discussion

While various apoptotic mediators and signalling pathways have been implicated in TGFβ-mediated apoptosis, most of these regulatory mechanisms appear to be cell type-dependent or tissue-specific [5]. This study defines a novel process of gene activation by the TGFβ-E2F1 signalling axis, and highlights the pRb-E2F1-P/CAF pathway as a wide-ranging and critical mediator of the TGFβ apoptotic program in multiple target tissues.

We identified a number of key pro-apoptotic TGFβ target genes that trigger the intrinsic apoptosis pathway through the induction of E2F1. While these genes are functionally interrelated, our results imply that TGFβ regulates the intrinsic apoptosis pathway at multiple levels, consistent with the strong pro-apoptotic effect played by 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 TGFβ-mediated cell death. Importantly, these results are corroborated using the E2F1 knockout mouse model, demonstrating that the TGFβ-E2F1 signalling pathway mediates TGFβ-induced cell death not only in a diseased state, but in a normal cell setting as well.

While 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 [139] . 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 [354]. 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 actyltransferase (P/CAF) to activate

transcription of pro-apototic genes in response to TGFβ. Indeed, analysis with dominant negative E2F1 mutants revealed that, in fact, pRb binding to E2F1 is required for TGFβ-mediated apoptosis.

Our results also indicate that TGFβ 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 [355], acetylation [356], and by the ubiquitin-proteasome pathways [357]. Binding of pRb to E2F1 protects E2F1 from ubiquitination and proteolytic degradation [358], thereby increasing its stability. As TGFβ maintains pRb in a hypophosphorylated form, causing E2F1 to remain bound to pRb and suppressing activation of E2F1-responsive cell cycle regulatory genes [359], it is likely that the TGFβ 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 TGFβ, 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 [356]. Thus, P/CAF binding to E2F1 in response to TGFβ 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 [360]. It is interesting to consider, then, whether TGFβ 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 demonstrate here to be required for TGFβ to activate E2F1-dependent pro-apoptotic target genes. The coordinated recruitment of E2F1, pRb, and P/CAF to pro-apoptotic gene promoters that we observed suggests the potential formation of a

transcriptionally active pRb–E2F1 complex, which mediates the regulation of TGFβ pro-apoptotic targets. Taken together, these results strongly support a pro-apoptotic role for the E2F1 pathway downstream of TGFβ and provide a potential mechanism for the activation of E2F1-responsive pro-apoptotic genes in response to TGFβ.

It is interesting to consider that TGFβ tumour suppressive effects might utilize the functional interplay among the E2F family members, which plays a role in affecting E2F activity. It is well-established that TGFβ prevents cell cycle progression, causing G1 arrest, by up-regulating expression of Cdk inhibitors and by inhibiting both cdc25a [163] and c-myc [162] by means of Smad-E2F4/5-pocket protein repressor complexes. The rapid surge in E2F1 that we observe in response to TGFβ may thus effectively initiate the TGFβ apoptotic program, without affecting cell cycle, since TGFβ maintains transcriptional repression of factors required for S phase entry through 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 hours of TGFβ treatment [350]. Thus, it is conceivable that TGFβ 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 TGFβ, further preventing cell cycle progression.

The present work delineates a novel process of gene activation by the TGFβ-E2F1 signalling axis and supports a role for the E2F family as potent co-transducers of TGFβ signals. Combined with previous studies from our lab and others, these findings highlight the crucial role for the E2F family in regulating TGFβ tumour suppressive effects and we propose the following model of E2F tumour suppressive action downstream of TGFβ (Figure 3.7):

(1) TGFβ induces E2F4/5 recruitment into classical repressive pRb–E2F–HDAC complexes which target key cell-cycle regulators, such as *cdc25a* [163] and *c-myc* [162], preventing cell cycle entry.

(2) TGFβ also induces E2F1 recruitment into repressive E2F–HDAC complexes, inhibiting hTERT expression and suppressing immortalization, as we have previously demonstrated [209].

(3) The current study demonstrates that TGFβ 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 tumour suppression. We can thus consider the E2F family as "super-mediators" of TGFβ tumour suppressive effects. A better understanding of the mechanisms by which both TGFβ and E2F1 exert their tumour suppressive roles may prove useful for the development of novel therapeutic strategies aimed at restoring the apoptotic or tumour suppressive response of the E2Fs in human cancer.

3.7 Acknowledgements

We thank Dr. Kristian Helin for kindly providing the mutant E2F1 expression vectors and Dr. Lili Yamasaki for generously providing wild-type and E2F1 knockout MEFs. This work was supported by grants from the Canadian Institutes for Health Research (CIHR, MOP-114904 to JJL). JJL is the recipient of the McGill Sir William Dawson Research Chair and JK holds a CIHR Frederick Banting and Charles Best Doctoral Research Award.

3.8 Figures for Chapter 3

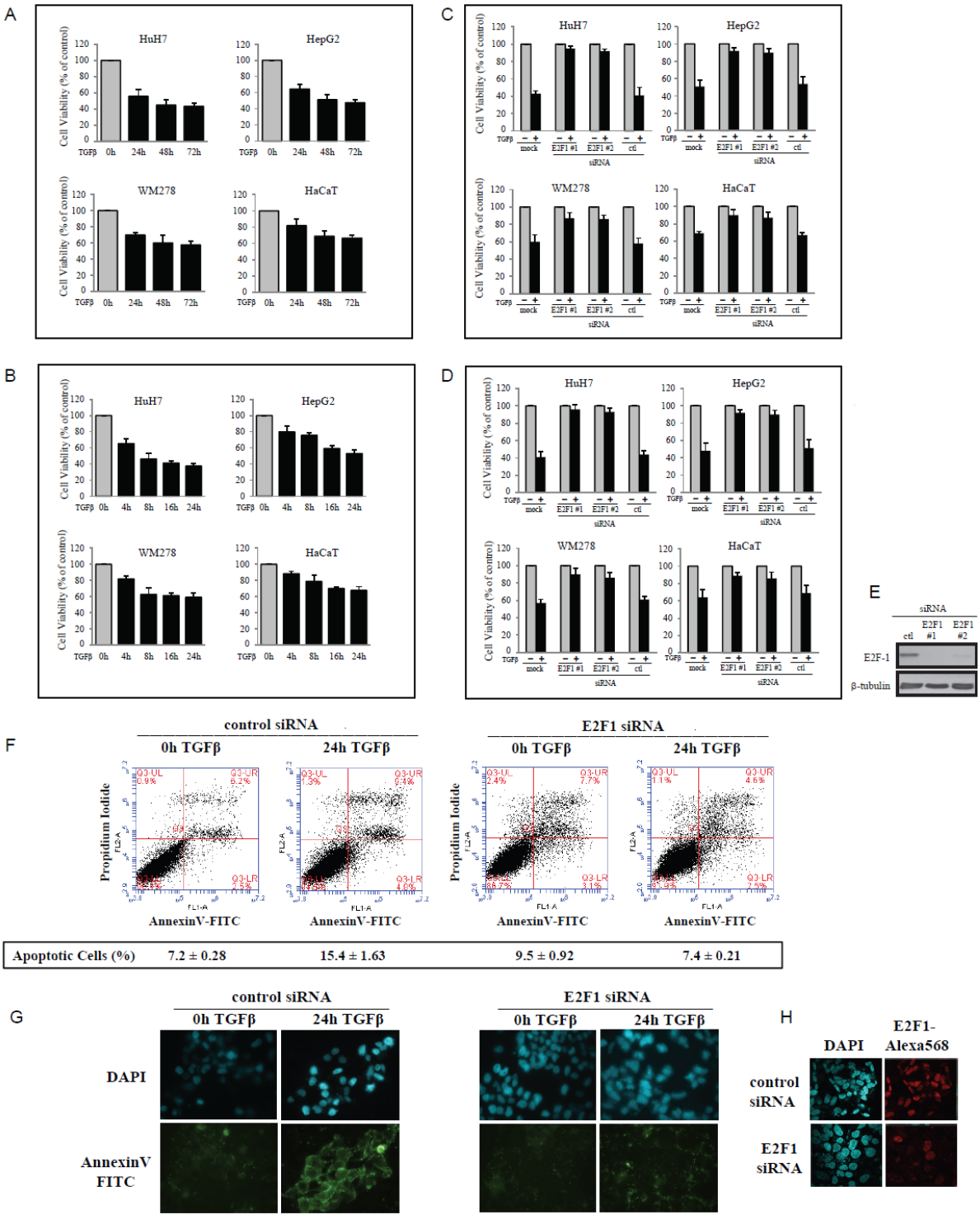


Figure 3.1: TGFβ-mediated apoptosis is dependent on E2F1

The specified cell lines were untreated or treated with TGFβ (100 pM) for the indicated times and assessed for cell viability by MTT (A) and Calcein-AM (B) assays. Data are represented as mean ± SD. (C, D) Cells were transiently transfected with two different siRNAs against E2F1 or a control non-silencing siRNA and assessed by MTT (C) and Calcein-AM assays (D). The efficiency of E2F1 knockdown by siRNA was verified by immunoblotting with an E2F1 specific antibody (E). Activation of the apoptotic program by TGFβ was assessed by AnnexinV staining followed by FACS (F) or fluorescence microscopy (G), 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 ± SD. (H) Expression of endogenous E2F1 in these cells was assessed by immunofluorescence.

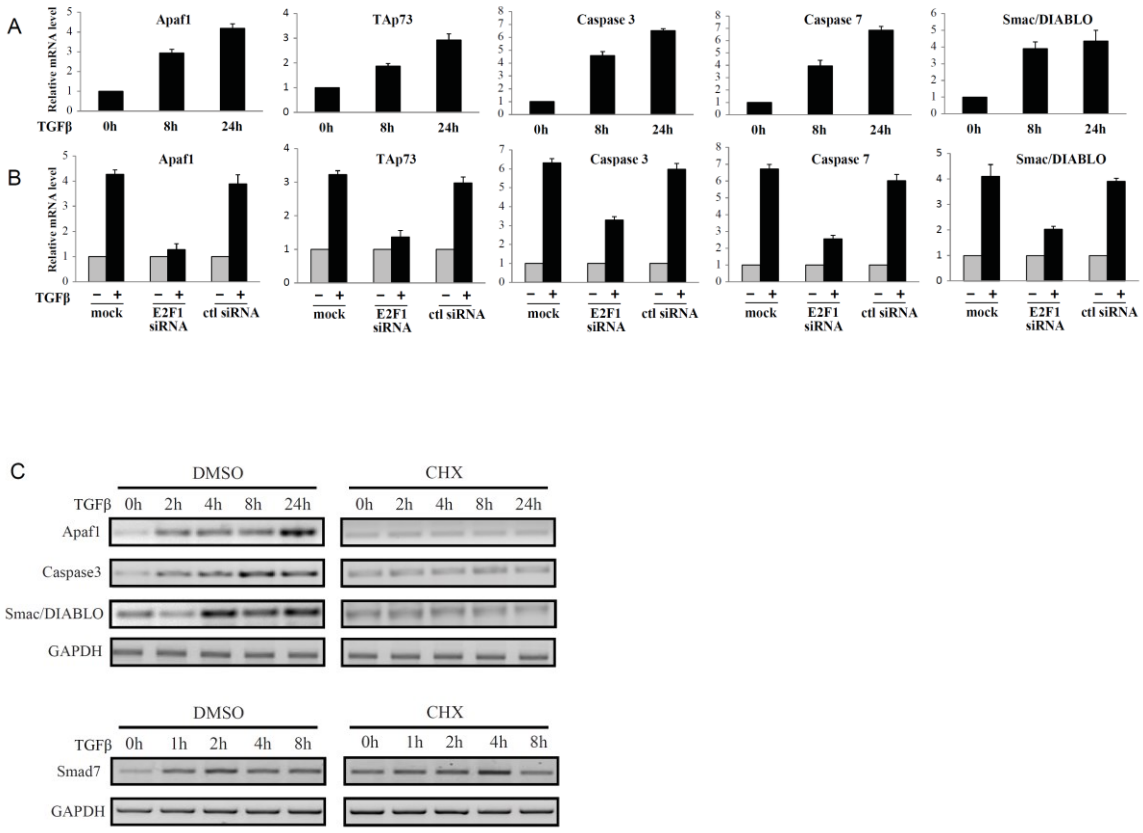


Figure 3.2: E2F1 is required for TGFβ-mediated regulation of pro-apoptotic genes

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* and shown relative to levels observed in untreated cells (set to 1). Data are represented as mean ± SD. B) HuH7 cells were transiently transfected with siRNA against E2F1 or a control non-silencing siRNA and stimulated with TGFβ (100 pM) for 24h. 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 amplified products were analyzed by DNA gel electrophoresis.

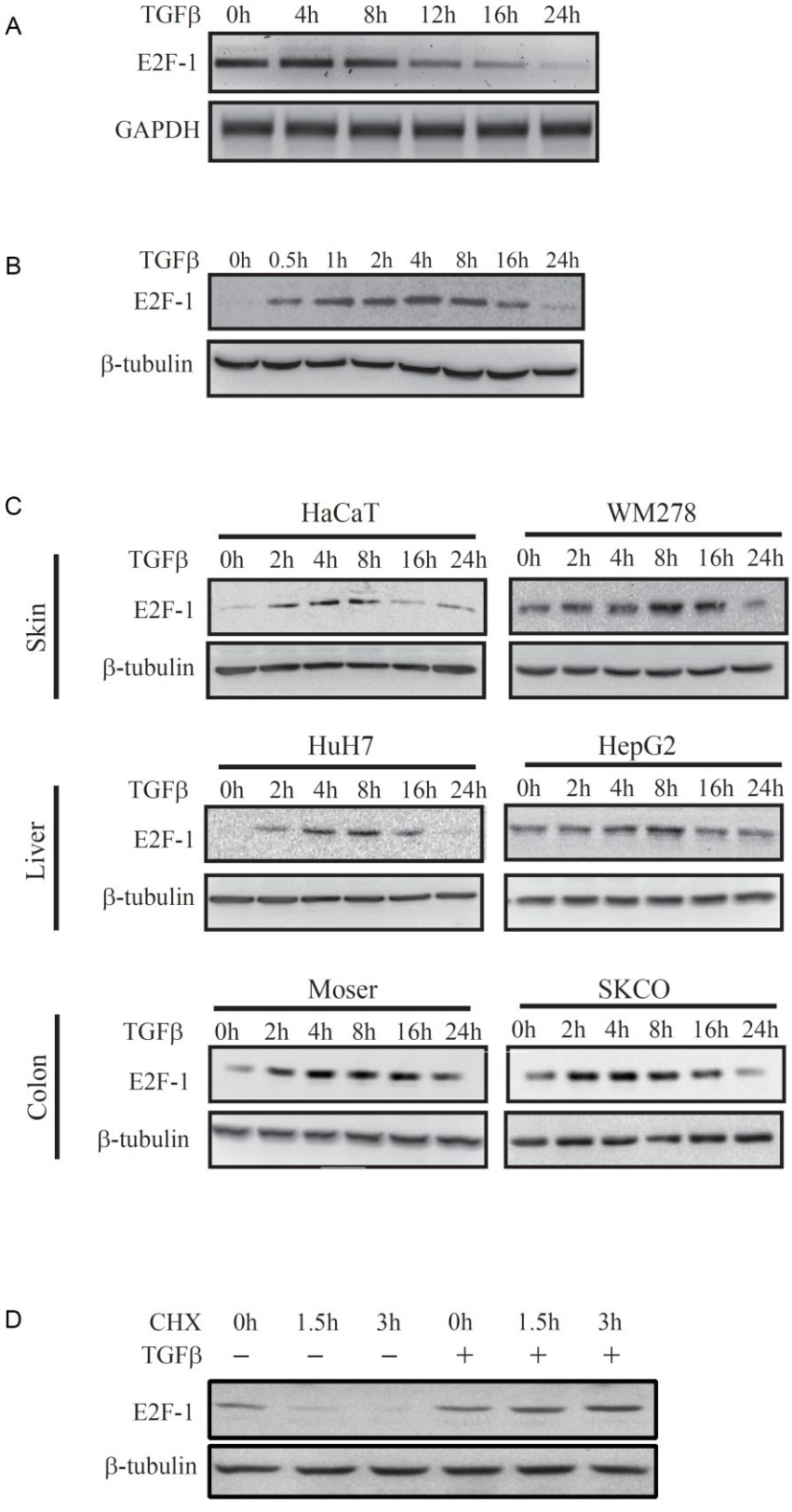
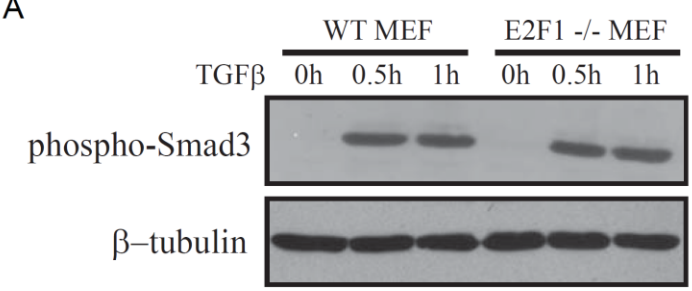

A
TGFβ 0h 4h 8h 12h 16h 24h
E2F-1
GAPDH
B
TGFβ 0h 0.5h 1h 2h 4h 8h 16h 24h
E2F-1
β-tubulin
C
HaCaT
WM278
TGFβ 0h 2h 4h 8h 16h 24h
Skin
E2F-1
β-tubulin
HuH7
HepG2
Liver
Moser
SKCO
Colon
D
CHX 0h 1.5h 3h 0h 1.5h 3h
TGFβ − − − + + +
E2F-1
β-tubulin

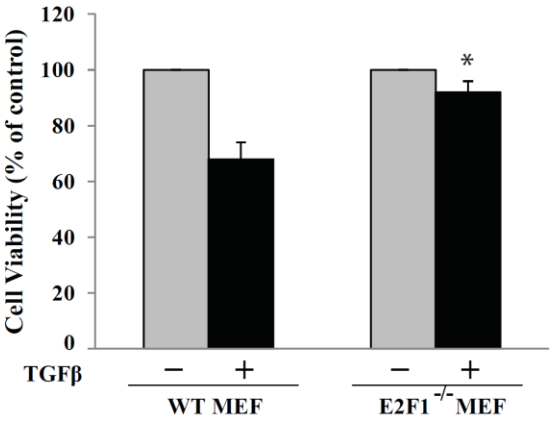
Figure 3.3: TGFβ rapidly and transiently induces E2F1 protein expression levels

HaCaT cells were stimulated with TGFβ (100 pM) for the indicated times and subjected to RT-PCR followed by DNA gel electrophoresis (A) and Western blotting (B) to measure E2F1 RNA and protein levels, respectively. C) Western blot analysis of total E2F1 protein levels in TGFβ-treated cells of various origins, as indicated. D) Cycloheximide (CHX) chase analysis in HaCaT cells to address the potential contribution of TGFβ in E2F1 post-translational stabilization. Cells were incubated with CHX (50 μg/mL) and treated or not with TGFβ (100pM) for the indicated times. Total cell lysates were analyzed for E2F1 protein levels by Western blotting.

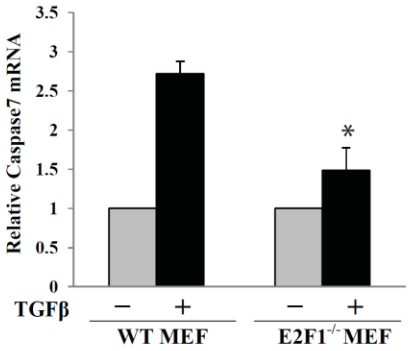
A


WT MEF
E2F1 -/- MEF
TGFβ 0h 0.5h 1h 0h 0.5h 1h
phospho-Smad3
β–tubulin

B


Cell Viability (% of control)
120
100
80
60
40
20
0
*
TGFβ
–
+
–
+
WT MEF
E2F1 -/- MEF

C


Relative Caspase7 mRNA
3.5
3
2.5
2
1.5
1
0.5
0
*
TGFβ
–
+
–
+
WT MEF
E2F1 -/- MEF

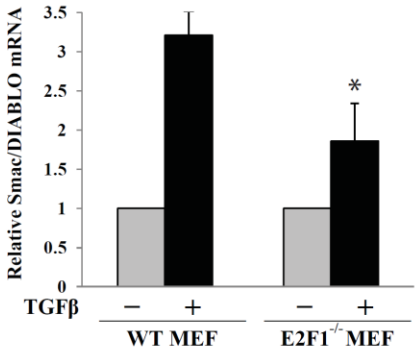
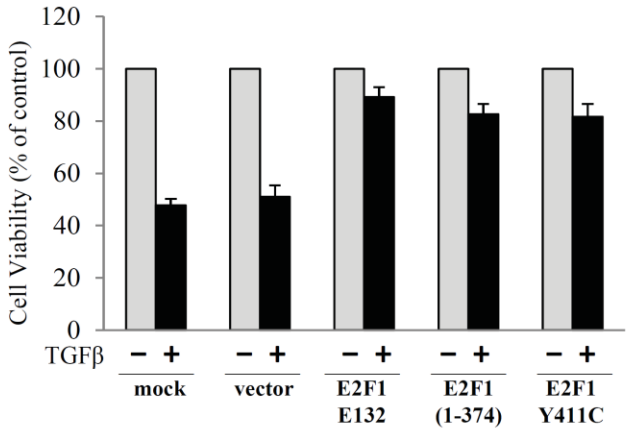

Relative Smac/DIABLO mRNA
3.5
3
2.5
2
1.5
1
0.5
0
*
TGFβ
–
+
–
+
WT MEF
E2F1 -/- MEF

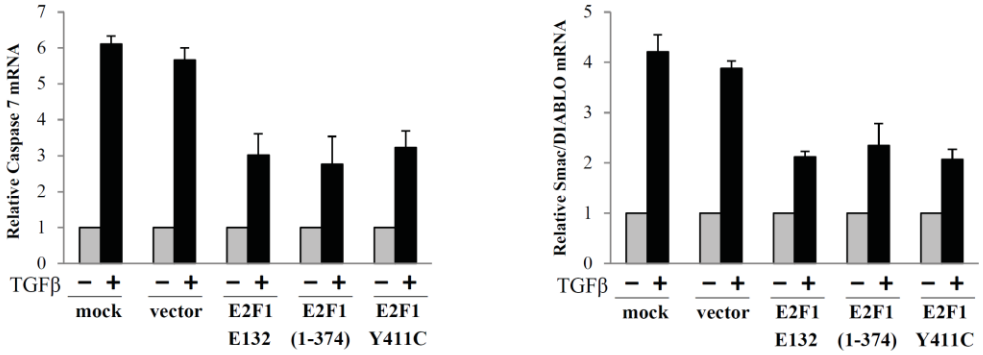
Figure 3.4: TGFβ pro-apoptotic effects are impaired in E2F1-null embryonic fibroblasts

(A) Wild-type (WT) and E2F1$^{-/-}$ mouse embryonic fibroblasts (MEFs) were untreated or treated with TGFβ (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 TGFβ (100 pM) for 24h and cell viability assessed by calcein-AM assay. (C) *Caspase 7* and *Smac/DIABLO* mRNA levels in TGFβ-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 ± SD, (* $p < 0.05$).

A


Cell Viability (% of control)
120
100
80
60
40
20
0
TGFβ
− +
mock
vector
E2F1 E132
E2F1 (1-374)
E2F1 Y411C

B


Relative Caspase 7 mRNA
Relative Smac/DIABLO mRNA
TGFβ
− +
mock
vector
E2F1 E132
E2F1 (1-374)
E2F1 Y411C

C

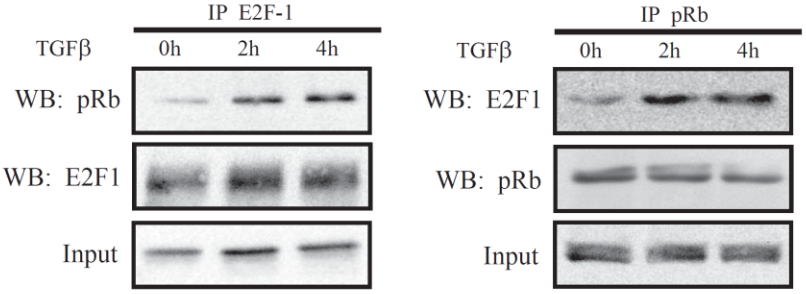

IP E2F-1
IP pRb
TGFβ
0h
2h
4h
WB: pRb
WB: E2F1
Input

Figure 3.5: E2F1 DNA-binding, transactivation and pRb-interaction are required for TGFβ-mediated apoptosis

HuH7 cells transiently transfected with empty vector or mutant E2F1 expression constructs as indicated were untreated or treated with 100 pM TGFβ for 24h. (A) Cell viability was assessed by calcein-AM assay, with bars representing means ± SD. (B) *Caspase 7* and *Smac/DIABLO* mRNA levels were measured by real-time qPCR analysis. Results are normalized to *GAPDH* and show the mean ± SD, expressed as relative to levels observed in untreated cells (set to 1). (C) HuH7 cells untreated or treated with TGFβ (100 pM) were subjected to immunoprecipitation (IP) with the specified antibodies followed by Western blotting (WB) to assess levels of associated E2F1 and pRb.

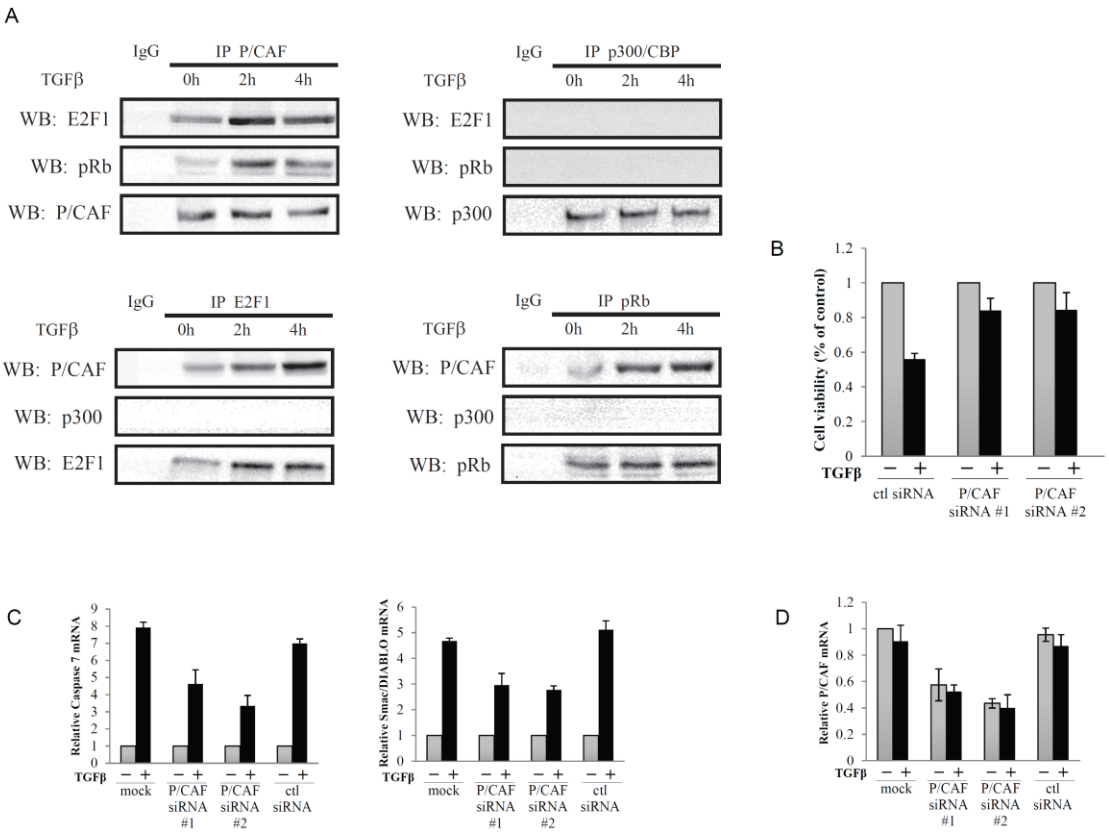

A
IgG
IP P/CAF
TGFβ
0h 2h 4h
WB: E2F1
WB: pRb
WB: P/CAF
IgG
IP p300/CBP
TGFβ
0h 2h 4h
WB: E2F1
WB: pRb
WB: p300
IgG
IP E2F1
TGFβ
0h 2h 4h
WB: P/CAF
WB: p300
WB: E2F1
IgG
IP pRb
TGFβ
0h 2h 4h
WB: P/CAF
WB: p300
WB: pRb
B
Cell viability (% of control)
TGFβ
ctl siRNA
P/CAF siRNA #1
P/CAF siRNA #2
C
Relative Caspase 7 mRNA
TGFβ
mock
P/CAF siRNA #1
P/CAF siRNA #2
ctl siRNA
Relative Smac/DIABLO mRNA
TGFβ
mock
P/CAF siRNA #1
P/CAF siRNA #2
ctl siRNA
D
Relative P/CAF mRNA
TGFβ
mock
P/CAF siRNA #1
P/CAF siRNA #2
ctl siRNA
E
Caspase Activity Luminescence (RLU x 10³)
ctl siRNA
P/CAF siRNA
0h
24h
48h
TGFβ treatment (hours)
Caspase Activity Luminescence (RLU x 10³)
ctl siRNA
E2F1 siRNA
0h
24h
48h
TGFβ treatment (hours)
F
TAp73
Apaf1
Smac/DIABLO
Caspase 7
TGFβ
0h 3h 6h
ChIP
E2F1
pRb
P/CAF
Input

Figure 3.6: TGFβ induces formation of a transcriptionally active complex between pRb/E2F1, and the acetyltransferase P/CAF, onto pro-apoptotic gene promoters

A) Untreated and TGFβ-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, C) HuH7 cells were transiently transfected with siRNA against p/CAF or a control non-silencing siRNA and treated with TGFβ (100pM) for 24h. Cell viability was assessed by calcein-AM assay (B), and *Caspase 7* and *Smac/DIABLO* mRNA levels were measured by real-time qPCR analysis (C). Results are normalized to *GAPDH* and shown relative to levels observed in untreated cells (set to 1). Data are represented as mean ± SD. 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 TGFβ (100pM) as indicated. Activation of Caspase 3/7 was measured by Caspase-Glo® 3/7 Assay (Promega). Data are represented as mean ± SD. F) HuH7 cells were untreated or treated with TGFβ (100 pM) for the indicated times, and the binding of E2F1, pRb, and p/CAF to the *p73*, *Apaf1*, *Smac/DIABLO*, and *Caspase7* gene promoters was determined by chromatin immunoprecipitation (ChIP).

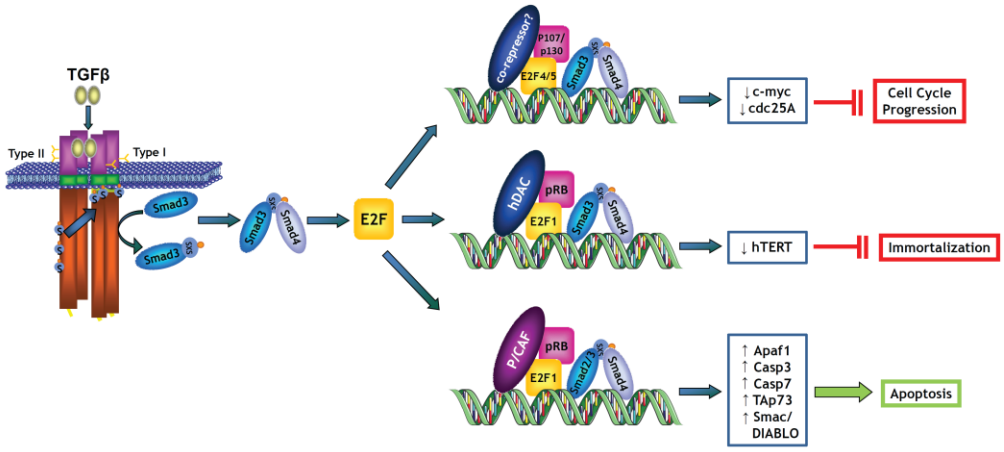


Figure 3.7: The pRb/E2F signalling pathway mediates three distinct arms of TGFβ tumour-suppressive effects.

See text for details

Chapter 4 | The pRb/E2F1 pathway mediates TGFβ-induced autophagy

Juliana Korah and Jean-Jacques Lebrun

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4.1 Preface

In Chapter 3, we established that TGFβ recruits E2F1 into transcriptionally active pRb–E2F1–P/CAF complexes, increasing the expression of multiple pro-apoptotic target genes and inducing apoptotic cell death. Intriguingly, the pRb/E2F pathway appears to act as a major signalling axis leading to multiple tumour suppressive responses downstream of TGFβ.

Recently, TGFβ has been reported to also induce programmed cell death type II, or autophagic cell death, in a context-dependent manner. Having previously identified the pRb/E2F pathway as a critical mediator of both hTERT inhibition and apoptotic induction in response to TGFβ , we thus investigated the potential role of pRb/E2F signalling to another arm of TGFβ-mediated tumour suppression. In this study, we assessed the contribution of the pRb/E2F pathway to autophagy activation in response to TGFβ.

4.2 Abstract

Transforming growth factor-β (TGFβ) is a multifunctional cytokine that regulates cell proliferation as well as cell death, acting as a key homeostatic mediator in various cell types and tissues. Autophagy is a programmed mechanism that plays a pivotal role in controlling cell fate and, consequently, many physiological and pathological processes, including carcinogenesis. Though autophagy is often considered a pro-survival mechanism that renders cells viable in stressful conditions and thus might promote tumour growth, emerging evidence suggests that autophagy is also a tumour suppressor pathway. The relationship between TGFβ signalling and autophagy is context-dependent and remains unclear. TGFβ-mediated activation of autophagy has recently been suggested to contribute to the growth inhibitory effect of TGFβ in hepatocarcinoma cells. In the present study, we define a novel process of TGFβ-mediated autophagy in various cancer cell lines. We found that autophagosome initiation and maturation by TGFβ is dependent on the pRb/E2F1 pathway, which we have previously established as a critical signalling axis leading to various TGFβ tumour suppressive effects. We further determined that TGFβ induces pRb/E2F1-dependent transcriptional activation of several autophagy-related genes that function at various stages in the autophagic process. Together, our findings suggest that TGFβ induces autophagy at least partially through the pRb/E2F1 pathway and transcriptional activation of autophagy-related genes, and further highlights the central relevance of the pRb/E2F1 pathway downstream of TGFβ signalling.

4.3 Introduction

Transforming growth factor-β (TGFβ) is a multifunctional cytokine that is involved in the regulation of numerous diverse fundamental biological processes, including cell proliferation, differentiation, immortalization, and apoptosis, in a context- and cell-specific manner [1,5]. TGFβ is a key mediator in the maintenance of homeostasis between cell growth and cell death and the growth inhibitory and pro-apoptotic effects of TGFβ have been described in various cell types, including epithelial cells, hepatocytes, hematopoietic cells, and lymphocytes [162,163,269,270,274].

TGFβ signals through serine/threonine kinase receptors that recruit and phosphorylate the canonical downstream mediators, the Smad proteins, inducing Smad nuclear translocation to regulate target gene expression. Nuclear Smad complexes interact with various co-activators or co-repressors, which are differentially expressed in different cell types and are thus thought to provide a basis for tissue- and cell type-specific functions of TGFβ ligands [19,28]. Alternatively, TGFβ activates other intracellular signalling pathways independently of the Smads. These include the stress-activated kinases, p38 and c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and phosphoinositide 3-kinase (PI3K)/Akt pathways [73].

In a recent study, we identified the pRb/E2F pathway as a major signalling axis leading to apoptosis downstream of TGFβ in a number of cell types and in both normal and cancer cells [361]. We found that TGFβ recruits E2F1 into transcriptionally active pRb–E2F1–P/CAF complexes, increasing the expression of multiple pro-apoptotic target genes and inducing programmed cell death. Intriguingly, the pRb/E2F pathway appears to be required for a number of TGFβ tumour suppressive effects. TGFβ-mediated growth inhibition involves E2F4/5 recruitment into repressive pRb–E2F–HDAC complexes which target key cell-cycle regulators, such as *cdc25a* [163] and *c-myc* [162,362], preventing cell cycle entry.

TGFβ also induces E2F1 recruitment into repressive E2F-HDAC complexes, inhibiting hTERT expression and suppressing immortalization [209]. Moreover, the assembly of complexes formed of E2F, pocket proteins (potentially pRb) and HDACs have been shown to repress hTERT gene expression [204], indicating that each of these is a key regulator of telomerase activity in human cells. Additionally, in certain cancer cell types, TGFβ transcriptionally suppresses the inhibitor of apoptosis survivin through Smad signalling and recruitment of a pRb–E2F4 repressive complex to the survivin promoter , thus promoting apoptosis.

Interestingly, TGFβ has recently been shown to also induce another form of programmed cell death, autophagy [303]. Autophagy is an evolutionarily conserved process by which a cell self-digests its own cytoplasmic materials, including long-lived or malfunctioning proteins and damaged organelles, through a lysosomal degradative pathway in response to various stress conditions, such as nutrient deprivation, growth factor depletion, or hypoxia [276,363]. Briefly, the general mechanism of autophagy involves the formation of a double membrane-bound vesicle called an autophagosome that envelops and sequesters a targeted region of the cell. The autophagosome then fuses with a lysosome, forming an autolysosome, in which hydrolases digest the sequestered contents to metabolites that are recycled for biosynthesis in the cell [364]. As such, autophagy is often considered a pro-survival mechanism, protecting cells and maintaining homeostasis under poor nutrient conditions or cell stress [277]. However, autophagy has in fact been implicated in several vital biological processes, including aging, cellular remodeling, pathogenic infection, and programmed cell death [276,365]. Indeed, autophagy resulting in total destruction of the cell is considered to be a form of programmed cell death (PCD type II) [278]. The manner in which autophagy affects cell death depends on the type and context of the cell.

Autophagy is regulated by numerous autophagy-related genes (ATGs) that function collaboratively in autophagy initiation and progression. Initiation of autophagy depends on the Unc-51-Like Kinases (ULK kinases), which are

mammalian homologues of ATG1, a serine-threonine protein kinase involved in initiating the isolation membrane of the autophagosome. ULK kinase activity is required for autophagy activation in various cell types [366-368]. ATG6, also known as Beclin-1, is similarly involved in initiating the autophagic process, and Beclin-1 deficiency inhibits autophagic activity [369,370]. Autophagosome membrane expansion and closure is mediated by two ubiquitin-like conjugation systems composed of various ATG proteins, which culminate in the conjugation of ATG12 to ATG5 and the conversion of microtubule-associated protein 1 light chain 3 (MAP1LC3, or simply LC3) from its soluble cytosolic form (LC3-I) to the phosphatidylethanolamine-conjugated, autophagosome membrane-bound form (LC3-II), which is characteristic to autophagy [284,285].

In addition to normal cell growth and homeostasis, autophagy has been implicated to play a protective role in preventing the progression of a number of human diseases, including muscular disorders, neurodegeneration, and cancer [277,279,280,286]. Autophagy in fact plays a dual and opposing role in cancer, demonstrating evidence of both tumour-promoting and tumour-suppressive functions in a context-dependent manner. While many studies support a role for autophagy in maintaining tumour cell survival in response to metabolic stress or hypoxia and thus promoting the growth of solid tumours [287-290], mounting evidence suggests that autophagy is intrinsically a tumour suppressor pathway. A number of tumour suppressor proteins, including p53, pRb, and PTEN, have been demonstrated to positively regulate autophagy [298-300,371]. Conversely, oncogenes such as PI3K, Akt, and Bcl-2, have been shown to inhibit autophagy [299,301,372]. Moreover, alterations in numerous key autophagy-related genes have been linked to various human cancers and mice deficient for these genes are prone to tumour development [295,296,302,373]. However, the mechanisms underlying how autophagy suppresses cancer have not been well established.

While TGFβ has been implicated in both sides of the ‘autophagic coin’, a recent study by Kiyono and colleagues demonstrated that activation of autophagy may in

fact contribute to TGFβ-mediated tumour suppressive effects. They found that TGFβ induces accumulation of autophagosomes and enhances the degradation of long-lived proteins. Moreover, they showed that induction of autophagy relies on both Smad-dependent and Smad-independent signalling and proceeds via transcriptional activation of a number of autophagic genes in hepatocellular carcinoma cells, potentiating the tumour-suppressive effects of TGFβ in these cells [303]. In addition to cancer cells, TGFβ has been shown to induce autophagy in mammary and renal epithelial cells as well as mesangial cells [304-306]. These studies provide emerging evidence for a novel TGFβ-mediated tumour suppressive pathway, though the precise mechanisms and therapeutic implications of which remain largely elusive.

Having previously identified the pRb/E2F pathway as a critical mediator of both hTERT inhibition [209] and apoptotic induction [361] in response to TGFβ , we investigated the potential role of this pathway to TGFβ-mediated autophagy. We found that TGFβ activates autophagy in various cancer cells lines and that these effects are dependent on E2F1 and pRb. Moreover, our results indicate that TGFβ may regulate autophagy through pRb/E2F1-dependent transcriptional activation of multiple autophagy-related genes that function at various stages in the autophagic process. These data further support the crucial role for pRb/E2F signalling as a potent tumour suppressive pathway downstream of TGFβ.

4.4 Materials and Methods

Cell Culture & Transfections

HuH7, HepG2, and Hep3B cell lines, as well as H1299 cells stably expressing GFP–LC3 (gift from Dr Gordon Shore, McGill University) were cultured in DMEM (HyClone), and WM278 cells in RPMI-1640 (HyClone). Medium for all cells was supplemented with 10% fetal bovine serum (FBS) (HyClone) and 2mM L-glutamine (GIBCO) and cells were grown at 37°C in 5% CO_2 conditions. To

generate HuH7 cell lines stably expressing GFP–LC3, cells were transfected with pEGFP-LC3 (Addgene plasmid 21073) using Lipofectamine™ 2000 reagent (Invitrogen) and G418-resistent colonies were screened for expression of GFP-LC3. Prior to treatment, cells were serum-starved for 24 hours and all stimulations were done in serum-free medium containing 100pM TGFβ1 (Peprotech). Cells were transiently transfected with different siRNAs against E2F1, E2F4 (Ambion), pRb, or P/CAF (Sigma-Aldrich), or with wild-type and mutant E2F1 expression vectors using Lipofectamine™ 2000 reagent (Invitrogen), according to the manufacturer's instructions.

Immunoblotting

Cells were lysed in cold RIPA buffer (50mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA), containing 1 mM sodium orthovanadate, 1mM 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-Beclin1 (Novus Biologicals), anti-LC3 (Novus Biologicals), anti-p62 (Santa Cruz Biotechnology), anti-E2F1 (KH95) (Santa Cruz Biotechnology), anti-β-tubulin (Sigma). Following primary antibody incubation, membranes were washed twice in TBST (50mM Tris-HCl at pH 7.6, 200mM NaCl, 0.05% Tween20), and incubated with secondary antibody coupled to horseradish peroxidase (Sigma) at 1:10,000 dilution for 1h 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).

Subcellular localization of LC3

Cells expressing EGFP-LC3 were fixed with 4% paraformaldehyde and the change in the LC3 localization was examined using a Zeiss LSM-510 Meta Axiovert confocal microscope. Autophagy was measured by quantitation of the GFP-LC3 puncta as described by Klionsky *et al.* [374] The areas of GFP-LC3 puncta

were analyzed using ImageJ software (NIH) and a minimum of 100 cells from three independent experiments was counted for quantitative analysis.

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 (BioRad) in a RotorGene 6000 PCR detection system (Corbett Life Science). Conditions for qPCR were as follows: 95°C for 30sec, 40 cycles of 95°C for 5sec and 60°C for 20sec. Primer sequences are listed in Table 1.

Statistical Analysis

Results are expressed as mean ± standard deviation of at least 3 independent experiments. Statistical differences were determined by two-tailed unpaired *t*-test. $p < 0.05$ was considered statistically significant.

Table 4: PCR Primer Sequences

Beclin1 (2) forward	5'- TGTCACCATCCAGGAACTCA -3'
Beclin1 (2) reverse	5'- CTGTTGGCACTTTCTGTGGA -3'
ULK1 forward	5'- TCGAGTTCTCCCGCAAGG -3'
ULK1 reverse	5'- CGTCTGAGACTTGGCGAGGT -3'
ULK2 forward	5'- GGCTCTCCTACTAAGACCACAG -3'
ULK2 reverse	5'- GACGAGTAACCAAGGCTAACAG -3'
UVRAG forward	5'- CTGTTTGGATGGGCTGAAAT -3'
UVRAG reverse	5'- TGCGAACACAGTTCTGATCC -3'
ATG14 forward	5'- ATGAGCGTCTGGCAAATCTT -3'
ATG14 reverse	5'- CCCATCGTCCTGAGAGGTAA -3'
PIK3C3 forward	5'- AAGCAGTGCCTGTAGGAGGA -3'
PIK3C3 reverse	5'- TGTCGATGAGCTTTGGTGAG -3'
MAP1LC3A forward	5'- CGTCCTGGACAAGACCAAGT -3'
MAP1LC3A reverse	5'- CTCGTCTTTCTCCTGCTCGT -3'
MAP1LC3B forward	5'- AGCAGCATCCAACCAAAATC -3'
MAP1LC3B reverse	5'- CTGTGTCCGTTCACCAACAG -3'
ATG4B forward	5'- GCCGAGATTGGAGGTG -3'

ATG4B reverse	5'- GCCTATGGACTTGCCTTC -3'
GABARAPL1 forward	5'- TTTGGTGCCCCTTATCTCAC -3'
GABARAPL1 reverse	5'- GGCCATCATGTAGCATTCCTT -3'
ATG12 forward	5'- AGTAGAGCGAACACGAACCATCC -3'
ATG12 reverse	5'- AAGGAGCAAAGGACTGATTCACATA -3'
BCL2 forward	5'- GAGTTCGGTGGGGTCATGT -3'
BCL2 reverse	5'- GCCGGTTCAGGTACTCAGTC-3'
c-myc forward	5'- TTCGGGTAGTGGAAAACCAG -3'
c-myc reverse	5'- CAGCAGCTCGAATTTCTTCC-3'
E2F1 forward	5'-TGCAGAGCAGATGGTTATGG-3'
E2F1 reverse	5'-ATCTGTGGTGAGGGATGAGG-3'
E2F4 forward	5'- GTGCCACCACCTGAAGATTT-3'
E2F4 reverse	5'- TGAGCTCACCACTGTCCTTG-3'
GAPDH forward	5'-GCCTCAAGATCATCAGCAATGCCT-3'
GAPDH reverse	5'-TGTGGTCATGAGTCCTTCCACGAT-3'

4.5 Results

4.5.1 TGFβ induces autophagy in human hepatocarcinoma cell lines

We first assessed the autophagic response to TGFβ in two human hepatocarcinoma cell lines, HuH7 and HepG2. For this, we initially examined whether TGFβ treatment induced the conversion of cytosolic LC3-I to the lipidated LC3-II form, which is localized in the autophagosome membrane, and is widely used as a marker of autophagy in mammalian cells. In fact, LC3-II is the only identified mammalian protein to date that stably associates with autophagosome membranes, thus LC3-II accumulation is a hallmark of autophagy [284,375]. Immunoblotting analyses revealed an accumulation of endogenous LC3-II in cells treated with TGFβ for 24 hours (Figure 4.1a). We also examined the expression of two key autophagic regulators, Beclin-1 and Sequestosome-1 (SQSTM1, best known as p62), in response to TGFβ in both cell lines. While Beclin-1 is required for the initiation of autophagosome formation [370], p62 links ubiquitinated proteins to the autophagy machinery and enables their degradation in lysosomes [376,377]. As such, p62 levels are preferentially degraded by autophagy.

Accordingly, we found that TGFβ treatment in HuH7 and HepG2 cells resulted in an increase in Beclin-1 and loss of p62 protein expression levels, supporting that TGFβ induces autophagy in these cells.

To further corroborate the conversion of LC3-I to LC3-II, and thus activation of autophagy, we employed a GFP-tagged LC3 plasmid (pEGFP-LC3) to monitor the cellular localization of LC3. Fluorescent GFP-LC3 is used extensively to measure autophagy as it displays a diffuse cytoplasmic distribution (corresponding to the LC3-I form) under normal conditions and a distinctly punctate distribution once LC3 is lipidated and integrated into the autophagosome membrane (LC3-II form) under autophagy-inducing conditions [375]. For this, HuH7 cells were stably transfected with pEGFP-LC3 and the expression pattern of LC3 was examined before and after TGFβ treatment. In the absence of TGFβ, only occasional puncta were detected, representing a basal level of autophagy. In contrast, TGFβ treatment markedly increased punctate distribution (Figure 4.1b). Taken together, these data indicate that TGFβ indeed induces autophagy in these cells. Ultimately, it would be interesting to confirm these data by an ultrastructual study of the cells with transmission electron microscopy, to observe an accumulation of membrane-rimmed vacuoles with characteristics of autophagosomes following TGFβ treatment.

4.5.2 TGFβ transcriptionally mediates autophagy in an E2F1-dependent manner

We then investigated whether TGFβ transcriptionally modulates the expression levels of numerous genes involved in various steps of the autophagic process. Our results indicate that TGFβ regulates several autophagy-related genes in HuH7 cells (Figure 4.2a, upper panel), but that there is in fact no functional distinction in the genes that are regulated. This suggests that TGFβ plays a more general role in promoting autophagy rather than specifically targeting one phase or function. Importantly, in addition to assessing known TGFβ target genes (Beclin-1, DAPK,

ATG5, ATG7, MAP1LC3), we uncovered a number of novel TGFβ-targeted autophagic regulators (PIK3C3, ULK1, ULK2, GABARAP, ATG4b, ATG12, ATG14), some of which are quite potently induced in response to TGFβ treatment. Many of these genes were also transcriptionally induced by TGFβ in HepG2 cells, as well as human non-small cell lung carcinoma cells (H1299) and melanoma cells (WM278 and WM793B, data not shown), albeit less potently than in HuH7 cells, indicating that the autophagic response to TGFβ is not limited to hepatocarcinoma (Figure 4.2a, middle and lower panels). In addition, we measured the expression levels of Bcl-2, an anti-apoptotic protein that inhibits autophagy by binding and sequestering Beclin-1 [372] . We found that TGFβ treatment transcriptionally repressed Bcl-2 expression, potentially further contributing to the activation of autophagy in these cells.

In previous studies from our laboratory, we identified E2F1 as a critical transcriptional co-regulator of multiple TGFβ-targeted genes involved in tumour suppression [209,361]. We therefore wanted to assess whether E2F1 could also contribute to the transcriptional regulation of autophagy-related genes in response to TGFβ. Quite remarkably, loss of E2F1 expression by siRNA significantly impaired the TGFβ-mediated regulation of many of these target genes in both HuH7 and H1299 cells (Figure 4.2b). Thus, the transcriptional activation of multiple autophagic genes by TGFβ is indeed E2F1-dependent. In addition to providing novel TGFβ targets, these data provide a novel pathway by which TGFβ regulates autophagic regulatory genes and potentially the autophagic process itself.

To further address this, we silenced E2F1 expression and examined the activation of autophagy by TGFβ, as assessed previously. Consistent with our previous results, E2F1 knockdown attenuated the autophagic response to TGFβ. By immunoblotting analysis, we found that loss of E2F1 expression prevented TGFβ-mediated accumulation of LC3-II, induction of Beclin-1, and degradation of p62 (Figure 4.2c, left panel). Moreover, E2F1 knockdown markedly reduced the

number of GFP-LC3 puncta observed in response to TGFβ treatment (Figure 4.2c, right panel).

Inhibition of mRNA synthesis by actinomycin D treatment has been shown to inhibit TGFβ-induced GFP-LC3 puncta formation [303], indicating the importance of gene transcription for autophagy activation by TGFβ. Our results here suggest that E2F1 may regulate TGFβ-mediated autophagy at least partially through the transcriptional control of autophagy-related genes.

Polager and colleagues established a role for E2F1 in DNA damage-induced autophagy, identifying ATG5, MAP1LC3, ULK1, and DNA damage regulated autophagy modulator (DRAM) as E2F1 transcriptional targets and demonstrating that endogenous E2F1 is associated with these gene promoters [378]. Interestingly, they also detected endogenous E2F4 at these same gene promoters. Given their results, and since E2F4 also acts downstream of TGFβ signalling to regulate cell growth arrest through transcriptional modulation of cell-cycle genes [162,350], we investigated whether E2F4 could also regulate these autophagic genes in response to TGFβ. We found that silencing E2F4 expression by siRNA in fact had no significant effect on the regulation of these genes by TGFβ (Figure 4.2d). As a control, we also measured c-myc expression levels and, as expected, E2F4 knockdown partially impeded the downregulation of c-myc by TGFβ. Taken together, these data indicate that E2F1, but not E2F4, plays a role in TGFβ-mediated induction of autophagy-related genes, suggesting that the autophagic response to TGFβ is not only dependent on but also specific to E2F1.

4.5.3 TGFβ-induced activation of autophagy acts through pRb/E2F1 signalling and requires the histone acetyltransferase P/CAF

Having previously established that TGFβ induces numerous apoptotic genes via a transcriptionally active pRb–E2F1–P/CAF complex [361], we sought to determine whether these components could also be involved in regulating autophagic genes

in response to TGFβ. Recently, pRb overexpression has been shown to induce autophagy [371]. Using pRb restoration in pRb-deficient cells, Jiang and colleagues demonstrated that pRb activates the autophagic response and that pRb binding to E2F1 is required for autophagy induction. Accordingly, we first speculated whether an E2F1 mutant that is deficient for pRb binding (E2F1 Y411C) would lose the capability to induce autophagy in response to TGFβ. We found that ectopic expression of E2F1 Y411C suppressed the transcriptional activation of numerous autophagic genes in response to TGFβ (Figure 4.3a), suggesting that pRb binding to E2F1 is indeed required for TGFβ-induced autophagy. This parallels our previous study in which we found pRb–E2F1 association to be required for TGFβ-induced apoptosis.

We subsequently addressed the contribution of pRb to TGFβ-mediated autophagy. As shown in Figure 4.3b, the transcriptional induction of a number of autophagic genes in response to TGFβ was also notably impaired when pRb expression was silenced by RNA interference. Moreover, knockdown of pRb expression significantly reduced the number of GFP-LC3 puncta observed in response to TGFβ, indicating impaired autophagosome formation by TGFβ in the absence of pRb (Figure 4.3c). Thus, pRb is indeed required for the TGFβ autophagic response in HuH7 cells. Interestingly, we also found that these same autophagy-related genes were not significantly regulated by TGFβ in Hep3B cells which are pRb-deficient (data not shown), further supporting that proper pRb function and/or expression is required for this regulation.

Gene transcription downstream of TGFβ signalling may be co-regulated by histone acetyltransferases such as p300/CBP and p300/CBP-associated factor (P/CAF) [62,64]. In a recent study, we have shown P/CAF to be involved in the transcriptional induction of apoptotic genes in response to TGFβ. We thus assessed the requirement for this histone acetyltransferase to TGFβ-mediated autophagy. As shown in Figure 4.3d, loss of P/CAF expression similarly suppressed the induction of multiple autophagic genes by TGFβ. The role and

contribution of P/CAF as well other histone acetyltransferases to TGFβ-mediated autophagy activation remains to be clarified in our further studies.

Collectively, these findings support a role for pRb and P/CAF downstream of TGFβ in the transcriptional activation of autophagic genes and the mediation of autophagy. Further investigation, including ChIP analysis of the specific autophagic gene promoters to validate these findings would further support that a transcriptionally active pRb–E2F1 complex, similar to that observed in our previous study, plays a role in mediating the TGFβ autophagic response.

4.6 Discussion

The balance between cell proliferation and cell death is central to many fundamental physiological processes. As such, deregulation of this balance has been linked to the pathogenesis of multiple diseases. Mounting evidence indicates that autophagy is a key programmed mechanism that controls cell survival and cell death, thus playing a pivotal role in maintaining cellular homeostasis and, consequently, in many biological and pathological processes, including carcinogenesis [288].

Autophagy has been implicated in promoting tumour development by enabling tumour cells to survive stressful environmental conditions. Paradoxically, several studies have also demonstrated a tumour suppressive function for autophagy. Indeed, several genetic links have emerged between perturbations of autophagy and cancer development. For instance, mono-allelic deletion of Beclin-1 is frequently observed in human breast, ovarian, and prostate cancers [295,302]. Correspondingly, heterozygous disruption of Beclin-1 in mice has been shown to promote spontaneous tumour formation [296,297]. Moreover, deletion of the Beclin-1-binding protein UVRAG [379] or other autophagy-related genes, such as ATG4C [373], ATG5, and ATG7 [380], also resulted in accelerated tumourigenesis.

TGFβ is a multifunctional cytokine that regulates cell proliferation as well as cell death, acting as an essential homeostatic mediator in various cell types and tissues. Similar to autophagy, TGFβ plays bidirectional and paradoxical roles in tumour suppression and tumour progression. Further adding to this dichotomy, TGFβ-mediated induction of autophagy may lead to cell survival or cell death, in a context-dependent manner. The mechanisms by which TGFβ regulates autophagy are not well established. TGFβ generally signals in a cell-type and context-specific manner and its regulation of autophagy is no exception. In primary mouse mesangial cells, TGFβ induces autophagy through activation of the TAK1-MKK3-p38 signalling axis, acting as a cytoprotective mechanism against serum deprivation-induced apoptosis [304,381]. Conversely, TGFβ induces autophagy in bovine mammary gland epithelial cells and neonatal piglet gut epithelium, leading to autophagic cell death (PCD type II) [305,382]. Moreover, autophagy activation by TGFβ in hepatocellular carcinoma cells is mediated through the Smad and JNK pathways, and contributes to the growth inhibitory effect of TGFβ in these cells [303]. Clearly, the underlying mechanisms induced by TGFβ to regulate cell fate remain to be fully elucidated.

In the present study, we uncovered a novel pathway of TGFβ-mediated autophagy in various cancer cell lines. We found that autophagosome initiation and maturation by TGFβ is dependent on the pRb/E2F1 pathway, which we have previously established as a critical signalling axis leading to TGFβ tumour suppressive effects. We further determined that TGFβ potentially regulates autophagy through transcriptional activation of numerous autophagy-related genes, also in a pRb/E2F1-dependent manner, and that this transcriptional effect might also rely on the histone acetyltransferase P/CAF.

During the autophagic process, autophagosome formation engages relocalization of LC3 from the cytosol to autophagic membranes and this involves the conversion of cytosolic LC3-I to the phospholipid-conjugated form LC3-II, which

is characteristic to autophagy [15]. Our data demonstrate that TGFβ treatment resulted in increased endogenous LC3-II protein levels as well as increased punctate distribution of LC3 by immunofluorescence, indicating the formation of autophagosomes, and thus activation of autophagy by TGFβ in our tested cell lines. Moreover, we found that TGFβ treatment resulted in increased Beclin-1 protein expression as well as loss of p62 protein expression, consistent with autophagic activation. Importantly, we also determined that loss of E2F1 expression by RNA interference abrogated the TGFβ autophagic response in these cells, highlighting the relevance of this transcription factor to TGFβ-mediated autophagy.

As a major regulatory mechanism that dictates cell fate, the autophagic process must be tightly controlled. Numerous studies have identified various mechanisms that regulate autophagy, largely involving phosphorylation and ubiquitination-like events [383-385]. Recently, however, it has become increasingly evident that, in addition to these post-translational protein modifications, autophagy is also regulated at the transcriptional level. Indeed, several transcription factors have been implicated in regulating various autophagy-related genes. The forkhead box O3 (FoxO3) transcription factor has been shown to induce transcription of multiple autophagy genes, including Beclin-1, ATG4, and PIK3C3 [386]. Moreover, FoxO3 directly binds to the promoters of MAP1LC3, GABARAP, and ATG12 to activate gene transcription and induce autophagy [387]. Autophagy activation by p53 is mediated through transcriptional activation of DRAM [388,389] and ULK1/2 genes [390]. Similarly, the p53 family member p73 transcriptionally regulates DRAM [391] as well as ATG5, ATG7, UVRAG, GABARAP, and PIK3C3 [392-394]. TGFβ-mediated autophagy has also been demonstrated to rely on the execution of specific transcriptional programs. In hepatocarcinoma cells, transcriptional activation of Beclin-1, DAPK, ATG5, and ATG7 is both Smad- and JNK-dependent [303]. In contrast, induction of MAP1LC3 expression by TGFβ in MMC is in fact independent of Smad signalling, and is mediated by TAK1 and the PI3K/Akt pathway [304,381].

To identify novel features of TGFβ-induced autophagy, we investigated the transcriptional regulation of a wide range of autophagy-related genes in response to TGFβ treatment. In addition to known TGFβ target genes, our analysis revealed a number of novel and potent TGFβ transcriptional targets, namely PIK3C3, ULK1, ULK2, GABARAP, ATG4b, ATG12, and ATG14. These autophagy-related gene products function cooperatively at multiple steps to facilitate autophagy initiation and maturation, indicating that the transcriptional regulation of autophagy by TGFβ is multifaceted. Moreover, our data provide further support for the premise that autophagy is regulated at the transcriptional level.

In addition to being a novel TGFβ target, ULK1 is of particular interest, as its upregulation alone is sufficient to induce high levels of autophagy and contributes to subsequent cell death in response to DNA damage [390]. Autophagic induction by ULK1 is dependent on its kinase activity. Active ULK1 directly phosphorylates Beclin-1 and activates the pro-autophagic vacuolar sorting protein 34 (VPS34) complexes to induce autophagy [368]. In fact, ULK1 interacts with various components of the autophagic machinery, either by direct association, phosphorylation, or due to subcellular relocalization events [395-397], and may thus act as a central mediator for controlling multiple steps in the autophagic process. Given the functional importance of this autophagic regulator and its potent transcriptional activation in response to TGFβ, it would be interesting to further investigate the role and contribution of ULK1, in terms of its expression and kinase activity, to TGFβ-mediated autophagy.

Our previous work established a central role for the pRb/E2F1 pathway as a major regulatory signalling axis through which TGFβ induces the expression of numerous pro-apoptotic genes, leading to apoptotic cell death. We thus investigated the potential role of this pathway to TGFβ-mediated autophagy. In the present study, we demonstrate that the transcriptional regulation of several

autophagic genes by TGFβ is also E2F1- and pRb-dependent. Moreover, activation of the autophagic process by TGFβ also relies on E2F1 and pRb. Our data provide strong evidence for the importance and specificity of E2F1 in the autophagic response to TGFβ and provide a novel pathway by which TGFβ regulates autophagy-related genes and autophagy activation. Further study will be required to determine the precise mechanisms by which pRb/E2F signalling mediates TGFβ-induced autophagy.

The E2F family of transcription factors have been reported to play an essential role in autophagy activation. The transcriptional activity of E2F1 was previously shown to be required for DNA damage-induced autophagy in osteosarcoma cells, by upregulating the expression of ATG5, MAP1LC3, ULK1, and DRAM [378]. Moreover, the Bcl-2/adenovirus E1B 19kD protein-interacting protein 3 (BNIP3) was identified as a direct transcriptional target of E2F1 that is involved in hypoxia-induced autophagic cell death [398]. Interestingly, Beclin-1 appears to be regulated by multiple E2F members, as most E2F members occupy the Beclin-1 promoter [399] and both E2F1 and E2F3 have been shown to transactivate the Beclin-1 promoter by reporter assay [400]. Recently, Kusama and colleagues amplified and cloned the putative promoter regions of 23 human autophagic genes, more than half of which were found to be regulated by E2F1 in HeLa cells [401]. These studies strongly support that the E2F transcription factors potentially act as major autophagic regulators. Since our data implicates E2F signalling as an essential regulatory axis through which TGFβ mediates autophagy, this further suggests that TGFβ may also represent a major player in autophagic regulation. Though our data suggests that E2F4 is not involved in the induction of autophagy-related genes by TGFβ, further investigation of the contribution of other E2F members to TGFβ-mediated autophagy would be of interest. In particular, our results indicate that TGFβ upregulates Beclin-1 expression, both at the transcript and protein level. Given that multiple E2F members have previously been implicated in its regulation, it is plausible that other E2F species may be involved downstream of TGFβ in mediating these effects. As mentioned previously, ChIP

analysis to assess whether E2F members are present at autophagic gene promoters in response to TGFβ warrants investigation, and would further support a transcriptional role for the pRb/E2F pathway in mediating the TGFβ autophagic response.

Results of our current study, taken together with our previously established role of pRb/E2F1 in TGFβ-mediated apoptosis, suggest that the pRb/E2F1 pathway potentially regulates both apoptosis and autophagy in response to TGFβ. Thus, it is conceivable that autophagy and apoptosis are co-regulated at the transcriptional level downstream of TGFβ signalling.

Accordingly, an increasing number of reports suggest that there is indeed a complex interplay between autophagy and apoptosis, evidenced by the extensive molecular crosstalk between autophagy-related and apoptosis-related proteins. In this regard, depletion of key autophagic regulators or pharmacological interference with autophagy has been shown to prevent apoptosis. Inhibition of autophagy by knockdown of Beclin-1 or Atg7, or by treatment with 3-methyladenine (3-MA), reduces caspase activity and mitigates apoptosis. In addition, Atg5 knockdown dramatically decreases DNA damage-induced apoptosis [389]. Moreover, several Atg proteins have been reported to engage the apoptotic pathway. For instance, Atg5 was demonstrated to bind Fas-associated protein with death domain (FADD), promoting IFN-γ-induced cell death through the extrinsic pathway [402]. Moreover, treatment with obatoclax, a Bcl-2 inhibitor, resulted in Beclin-1-induced apoptosis through caspase-dependent and -independent mechanisms [403]. Additionally, ATG12 conjugation to ATG3 sensitizes cells to apoptosis through the mitochondrial pathway [404]. Reciprocally, several apoptotic proteins also regulate the autophagic process. As mentioned previously, anti-apoptotic Bcl-2 directly binds Beclin-1, inhibiting its autophagic activity [372]. Interestingly, a number of pro-apoptotic proteins promote autophagy induction by enabling the dissociation of Beclin-1 from Bcl-2. This is mediated either through competition with Beclin-1 for Bcl-2 binding by various BH3-only

proteins, such as Bad, tBid, and BNIP3 [405,406], or through post-translational modifications of Beclin-1 or Bcl-2 mediated by DAPK, JNK, or TRAF6 [407-409]. While autophagy and apoptosis are correlated in a number of contexts, the causal relationships often remain unclear.

In the context of TGFβ signalling, Kiyono and colleagues proposed that autophagy activation partially contributes to TGFβ-mediated growth inhibition in hepatoma cells [303]. The mechanisms by which the autophagic process might be coupled to apoptotic cell death will be investigated in our future studies with the aim of identifying candidate molecules linking autophagy to apoptosis downstream of TGFβ signalling. Specifically, the potential molecular crosstalk between the E2F-mediated autophagy-related and apoptosis-related genes downstream of TGFβ signalling that we have identified here and in our previous study will be assessed. Interestingly, many of these identified targets have already been implicated in cross-regulating these two pathways, thus it is likely that such crosstalk does indeed occur and is conceivably dependent on the pRb/E2F pathway.

In summary, our data suggests that TGFβ might induce autophagy at least partially through the pRb/E2F1 pathway and transcriptional activation of autophagy-related genes. These findings provide new insights for mechanisms of autophagy regulation downstream of TGFβ signalling. Notably, our data also suggest that we have potentially uncovered yet another arm of TGFβ tumour suppressive effects that is mediated through pRb/E2F signalling, further supporting the importance of this pathway downstream of TGFβ signalling.

Both TGFβ and autophagy are involved in numerous diverse physiological effects that may influence multiple important fields beyond cancer research, such as immune regulation and neurodegeneration. Accordingly, autophagy might contribute to various TGFβ-mediated biological functions. Further elucidation of the molecular mechanisms by which TGFβ regulates cell fate through autophagy

may thus provide a better understanding of the physiological and pathological roles of TGFβ signalling pathways.

4.7 Acknowledgements

We thank Dr. Gordon Shore for generously providing the H1299 GFP-LC3 cells and Dr. Kristian Helin for kindly providing the mutant E2F1 expression vectors. This work was supported by grants from the Canadian Institutes for Health Research (CIHR, MOP-114904 to JJL). JJL is the recipient of the McGill Sir William Dawson Research Chair and JK holds a CIHR Frederick Banting and Charles Best Doctoral Research Award.

4.8 Figures for Chapter 4

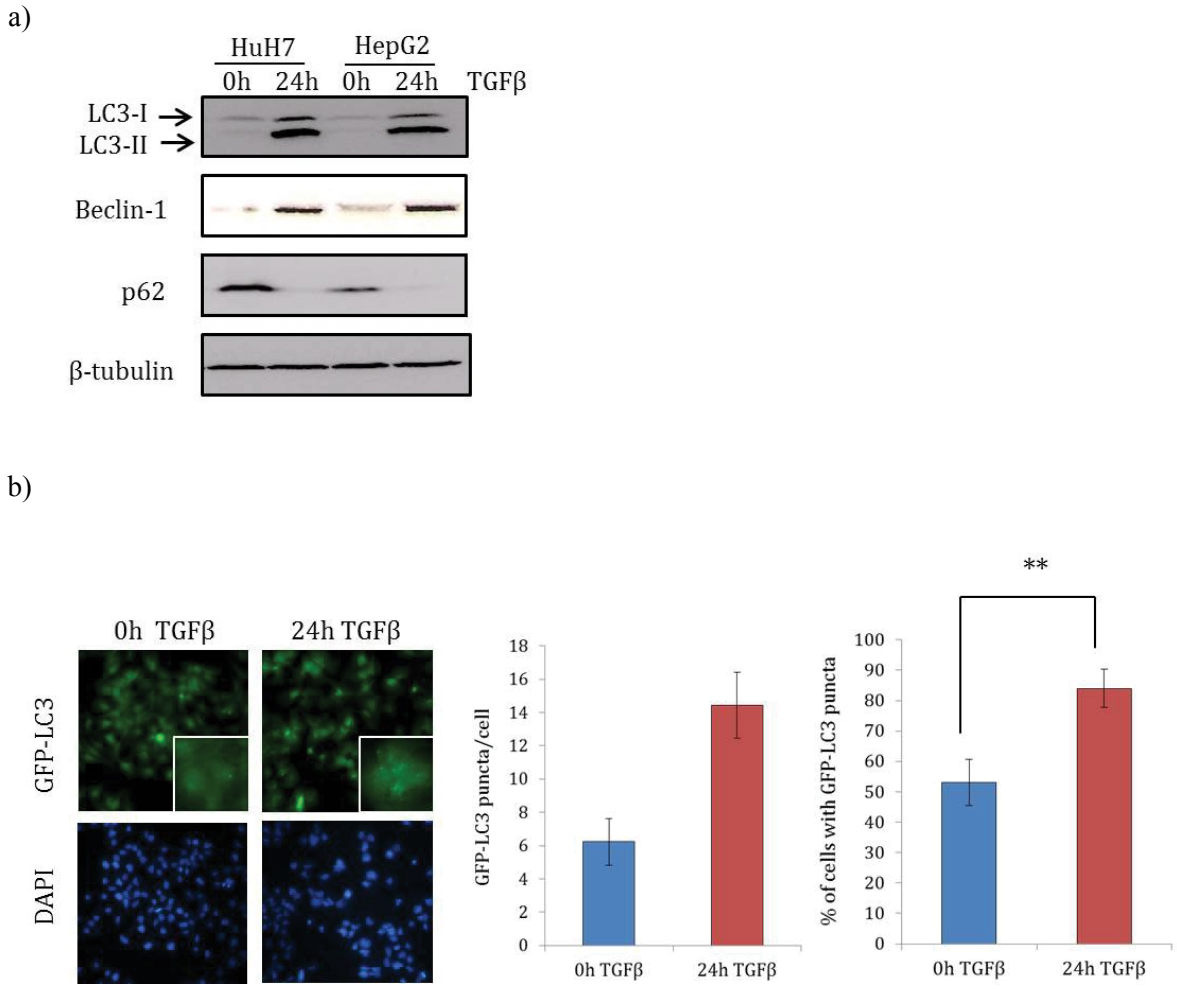
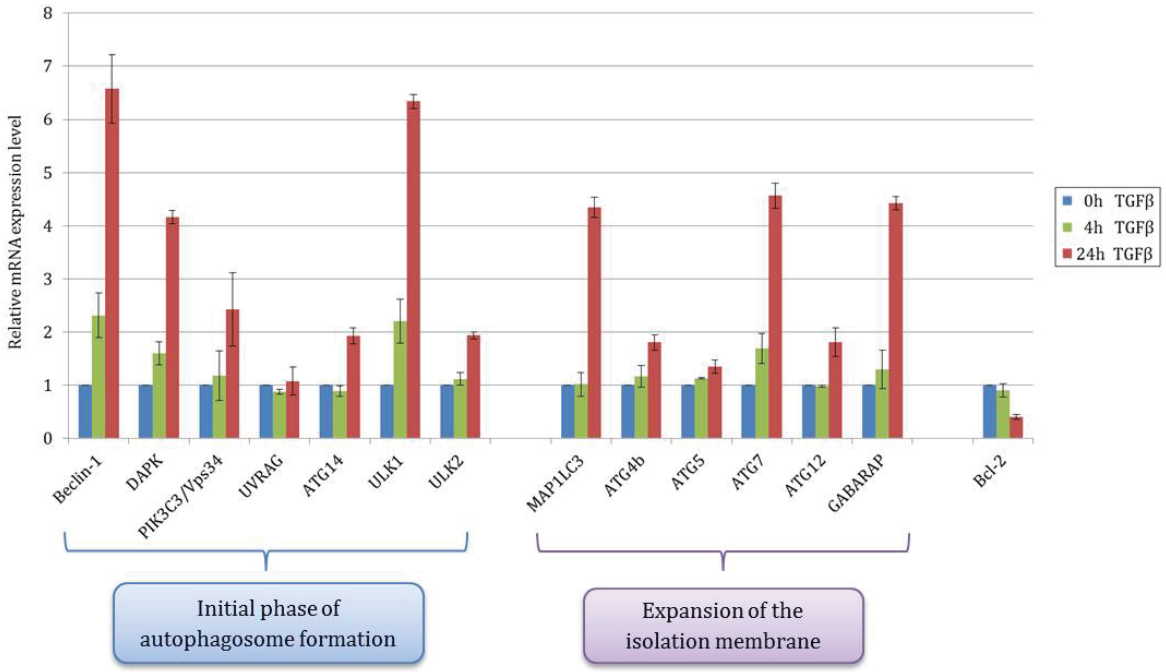
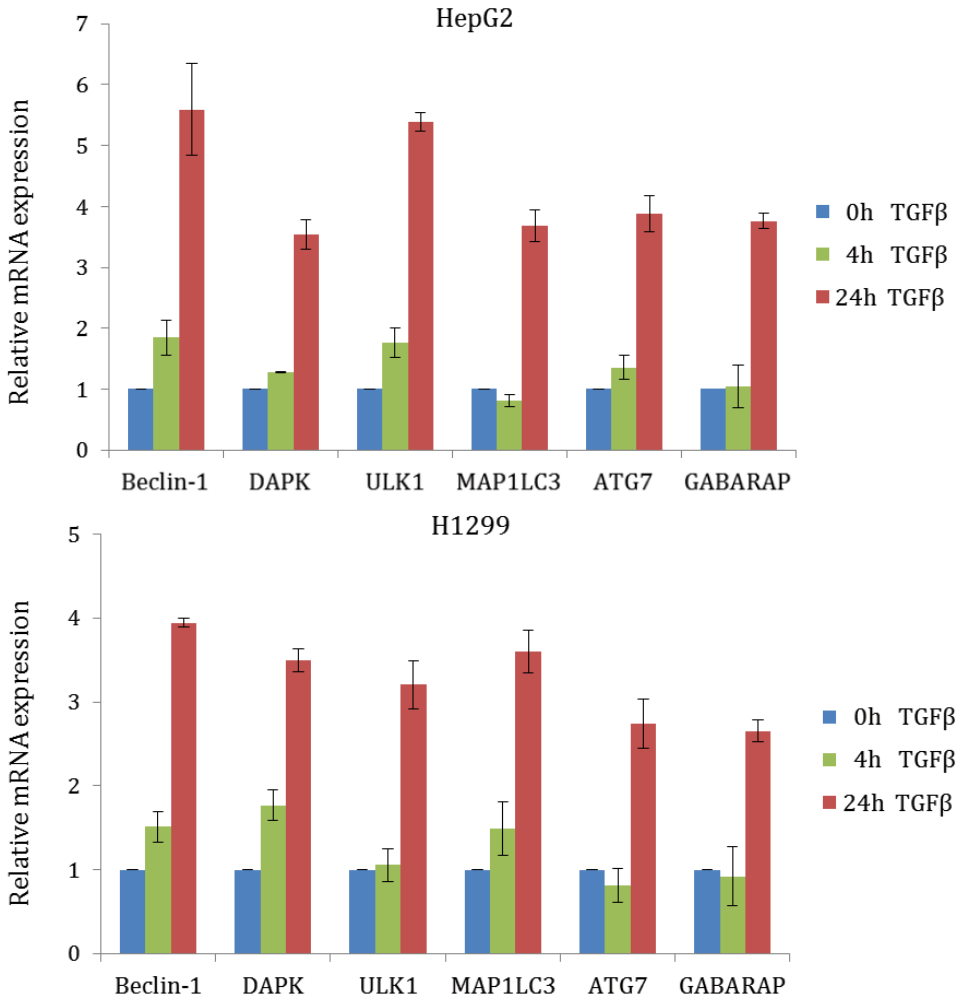


Figure 4.1: TGFβ induces autophagy in human hepatocarcinoma cell lines.

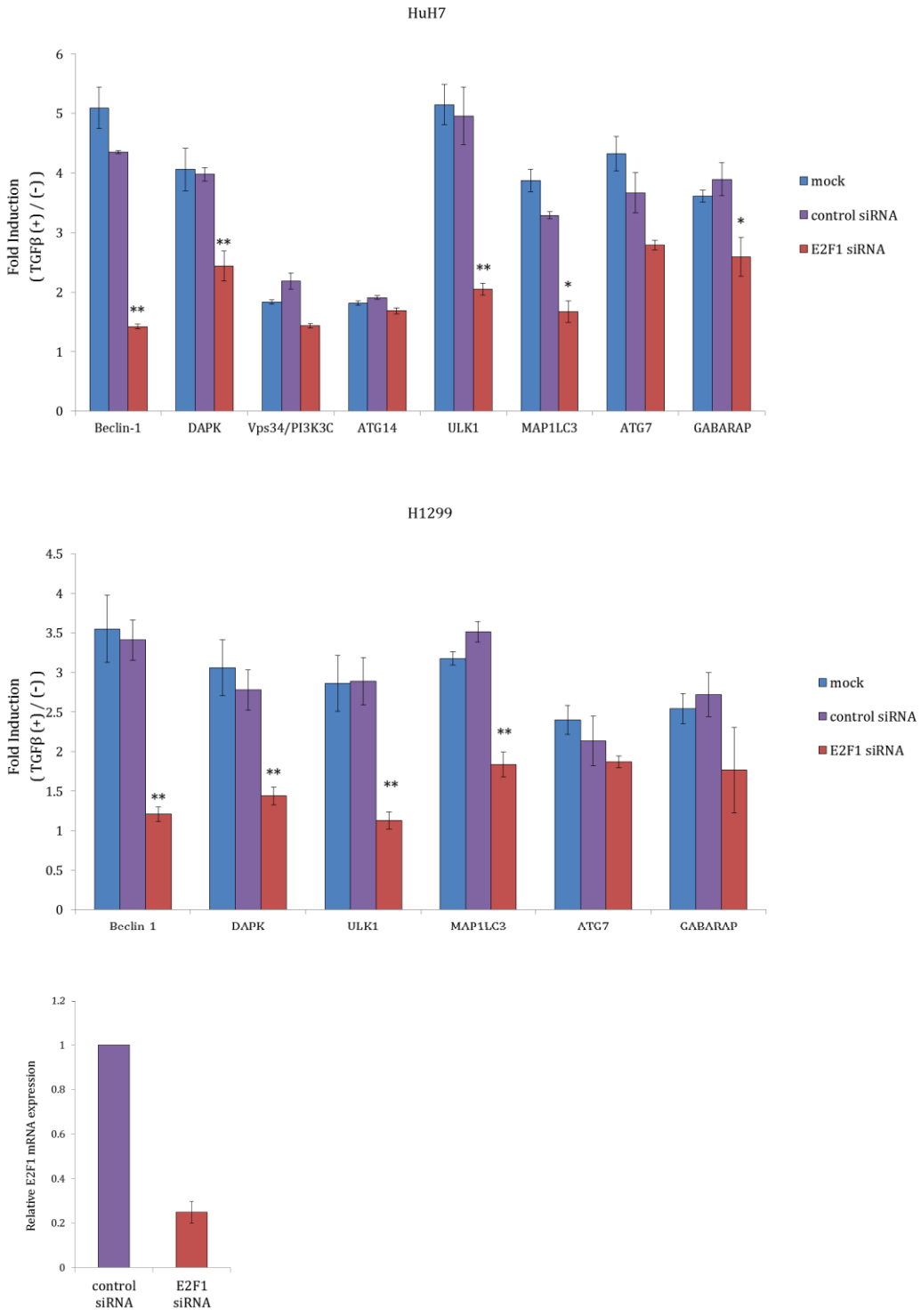
a) Immunoblot analysis of the conversion of endogenous LC3 (LC3-I to the more rapidly migrating LC3-II), as well as expression levels of Beclin-1 and p62, in HuH7 and HepG2 cells treated with TGFβ for 24h. b) HuH7 cells stably expressing GFP-LC3 were treated with TGFβ as indicated and the relocalization of GFP-LC3 to autophagosomes was detected as punctate formation, visualized by fluorescence microscopy (representative images at *left*). The number of GFP-LC3 puncta per cell and percentage of cells exhibiting more than five GFP-LC3 puncta were quantified (*right*). Data are represented as mean ± SD, (** $p < 0.01$).

a)


Relative mRNA expression level
0h TGFβ
4h TGFβ
24h TGFβ
Beclin-1
DAPK
PIK3C3/Vps34
UVRAG
ATG14
ULK1
ULK2
MAP1LC3
ATG4b
ATG5
ATG7
ATG12
GABARAP
Bcl-2
Initial phase of autophagosome formation
Expansion of the isolation membrane


HepG2
Relative mRNA expression
0h TGFβ
4h TGFβ
24h TGFβ
Beclin-1
DAPK
ULK1
MAP1LC3
ATG7
GABARAP
H1299
Relative mRNA expression
0h TGFβ
4h TGFβ
24h TGFβ
Beclin-1
DAPK
ULK1
MAP1LC3
ATG7
GABARAP

b)


HuH7
Fold Induction
(TGFβ (+) / (-))
mock
control siRNA
E2F1 siRNA
Beclin-1
DAPK
Vps34/PI3K3C
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ULK1
MAP1LC3
ATG7
GABARAP
H1299
Fold Induction
(TGFβ (+) / (-))
mock
control siRNA
E2F1 siRNA
Beclin 1
DAPK
ULK1
MAP1LC3
ATG7
GABARAP
Relative E2F1 mRNA expression
control siRNA
E2F1 siRNA

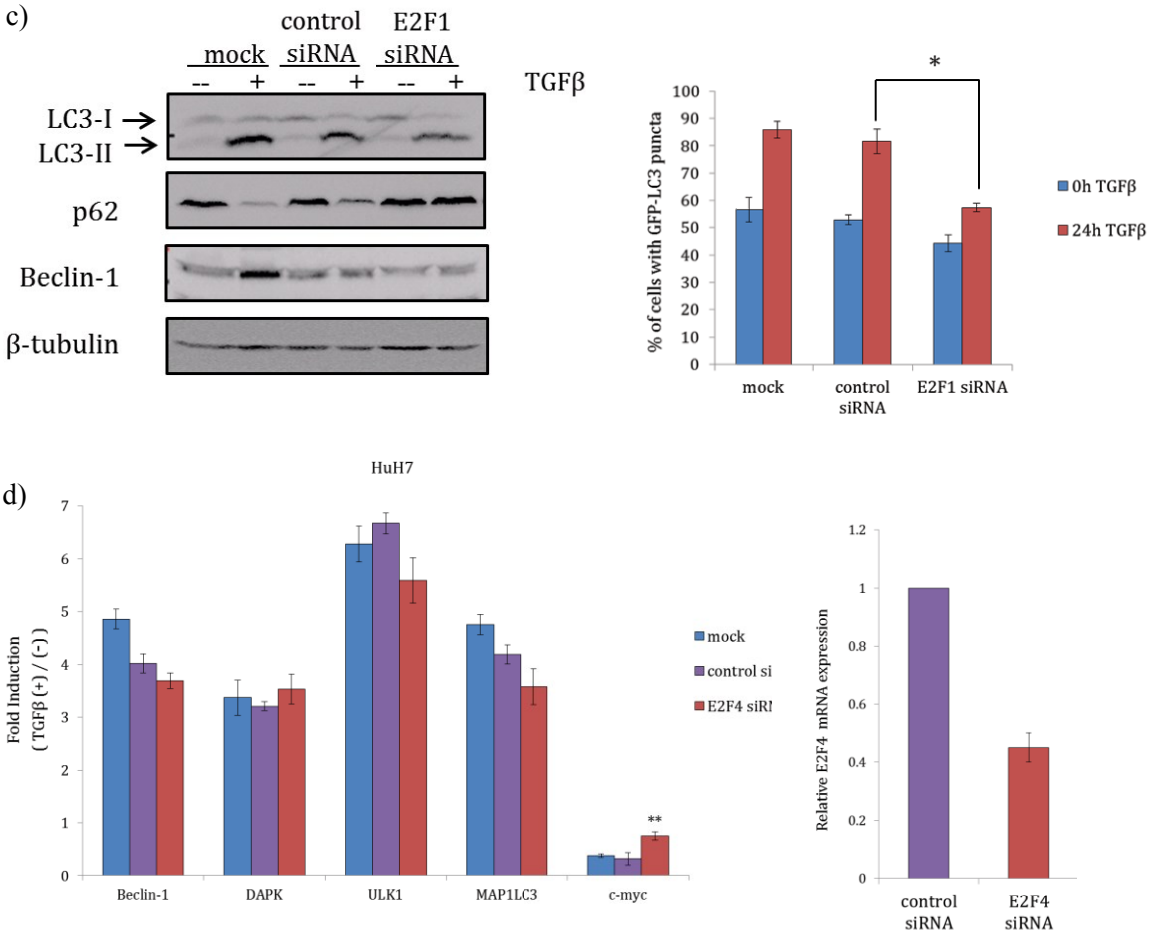
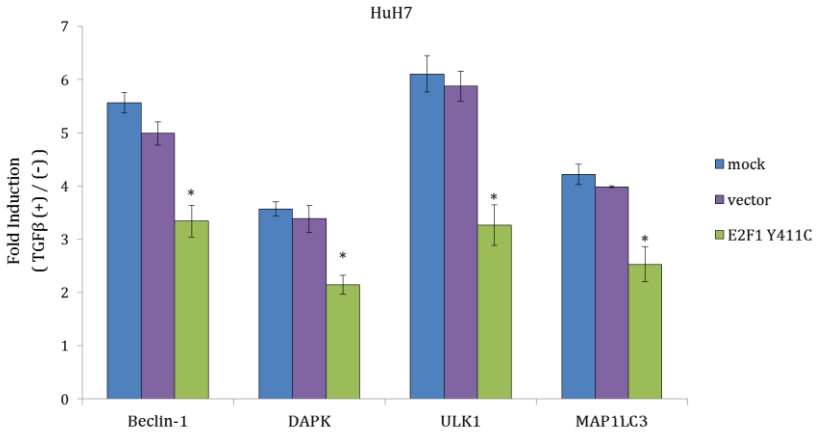


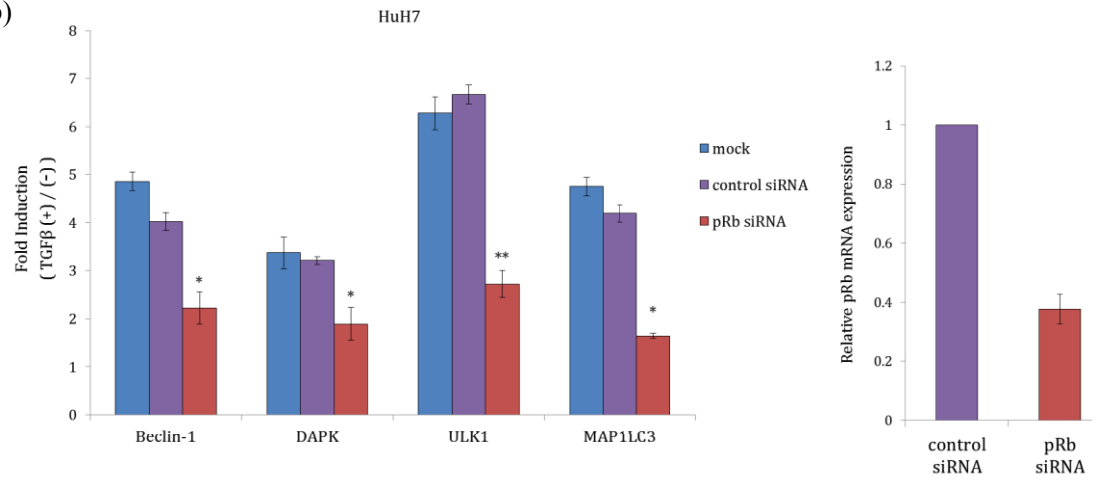
Figure 4.2: TGFβ transcriptionally mediates autophagy in an E2F1-dependent manner

a) HuH7, HepG2, and H1299 cells were stimulated with TGFβ as indicated and mRNA levels for the specified genes 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 ± SD. b) HuH7 and H1299 cells were transiently transfected with siRNA against E2F1 or a control non-silencing siRNA and stimulated with TGFβ for 24h. The mRNA levels for the indicated genes were measured as in (a). c) Following siRNA transfection, HuH7 cells were treated with TGFβ for 24h and LC3 conversion (*left*) and GFP-LC3 puncta formation (*right*) were analyzed by immunoblotting and fluorescence microscopy, respectively. Effect of E2F1 knockdown on Beclin-1 and p62 expression was also evaluated by immunoblotting (*left*). d) HuH7 cells were transiently transfected with siRNA against E2F4 or a control non-silencing siRNA and stimulated with TGFβ for 24h. The mRNA levels for the indicated genes were measured as in (a), (* $p < 0.05$, ** $p < 0.01$).

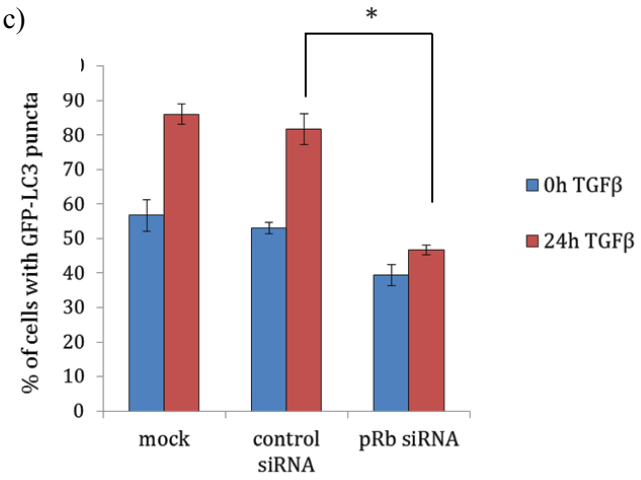
a)


HuH7
Fold Induction
(TGFβ (+) / (-))
mock
vector
E2F1 Y411C
Beclin-1
DAPK
ULK1
MAP1LC3

b)


HuH7
Fold Induction
(TGFβ (+) / (-))
mock
control siRNA
pRb siRNA
Beclin-1
DAPK
ULK1
MAP1LC3
Relative pRb mRNA expression
control siRNA
pRb siRNA

c)


% of cells with GFP-LC3 puncta
0h TGFβ
24h TGFβ
mock
control siRNA
pRb siRNA

d)

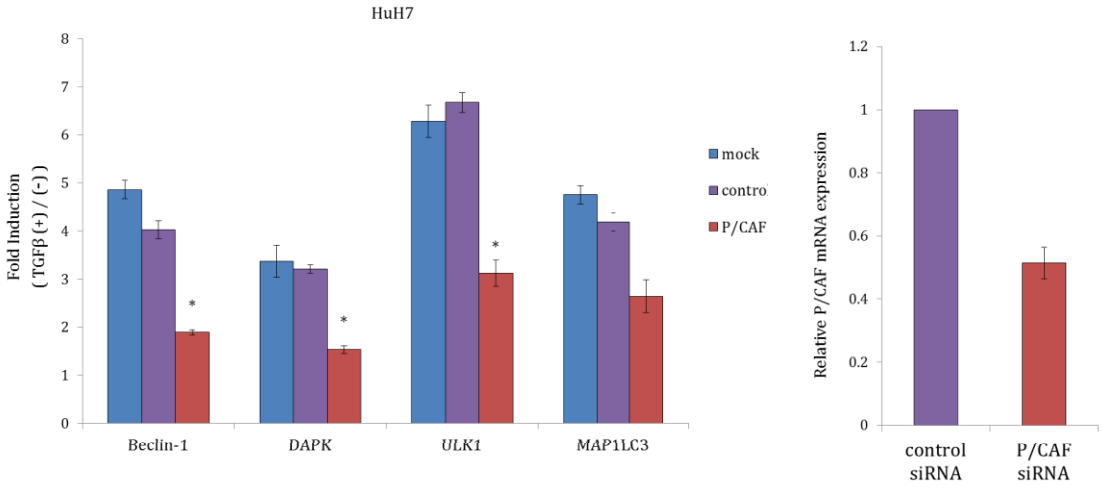


Figure 4.3: TGFβ-induced activation of autophagy acts through pRb/E2F1 signalling and requires the histone acetyltransferase P/CAF.

a) HuH7 cells transiently transfected with empty vector or an E2F1 mutant deficient for pRb binding (E2F1 Y411C) were treated with TGFβ for 24h. Levels of mRNA expression of the specified genes were determined by real-time qPCR analysis. Results are normalized to GAPDH and shown relative to levels observed in untreated cells (set to 1). b, d) HuH7 cells were transiently transfected a control non-silencing siRNA or siRNA against pRb (b) or P/CAF (d) and stimulated with TGFβ for 24h. The mRNA levels for the indicated genes were determined by real-time qPCR analysis. c) GFP-LC3 puncta formation was analyzed by fluorescence microscopy and quantified as described previously. Data are represented as mean ± SD, (* $p < 0.05$, ** $p < 0.01$).

Chapter 5 | General Discussion

5.1 General Discussion

The balance between cell proliferation and cell death is critical for the maintenance of tissue homeostasis, and its deregulation is involved in numerous human pathologies, including cancer. Indeed, uncontrolled cell growth and limitless replicative potential are defining features of tumourigenesis.

In normal cells, cell cycle progression is tightly monitored by a series of checkpoints. While minor irregularities are amended by repair mechanisms, more damaging defects elicit the execution of failsafe processes such as programmed cell death or cell senescence. Cancer cells, however, proliferate beyond these constraints by adopting capabilities that allow them to circumvent or override these intrinsic cell surveillance systems. In addition to achieving autonomous growth, insensitivity to anti-growth signals, and resistance to apoptosis, tumour cells also breach inherent cell replication limits and attain immortalization through telomere maintenance mechanisms.

As a major premise throughout this thesis, TGFβ plays a critical role in mediating fundamental cell homeostatic processes and, consequently, acts as a potent tumour suppressive agent that prevents uncontrolled cell proliferation and immortalization in various cell types. As such, it is unsurprising that many cancers target this pathway for disruption. Indeed, many tumour cells of different origin acquire resistance to the tumour suppressive effects of TGFβ through downregulation or mutational inactivation of the TGFβ receptors or aberrations in various downstream TGFβ signalling effectors [2,125,410,411]. Paradoxically, these genetic and epigenetic alterations conspire to convert TGFβ from a tumour suppressor to an oncogenic factor, promoting tumour growth, survival, and metastasis [412].

Intriguingly, it appears that the pRb/E2F pathway is potentially at the core of TGFβ-mediated tumour suppression. Indeed multiple different E2F family

members act downstream of TGFβ signalling to elicit these tumour suppressive responses (summarized in Figure 5.1). Combined with studies from other labs, the work presented in the present thesis supports a pivotal function for the E2F family as potent co-transducers of TGFβ signalling and highlights the prominent role for the pRb/E2F pathway in regulating TGFβ tumour suppressive effects. Based on our studies, we deem the E2F transcription factors to be 'supermediators' of TGFβ tumour suppressive responses and propose the following model of E2F tumour suppressive action downstream of TGFβ:

(1) TGFβ induces E2F4/5 recruitment into classical repressive pRb–E2F–HDAC complexes, targeting the key cell-cycle regulators *cdc25a* [163] and *c-myc* [162,362], thus preventing cell cycle progression;

(2) TGFβ transcriptionally inhibits hTERT expression though E2F1 and HDAC activity [209], suppressing immortalization (Chapter 2);

(3) TGFβ can also recruit E2F1 into transcriptionally active pRb–E2F1–P/CAF complexes, increasing the expression of several pro-apoptotic target genes and inducing programmed cell death [361] (Chapter 3);

(4) In certain cancer cell types, TGFβ also transcriptionally suppresses the inhibitor of apoptosis survivin through Smad signalling and recruitment of a pRb–E2F4 repressive complex to the survivin promoter [266], thus promoting apoptosis;

(5) TGFβ induces autophagy through pRb-, E2F1-, and potentially P/CAF-dependent transcriptional activation of numerous autophagy-related genes (Chapter 4).

Interestingly, the E2F family acts via distinct pathways to regulate specific genes, yet all toward a global action of tumour suppression. We might speculate, then,

that the different E2F species may potentially function collaboratively to maintain or ensure proper cell growth, proliferation, and appropriate cell death downstream of TGFβ signalling. A better understanding of the mechanisms by which both TGFβ and pRb/E2F signalling mediate their tumour suppressive roles may thus prove useful for the development of novel therapeutic strategies aimed at restoring these tumour suppressive responses in human cancer.

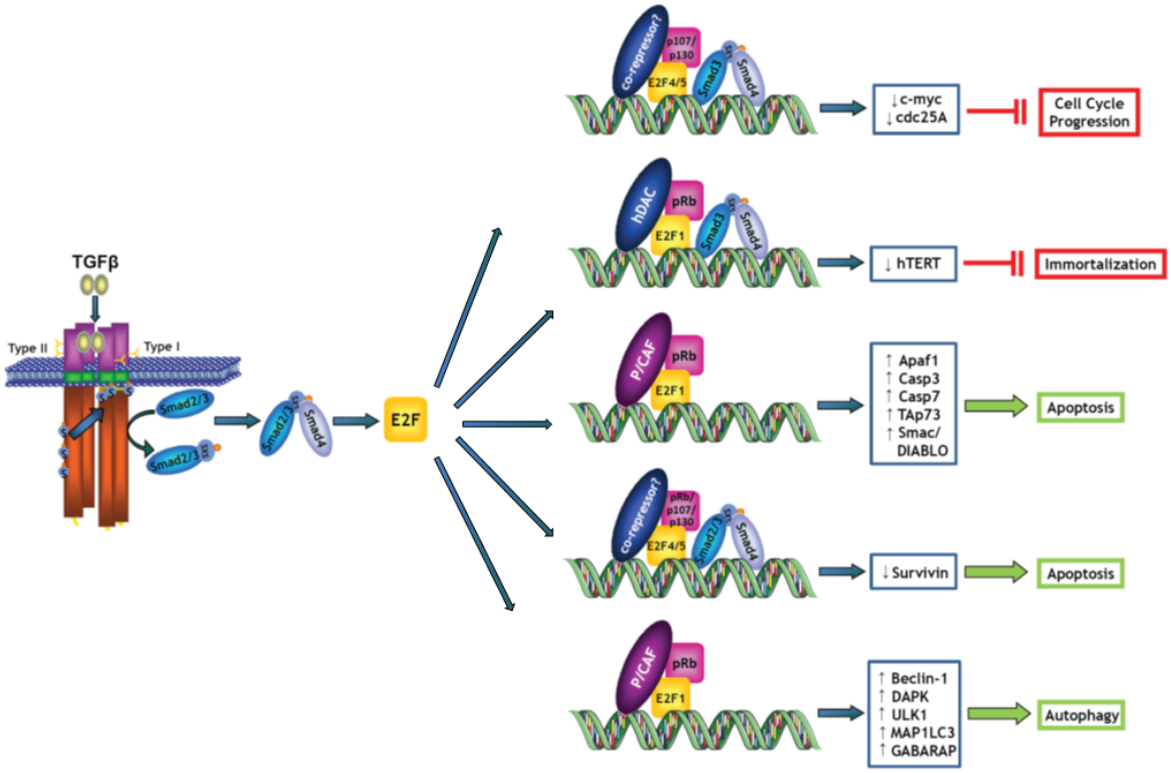


Figure 5.1: Model of E2F tumour suppressive action downstream of TGFβ signalling

Indeed, the elimination of cancer cells is the ultimate goal of cancer therapy and one of the many challenges in designing effective chemotherapeutics is to identify potential targets of therapeutic action. In recent years, a number of pre-clinical

studies have investigated whether E2F1 may be utilized as an anti-cancer therapeutic. The frequent deregulation of E2F1 in human cancer, along with its apoptotic potential and its stabilization following DNA damage, suggest that E2F1 may in fact contribute to the enhanced sensitivity of tumour cells to DNA damage-induced cell death [413]. Numerous *in vitro* and *in vivo* studies have evaluated the effect of E2F1 overexpression on tumour growth in several types of human cancer including glioma [414], melanoma [415], breast and ovarian carcinoma [416], head and neck squamous cell carcinoma [417], gastric carcinoma [418], pancreatic carcinoma [419], and nonsmall-cell lung carcinoma [420]. Remarkably, these studies clearly demonstrated that apoptosis induction by adenoviral-expressed E2F1 resulted in growth suppression of tumour cells without significantly affecting normal tissues. Moreover, increased E2F1 expression enhanced tumour cell chemosensitivity.

The compound β-lapachone, which is a natural product of the South American *Tabebuia avellanedae* tree, is a potential chemotherapeutic agent that exhibits anti-neoplastic and radiosensitizing activity [421-426]. Remarkably, β-lapachone has been shown to induce E2F1 expression and selectively activate the E2F1-mediated checkpoint pathway, directly triggering apoptosis in tumour cells without affecting normal cells [427]. Based on these properties, the biotechnology company ArQule has developed a synthetic, soluble pro-drug of β-lapachone called ARQ 761, which is currently being investigated in Phase I clinical trials (source www.clinicaltrials.gov). Interestingly, β-lapachone has also been shown to have a direct cytotoxic effect in human leukemia cells *in vitro,* associated with decreased telomerase activity [428].

Though these pre-clinical and clinical studies appear rather promising, targeting E2F1 itself may in fact not be suitable for all therapeutic purposes due to its dual role in cell proliferation and cell death. As such, identification of E2F1 target genes involved in programmed cell death may help to further elucidate the mechanisms by which E2F1 functions in tumour treatment.

In fact, many of the E2F-dependent TGFβ tumour suppressive target genes that we identified in the present thesis have previously been investigated for their potential diagnostic and therapeutic value in cancer. For instance, both hTERT and survivin are highly expressed in tumours but not in normal tissue, rendering them attractive candidates for targeted cancer therapy. Numerous studies have shown promising potential therapeutic effects using various telomerase inhibitors. For instance, the very potent hTR antagonist GRN163L has been demonstrated to inhibit telomerase activity and trigger senescence and widespread apoptosis after progressive telomere shortening in various cell lines, including human multiple myeloma and non-Hodgkin lymphoma cell lines [429]. Moreover, many studies have successfully used the hTERT promoter to drive pro-apoptotic genes *in vitro*, including *Bax* [430], *caspases 6-8* [431,432], and *TRAIL* [433]. This strategy selectively affects cells that are telomerase-positive, while sparing telomerase-negative cells. In contrast, survivin contributes to tumour maintenance and progression primarily by conferring apoptotic resistance. Antagonizing survivin expression or function has been shown to induce spontaneous apoptosis, sensitize tumour cells to chemotherapeutic agents, and reduce tumour growth potential [262,263]. Notably, Pennati *et al.* demonstrated that inhibiting survivin expression in human prostate cancer cells enhanced their susceptibility to cisplatin-induced apoptosis and prevented tumour formation when cells were xenografted in athymic nude mice [434]. They further showed that survivin suppression in human melanoma cells increased sensitivity to gamma-irradiation [435]. A better understanding of the role of both hTERT and survivin in tumour versus normal cells will be instrumental for the design of optimal strategies to selectively target these genes in cancer.

Additionally, the downstream TGFβ and E2F1 pro-apoptotic target p73 was established as a promising candidate for targeted cancer therapy. In a similar manner to E2F1, p73 overexpression induced apoptosis and increased the tumour cell sensitivity to chemotherapy, thus providing a basis for selective killing of cancer cells, including p53-defective tumour cells, by DNA-damaging agents

[436,437]. Since manipulating apoptotic signalling can produce abundant changes in cell death, the genes and proteins involved in controlling the apoptotic programme are thus potential therapeutic drug targets.

In effect, most anti-cancer therapies target tumour cells primarily by inducing apoptosis. However, as tumours often harbour or develop defects in the apoptotic pathway, this may render them resistant to conventional therapies based on apoptosis-mediated cell death [438,439]. For instance, overexpression of anti-apoptotic factors, including Bcl-2 family members and inhibitor of apoptosis proteins (IAPs), has been reported to significantly decrease the efficacy of apoptosis-inducing therapeutics [440-442]. Recently, several studies have investigated the prospect of activating alternative cell death pathways to selectively eliminate apoptosis-resistant cancer cells. Accumulating evidence suggests that autophagy may in fact be required for cell death in cancer cells with defects in apoptosis [443-445]. In this regard, the lack of autophagy diminishes or abolishes the effects of anti-cancer agents. These studies strongly suggest that autophagy may in fact play an important role in destroying cancer cells as well, thus modulation of autophagy may represent a novel therapeutic approach [385,446-448].

The role of autophagy in cancer therapy, however, is rather complex. Similar to its contradictory effects on tumour development and progression, autophagy may elicit a pro-survival or pro-death response to counteract or mediate the cytotoxic effects of therapeutic agents [449]. Indeed, autophagy may protect tumour cells from undergoing apoptosis in response to anti-cancer agents, but may also be a mechanism of cell death in apoptotic-defective tumour cells. Thus, the dichotomous roles of autophagy in tumourigenesis warrants careful consideration for the development of rational cancer therapies [450].

Autophagy is induced by many different forms of cancer therapy, including conventional chemotherapeutics, novel targeted cancer therapeutics, and ionizing radiation in various types of tumours [451]. Though the majority of the literature has

reported a cytoprotective role of autophagy in response to anti-cancer agents, in some cases autophagy has also been shown to facilitate or even induce robust cell death in tumour cells. For instance, the mTOR inhibitor rapamycin, which induces autophagy, suppresses the proliferation of malignant glioma cells [452]. Moreover, rapamycin-induced autophagy sensitizes prostate cancer cells lacking PTEN to radiation therapy [453]. Autophagy is thus considered a critical pathway by which rapamycin mediates its therapeutic activities through a pro-death mechanism [454].

Nevertheless, the role of autophagy in cancer therapy appears to be contextual. As such, targeting this pathway may require design of tumour-selective autophagy modulators that target the signalling pathways that regulate autophagy in specific contexts, as opposed to broadly targeting the autophagic machinery. In this regard, Wilkinson and colleagues recently identified a cell signalling pathway that is required for hypoxia-induced autophagy in tumour cells, while being seemingly dispensable for autophagy induced by other stimuli [455]. Since hypoxia is primarily a tumour-associated state, these findings may provide a basis for selective therapeutic targeting of autophagy in tumour cells, without affecting autophagy in normal cells.

Evidently, a better understanding of the molecular mechanisms governing the switch between cell-survival and cell-death is required for the clinical development of autophagy modulators as cancer therapeutics. Conceivably, the molecular cross-talk between autophagic and apoptotic pathways might also shed some light on more effective interventions in cancer therapy.

Intriguingly, the contrasting roles for autophagy during cancer development and progression seem to parallel the paradoxical functions of TGFβ in cancer. Indeed, both TGFβ and autophagy play bidirectional roles in tumour suppression and progression, by limiting tumour initiation but promoting tumour establishment and progression. Given the role of autophagy downstream of TGFβ signalling, it is interesting to consider that autophagy might in fact contribute to the

dichotomous nature of TGFβ in cancer. In this sense, autophagy might prevent tumour initiation in collaboration with other tumour suppressive pathways downstream of TGFβ signalling. Conversely, in later stages of tumour progression, TGFβ-induced autophagy might confer a survival advantage to cancer cells, after acquiring resistance to TGFβ-mediated cytostatic and pro-apoptotic responses.

On account of its crucial role in cancer regulation, many efforts have been made to target TGFβ signalling in cancer. However, one of the main challenges in targeted therapy is the off-target effects, and these are of particular concern when considering interfering with the TGFβ pathway due to its dual role in cancer. Indeed, activating TGFβ tumour suppressive pathways would only be advantageous if the therapy does not concomitantly promote the TGFβ pro-metastatic effects. Conversely, blocking the TGFβ tumour promoting pathways would only be favourable if it did not affect the tumour suppressive arm of TGFβ signalling. Clearly, predicting the final outcome of targeting TGFβ signalling for tumour treatment is particularly challenging. As such, targeting the TGFβ pathway may require the development of tumour- or context-specific treatments, or selectively modulating downstream signalling effectors of the TGFβ tumour promoting effects. Interestingly, in a recent study from our laboratory, we demonstrated that the cell cycle regulator p21 plays a prominent role in regulating TGFβ-mediated local tumour cell invasion in breast cancer. Using *in vitro* and *in vivo* approaches, we found that blocking p21 expression significantly alters the TGFβ tumour promoting effects, without affecting cell growth or tumour formation [167]. Moreover, numerous pre-clinical studies have supported the use of anti-TGFβ therapies, many of which are currently in clinical trials. While some of these strategies show promise for targeting the TGFβ pathway as an anti-cancer therapeutic, the greatest challenge for successful use of these therapies lies in the multifunctional nature of this pathway. In addition to the dichotomous role of TGFβ in cancer, it is also important to consider the ubiquitous nature of this

growth factor. As TGFβ is involved in so many normal biological processes, systemic inhibition of TGFβ may result in deleterious side effects [21].

In summary, the context and manner by which the TGFβ pathway dictates cell fate have implications for a better understanding of the physiological, pathological, and paradoxical roles of TGFβ signalling. Understanding the molecular mechanisms of TGFβ-mediated cell growth arrest, senescence, apoptosis, and autophagy may provide deeper insight into carcinogenesis, influence therapeutic strategies and, ultimately, lead to new therapeutic approaches based on resensitizing tumour cells to tumour suppressive responses. Moreover, though there is much evidence to support that various effectors of TGFβ and pRb/E2F tumour suppressive signalling can significantly and specifically kill cancer cells, their role as targets for cancer therapeutics depends on further elucidation of their precise regulatory mechanisms. The potential exploitation of these pRb/E2F and TGFβ signalling tumour suppressive pathways may provide new avenues for the development of novel cancer therapies and management of human cancers.

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Appendix | Articles in their Original Published Format

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Transforming growth factor-β inhibits telomerase through SMAD3 and E2F transcription factors

Annie Lacerte [a,1], Juliana Korah [a,1], Mélanie Roy [a], Xiang-Jiao Yang [b], Serge Lemay [a], Jean-Jacques Lebrun [a,*]

[a] *Hormones and Cancer Research Unit, Department of Medicine, Royal Victoria Hospital, McGill University, 687 Pine Avenue West, H3A 1A1, Montreal, Quebec, Canada*
[b] *Molecular Oncology Group, McGill University, Montreal, Quebec, Canada*

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Abstract

Cancer arises from multiple genetic changes within the cell, among which constitutive telomerase activity and attainment of immortality are central. Expression of *hTERT*, the protein component of telomerase, is increased in most cancer cells. Transforming growth factor-β (TGFβ), a potent tumor suppressor, has been reported to regulate *hTERT* expression. We found that TGFβ represses *hTERT* expression in normal and cancer cells and that this effect is mediated through Smad3 but also requires Erk1/2, p38 kinase and histone deacetylase activity. Furthermore, we identified four critical E2F transcription factor binding sites within the *hTERT* gene promoter that confer the TGFβ response. Finally, using the E2F-1 knockout model, we showed that loss of E2F-1 abolishes TGFβ inhibition of telomerase expression. These findings highlight the prominent role of TGFβ in regulating telomerase expression and identify Smad3 and E2F-1 as critical mediators of TGFβ effects in both normal and cancer cells.

Keywords: Telomerase; TGFβ; Smad; E2F-1; *hTERT*

1. Introduction

In humans, tumor formation and progression are characterized by several hallmarks [1]. Cancer cells acquire the ability to become resistant to growth arrest signals, to proliferate in the absence of growth factors and to benefit from increased vascularisation. They also evade apoptosis and escape the immune system, become invasive and attain immortalization. In normal human somatic cells, cell division occurs a limited number of times as the length of the ends of chromosomes (telomeres) shortens with each cell division, leading to senescence and cell death. Cancer cells are not limited by such a fixed number of replication but are instead immortalized. This is due to constitutive telomerase activity, which adds telomeric DNA repeats at the ends of newly duplicated telomeres, thereby preserving their length throughout successive replication cycles and protecting chromosomes from degradation [2].

The telomerase enzyme contains an RNA component, the human telomerase RNA template or hTER and a protein component, the human telomerase reverse transcriptase or *hTERT* [3]. The *hTERT* protein is a key determinant of telomerase activity, as its expression is normally suppressed in somatic cells [4]. However, ninety percent of human cancers show increased expression of telomerase, a process that leads to cell immortalization [5]. This is by far the most commonly observed abnormality acquired by tumor cells and is used as a diagnosis marker for cancer [6]. Reactivation of telomerase activity is mainly due to the loss of repression of the *hTERT* gene in cancer cells. Although telomerase activity is regulated at various levels, such as mRNA splicing and accessibility of the telomeres, the transcriptional control of the *hTERT* gene is a key event in the increased telomerase activity observed in cancer cells [7].

Growth factors from the TGFβ family have a profound impact on cell homeostasis and act as tumor suppressors, through

* Corresponding author. Hormones and Cancer Research Unit, Department of Medicine, Royal Victoria Hospital, 687 Pine Avenue West, H3A 1A1, Montreal, Canada. Tel.: +1 514 934 1934x34846; fax: +1 514 982 0893.
E-mail address: JJ.Lebrun@mcgill.ca (J.-J. Lebrun).
URL: http://www.hcru.mcgill.ca (J.-J. Lebrun).
[1] Both authors equally contributed to the manuscript.

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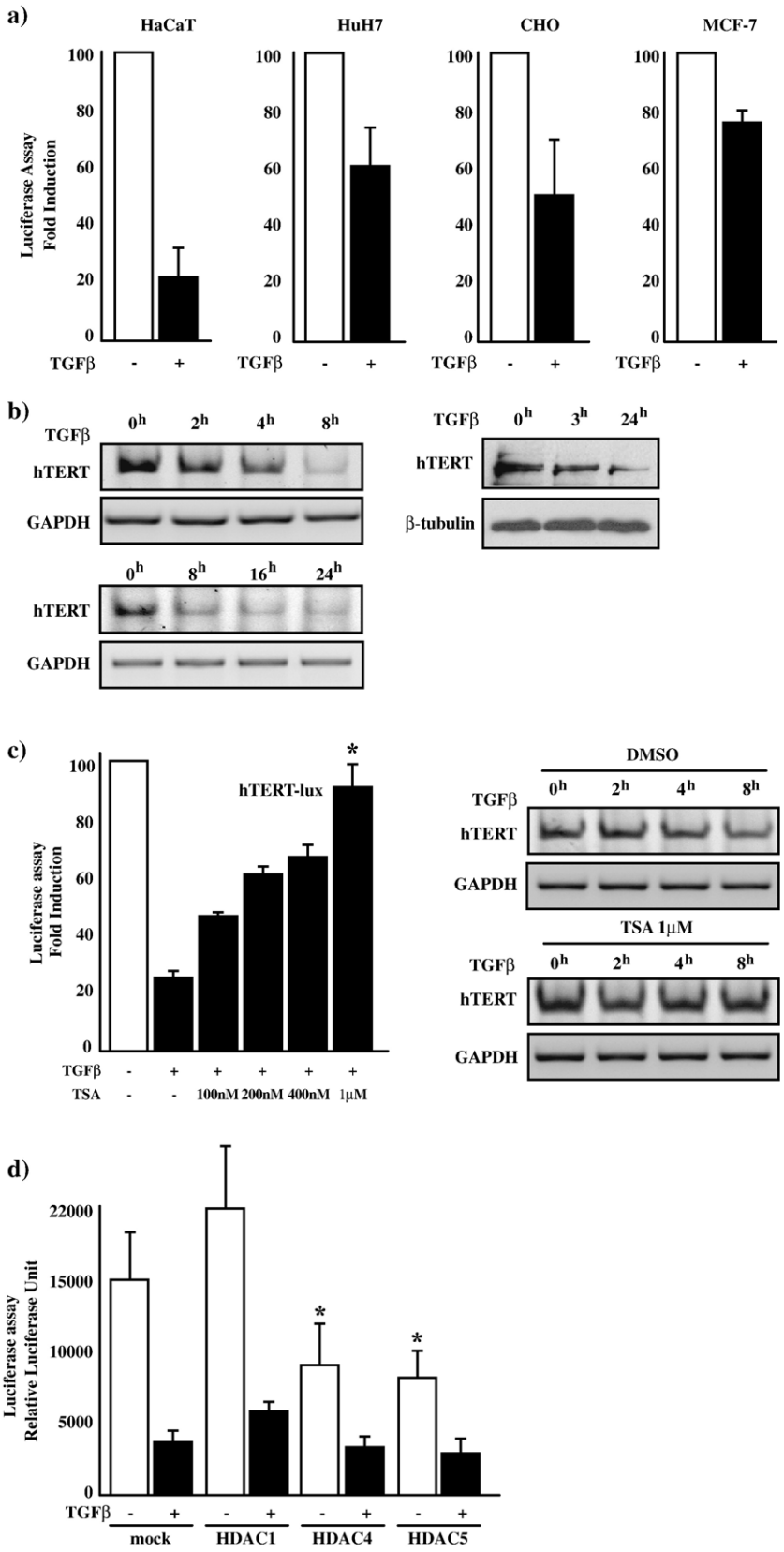
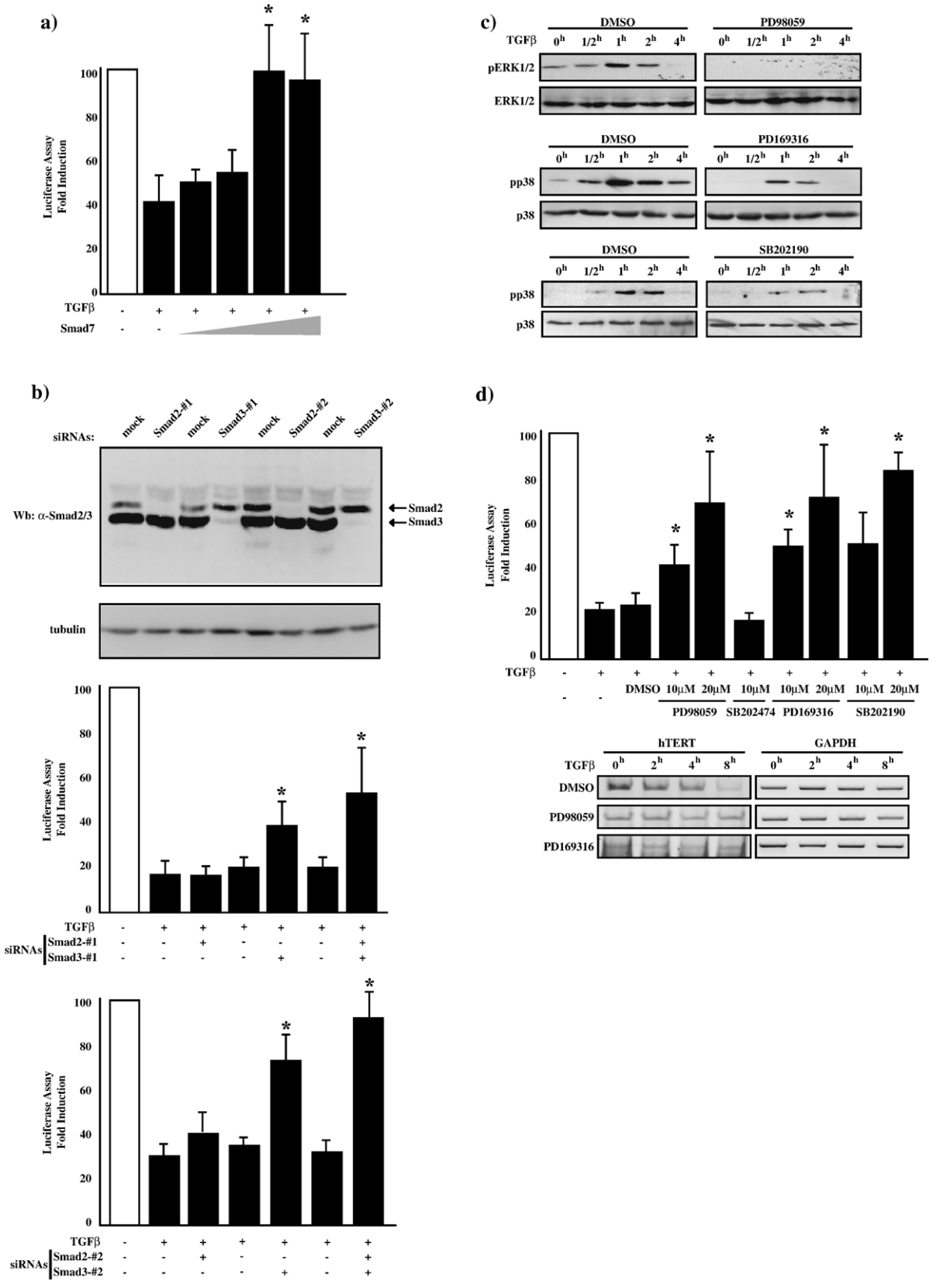


Fig. 1. TGFβ inhibits *hTERT* expression in a HDAC dependent manner. a) Cells were transfected with the *hTERT*-lux reporter, stimulated with TGFβ and assessed for luciferase. b) RT-PCR (left) and Western blot (right) analysis of *hTERT* mRNA and protein levels in HaCaT cells stimulated with TGFβ. c) *hTERT* (−1934)-lux transfected HaCaT cells were pre-treated with the indicated concentrations of TSA and stimulated with TGFβ before being assessed for luciferase (left) and *hTERT* mRNA levels by RT-PCR (right). d) HaCaT cells were transfected with *hTERT* (−1934)-lux and HDAC1, HDAC4 and HDAC5 expression vectors, as indicated, before being assessed for luciferase.

regulation of cell growth arrest and apoptosis. Interestingly, TGFβ has also been shown to regulate telomerase activity, through repression of the *hTERT* gene [8,9]. TGFβ 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, co-activators and/or co-repressors to regulate expression or repression of the target genes in a cell and tissue specific manner [10].

TGFβ inhibits telomerase activity in various cell lines, however, the precise mechanisms by which it represses *hTERT* expression remain unclear. Several factors have been shown to be involved downstream of TGFβ-mediated inhibition of telomerase activity. It has been proposed that TGFβ decreases human and rat *TERT* expression indirectly through inhibition of c-myc expression [9, 11]. However, a recent study in MCF-7 breast cancer cells suggested that the TGFβ inhibitory effect on *hTERT* was not mediated through inhibition of c-myc expression, but rather involved a direct interaction of Smad3 and c-myc on the *hTERT* promoter, leading to inhibition of c-myc transcriptional activity [12]. Another study suggested a role for the Smad interacting protein-1 (SIP1) in TGFβ-mediated *hTERT* repression [13]. Thus, the different mechanisms observed using different cell systems may reflect cell specific effects rather than a central mechanism.

In this paper, we show that TGFβ inhibitory effects on *hTERT* expression are mediated not only through the Smad pathway but also require the Erk1/2 and p38 kinase pathways, as well as histone deacetylase activity. Using truncation and deletion mutant forms of the *hTERT* promoter construct, we showed that TGFβ repressed *hTERT* gene promoter activity through the −252 to the +3 region, proximal to the start site. Interestingly, we found that several binding sites for E2F family members were critical for TGFβ-mediated inhibition of the *hTERT* gene promoter. We further demonstrated that interfering with E2F activity resulted in complete reversal of TGFβ-mediated *hTERT* inhibition, thus highlighting the E2F transcription factors as central mediators of the TGFβ inhibitory effects on telomerase activity. Finally, using the E2F-1 knockout mice model, we found that TGFβ-mediated inhibition of *hTERT* expression is reversed in the E2F-1 null mutant cells, thus highlighting E2F-1 itself as critical to TGFβ-mediated repression of telomerase activity. A better understanding of the mechanisms regulating *hTERT* gene expression in normal and cancer cells may prove useful for the development of cancer therapy.

2. Materials and methods

2.1. Plasmid constructions

hTERT-2k GFP reporter construct was digested with BamHI and KpnI to separate the *hTERT* promoter insert (−1934 to +78, ATG as +1, GenBank sequence gi: 4210970) from the pGFP vector. This *hTERT* promoter insert was ligated into pGL3-basic vector cut with BglII and KpnI. The resulting *hTERT* (−1934)-lux reporter construct was confirmed by sequencing. Sequential deletion mutants of *hTERT* promoter reporter were done using Erase-a-Base System (Promega, Madison, WI, USA) according to manufacturer instructions. All constructs were confirmed by sequencing.

2.2. RT-PCR

For reverse transcription reactions, total RNA was prepared from cells treated or not with TGFβ, using Trizol reagent (Invitrogen) according to the manufacturer's protocol. When used, inhibitors were added to the starvation media 30 min prior to starting the time course. cDNA synthesis was carried out using Superscript First Strand Synthesis System for RT-PCR (Invitrogen) using random primers and 5 μg of total RNA. Primers sequences used for the PCR reactions of the different human genes were as follows: *hTERT* (LT5: 5′CGGAAGAGTGTCTGGAGCAA-3′; LT6: 5′-GGATGAAGCGGAGTCTGGA-3′); GAPDH (sense: 5′-ACCACCATGGAGAAGGCTGG-3′; antisense: 5′-CTCAGTGTAGCCCAGGATGC-3′); E2F1 (sense: 5′-TGCAGAGCAGATGGTTATGG-3′; antisense: 5′-ATCTGTGGTGAGGGATGAGG-3′); Densitometry analysis was performed using Alpha Innotech Corporation (San Leandro, CA) Fluorochem 8000 software version 3.04. The linear amplification range of each PCR was tested on the adjusted cDNA. The conditions were chosen so that none of the RNA analyzed reached a plateau at the end of the amplification protocol, i.e. they were in the exponential phase of amplification.

2.3. Cell culture

HaCaT, HuH7, MCF-7, MEFs and CHO cells were cultured in DMEM (Hyclone Laboratories Inc., Logan, UT) in the presence of 10% fetal bovine serum (FBS) (Hyclone) and 2 mM L-glutamine. All stimulations were done in serum-free media containing 100 pM TGFβ (Peprotech), for the periods of time indicated in the figures.

2.4. siRNA transfections

Smad2 and Smad3 siRNAs were purchased from Ambion and introduced into HaCaT cells by reverse transfection using Lipofectamine 2000 reagent (Invitrogen). Briefly, 10 nM of each siRNA was mixed with Lipofectamine 2000 reagent and Optimem media (Invitrogen) directly into 60 mm^2 plates. 7.5×10^5 HaCaT cells were then added to each transfection mix and incubated 72 h at 37 °C in a 5% CO_2 incubator. Cells were then trypsinized and fed into 6-well plates for 16 h. siRNA transfected cells were then incubated in serum-free media 30 min before adding TGFβ for the periods of time indicated in the figures. siRNA-Smad2-#1 (sense GGUCUCAUCAAUUAAAGCA, antisense: UGCUUUAAUUGAUGAGACC), siRNA-Smad2-#2 (sense GGUAAUGUAUCAUGAUCCA, antisense: UGGAUCAUGAUACAUUACC), siRNA-Smad3-#1 (sense GCACAUAAUAACUUGGACC, antisense: GGUCCAAGUUAUUAUGUGC), siRNA-Smad3-#2 (sense GGCCCAGUGCAUAUGCAAU, antisense: AUUGCAUAUGCACUGGGCC).

2.5. Immunoblotting

Cells were starved overnight in the absence of serum before being stimulated by TGFβ or activin. Cells were then harvested in RIPA buffer supplemented with 100 mM sodium vanadate, 1 mM phenyl methylsulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 2 μg/ml pepstatin. Whole-cell lysates were separated on a polyacrylamide gel, transferred to nitrocellulose and incubated with the indicated specific antibody overnight at 4 °C: [anti-hTERT (Calbiochem); anti-β-tubulin, anti-flag (Sigma); anti-phospho-Erk, anti-Erk, anti-phospho-p38, anti-p38 (Cell Signaling Technology), anti-phospho-Smad3 (BioSource); anti-Smad4 and anti-Smad2/3 (SantaCruz)]. After the primary antibody incubation, membranes were washed twice in TBST (50 mM Tris–Cl at pH 7.6, 200 mM NaCl, 0.05% Tween 20), and incubated with the proper secondary antibody coupled to horseradish peroxidase (Sigma) at 1:10,000 dilution) for 1 h at room temperature. Then, membranes were washed four times for 15 min in TBST. Immunoreactivity was normalized by chemiluminescence (ECL reagent, Roche) according to the manufacturer's

Fig. 2. TGFβ requires the Smad, Erk and p38 MAPK pathways to inhibit *hTERT* promoter activity. a) HaCaT cells transfected with *hTERT*-lux and increasing amounts of Smad7 cDNA (0.1 to 0.8 μg) were stimulated or not with TGFβ before being assessed for luciferase. b) HaCaT cells were transfected with two different sets of siRNAs against human Smad2 or Smad3 and Smad2 and 3 protein levels were analyzed by Western blot using an anti-Smad2/3 antibody (upper panel). HaCaT cells were then transfected with *hTERT*-lux and the different Smad2/3 siRNAs, as indicated, stimulated with TGFβ and assessed for luciferase (middle and lower panels). c) HaCaT cells were treated with PD98059, PD169316 or SB202190 as indicated, stimulated with TGFβ and protein phosphorylation levels were monitored by anti-phospho-Erk and anti-phospho-p38 Western blot. Equal protein levels were verified by immunoblotting with anti-Erk1/2 and anti-p38. d) HaCaT cells were transfected with *hTERT*-lux with or without the different MAPK inhibitors, at the indicated concentrations for 30 min, stimulated or not with TGFβ for 16 h and assessed for luciferase (upper panel). Following treatment of HaCaT cells with MAPK inhibitors, *hTERT* and GAPDH mRNA levels were measured by RT-PCR 9 lower panel).

instructions and revealed using an Alpha Innotech Fluorochem Imaging system (Packard Canberra, Montreal, Quebec, Canada). Densitometry analysis was performed using Fluorochem 8000 software (Alpha Innotech, San Leandro, CA) that allows quantitative analysis of chemiluminescence under non-saturating conditions.

2.6. Luciferase assays

HaCaT cells were transfected using Lipofectamine 2000 (Invitrogen) with 0.5 μg of luciferase reporter construct, 0.5 μg of β-galactosidase (pCMV-lacZ) expression vector and 0.1 μg to 0.8 μg of the different Smad expression vectors. For siRNA transfection, 10 nM of each Smad siRNAs were added to the transfection. The next day, cells were stimulated or not with TGFβ (100 pM) in starvation media. When inhibitors were used, they were added 30 min prior to TGFβ treatment. All experiments were repeated independently six times and the luciferase activity normalized to β-galactosidase values.

2.7. Statistical analysis

Results are expressed as mean±standard deviation. Differences were assessed by one-way ANOVA or the unpaired *t* test, where appropriate. $p<0.05$ was considered significant.

3. Results

3.1. TGFβ family members repress hTERT gene expression in a HDAC-dependent manner

To study the effect of TGFβ on *hTERT* expression, we used human epithelial cancer cell lines originating from different tissues (skin, breast and liver), as well as CHO cells. We first analyzed the effect of TGFβ on *hTERT* gene promoter activity. For this, 2 kb (−1978 to +73) of the *hTERT* gene promoter region was cloned in front of the luciferase gene. The resulting *hTERT*-lux construct was then transfected in the different cell lines, and the cells were stimulated or not with TGFβ. As shown in Fig. 1a, TGFβ significantly decreased *hTERT* gene promoter activity in all cell lines tested to various extents. The strongest effect was observed in HaCaT cells (76%±8% inhibition), while more modest in HuH7 cells (32%±13% inhibition) and CHO cells (46%±24% inhibition) to weak in MCF-7 cells (24%±4% inhibition). We then examined if the decrease of *hTERT* gene promoter activity translated into reduced *hTERT* mRNA and protein levels. As illustrated in Fig. 1b, TGFβ treatment of HaCaT cells resulted in a strong and rapid decrease of *hTERT* mRNA levels (left panels) followed by a significant decrease in *hTERT* protein level (right panels). Together, our results indicate that TGFβ acts as a potent inhibitor of *hTERT* expression in epithelial cancer cells.

Previous reports indicated that histone deacetylases (HDACs) repressed telomerase activity [7]. To assess whether inhibition of *hTERT* expression by TGFβ requires HDAC activity, we examined the effect of Trichostatin A (TSA), a class I and II HDAC inhibitor, on *hTERT* promoter activity. As shown in Fig. 1c, increasing concentrations of TSA fully reversed the inhibitory effect of TGFβ on *hTERT* promoter activity and endogenous *hTERT* mRNA level. To further elucidate whether both class I and II HDAC proteins were involved in *hTERT* regulation, we overexpressed class I (HDAC1) and class II (HDAC4, HDAC5) cDNAs in HaCaT cells and analyzed their effect on *hTERT* gene promoter activity in the presence or the absence of TGFβ. Interestingly, while class I HDAC1 did not have any significant effect on *hTERT* promoter activity (Fig. 1d), the two class II HDAC4 and HDAC5 significantly repressed *hTERT* gene promoter activity.

3.2. The Smad, Erk and p38 MAPK pathways are required for TGFβ-mediated hTERT inhibition

The receptor regulated (R-Smads) Smad2 and Smad3 are central to TGFβ signaling [14]. Blocking TGFβ receptor signaling using Smad7, a potent inhibitor that restrains Smad2/3 phosphorylation by the TGFβ type I receptor [15] and further targets the receptor complex to degradation [16,17] completely reversed the TGFβ inhibition of *hTERT* (Fig. 2a). This demonstrated that functional TGFβ receptors and proper Smad2/3 signaling are required for inhibition of *hTERT* expression. To further address the relative contribution of Smad2 and Smad3, we used specific siRNAs (2 sets for each Smad) to efficiently and selectively block their expression. As shown in Fig. 2b (upper panel), transfection of specific human Smad2 or Smad3 siRNAs led to potent inhibition of their relative expression. Importantly, the siRNAs were highly specific since Smad2 siRNAs did not affect the expression of the Smad3 protein and vice versa. Interestingly, blocking expression of Smad3, but not Smad2 led to a partial but significant reversal of the TGFβ inhibitory effect of *hTERT* gene promoter activity (Fig. 2b, middle and lower panels), indicating that the TGFβ inhibitory effect on *hTERT* promoter activity is specifically mediated through Smad3 and is independent of Smad2. This is consistent with a recent study showing that TGFβ-mediated inhibition of *hTERT* is Smad3-dependent [12]. The partial reversal observed with the Smad3 siRNA also suggested that the Smad pathway is required but not sufficient for TGFβ to inhibit *hTERT* expression and suggested the requirement of additional pathways. TGFβ signals through activation of the canonical Smad pathway, but has also been reported to use other intracellular signaling cascades such as the p38 and Erk MAP kinases [14]. To determine whether these pathways were activated by TGFβ, HaCaT cells were stimulated for different periods of time with TGFβ and the levels of Erk1/2 and p38 phosphorylation examined using specific phospho-Erk1/2 or phospho-p38 antibodies. As shown in Fig. 2c, both Erk1/2 and p38 kinases were strongly activated in response to TGFβ and these effects were specifically blocked when cells were treated with either a specific Erk1/2 inhibitor (PD98059) or two different specific p38 kinase inhibitors (PD169316 and SB202190).

To address the contribution of these two pathways in TGFβ-mediated regulation of the *hTERT* promoter, HaCaT cells were transfected with *hTERT*-lux and stimulated or not with TGFβ in the presence or absence of increasing concentrations of the specific inhibitors. As shown in Fig. 2d (upper panel), TGFβ-mediated inhibition of the *hTERT* promoter was significantly reversed when cells were pretreated with the Erk1/2 (PD98059) or with the two p38 (PD169316 and SB202190) inhibitors, while not affected in cells treated with a non-functional analog of the PD169316 inhibitor (SB202474). We then determined if this effect on the *hTERT* promoter was also observed at the mRNA level. Pre-treatment of HaCaT cells with PD98059 or PD169316

resulted in lower *hTERT* mRNA basal levels, as compared to cells treated with DMSO alone. However, the TGFβ effect on *hTERT* mRNA repression observed in cells treated with DMSO appeared to be blocked in cells treated with the two inhibitors (Fig. 2d, lower panel), further suggesting a role for Erk and p38 kinases in regulating *hTERT* inhibition by TGFβ.

3.3. The hTERT core promoter region is required for TGFβ inhibition

To further identify the *hTERT* gene promoter elements which confer the TGFβ response, progressive deletion mutants of the *hTERT* promoter were generated and assessed for their TGFβ responsiveness by luciferase assays. As shown in Fig. 3a, the results clearly indicate that the critical regulatory region for TGFβ-mediated inhibition of *hTERT* promoter activity was located between nucleotides −252 and +3. Interestingly, this 255 bp sequence of the *hTERT* promoter corresponds to the previously reported minimal promoter sequence necessary for its activity [7]. An internal deletion of this important regulatory region (−252 to +3 bp) was then introduced in the full length *hTERT* (−1934) gene promoter construct. As shown in Fig. 3b, this deletion mutant (*hTERT* Δ-252,+3-lux) construct lost its ability to respond to TGFβ. Interestingly, deletion of either half of the 255 bp region of the promoter (mutants *hTERT* Δ-252, −116-lux and Δ-116,+3-lux) did not reverse the TGFβ effect, indicating that both regions play a role in TGFβ-mediated inhibition of the *hTERT* promoter and suggesting the presence of redundant binding sites within the core region of the *hTERT* promoter (Fig. 3b).

3.4. E2F DNA binding elements are involved in TGFβ-mediated inhibition of the hTERT promoter

The −252 to +3 *hTERT* promoter region contains two E-box DNA binding sites and five GC-boxes. E-box DNA elements of the *hTERT* promoter are recognized by the Myc/Mad/Max transcription factor family. In many cases, c-myc expression parallels *hTERT* expression, in that both are increased in highly dividing cells and down regulated during differentiation. On the other hand, Mad overexpression results in decreased *hTERT* promoter activity [18]. Both c-myc and Mad protein expression levels are known to be controlled by TGFβ signaling [19–21]. For this reason and as it was previously suggested that TGFβ inhibits *hTERT* through a decrease of c-myc protein [9], we evaluated the importance of each E-box in the TGFβ-mediated decrease of *hTERT* promoter activity by specific point mutations resulting in the disruption of DNA binding [22]. Mutation of either or both E-box sites did not reverse the TGFβ inhibition of the promoter activity (Fig. 4a). The GC-boxes are DNA binding elements for the Sp1 transcription factor family. Sp1 transcription factors are known to cooperate with Smad proteins to

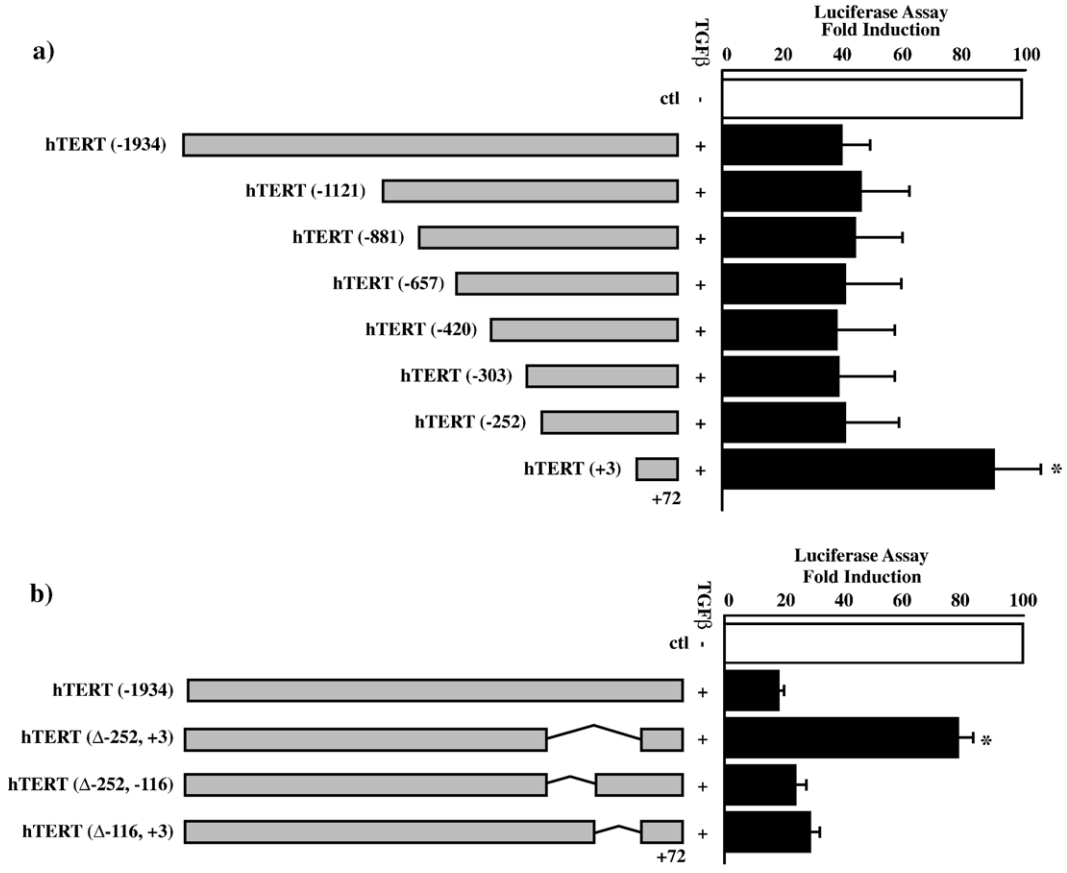


Fig. 3. The −252 to +3 region of the *hTERT* gene promoter is required for TGFβ-mediated inhibition of *hTERT*. Progressive deletion mutants a) or internal deletion mutants b) of the *hTERT* gene promoter were transfected in HaCaT cells and assessed for luciferase in response to TGFβ.

regulate expression of several target genes [23]. However, mutations of the GC-box sites within the *hTERT* promoter, alone or in combination, did not affect TGFβ-mediated decrease of *hTERT* promoter activity, also ruling out the involvement of these sites in the TGFβ response (data not shown).

The −252 to +3 region also contains four E2F DNA binding elements and the dynamic assembly of the E2F/pocket protein/HDAC complex has been suggested to play a role in the regulation of *hTERT* [24]. Thus, we evaluated the potential role of the E2F DNA binding sites in TGFβ-induced *hTERT* repression by

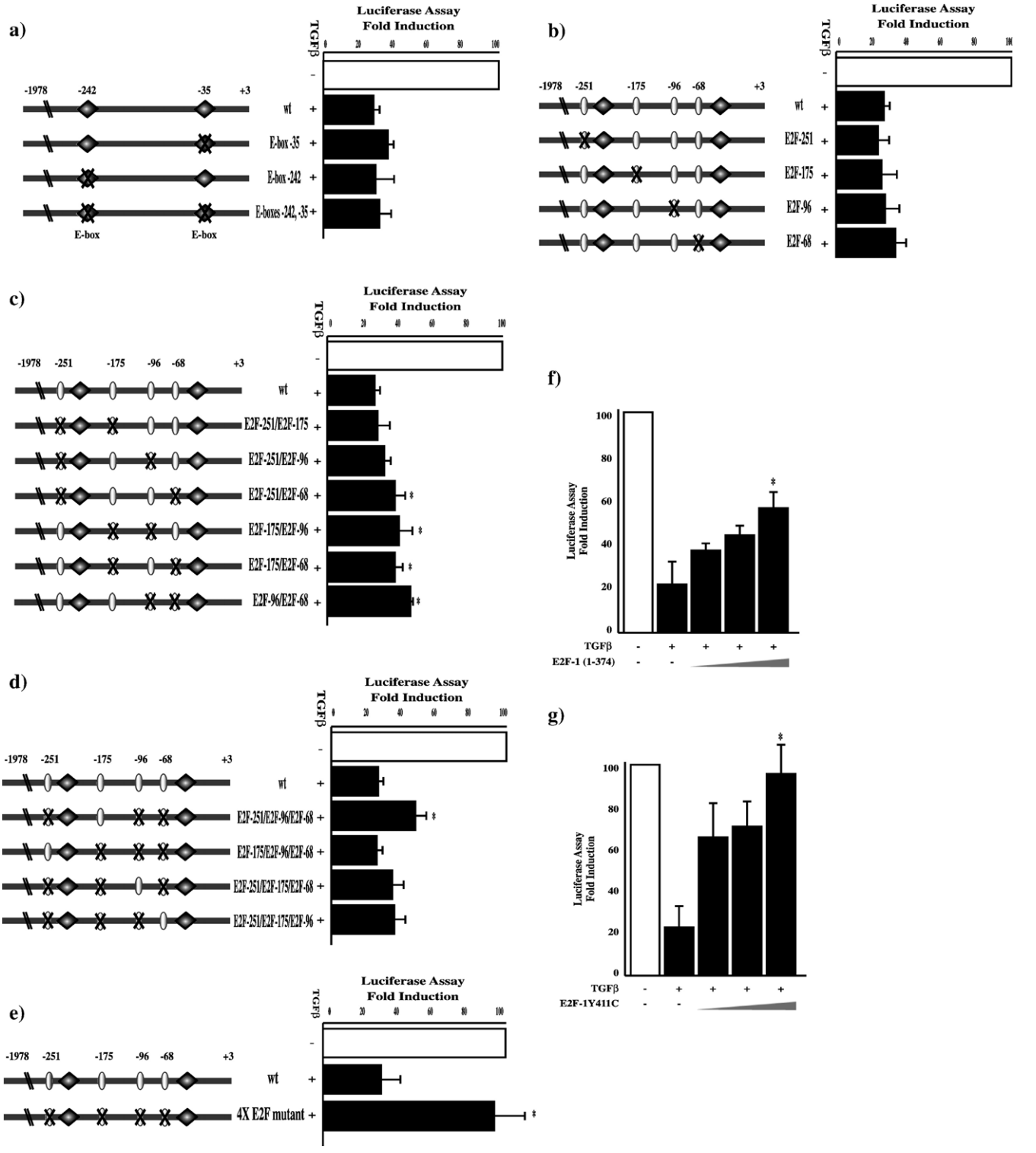


Fig. 4. E2F is required for TGFβ-mediated inhibition of *hTERT*. E-box mutants a) and single b), double c), triple d) or quadruple e) E2F binding site mutants of the *hTERT* (−1934)-lux construct were transfected in HaCaT cells as indicated and assessed for luciferase in response to TGFβ. f, g) Dominant negative forms of E2F, f) (E2F-1 (1–374) and g) E2F-1 (Y411C) were transfected in HaCaT cells and luciferase activity assessed in response to TGFβ.

mutational analysis. Interestingly, while single, double and triple mutations of the E2F binding sites had little or no effect (Fig. 4b, c and d, respectively), removal of all four E2F DNA sites (4XE2F mutant) completely reversed the TGFβ inhibitory response (Fig. 4e), suggesting that TGFβ inhibitory effect on *hTERT* promoter activity is mediated through several E2F binding sites located throughout the 255 bp core promoter region.

To then investigate whether E2F transcription factors are required for TGFβ to inhibit *hTERT* expression, we used two dominant negative forms of E2F. We first used E2F-1(1–374), which only contains the DNA binding domain. Overexpression of this mutant was previously shown to act as a dominant negative by displacing endogenous E2F-complexes from E2F DNA binding sites [25]. Transcriptional activity of E2F family members is regulated by interactions with pocket proteins (Rb, p107, p130) that recruit HDAC proteins to repress target genes [26,27]. Thus, we also used a mutated form of E2F-1 (Y411C) which is unable to bind pocket proteins [25]. Interestingly, overexpression of increasing amounts of either dominant negative E2Fs significantly reversed TGFβ-mediated inhibition of the *hTERT* promoter (Fig. 4f, g). Altogether, our results support the hypothesis that TGFβ inhibits telomerase activity through binding of an E2F/repressor complex, within the proximal region of the *hTERT* promoter.

3.5. TGFβ-mediated repression of hTERT is lost in embryonic fibroblasts E2F-1 null mutant mice

To further define the role and contribution of E2F downstream of TGFβ in normal cells, we used mouse embryonic fibroblasts (MEFs) isolated from the E2F-1 knockout mice [28]. Wild type (+/+) or E2F-1 null mutant (−/−) MEFs were stimulated or not with TGFβ and the level of Smad phosphorylation assessed by Western blot. As shown in Fig. 5a, the wild type and E2F-1 (−/−) MEFs responded to TGFβ. To next analyze the contribution of E2F-1 to TGFβ-mediated inhibition of *mTERT*, MEFs from wild type and E2F-1 null mice were stimulated with TGFβ for different periods of time and *mTERT* mRNA and protein levels were analyzed by RT-PCR and Western blot respectively. As shown in Fig. 5b and c, while TGFβ potently inhibited both *mTERT* mRNA and protein levels in wild type cells, this effect was lost in the E2F-1 knockout cells, further highlighting the critical role played by E2F-1 in TGFβ-mediated repression of *mTERT* expression.

4. Discussion/conclusions

In this study, we show that TGFβ inhibits *hTERT* expression in both normal and cancer cells. Our results indicate that the TGFβ effects on *hTERT* repression are mediated through the canonical Smad pathway but also require the activation of p38 and Erk kinases. Activation of these three pathways is necessary to decrease *hTERT* expression in response to TGFβ. We also found that E2F and HDAC activity are necessary for the mediation of the TGFβ inhibitory effects on *hTERT* expression. We further identified four critical E2F binding sites, within the proximal region of the core *hTERT* promoter, that confer the TGFβ response. Finally, using the E2F-1 (−/−) MEFs we show that the loss of E2F-1 abolishes the TGFβ inhibitory effect on TERT expression in normal mouse embryonic fibroblasts. Together, our results highlight the prominent role played by TGFβ in regulating telomerase activity and place E2F-1 center stage in the mediation of these effects in both normal and cancer cells.

Replicative senescence is a telomere-dependent mechanism that defines a limited number of successive cell divisions in somatic cells [29]. All dividing cells exhibit a progressive shortening of their telomeres due to the lack of *hTERT* expression observed in most human somatic cells [29]. Critically shortened telomeres then lead to permanent growth arrest or senescence. Our results indicate that TGFβ signaling plays a major role in suppressing *hTERT* expression and as most normal human cell types respond to TGFβ, this suggests that this growth factor provides a protective barrier against abnormal *hTERT* expression, thereby contributing to replicative senescence in normal somatic cells.

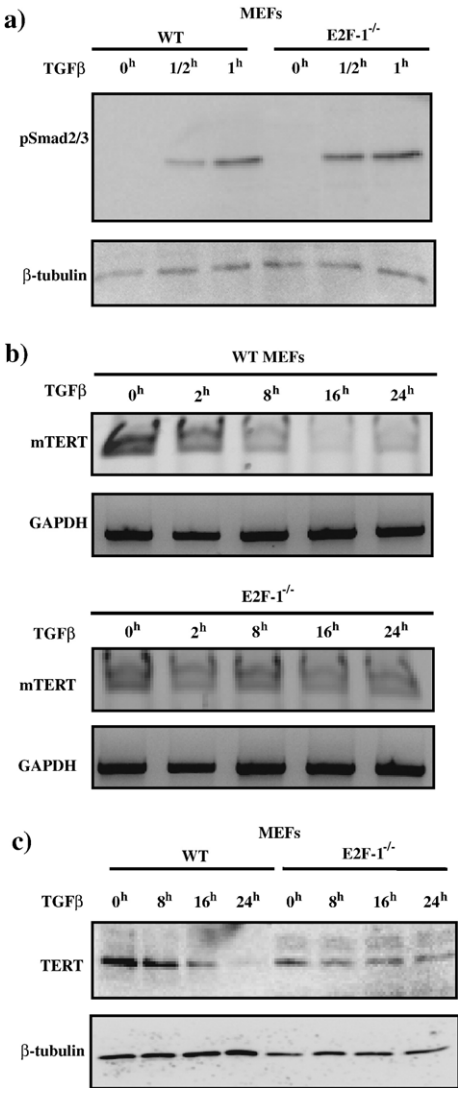


Fig. 5. TGFβ-mediated repression of *mTERT* is lost in the E2F-1 null mutant mice. Wild type and E2F-1 (−/−) MEFs were stimulated or not with TGFβ for the indicated times and phospho-Smad3 a), *TERT* mRNA b) and *TERT* protein c) levels were assessed by Western blotting and RT-PCR.

Our data indicate that Smad3 but not Smad2 is important for *hTERT* gene regulation by TGFβ. This is in agreement with previous observations by Li et al. highlighting *hTERT* as a Smad3-specific target gene [12]. Smad3 is essential but not sufficient for TGFβ to repress *hTERT* gene expression, which also requires both the Erk and p38 kinase pathways. In other cell systems, such crosstalk between these three pathways have been described to be important for the activation of the aggrecan gene [30] and the collagenase-3 gene [31], downstream of TGFβ. These results further strengthen the current paradigm that, in addition to the canonical Smad pathway, TGFβ signals through different cascades in a cell type dependent manner [31].

Previous studies investigating the role of E2F-1 in *hTERT* gene regulation have generated some controversial results. While some studies suggested that E2F-1 was required for telomerase activity in mouse and human cancer cells [32], others showed that E2F-1 induced repression of the *hTERT* gene [24, 33,34]. It has also been proposed that E2F-1 exerts opposing regulatory roles in *hTERT* gene expression, by repressing *hTERT* in cancer cells, while activating the *hTERT* gene in normal somatic cells [35]. Our results indicate that the transcription factor E2F-1 plays a central role in regulating telomerase activity and that E2F-1 effects, at least downstream of TGFβ signaling, clearly lead to *hTERT* repression in normal and cancer cells.

A previous report, using the breast cancer cell line MCF7, suggested that SIP1, a TGFβ downstream effector, plays a role in regulating the *hTERT* promoter [13]. In another study, also using MCF7 cells, a Smad binding element (SBE) located between −262 and −259 of the *hTERT* gene promoter was shown to be involved in TGFβ inhibition of the *hTERT* promoter [12]. Our results, however, indicate that this particular SBE is not critical for TGFβ-mediated *hTERT* repression in human keratinocyte (HaCaT) cells. As seen in Fig. 1, the TGFβ effect on *hTERT* repression in MCF7 is much weaker that that observed in other cell types, thus it is conceivable that the TGFβ effects in that particular cell line may differ from those observed in other cell types. Downregulation of c-myc has also been previously suggested as a mechanism by which TGFβ could regulate *hTERT*. However, our results indicate otherwise, at least in the cell lines used in our studies. Indeed, TGFβ still potently repressed transcription of the *hTERT* gene promoter when both c-myc binding sites are removed. This is consistent with a recent study in MCF-7 cells showing that TGFβ negatively regulates telomerase activity via Smad3 interactions with c-myc and the *TERT* gene promoter, independent of c-myc downregulation [12]. However, in light of our results, it is unlikely that a Smad3/c-myc interaction plays a role in *hTERT* repression in human keratinocytes. This suggests that TGFβ utilizes distinct mechanisms to repress telomerase activity in a cell specific manner.

Recent studies have suggested a role for HDACs in *hTERT* gene repression in normal cells. Trichostatin A treatment results in increased telomerase activity [7, 36–38] and HDAC complexes are shown to be recruited to the *hTERT* promoter via uncharacterized factors; Sp1 and/or Rb/E2F being potential candidates. Furthermore, a recent and elegant study has demonstrated that the assembly of complexes made up of E2F, pocket proteins and HDAC regulates *hTERT* gene expression in normal human fibroblasts [24]. A role for E2F in regulating *hTERT* activity was previously suggested, as E2F overexpression in human cells led to telomerase repression [36]. Another study demonstrated that endogenous p53 represses *hTERT* expression through a p21-and E2F/Rb-dependent pathway [37]. p53-induced p21 expression leads to decreased pRb phosphorylation and induces the recruitment of E2F family members and histone deacetylases to form complexes that inhibit transcription [37]. These data are complementary to our results and combined, these studies highlight E2F and HDAC proteins as central mediators of TGFβ-induced telomerase repression.

TGFβ is a very potent negative regulator of the cell cycle, which can activate the expression of the cyclin-dependent kinase inhibitors p15 and p21, and repress Id proteins and c-myc in most cell types. Repression of c-myc by TGFβ has been extensively characterized. It involves interactions between Smad3, E2F4/5, the co-repressor p107 and HDAC activity [39]. Moreover, repression of cdc25A also involves E2F and HDAC [40]. It is interesting to note that in addition to c-myc and cdc25A, two critical cell cycle regulators, TGFβ also modulates *hTERT* expression through cooperation between the Smad proteins and E2F transcription factors. Thus these transcription factors appear to be critical regulators of TGFβ cytostatic responses.

By inhibiting the cell cycle, inducing apoptosis and preventing immortalization through the inhibition of telomerase activity, TGFβ exerts strong tumor suppressive effects. TGFβ-mediated inhibition of telomerase activity is of profound impact for this growth factor's tumor suppressive role. While *hTERT* involvement in cell immortalization is well characterized, recent studies indicated that telomerase possesses additional functions that are not related to net telomere length. In fact, increased telomerase expression enhances tumor formation even in the presence of very long telomeres in mice [41–44]. Overexpression of *hTERT* in human epithelial or neural cultured cells induces resistance to pro-apoptotic or anti-proliferative signals, including TGFβ [45]. In fibroblast cells, overexpression of *hTERT* with H-Ras produced tumors in nude mice, while a defective form of *hTERT*, which is unable to lengthen telomeres, is still able to cooperate with H-Ras to induce tumor formation. This suggests that the *hTERT* effect on tumor progression includes non-telomere function [46]. A recent study using microarrays demonstrated that telomerase is able to stimulate proliferation of epithelial cells by controlling expression of genes involved in cell proliferation [47]. Thus, the TGFβ inhibitory effect on *hTERT* activity not only leads to repression of immortalization, but might also represent an important component of the cytostatic program induced by this growth factor to inhibit cell proliferation.

Deregulation of E2F-1 function is common in most human cancers and interestingly, like TGFβ, E2F-1 plays a dual role in cancer, acting as both a tumor suppressor and a tumor promoting agent. The oncogenic properties of E2F-1 and its regulatory role in the transition of the cell cycle from G1 to S phase, in order to activate genes required for DNA synthesis and cell cycle control are well characterized [48]. However, mice studies have revealed that E2F-1 could also act as a tumor suppressor gene. E2F-1

knockout mice exhibit apoptosis defects in thymocytes and develop highly malignant tumors [28, 49], whereas transgenic mice expressing E2F-1 display aberrant cell apoptosis [50]. Thus, it is conceivable that, in addition to its inhibitory role on telomerase activity, E2F-1 may mediate some of the TGFβ pro-apoptotic responses. This role of E2F-1 in cell death and tumor suppression raises an interesting prospect as to its potential use in targeted therapy for human cancer.

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

J Korah[1], N Falah[1], A Lacerte[1] and JJ Lebrun*[,1]

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 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 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 types[5,6] 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

[1]Division 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; TGFβ, 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 TGFβ-mediated inhibition of hTERT (human telomerase reverse transcriptase)[11] and that TGFβ 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 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 onto 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 (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 TGFβ treatment in a time-dependent manner (Figures 1a and b). To address the contribution of E2F1 in mediating this TGFβ response, we used RNA interference to reduce the expression of endogenous E2F1. Interestingly, we found that the effect of TGFβ 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 TGFβ pro-apoptotic response in multiple cell lines of various origins.

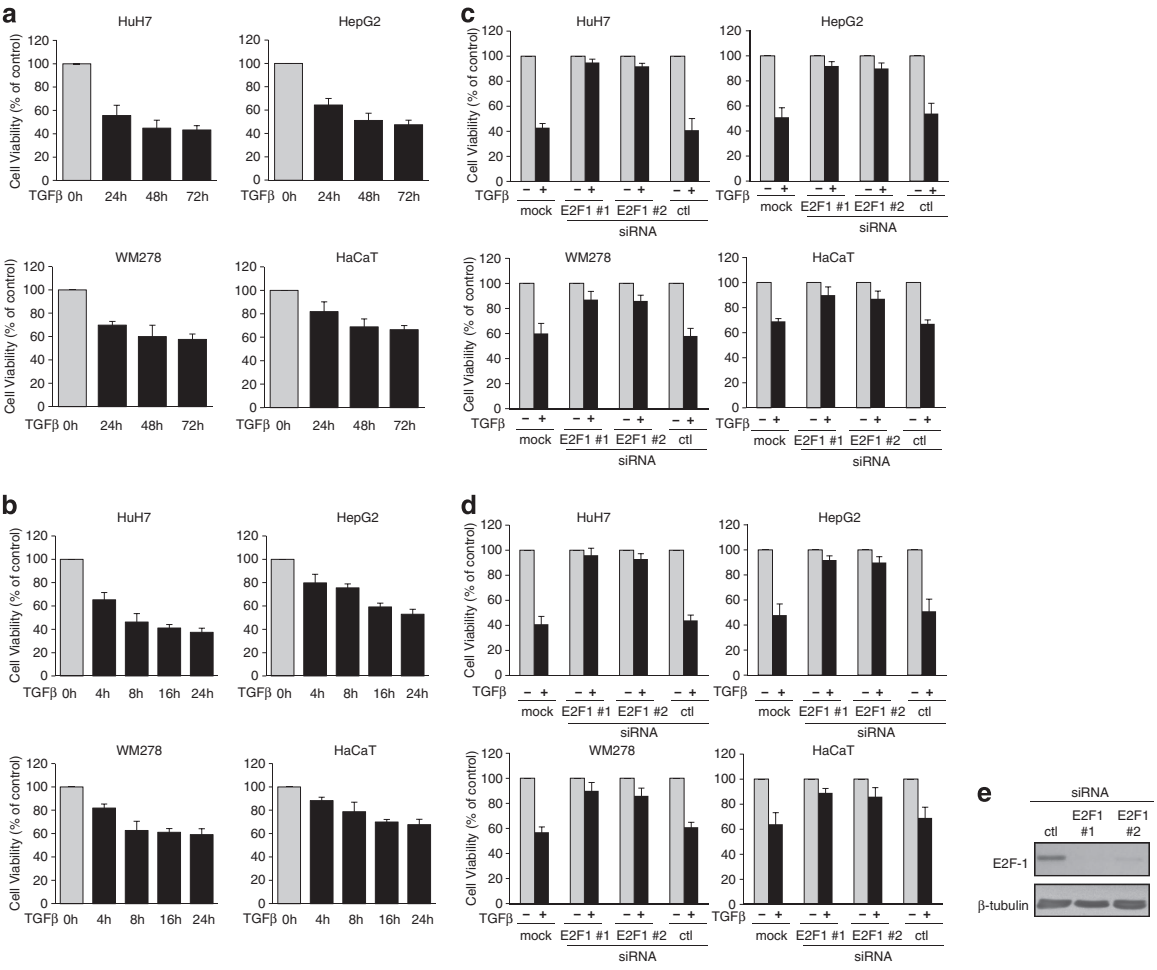
To further investigate the role of E2F1 in TGFβ-mediated apoptosis, we performed fluorescence-activated cell sorting (FACS) following AnnexinV and propidium iodide staining. Although TGFβ treatment markedly increased the number of apoptotic cells in control siRNA-transfected HuH7 cells (Figure 1f, left 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 TGFβ has a strong pro-apoptotic function in various cell lines and that these effects require the transcription factor E2F1.

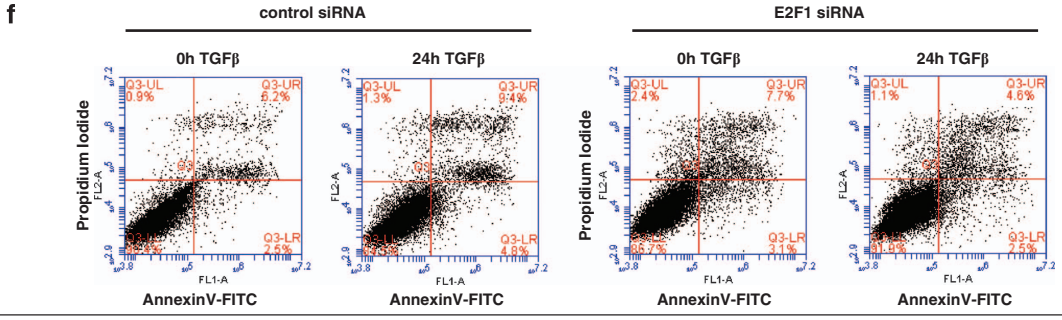
E2F1 is required for TGFβ-mediated regulation of pro-apoptotic target genes. TGFβ signaling activates multiple pro-apoptotic genes and pathways in a cell- and tissue-specific manner.[4] Independently of TGFβ, the E2F pathway is also involved in multiple distinct apoptotic mechanisms. In varying cell 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 TGFβ and E2F1 share any common downstream apoptotic targets, we examined the regulation of representative E2F1-responsive pro-apoptotic genes in TGFβ-treated human hepatoma HuH7 cells, which express both functional p53 and pRb. As shown in Figure 2a, TGFβ potently induced mRNA expression of *Apaf1*, *Caspase 3*, *Caspase 7*, *p73*, and *Smac/DIABLO*, suggesting that TGFβ induces apoptosis in HuH7 cells by the intrinsic mitochondrial pathway. Importantly, this analysis also revealed *Smac/DIABLO* as a novel TGFβ target. Loss of E2F1 expression markedly impaired the TGFβ-mediated induction of each of these target genes (Figure 2b), indicating that E2F1 is required for TGFβ-mediated regulation of its pro-apoptotic downstream target genes. Moreover, these data provide a novel pathway by which TGFβ regulates these genes and reveals E2F1 as a widespread co-transducer of TGFβ-induced activation of the intrinsic mitochondrial pathway.

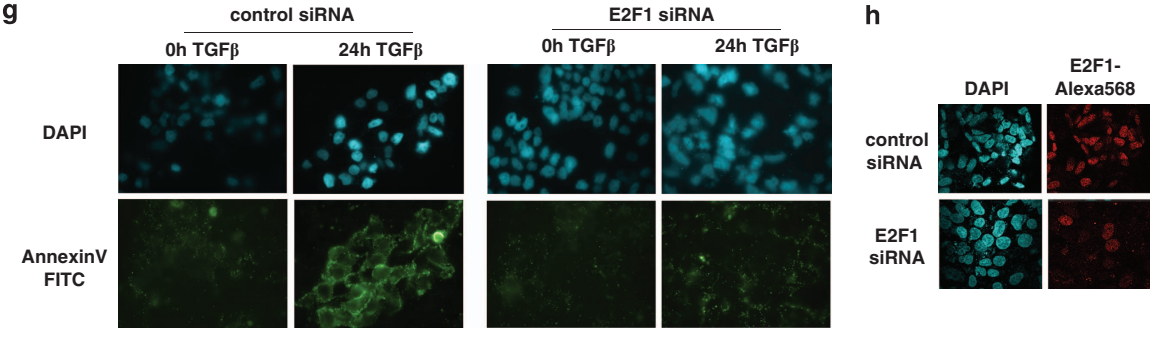
To then examine whether these pro-apoptotic genes are direct targets of TGFβ, cells were treated or not with the

Figure 1 TGFβ-mediated apoptosis is dependent on E2F1. (**a** and **b**) The specified cell lines were untreated or treated with TGFβ (100 pM) for the indicated times and assessed for cell viability by (**a**) MTT and (**b**) calcein-AM assays. Data are represented as mean ± 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 TGFβ 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 ± S.D. (**h**) Expression of endogenous E2F1 in these cells was assessed by immunofluorescence





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



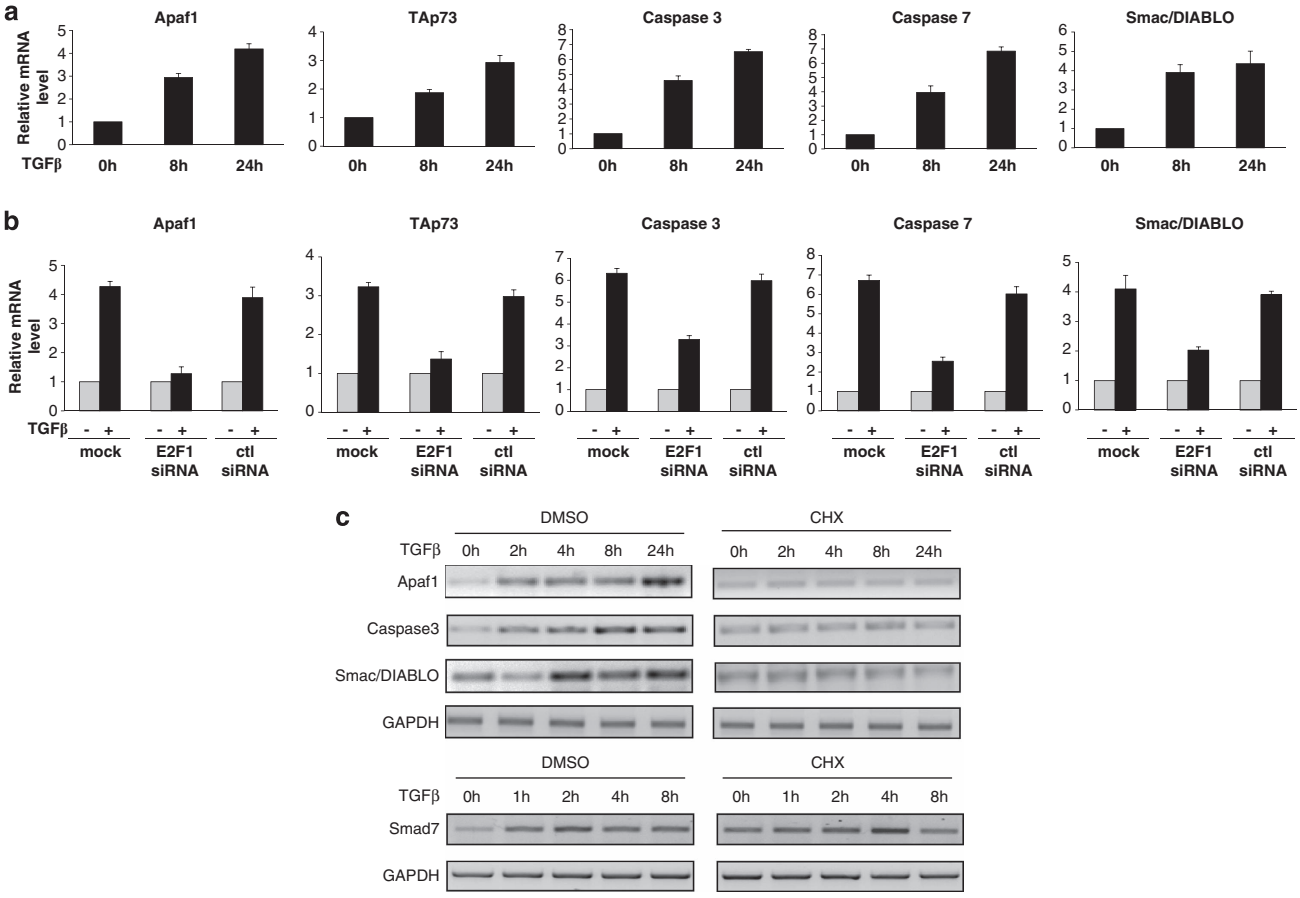


Figure 2 E2F1 is required for TGFβ-mediated regulation of proapoptotic genes. (**a**) HuH7 cells were stimulated with TGFβ (100 pM) and mRNA levels for the indicated genes were measured by real-time qPCR. Results are normalized to *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and shown relative 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 down-stream of TGFβ-mediated cell death in normal cells. For this,

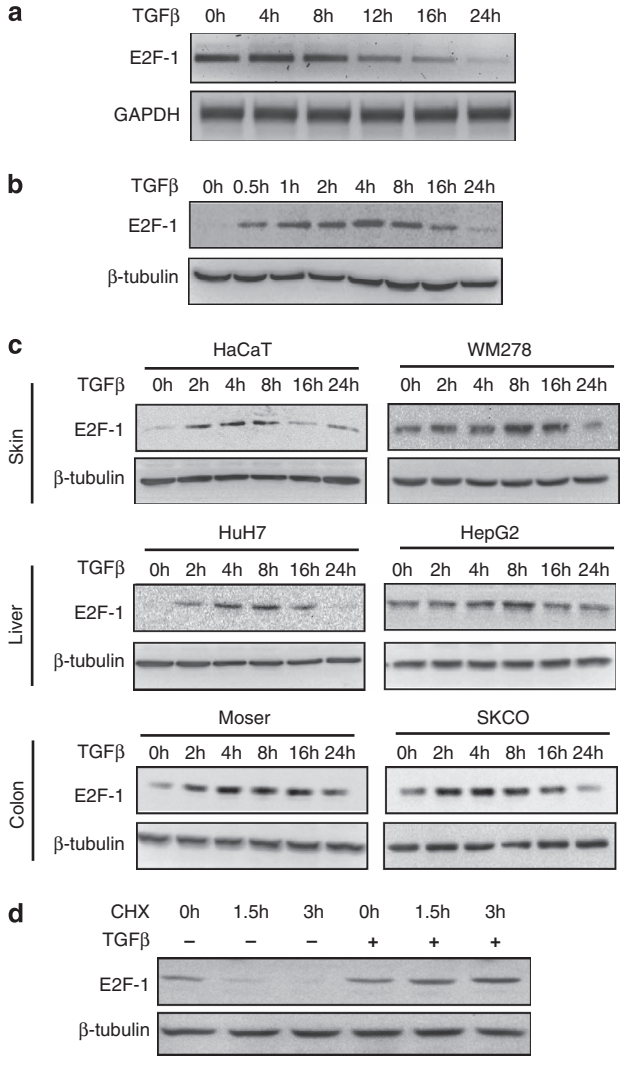


Figure 3 TGFβ rapidly and transiently induces E2F1 protein expression levels. HaCaT cells were stimulated with TGFβ (100 pM) for the indicated times and subjected to (**a**) RT-PCR followed by DNA gel electrophoresis and (**b**) western blotting to measure E2F1 RNA and protein levels, respectively. (**c**)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

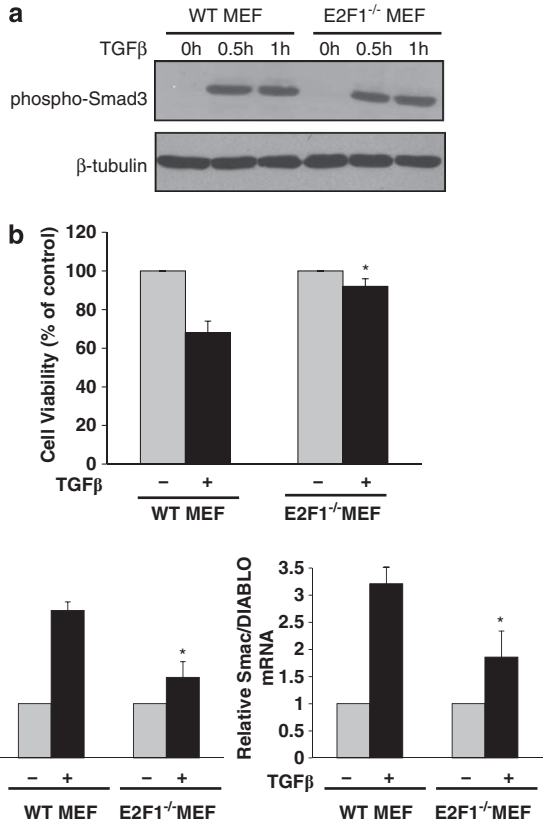


Figure 4 TGFβ pro-apoptotic effects are impaired in E2F1-null embryonic fibroblasts. (**a**) Wild-type (WT) and E2F1$^{-/-}$ mouse embryonic fibroblasts (MEFs) were untreated or treated with TGFβ (100 pM) for the indicated times and phospho-Smad3 levels of total cell lysates were analyzed by western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

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 TGFβ stimulation, as assessed by the induction of Smad phosphorylation (Figure 4a). The pro-apoptotic effect of TGFβ, however, greatly differed in these two cell types. Athough cell viability of the wild-type E2F1$^{+/+}$ MEFs was potently decreased in response to TGFβ, this effect was severely impaired in the E2F1$^{-/-}$ MEFs (Figure 4b). Correspondingly, TGFβ-induced expression of *Caspase 7* and *Smac/DIABLO* was significantly reduced in the E2F1$^{-/-}$ MEFs (Figure 4c). Together, these findings highlight a critical role for E2F1 downstream of TGFβ 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 TGFβ-mediated apoptosis. To further understand the molecular mechanisms underlying the role of E2F1 in the induction of programmed cell death by TGFβ, 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 TGFβ on cell viability in HuH7 cells (Figure 5a). The antagonistic effects of these E2F1 mutants were further

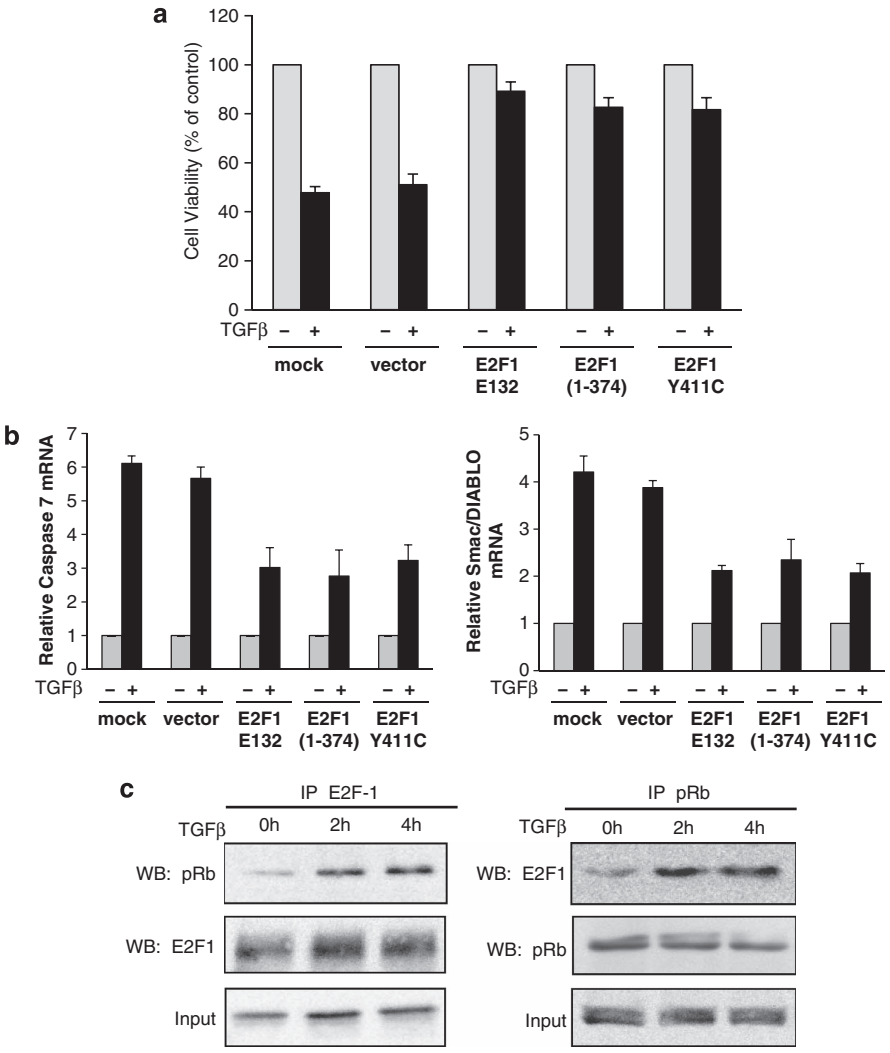


Figure 5 E2F1 DNA-binding, transactivation, and pRb-interaction are required for TGFβ-mediated apoptosis. HuH7 cells transiently transfected with empty vector or mutant E2F1 expression constructs as indicated were untreated or treated with 100 pM TGFβ for 24 h. (**a**) Cell viability was assessed by calcein-AM assay, with bars representing means ± 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 ± S.D., expressed as relative to levels observed in untreated cells (set to 1). (**c**) HuH7 cells untreated or treated with TGFβ (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 TGFβ-induced *Caspase 7* and *Smac/DIABLO* mRNA levels (Figure 5b). These results indicate that TGFβ 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 TGFβ could induce association between endogenous E2F1 and pRb using co-immunoprecipitation studies. As shown in Figure 5c, TGFβ treatment indeed promotes the association between E2F1 and pRb. Altogether, these results indicate that pRb-E2F binding is required for TGFβ to induce apoptosis and that this association is induced by TGFβ itself, strongly supporting the fact that the pRb-E2F1 protein complex has a role downstream of TGFβ-mediated cell signaling, leading to apoptosis.

TGFβ 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 TGFβ while remaining in its seemingly transcriptionally repressive pRb-E2F complex. Thus, we assessed whether TGFβ could in fact recruit positive regulators of transcription to the pRb-E2F1 complex. As TGFβ 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* TGFβ-treated cells. Interestingly, as shown

in Figure 6a, we found that TGFβ 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 TGFβ. Loss of P/CAF expression by RNA interference dramatically reduced the TGFβ pro-apoptotic effect in these cells (Figure 6b). Moreover, the TGFβ-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

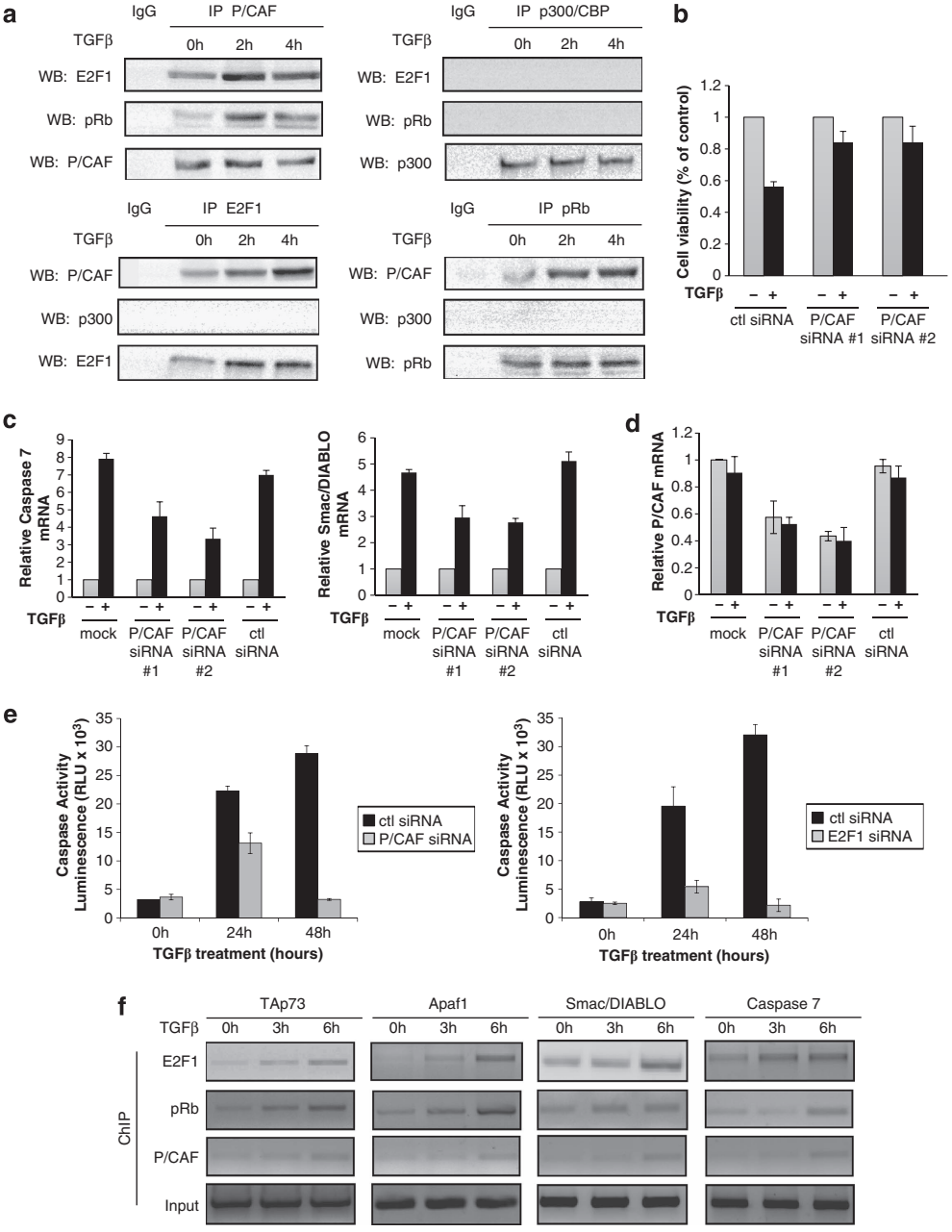


Figure 6 TGFβ induces formation of a transcriptionally active complex between pRb/E2F1 and the acetyltransferase P/CAF, onto pro-apoptotic gene promoters. (**a**) Untreated and TGFβ-treated HuH7 cells were subjected to immunoprecipitation (IP) with the specified antibodies followed by western blotting (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 TGFβ (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 TGFβ (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 TGFβ (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 TGFβ-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 TGFβ-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 TGFβ-induced caspase activation. Collectively, these findings support a critical role for P/CAF downstream of TGFβ in the E2F1-dependent activation of pro-apoptotic genes and the mediation of programmed cell death.

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

Discussion

Although various apoptotic mediators and signaling pathways have been implicated in TGFβ-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 TGFβ–E2F1 signaling axis, and highlights the pRb–E2F1–P/CAF pathway as a wide-ranging and critical mediator of the TGFβ apoptotic program in multiple target tissues.

We identified a number of key pro-apoptotic TGFβ target genes that trigger the intrinsic apoptosis pathway through the induction of E2F1. Although these genes are functionally interrelated, our results imply that TGFβ 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 TGFβ-mediated cell death. Importantly, these results are corroborated using the E2F1 knockout mouse model, demonstrating that the TGFβ–E2F1 signaling pathway mediates TGFβ-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 actyltransferase (P/CAF) to activate transcription of pro-apototic genes in response to TGFβ. Indeed, analysis with dominant-negative E2F1 mutants revealed that, in fact, pRb binding to E2F1 is required for TGFβ-mediated apoptosis.

Our results also indicate that TGFβ 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 TGFβ 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 TGFβ 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 TGFβ, 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 TGFβ 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 TGFβ 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 TGFβ 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 TGFβ pro-apoptotic targets. Taken together, these results strongly support a pro-apoptotic role for the E2F1 pathway downstream of TGFβ and provide a potential mechanism for the activation of E2F1-responsive pro-apoptotic genes in response to TGFβ.

It is interesting to consider that TGFβ tumor-suppressive effects might utilize the functional interplay among the E2F family members, which affects E2F activity. It is well-established that TGFβ 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-myc[41] by means of Smad–E2F4/5–pocket protein repressor complexes. The rapid surge in E2F1 that we observe in response to TGFβ may thus effectively

initiate the TGFβ apoptotic program, without affecting cell cycle, as TGFβ 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 TGFβ treatment.[28] Thus, it is conceivable that TGFβ 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 TGFβ, further preventing cell-cycle progression.

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

(1) TGFβ 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) TGFβ 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 TGFβ 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 TGFβ tumor-suppressive effects. A better understanding of the mechanisms by which both TGFβ 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% CO_2 conditions. Before treatment, cells were serum-starved for 24 h and all stimulations were done in serum-free medium containing 100 pM TGFβ1 (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 pM TGFβ. Mitochondrial viability was determined by MTT colorimetric assay. Briefly, following 24–72 h of TGFβ 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 TGFβ treatment, original culture medium was replaced with serum-free medium containing 2 μg/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 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin.

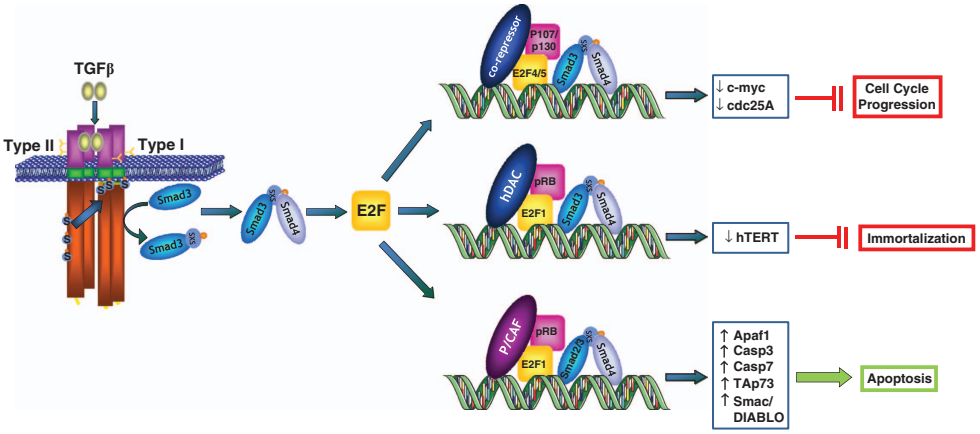


Figure 7 The pRb/E2F signaling pathway mediates three distinct arms of TGFβ tumor-suppressive effects. See text for details

Table 1 PCR primer sequences

Apaf1 forward	5′-CTCTCATTTGCTGATGTCGC-3′
Apaf1 reverse	5′-TCGAAATACCATGTTTGGTCA-3′
TAp73 forward	5′-CATGGAGACGAGGACACGTA-3′
TAp73 reverse	5′-CTGTAACCCTTGGGAGGTGA-3′
Caspase3 forward	5′-AGCGAATCAATGGACTCTGG-3′
Caspase3 reverse	5′-CGGCCTCCACTGGTATTTTA-3′
Caspase7 forward	5′-GCAGTGGGATTTGTGCTTCT-3′
Caspase7 reverse	5′-CCCTAAAGTGGGCTGTCAAA-3′
Smac/DIABLO forward	5′-AATGTGATTCCTGGCGGTTA-3′
Smac/DIABLO reverse	5′-AGCTGGAAACCACTTGGATG-3′
GAPDH forward	5′-GCCTCAAGATCATCAGCAATGCCT-3′
GAPDH reverse	5′-TGTGGTCATGAGTCCTTCCACGAT-3′
Smad7 forward	5′-TCCTGCTGTGCAAAGTGTTC-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 TGFβ treatment, cells were collected by trypsinization, pelleted by centrifugation, washed with PBS, and each sample was incubated with 0.5 μg Annexin V-FITC and 10 μl propidium iodide (50 μg/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 TGFβ 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 μl in medium supplemented with 2% FBS, and in the presence or absence of 100 pM TGFβ. 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 TGFβ 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-mm^2 plates and grown to 85% confluence. Following overnight serum-starvation, the cells were incubated, in the presence or absence of 100 pM TGFβ, with 50 μg/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′-TTTGGGCACTTGGAGCGCG-3′
Caspase7 reverse	5′-AAGAGCCCAAAGCGACCCGT-3′
Smac/DIABLO forward	5′-TTCCCTTCAAGCCCTGGCCCGAAC-3′
Smac/DIABLO reverse	5′-ACGCCCCCACCCAAGGAAGCAGTC-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 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/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 μg of the indicated antibodies. Antibody–protein–DNA complexes were then isolated by immunoprecipitation with 40 μl 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 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 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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