Chapter 5
TGF-β as Tumor Suppressor: In Vitro Mechanistic Aspects of Growth Inhibition

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Abstract Over time, TGF-β has emerged as both a tumor promoter and a tumor suppressor, depending on the cell type. Although it was first characterized as having the capacity to transform mesenchymal cells, many studies have shown it to exert an anti-proliferative effect on epithelial, endothelial, and immune cells. Most of the functional studies performed during the two decades following its discovery focused on its tumor-suppressive role and addressed a detailed understanding of the signaling events and molecular mechanisms by which TGF-β orchestrated cell growth arrest and apoptosis. This chapter entitled "TGF-β as tumor suppressor: in vitro mechanistic aspects of growth inhibition" depicts how TGF-β protects cells from transformation at the transcriptional, translational, and posttranslational levels. After a detailed view of the cytostatic program elicited by TGF-β to control the cell cycle, the focus shifts to TGF-β signaling pathways and biochemical events by which this cytokine governs the apoptotic program.

Keywords Apoptosis • Cell cycle regulation • Cytostasis • Posttranslational events • Smad signaling • Smad-independent pathways • Transcriptional regulation

Abbreviations

ATF/CREB	Activation transcription factor/cAMP responsive element-binding
bHLH	Basic helix-loop-helix
CDKs	Cyclin-dependent kinases

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A. Moustakas and K. Miyazawa (eds.), *TGF-β in Human Disease*,
DOI 10.1007/978-4-431-54409-8_5,

ChIP	Chromatin immunoprecipitation
CKIs	Cyclin-dependent kinase inhibitors
CTGF	Connective tissue growth factor
eEF1A1	Eukaryotic elongation factor 1A1
eIF4E	Eukaryotic translation initiation factor-4E
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FHBE	Forkhead-binding element
HDAC1	Histone deacetylase 1
HIPK2	Homeodomain-interacting protein kinase 2
IAP	Inhibitor of apoptosis
ID	Inhibitor of DNA binding/inhibitor of differentiation
INK4	Inhibitor of CDK4
JNK	c-Jun N-terminal kinase
MAP	Mitogen-activated protein
MAPK(K)	Mitogen-activated protein kinase (kinase)
MOMP	Mitochondrial outer membrane permeabilization
NGF	Nerve growth factor
PDGF	Platelet-derived growth factor
RB	Retinoblastoma protein
SBE	Smad binding element
TAK1	TGF-β-activated kinase 1
TGF-β	Transforming growth factor β
TIE	TGF-β inhibitory element
TIEG1	TGF-β-induced early gene 1
TNF	Tumor necrosis factor
TSS	Transcriptional start site
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
4E-BP1	eIF4E-binding protein 1

5.1 Introduction

Transforming growth factor-β (TGF-β) was first discovered as a tumor-promoting factor by supporting anchorage-independent growth of untransformed rat fibroblasts (Roberts et al. 1980; Roberts et al. 1981) and was further demonstrated to stimulate proliferation of different cell types in vitro such as mesenchymal-derived cells, like osteoblasts (Battegay et al. 1990), or glial cells, such as Schwann cells (Guenard et al. 1995; Ridley et al. 1989). However, the proliferative response induced by TGF-β in these cells appeared later on to be mostly mediated indirectly through autocrine secretion of other soluble factors, such as platelet-derived growth factor (PDGF) (Battegay et al. 1990; Leof et al. 1986; Soma and Grotendorst 1989) or connective tissue growth factor (CTGF) (Igarashi et al. 1993), and TGF-β has

then been proved to act mainly as a growth-inhibitory factor rather than a growth-promoting cytokine.

The TGF-β-mediated inhibition of cell growth was initially observed in vitro in a study focusing on the purification of growth inhibitors from the culture medium of BSC-1 epithelial kidney cells from African green monkey (Holley et al. 1980). Indeed, BSC-1-cell-conditioned medium was found to inhibit growth of Hs578T human mammary tumor cells (Holley et al. 1983), and TGF-β was identified as the mediator of this anti-proliferative effect (Tucker et al. 1984). It has been clearly established in the same period that TGF-β caused growth inhibitory responses in different cell types including epithelial (Masui et al. 1986; Tucker et al. 1984), endothelial (Baird and Durkin 1986; Frater-Schroder et al. 1986; Heimark et al. 1986), hematopoietic (Kehrl et al. 1986a; Kehrl et al. 1986b), neural cells as well as certain mesenchymal cells (reviewed in (Massague 1990)) and primary embryonic fibroblasts (Anzano et al. 1986). One of the first compelling evidence showing that TGF-β was able to inhibit cell growth in vivo was produced in 1987, when Silberstein et al. reported that implantation of ethylene vinyl acetate copolymer pellets allowing the slow release of TGF-β into small zones of the mammary gland could induce a potent inhibition of ductal growth (Silberstein and Daniel 1987).

In addition to its growth-inhibitory properties, the tumor-suppressive action of TGF-β was also shown to be mediated by its ability to induce apoptosis in different cell types both in vivo (Nguyen and Pollard 2000) and in vitro. Interestingly, TGF-β-mediated apoptosis concerns not only normal (Oberhammer et al. 1992) but also tumor cells (Lafon et al. 1996; Yanagihara and Tsumuraya 1992) and occurs in vitro concomitantly with the TGF-β-induced growth-inhibitory response in a cell culture (Kim et al. 1998; Rotello et al. 1991).

After the initial observation and characterization of the tumor-suppressive functions of TGF-β in the 1980s, the two last decades have been more dedicated to the detailed understanding of the signaling events and molecular mechanisms by which TGF-β orchestrated cell growth arrest and apoptosis. Hence, this chapter entitled "TGF-β as tumor suppressor: in vitro mechanistic aspects of growth inhibition" depicts how TGF-β protects cells from transformation at the transcriptional, translational, and posttranslational levels. After a detailed view of the cytostatic program elicited by TGF-β to control the cell cycle, the focus shifts to TGF-β signaling pathways and biochemical events governing the apoptotic program.

For a comprehensive review of mouse models with impaired TGF-β signaling and what they have taught us about the tumor-suppressive role of TGF-β, the reader is invited to read Chapter 6.

5.2 Control of the Cell Cycle: The Cytostatic Program

The growth-inhibitory response elicited by TGF-β has been extensively explored in epithelial cells. It involves many key cell-cycle regulators directly or indirectly regulated at the transcriptional level via the Smad signaling pathway or by

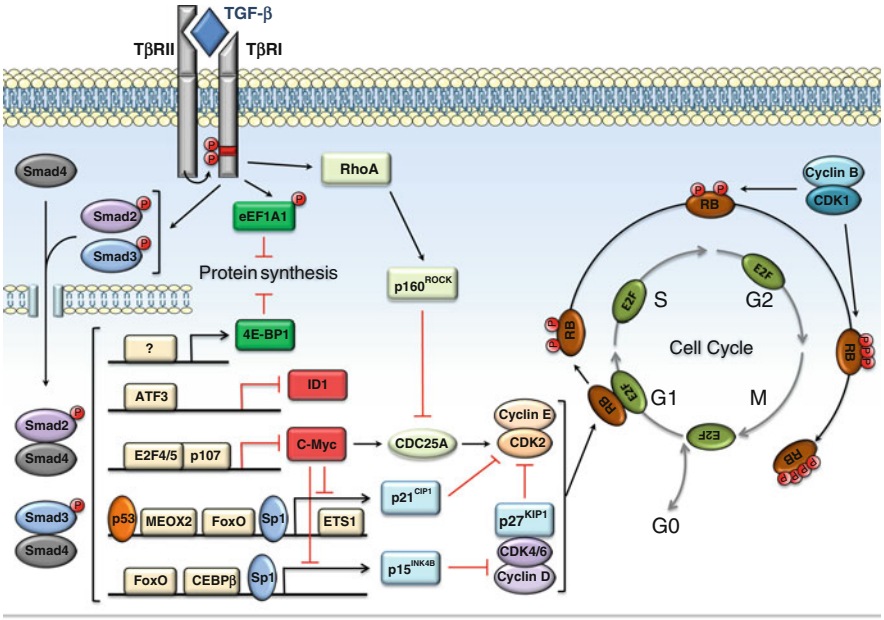

Fig. 5.1 The cytostatic program orchestrated by TGF-β. *Note*: The figure was illustrated using Servier Medical Art

posttranslational modifications mediated by the TGF-β receptor complex. The different targets of the TGF-β/Smad pathway thus cooperate to slow down the cell cycle and to eventually arrest cell growth, as is detailed in the following sections.

5.2.1 Control of the G1/S-Phase Transition by TGF-β

This growth inhibition is achieved by maintaining the retinoblastoma (RB) protein in a hypophosphorylated state (Laiho et al. 1990). Accordingly, ectopic expression of DNA virus oncoproteins sequestering the hypophosphorylated form of RB, such as the Adenovirus 5 protein ElA or the SV40 large T antigen, abolishes TGF-β-mediated growth inhibition (Pietenpol et al. 1990b). The requirement of a functional RB protein for TGF-β-mediated growth suppression has been confirmed in RB-deficient cells, in which TGF-β-mediated growth inhibition is abolished (Herrera et al. 1996). TGF-β inhibits cell growth by arresting the cell cycle at the early G1 phase (Holley et al. 1980; Laiho et al. 1990). Much effort has been devoted to understanding how TGF-β maintains the RB protein in a hypophosphorylated state and to identifying, cloning, and characterizing regulators of the cell cycle machinery (Fig. 5.1).

RB phosphorylation is catalyzed by cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases that are activated at specific points in the cell cycle. The enzymatic activity of CDKs requires the presence of cell-cycle-dependent regulators, the cyclins. Cell cycle progression from the G1 phase to the S phase is achieved through the successive action of the cyclin D-CDK4 and/or cyclin D-CDK6 and cyclin E/CDK2 complexes. Changes in cyclin levels, selective dephosphorylation of specific residues in CDKs, and interactions with stoichiometric inhibitors (the cyclin-dependent kinase inhibitors, CKIs) all contribute to tightly regulating the activity of these complexes (Sherr and Roberts 1999; Vermeulen et al. 2003). CKIs are classified into two families according to their mode of action: the CIP/KIP family and the INK4 (inhibitor of CDK4) family. The CIP/KIP family includes $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, whereas the INK4 family comprises $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$. The INK4 proteins prevent the interaction of cyclin D with CDK4/6. The CIP/KIP proteins can form ternary complexes with G1-phase cyclin-CDK complexes, but their role depends on the target: on the one hand, CIP/KIP proteins interact with cyclin E-CDK2 and inhibit its enzymatic activity by physically occluding the catalytic cleft of CDK2; on the other hand, they interact with and stabilize the cyclin D-CDK4/6 complex without necessarily inhibiting cyclin D-associated kinase activity (Sherr and Roberts 1999).

In epithelial cells, TGF-β has been shown to decrease the enzymatic activities of both cyclin D-CDK4/6 (Hannon and Beach 1994) and cyclin E-CDK2 (Koff et al. 1993). In Mv1Lu lung epithelial cells, it has also been shown to induce growth arrest by inhibiting CDK4 synthesis (Ewen et al. 1993). Extensive in vitro studies have been conducted, mostly on epithelial cells, in order to decipher the molecular mechanisms involved in the regulation of CDK activities and/or synthesis. Findings have led to identifying a limited number of factors involved in the growth-suppressive program governed by TGF-β, called the cytostatic program, which is shared by many lineages of epithelial origin in skin, lung, or mammary gland. The cytostatic program governed by TGF-β is orchestrated mainly at the transcriptional level, through the Smad transcription factors, which convey cues from the membrane to the nucleus. TGF-β-mediated transcriptional effects resulting in growth inhibition include (Fig. 5.1):

1. Transcriptional repression of the growth-promoting c-MYC transcription factor and of the differentiation-inhibiting ID nuclear factors (ID1 to −3).
2. Transcriptional activation of the genes encoding the cyclin-dependent kinase inhibitors $p15^{INK4B}$, $p21^{CIP1}$, and $p57^{KIP2}$.

TGF-β also exerts its cytostatic action via posttranslational events, such as protein modifications (phosphorylations) and changes in protein complex stoichiometry (stimulation of the inhibitory action of the $p27^{KIP1}$ CKI). The following paragraph presents the studies having led to major breakthroughs in this field.

5.2.2 *Smad-Mediated Transcriptional Repression of the* c-MYC *and* ID *Genes*

5.2.2.1 Regulation of the *c-MYC* Gene by the Smad Pathway

c-MYC was the first direct transcriptional target gene of TGF-β identified as being involved in the cell growth arrest induced by this cytokine in epithelial cells (Pietenpol et al. 1990a). In proliferating cells, *c-MYC* is transcriptionally active and its proto-oncoprotein product promotes the expression of genes essential to progression from the G1 to the S phase, such as those encoding cyclin D2 (Bouchard et al. 1999) or CDK4 (Hermeking et al. 2000). An important breakthrough in efforts to understand the cytostatic effect of TGF-β on epithelial cells was the finding that TGF-β can rapidly down-regulate *c-MYC* expression (Pietenpol et al. 1990a). Inversely, forced expression of *c-MYC* in epithelial cells impairs the cytostatic effect mediated by TGF-β (Alexandrow et al. 1995).

Repression of *c-MYC* transcription after TGF-β treatment is direct and involves the Smad signaling pathway. The activated Smad3/4 complex is recruited to a TGF-β inhibitory element (TIE) located between the two transcription start sites of the human *c-MYC* promoter, repressing its transcriptional activity (Chen et al. 2001). This event also involves the input of a repressive complex containing the E2F4 or E2F5 transcription factors and an additional co-repressor, the p107 pocket protein (Chen et al. 2002) (Fig. 5.1). Upon TGF-β stimulation and the subsequent phosphorylation of Smad3, the pre-formed Smad3-E2F4/5-p107 complex translocates into the nucleus, interacts with Smad4, and the Smad3/4-E2F4/5-p107 complex binds to the TIE of the *c-MYC* promoter (Chen et al. 2002; Frederick et al. 2004). The C/EBPβ transcription factor, which is a member of the basic leucine-zipper protein family, is also involved in TGF-β-mediated repression of *c-MYC* promoter activity, being recruited to the TIE sequence along with the Smad3/4-E2F4/5-p107 complex (Gomis et al. 2006b). TGF-β thus allows recruitment of a repressive transcriptional complex to the *c-MYC* promoter, causing cell cycle arrest through a rapid drop in the intracellular level of c-MYC protein.

5.2.2.2 Regulation of *ID* Genes by the TGF-β-Smad Pathway

Another set of target genes of the TGF-β/Smad signaling pathway has been implicated in the cytostatic response of epithelial cells: the ID (inhibitor of DNA binding) family of transcriptional regulators. ID proteins are inhibitors of cell differentiation belonging to the family of basic helix-loop-helix (bHLH) transcription factors (Perk et al. 2005). Classically, bHLH proteins bind to DNA as either homodimers or heterodimers and regulate gene transcription, either positively or negatively, depending on the dimer recruited to the DNA. As ID proteins lack a DNA-binding domain but have a dimerization domain, they act as dominant-negative proteins for other bHLH factors, preventing their binding to DNA and thus inhibiting their function (Perk et al. 2005).

The proteins ID1 to −3 are important positive regulators of cell growth (Fig. 5.1):

1. IDs indirectly promote inactivation of the RB protein by down-regulating CKI expression. They inhibit the transcriptional activity of bHLH proteins that positively regulate the CKI-encoding genes $p15^{INK4B}$, $p16^{INK4A}$, and $p21^{CIP1}$ by preventing their interaction with E-box DNA sequences contained in the promoters of CKI genes (Yokota and Mori 2002).
2. Alternatively, ID2 can block the cell-cycle-inhibiting action of retinoblastoma-family pocket proteins. This function correlates with the ability of ID2 to associate physically with active, hypophosphorylated forms of the pocket proteins (Lasorella et al. 1996; Lasorella et al. 2000). Like the E2F factors, ID2 can interact with the pocket domains of hypophosphorylated RB, p107, and p130. The formation of ID2-pocket protein complexes results in release of E2F from its repressive state, allowing progression of the cell cycle from G1 to S.

TGF-β causes a rapid decrease of ID1, −2, and −3 in various cell systems, such as mammary epithelial cells (NMuMG and MCF10A), normal epithelial lung cells (HPL-1), carcinoma-derived lung cells (A549), keratinocytes (HaCaT) (Kang et al. 2003; Kowanetz et al. 2004; Pardali et al. 2005; Siegel et al. 2003), and mouse embryonic fibroblasts (Lasorella et al. 2000). The mechanism by which TGF-β mediates this transcriptional repression is different for *ID1* and *ID2*.

The human *ID1* promoter contains three Smad binding elements (SBE) motifs downstream of an ATF/CREB binding site, suggesting transcriptional cooperation of Smad proteins with a member of the ATF/CREB (*activation transcription factor/cAMP responsive element-binding*) protein family (Kang et al. 2003). ATF-3, being rather a repressor than an activator of gene transcription, appears to be involved in TGF-β-mediated *ID1* gene repression. Interestingly, the Smad pathway directly regulates expression of the *ATF-3* gene, whose product interacts physically with the activated Smad3/4 complex and binds to the *ID1* promoter (Kang et al. 2003) (Fig. 5.1).

TGF-β-mediated regulation of *ID2* gene expression involves a different mechanism (Siegel et al. 2003). The *ID2* promoter contains E-box DNA sequences constituting a consensus binding site for bHLH transcription factors such as c-MYC. In proliferating cells, c-MYC has been shown to induce *ID2* expression through its recruitment to these E-box motifs (Lasorella et al. 2000), along with its partner Max (Siegel et al. 2003). Transcriptional repression of c-MYC and the subsequent drop in the c-MYC protein level might explain TGF-β-mediated inhibition of *ID2* gene transcription. Yet the time course of *ID2* down-regulation does not necessarily correlate with *c-MYC* repression in certain cell types (e.g., mammary epithelial cells), suggesting that other factors are required to negatively control *ID2* transcription. MAD proteins (MAD1-4) act as antagonists of c-MYC by replacing it in c-MYC-MAX transcriptional complexes. This leads to competition between complexes for the same regulatory sequences. TGF-β induces expression of MAD2/4 proteins in epithelial cells, causing increased association of MAD-MAX heterodimers with the *ID2* promoter (Siegel et al. 2003). Together, the TGF-β-mediated down-regulation of c-MYC and increased recruitment of MAD-MAX complexes to the *ID2* promoter contribute to the sustained transcriptional repression of this gene.

To our knowledge, the mechanism by which TGF-β causes transcriptional repression of *ID3* has not been elucidated to date. Unexpectedly, ID3 synthesis is reported to be induced upon TGF-β stimulation in B lymphocyte progenitor cells to mediate growth inhibition (Kee et al. 2001). This suggests that regulation of ID-factor expression by TGF-β might be cell- or context-specific.

5.2.3 *Transcriptional Activation of* $p21^{CIP1}$- *and* $p15^{INK4B}$- *Encoding Genes by Smad Complexes*

Repression of *c-MYC* and *ID* genes is generally not sufficient to ensure TGF-β-mediated growth inhibition and requires the activation of cell-cycle inhibitors. In epithelial cells, TGF-β induces a rapid increase in $p15^{INK4B}$ (Hannon and Beach 1994; Sandhu et al. 1997) and $p21^{CIP1}$ (Datto et al. 1995) protein levels, whereas in hematopoietic cells, it causes growth arrest through $p57^{KIP2}$ induction (Scandura et al. 2004). TGF-β directly stimulates transcriptional activation of the genes encoding the $p15^{INK4B}$, $p21^{CIP1}$, and $p57^{KIP2}$ CKIs through the Smad signaling pathway. The regulation of $p15^{INK4B}$ and $p21^{CIP1}$ promoter activity by the TGF-β/Smad pathway has been extensively analyzed, whereas the mechanism of TGF-β-mediated transcriptional regulation of $p57^{KIP2}$ is not yet fully understood.

Interestingly, the $p15^{INK4B}$ and $p21^{CIP1}$ promoters are similarly regulated. Under growth-promoting conditions, they are repressed by a complex containing c-MYC. This repressive complex must be released to allow recruitment of transactivators interacting with Smad proteins in TGF-β-stimulated cells. These transcriptional regulatory mechanisms are described below.

Forced expression of c-MYC blocks the transcriptional activation of $p15^{INK4B}$ (Warner et al. 1999) as well as the induction of $p21^{CIP1}$ (Claassen and Hann 2000) in epithelial cells, suggesting that c-MYC may hinder transcriptional activation of the genes encoding these CKIs. This appears to be the case, since in proliferating cells, c-MYC is expressed to a high level and tethered to the $p21^{CIP1}$ and $p15^{INK4B}$ promoters via the zinc finger protein Miz-1, thus interfering with transcriptional activation by TGF-β (Seoane et al. 2001; Seoane et al. 2002; Staller et al. 2001). In the $p15^{INK4B}$ promoter, the c-MYC-Miz-1 complex binds directly to the transcriptional initiator element to block transcriptional activation (Staller et al. 2001). It is noteworthy that c-MYC-mediated repression of $p21^{CIP1}$ occurs in other contexts besides TGF-β signaling. Binding of the c-MYC-Miz-1 complex also impedes transcriptional activation of $p21^{CIP1}$ by other transactivators, such as p53 (in the context of the response to DNA damage) (Seoane et al. 2002).

In this context, c-MYC down-regulation mediated by TGF-β signaling (Fig. 5.1) is essential to rendering the $p21^{CIP1}$ and $p15^{INK4B}$ promoters competent for transcriptional activation. Yet relief from inhibition by c-MYC is not sufficient for activation of these promoters. Transcriptional activation of both promoters by TGF-β requires the input of other transactivators. A precise analysis of the $p21^{CIP1}$ and $p15^{INK4B}$

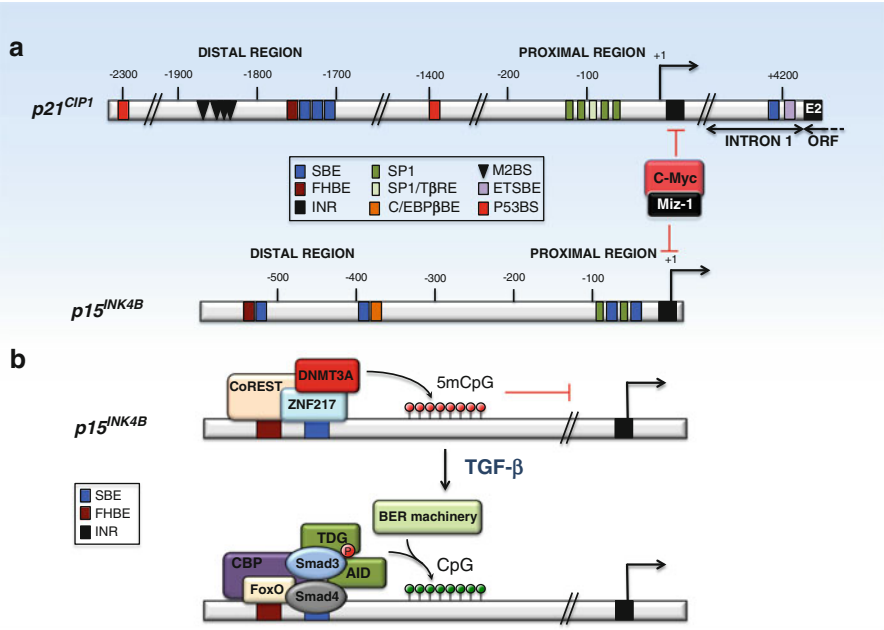

Fig. 5.2 (**a**) Regulatory elements of the $p15^{INK4B}$ and $p21^{CIP1}$ promoters involved in the TGF-β/Smad-mediated transcriptional control. (**b**) Active demethylation of the $p15^{INK4B}$ promoter in response to the TGF-β/Smad signaling pathway. SBE, Smad-binding element; FHBE, Forkhead-binding element; Sp1, Sp1-binding site; TβRE, TGF-β-responsive element; INR, transcriptional initiator element; ETS, ETS-binding element; P53BS, p53-binding site; M2BS, MEOX2-binding site (putative); C/EBPβBE, C/EBPβ-binding element. *Note*: The figure was illustrated using Servier Medical Art

promoters of different species has contributed to identifying the transcriptional regulators that orchestrate transcriptional activation in response to TGF-β.

The mechanisms involved would appear to be the same for $p21^{CIP1}$ and $p15^{INK4B}$ and to be shared by many epithelial cells:

1. The $p21^{CIP1}$ (Moustakas and Kardassis 1998) and $p15^{INK4B}$ (Feng et al. 2000) genes contain not only a GC-rich rich region in the proximal promoter region but also SBEs in more distal enhancer elements (Gomis et al. 2006a; Seoane et al. 2004), where activated Smad3/Smad4 complexes can bind upon TGF-β stimulation (Fig. 5.2a).
2. The SBEs in the distal enhancer elements are located close to a consensus site for binding of forkhead-family proteins. The $p21^{CIP1}$ promoter contains one forkhead-binding element (FHBE) adjacent to three closely spaced SBEs, whereas the $p15^{INK4B}$ promoter contains an FHBE followed by one SBE (Fig. 5.2a). Upon TGF-β stimulation, activated Smad3/4 forms a complex with FoxO proteins (FoxO1, FoxO3, and FoxO4), recruiting them to the promoters and thus activating transcription (Gomis et al. 2006a; Seoane et al. 2004).

3. The transcriptional activator complex also involves Sp1 transcription factors, interacting with Smad proteins in the proximal regions of both promoters (Fig. 5.2a). In the *p15*^{INK4B} promoter, two Sp1 binding sites are followed by one SBE (Feng et al. 2000), whereas the GC-rich proximal part of the *p21*^{CIP1} promoter contains one SBE and is flanked by several Sp1 binding sites (Moustakas and Kardassis 1998; Pardali et al. 2000). In this particular region, interestingly, a Smad2/3-Sp1 complex also recruits the c-MYC factor (Feng et al. 2002) to the *p15*^{INK4B} promoter, suggesting that in the case of this promoter at least, the Sp1 complex might be involved in both positive and negative regulatory mechanisms (in the latter case, via the c-MYC-Miz-1 complex) (Feng et al. 2002). Accordingly, the transcriptional repression mediated by c-MYC is lost when the Sp1-binding sites are mutated. Conversely, when c-MYC-mediated repression is relieved upon TGF-β signaling, Miz-1 may cooperate with the Smad-Sp1 complex to elicit full activation of *p15*^{INK4B} (Seoane et al. 2001). Taken together, these findings suggest that Sp1-binding elements serve as docking sites for both repressor and activator complexes.
4. The *p15*^{INK4B} promoter (but not the *p21*^{CIP1} promoter) contains a third TGF-β-responsive region, in its distal part, where an SBE is immediately followed by a C/EBPβ binding site (Fig. 5.2a). Upon stimulation, a Smad3/4-C/EBPβ complex is recruited to this promoter region to enable transcriptional activation. As a regulator of both c-MYC repression and p15^{INK4} activation, C/EBPβ appears as a crucial mediator of the cytostatic program governed by TGF-β (Gomis et al. 2006b).
5. p53, p63, and p73—major transactivators of *p21*^{CIP1} expression—have also been implicated in TGF-β-mediated transcriptional activation of the *p21*^{CIP1} promoter. Indeed, down-regulation of p53 or p63 in epithelial cells abolishes *p21*^{CIP} gene induction by TGF-β (or activin). The p53 transactivator interacts with activated Smads at the distal region of the *p21*^{CIP} promoter to promote robust induction of *p21*^{CIP} expression (Cordenonsi et al. 2003) (Figs. 5.1 and 5.2).
6. The induction of *p21*^{CIP1} expression in response to TGF-β also requires the involvement of MEOX2 (mesenchyme homeobox2). *MEOX2* is a target gene of the TGF-β signaling pathway, whose product has been shown to interact physically with Smad3 and Smad4 and to bind to the distal region of the *p21*^{CIP1} promoter (Figs. 5.1 and 5.2) (Valcourt et al. 2007).
7. Chromatin immunoprecipitation (ChIP) experiment followed by microarray (ChIP on chip) analysis identified two strong Smad-binding regions in the first intron of the *p21*^{CIP1} gene downstream from the translation start site (Figs. 5.1 and 5.2). The binding of Smad2/3 proteins in those intronic regions is strengthened by the presence of the ETS1 factor, the v-ets erythroblastosis virus E26 oncogene homolog (Koinuma et al. 2009).
8. Both *p15*^{INK4B} and *p21*^{CIP1} promoters contain a CpG island within 1 kb of the transcriptional start site (TSS). In proliferating cells, core CpG islands are highly methylated contributing to the silencing of the two promoters. Upon stimulation, TGF-β rapidly triggers an active demethylation of both promoters (Thillainadesan et al. 2012). In the case of the *p15*^{INK4B} promoter, the CpG island is located

adjacently to SBE/FHBE sequences (Fig. 5.2b). The promoter methylation and subsequent gene silencing is mediated by the constitutive binding of a repressive complex consisting of the oncogene ZNF217, coREST (corepressor of REST), and the DNA methyltransferase DNMT3A. TGF-β induces the displacement of the CoREST-ZNF217-DNMT3A complex, and the subsequent recruitment of the activated Smad3/4 proteins, together with the CBP co-activator and enzymes regulating DNA methylation. Indeed, the Smad3/4 complex recruits the cytidine deaminase AID and the DNA glycolases TDG and MBD4, as well as components of the base excision repair (BER) machinery (Thillainadesan et al. 2012) (Fig. 5.2b). It is important to highlight that this observation is the first showing that epigenetics is a dynamic process that could be regulated by intracellular signaling molecules.

The cytostatic activity of TGF-β also results from additional cooperation between Smad and other signaling pathways. For instance, TGF-β induces Notch signaling to mediate $p21^{CIP1}$ expression (Niimi et al. 2007). Indeed, the TGF-β/Smad pathway induces the expression of the Notch transmembrane ligand Jagged1, which in turn triggers the Notch intracellular signaling pathway in adjacent cells. This process ultimately stimulates the expression of the $p21^{CIP1}$ gene, through the activity of the CLS transcription factor, an effector and a partner of the Notch intracellular signaling domain.

In hematopoietic cells, TGF-β induces robust expression of $p57^{KIP2}$ rather than $p21^{CIP1}$ and $p15^{INK4B}$ (Scandura et al. 2004). TGF-β-mediated up-regulation of $p57^{KIP2}$ expression is direct and requires the action of the Smad3/4 complex on a proximal region of the $p57^{KIP2}$ promoter. Surprisingly, this promoter contains no consensus SBE but includes putative binding sites for other transcription factors, such as YY1, RUNX1, and Sp1, known to interact physically with Smad proteins. Whether these factors cooperate with Smads in regulating $p57^{KIP2}$ expression in hematopoietic cells remains to be elucidated.

Thus, TGF-β/Smad signaling involves (1) several transcription factors, acting both at the proximal promoter and at distal enhancer elements, as well as (2) the DNA demethylation machinery, to mediate rapid and sustained expression of both $p21^{CIP1}$ and $p15^{INK4B}$. The functional consequence of $p15^{INK4B}$, $p21^{CIP1}$, and $p57^{KIP2}$ induction is the abolition of CKD4/6 and CDK2 kinase activities, causing an arrest of G1-to-S cell cycle progression by impairing RB phosphorylation.

5.2.4 Stimulation by TGF-β of the $p27^{KIP1}$ Inhibitory Function

In growth-stimulated epithelial cells, $p27^{KIP1}$ interacts with active cyclin D-CDK4/6 complexes, and its expression level is not altered by TGF-β in the course of the cell cycle (Reynisdottir et al. 1995). TGF-β regulates $p27^{KIP1}$ activity by increasing its bioavailability for binding to the cyclin E-CDK2 complex. By increasing the pool of $p15^{INK4B}$ protein, TGF-β promotes the formation of $p15^{INK4B}$-CDK4/6 complexes

at the expense of ternary $p27^{KIP1}$-cyclin D-CDK4/6 complexes, and thus causes $p27^{KIP1}$ release. This leads to the recruitment of $p27^{KIP1}$ to available cyclin E-CDK2 complexes, which become inhibited (Reynisdottir et al. 1995). Together with the concomitant induction of $p21^{CIP1}$, the displacement of $p27^{KIP1}$ protein from active cyclin D-CDK4/6 to cyclin E-CDK2 complexes ensures robust inhibition of CDK2 catalytic activity, prevents phosphorylation of RB, and causes cell cycle arrest at the G1-to-S transition (Fig. 5.1).

5.2.5 *TGF-β-Mediated Down-Regulation of CDC25A Prevents CDK2 Activation*

TGF-β also regulates the activity of the G1-phase CDKs through posttranslational modifications. The activity of CDKs is negatively regulated by their phosphorylation on specific threonine (Thr14) or tyrosine (Tyr15) residues (Donzelli and Draetta 2003). In the G1 phase, the CDK dual-specificity phosphatase CDC25A dephosphorylates CDK2, allowing subsequent activation of cyclin E-CDK2 complexes (Donzelli and Draetta 2003). This activation step is crucial to subsequent cell cycle progression to the S phase. TGF-β has been shown to decrease the expression of CDC25A in epithelial cells, both in vitro, in cultured mammary epithelial cells (Iavarone and Massague 1997), and in vivo, in mice characterized by specific overexpression of TGF-β1 in hepatocytes (Bouzahzah et al. 2000). More precisely, TGF-β causes direct transcriptional repression of the *CDC25A* gene through recruitment of an E2F4-p130 repressive complex, together with the histone deacetylase 1 (HDAC1), to a region located immediately upstream of the transcription start site of the *CDC25A* promoter (Bouzahzah et al. 2000; Iavarone and Massague 1999). Furthermore, the proto-oncogene c-MYC has been shown to cooperate with its partner Max to directly activate transcription of *CDC25A* (Galaktionov et al. 1996). In this context, TGF-β might also regulate indirectly the transcriptional repression of *CDC25A*, by facilitating Smad-mediated transcriptional repression of the c-*MYC* gene. Alternatively, another TGF-β-dependent mechanism might negatively regulate CDC25A activity. TGF-β activates the RhoA-$p160^{ROCK}$ axis, causing an inhibitory phosphorylation of CDC25A (Bhowmick et al. 2003). In conclusion, CDC25A is targeted by TGF-β via both transcriptional and posttranslational events so as to inhibit CDK2 activation (Fig. 5.1).

5.2.6 *TGF-β-Mediated Inhibition of the Translational Machinery is Involved in Cell-Growth Arrest*

Protein synthesis is a tightly regulated process involving more than a hundred of different proteins. Regulators of the translational machinery have emerged as new players in cell-autonomous tumor-suppressive function of TGF-β. Two studies have

linked actors of the translation process to the cytostatic program governed by TGF-β. As detailed below, the first mechanism involves transcriptional activation of the translation-inhibiting protein 4E-BP1 (eIF4E-binding protein 1), mediated by the Smad signaling pathway (Azar et al. 2009); the second relies on catalytic inactivation of the translation initiation factor eEF1A1 (eukaryotic elongation factor 1A1) by the TGF-β type I receptor (TβRI) (Lin et al. 2010) (Fig. 5.1).

The translation-inhibiting protein 4E-BP1 blocks the function of the cap-binding eukaryotic translation initiation factor-4E (eIF4E), which docks the ribosome at the 5′-end of mRNA in order to initiate protein synthesis. 4E-BP1 is required for TGF-β-mediated growth arrest in pancreatic carcinoma cells, mouse embryonic fibroblasts, and keratinocytes (Azar et al. 2009). The gene encoding 4E-BP1 appears as a direct target of TGF-β signaling, its transcription being activated by TGF-β. Smad4 has been shown to bind to a conserved SBE located in the first exon of this gene. Artificial down-regulation of *4E-BP1* expression abolishes TGF-β-induced cell cycle arrest in pancreatic epithelial cells and embryonic fibroblasts (Azar et al. 2009). Thus, by increasing the level of 4E-BP1, TGF-β contributes to inhibiting the translational machinery and to causing cell growth arrest.

From a phage library screen followed by phosphorylation assays performed with a soluble form of recombinant TGF-β type I receptor, the eukaryotic elongation factor 1A1 (eEF1A1) has emerged as a novel direct substrate of TβRI kinase activity (Lin et al. 2010). TβRI phosphorylates eEF1A1 at serine 300, an amino acid crucial to efficient binding of amino-acyl-tRNA to the elongation factor. In vitro, TGF-β-mediated phosphorylation of serine 300 causes efficient inhibition of protein synthesis and subsequent growth arrest in the breast cancer cell line MCF-7 (Lin et al. 2010). This is the first evidence of a direct link between TGF-β receptors and the regulation of protein synthesis in the control of cell proliferation.

TGF-β has recently been shown to regulate epithelial-to-mesenchymal transition (EMT) by stimulating the translation elongation of selected transcripts (Hussey et al. 2011). More precisely, the translation of several genes essential for EMT is inhibited by the heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1), which blocks eEF1A1 release during translation elongation. Phosphorylation of hnRNP E1 in response to TGF-β signaling disrupts the hnRNP E1-eEF1A1 interaction, triggering EMT. Whether a similar mechanism is employed by TGF-β in order to regulate cytostasis has not been investigated yet.

5.3 The Apoptotic Program

5.3.1 Overview of Apoptotic Pathways

Apoptosis or programmed cell death (Conradt 2009; Joza et al. 2002) is a process leading to ordered disruption of the cell without leakage of cell components. It is absolutely required both during embryogenesis (elimination of interdigital structures, regulation of neuron cell numbers) and in adult life (destruction of immature

lymphocytes, regulation of regenerative growth of the liver, involution of the mammary gland after lactation). Apoptosis is characterized by activation of several pathways leading to activation of caspase-family cysteine proteases (Brenner and Mak 2009; Kroemer et al. 2007). Caspases are synthesized as pro-enzymes requiring activation in order to trigger apoptosis.

Two caspase apoptotic pathways have been described:

1. Extrinsic apoptosis is a form of cell death induced by extracellular signals resulting in binding of ligands to specific transmembrane receptors of the tumor necrosis factor/nerve growth factor (TNF/NGF) family, called death receptors. Upon ligand binding, receptor oligomerization allows recruitment of adaptor proteins and subsequent formation of a death initiation signaling complex (DISC) causing activation of the caspase cascade (initiated by caspase-8) (Lavrik and Krammer 2012).
2. The intrinsic pathway is activated in response to internal stresses, such as DNA damage and accumulation of free oxidant species. The various stress-induced signals converge on the mitochondria and determine mitochondrial outer membrane permeabilization (MOMP), which in turn results in dissipation of the mitochondrial membrane potential and hence the release of proteins, such as cytochrome C and the apoptosis-inducing factor (AIF), contributing to caspase-9 activation. The local regulation and occurrence of MOMP involve proteins of the BCL-2 family, which comprises pro-apoptotic factors (BAX, BAD, BIM) whose activity is finely tuned by its anti-apoptotic members (BCL-2, BCL-XL) (Green and Kroemer 2004).

5.3.2 TGF-β-Mediated Apoptosis

TGF-β-induced apoptosis plays important roles in many in vivo biological processes, such as wound healing (Amendt et al. 2002) and involution of the mammary epithelium after lactation (Nguyen and Pollard 2000) (see Chapter 6). It also plays a crucial role in tumor suppression, by eliminating cells with a malignant potential (Guasch et al. 2007; Yang et al. 2006). In vitro, a pro-apoptotic activity of TGF-β has been observed in many different cell types, such as B and T lymphocytes, prostate, mammary, and lung epithelial cells, hepatocytes, and neurons (reviewed in (Schuster and Krieglstein 2002)). Yet TGF-β has also been shown to promote survival of highly differentiated cells, such as cells of the nervous system (Sanchez-Capelo 2005).

TGF-β-induced apoptosis occurs mainly via the mitochondrial pathway (Schuster and Krieglstein 2002) (Fig. 5.3). In cell types where TGF-β (or activin) induces an apoptotic event, programmed cell death is usually associated with (1) down-regulation of anti-apoptotic factors such as BCL-2 or BCL-XL and/or (2) up-regulation of apoptosis-inducing factor such as BAX or BIK (Saltzman et al. 1998; Schuster and Krieglstein 2002). In contrast to the TGF-β cytostatic program, which

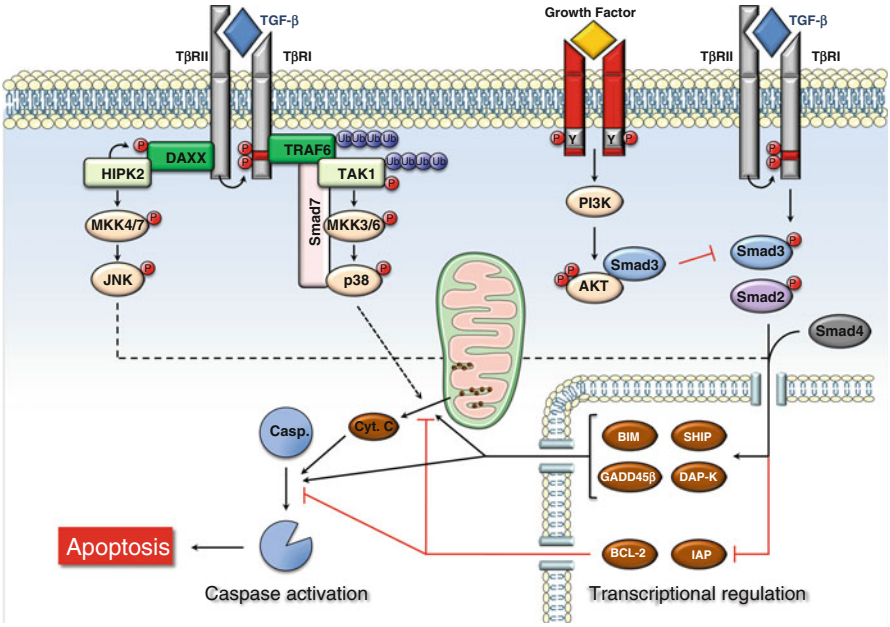

Fig. 5.3 The apoptotic program in response to TGF-β. *Dashed arrows* represent indirect biochemical events. *Note*: The figure was illustrated using Servier Medical Art

is shared by many cell types and involves a limited set of genes and regulators, the TGF-β apoptotic program is mostly cell-type specific and involves a plethora of regulators. It is also less well known and understood.

TGF-β regulates apoptosis via two distinct means that are not mutually exclusive (Fig. 5.3):

1. Smad-dependent transcription control of pro-apoptotic genes whose products are directly involved in the death pathway.
2. Smad-independent pathways initiated by enzymatic or non-catalytic activities of the TGF-β receptor complex.

The following sections present selected studies illustrating the variety of mechanisms that TGF-β can regulate to trigger cell death.

5.3.2.1 The Apoptotic Gene Program Regulated by the Smad Pathway

One of the first evidence of Smad pathway involvement in TGF-β-triggered apoptosis was the identification, in 1997, of the TGF-β-induced early gene 1 (TIEG1) as an immediate target gene of the transcription-regulating action of TGF-β. TIEG1 is an Sp1/Krüppel-like zinc finger transcription factor shown to induce apoptosis in pancreatic epithelial cells (Tachibana et al. 1997), mink lung epithelial cells (Chalaux

et al. 1999), and hepatocytes (Ribeiro et al. 1999). The mechanism by which TIEG induces apoptosis is believed to rely on generation of reactive oxygen species, which would be the initiating death signals leading to a decreased BCL-2 protein level and subsequent loss of mitochondrial membrane potential.

In hematopoietic cells, TGF-β induces expression of the inositol phosphatase SHIP (Src homology 2 (SH2) domain-containing 5′-inositol phosphatase), a central regulator of phospholipid metabolism. Smad-mediated transcriptional activation of the *SHIP* gene results in inhibition of an activating phosphorylation of the survival factor AKT (also known as protein kinase B, PKB), associated with subsequent cell death. Artificial down-regulation of SHIP compromises TGF-β-triggered death of myeloma cells (Valderrama-Carvajal et al. 2002).

Upon TGF-β stimulation, the Smad2/3-4 complex activates transcription of the gene encoding the death-associated protein kinase (DAP-K), an enzyme directly involved in regulating apoptosis. By facilitating cytochrome C release from the mitochondria and dissipation of the mitochondrial membrane potential, TGF-β-induced DAP-K expression causes apoptosis of Hep3B hepatocellular carcinoma cells (Jang et al. 2002).

TGF-β stimulates transcription of GADD45β, a member of the stress- and cytokine-induced factor family, via the action of activated Smad3/4 complex on the proximal region of the *GADD45β* promoter (Yoo et al. 2003) and on an enhancer element located in the third intron (Major and Jones 2004). GADD45β was first identified in a complex containing the p38-activating kinase MEKK4/MTK1 (a mitogen-activated protein (MAP) kinase kinase kinase). By phosphorylating the MAPKK MKK3/6, the GADD45β-MEKK4 complex promotes activation of the MAPK p38, thus regulating p38-mediated biological functions such as apoptosis (Takekawa et al. 2002). In hepatocytes, the involvement of the p38 signaling pathway in TGF-β/GADD45β-triggered apoptosis is supported by the fact that apoptosis is inhibited by a small-molecular-weight inhibitor selectively blocking p38 kinase activity (Yoo et al. 2003). Expression of GADD45β in hepatocytes is sufficient to trigger apoptosis in the absence of TGF-β stimulation, whereas down-regulation of GADD45β expression inhibits TGF-β-induced p38 activation and apoptosis (Yoo et al. 2003).

The Smad pathway has been shown to control directly or indirectly the transcriptional expression of BCL-2-family members, key regulators of the intrinsic apoptotic pathway. A first example concerns down-regulation of the prototypic anti-apoptotic protein BCL-2 by TGF-β in vivo. Ectopic expression of Smad3 in transgenic mice protects the liver from chemically induced carcinogenesis (Yang et al. 2006). Indeed, Smad3 exerts its protective role by inducing apoptosis in hepatocytes in order to prevent their malignant conversion. Smad3 triggers apoptosis by down-regulating the transcription of the gene encoding the anti-apoptotic protein BCL-2. This repression is exerted upon binding of a Smad3-containing transcriptional complex to a GC-rich TGF-β-responsive element in the *Bcl-2* promoter (Yang et al. 2006). Interestingly, Smad3-mediated apoptosis occurs concomitantly with p38 phosphorylation in both liver tumors of transgenic mice and primary hepatocytes cultured in the presence of TGF-β (Yang et al. 2006). As p38 MAPK activity

appears also to be involved in TGF-β-mediated apoptosis (see above), these findings indicate that Smad-dependent and Smad-independent pathways may cooperate in the same cell to strengthen death-initiating cues.

While down-regulating anti-apoptotic factors, TGF-β also positively regulates pro-apoptotic factors of the BCL-2 family. For example, it increases the level of the pro-apoptotic BIM protein in B lymphocytes (Ramesh et al. 2008; Wildey et al. 2003) and hepatocytes (Wildey and Howe 2009) in a Smad3-dependent manner so as to trigger apoptosis via the mitochondrial pathway. TGF-β can control the BIM protein level via two distinct mechanisms:

1. The canonical TGF-β/Smad3 pathway indirectly regulates transcriptional activation of the *BIM* gene, inducing expression of transcription factor RUNX1, which cooperates with FoxO3 and binds to the *BIM* promoter to trigger transcriptional activation in hepatocytes (Wildey et al. 2003). Despite the presence of a putative SBE in the *BIM* promoter, cooperation of activated Smad3/4 with the RUNX1-FoxO3 complex has not yet been established.
2. TGF-β indirectly promotes BIM protein stabilization. Extracellular signal-regulated kinase (ERK)-regulated phosphorylation of BIM targets it for degradation by the proteasome machinery. This posttranslational event is inhibited by the MAPK phosphatase MKP2, which dephosphorylates and thus inactivates the ERK kinase. The *MKP2* gene responds directly to TGF-β. By stimulating transcriptional activation of *MKP2*, the Smad3/4 complex causes stabilization of the BIM protein (Ramesh et al. 2008).

Survivin, a member of the mammalian inhibitor of apoptosis (IAP) family of endogenous caspase inhibitors, is also transcriptionally down-regulated by TGF-β (Yang et al. 2008). Interestingly, TGF-β represses transcription o*f* the *Survivin* gene by a peculiar mechanism. The proximal region of the *Survivin* promoter contains two canonical SBEs and two cell cycle repressor elements: a cell-cycle-dependent element (CDE) and a cell-cycle gene homology region (CHR). Both the CDE and the CHR are indispensable to TGF-β-mediated transcriptional repression in reporter assays. TGF-β causes recruitment of an RB-E2F4 repressive complex to the CDE/CHR elements of the *Survivin* promoter. Although Smad2/3 signaling is absolutely required for TGF-β-mediated repression of *Survivin* and although Smad3 binds to the minimal proximal region of the *Survivin* promoter, these SBEs do not seem to be the key elements mediating repression, since Smad4 does not bind to this particular promoter region and since mutations within the two SBEs do not abolish TGF-β-mediated transcriptional repression in reporter assays. Smad proteins might be required in this transcriptional repression indirectly, for their ability to maintain the RB protein in a hypophosphorylated state in a complex with E2F4 factors (in the framework of the TGF-β-governed cytostatic program). Alternatively, a Smad complex might interact physically with unidentified SBEs located elsewhere in the promoter or in other regulatory elements (Yang et al. 2008).

AKT overexpression is sufficient to block TGF-β-mediated apoptosis, independently of AKT kinase activity (Conery et al. 2004; Remy et al. 2004). This inhibition results from direct interaction of AKT with Smad3, preventing phosphorylation of

Smad3 by TβRI (titration). Hence, the Smad3:AKT ratio is a crucial determinant of the sensitivity to apoptosis in response to TGF-β, stronger apoptotic responses being observed in cells with higher Smad3:AKT ratios (Conery et al. 2004). More interestingly, the growth of all cell lines tested is potently inhibited by TGF-β, while their response to apoptosis depends on the Smad3-to-AKT ratio (Conery et al. 2004). The AKT-Smad3 complex might be part of a regulatory mechanism enabling a given cell to switch from the cytostatic program to the apoptotic program in response to TGF-β (Fig. 5.3).

5.3.2.2 Smad-Independent Pathways Controlling TGF-β-Mediated Apoptosis

TGF-β can also induce apoptosis through Smad-independent signaling pathways. In this case, to promote cell death, TGF-β signals through the p38 and JNK (c-Jun N-terminal kinase) pathways (Fig. 5.3), both classically associated with responses to cell stress and apoptosis. TGF-β has been known for long time to facilitate activation of JNK (Atfi et al. 1997; Parkinson et al. 2001) and p38 (Hyman et al. 2002; Takekawa et al. 2002; Yoo et al. 2003) in various cell types, but for many years the biochemical links between TGF-β receptors and the actors of the MAPK cascade remained unelucidated.

From a two-hybrid screen, the adaptor protein DAXX has emerged as a target protein interacting physically with TGF-β type II receptor (TβRII) used as bait (Perlman et al. 2001). DAXX is a FAS-receptor-associated protein mediating FAS-induced programmed cell death by activating the JNK pathway. In vitro, DAXX interacts directly with the cytoplasmic domain of TβRII, but not with TβRI. In vivo, it has been found to associate with the TβRI/II receptor complex upon TGF-β stimulation. This association requires the kinase activity of the TβRII receptor, since a kinase-dead mutant of TβRII fails to interact with DAXX (Perlman et al. 2001). DAXX is crucial to TGF-β-induced apoptosis in both B-cell lymphoma cells and mouse hepatocytes, since overexpression of a dominant-negative form of DAXX or artificial *DAXX* silencing inhibits TGF-β-mediated apoptosis in these cells (Perlman et al. 2001). Upon TGF-β stimulation, interestingly, DAXX acts as an adaptor protein facilitating the phosphorylation and subsequent activation of JNK and triggering cell death. DAXX can interact physically with HIPK2 (homeodomain-interacting protein kinase 2), a serine/threonine kinase involved in transcriptional regulation and also apoptosis (Hofmann et al. 2003). The HIPK2-DAXX complex facilitates activation of the JNK protein by the upstream mitogen-activated protein kinase kinases MKK4 and MKK7. Overexpression of both DAXX and HIPK2 leads to potentiation of TGF-β-induced cell death. This effect is mediated by the kinase function of HIPK2, since expression of a kinase-deficient HIPK2 protein fails to increase TGF-β-induced cell death in these cells. Interestingly, HIPK2 also phosphorylates the DAXX protein (Hofmann et al. 2003), but whether this phosphorylation event is required for the adaptor function of DAXX in the specific context of TGF-β-induced apoptosis has not been explored. These findings constitute the

first evidence of a biochemical link between TGF-β receptors and the JNK pathway, leading to the apoptotic machinery (Fig. 5.3).

Concerning activation of the p38 MAPK by TGF-β, previous studies have indicated that TAK1 (TGF-β-activated kinase 1) might be an indirect link between p38 and the TGF-β receptor complex (Shibuya et al. 1996; Yamaguchi et al. 1995). TAK1 is a member of the MAP kinase kinase kinase family, identified as a TβRI-interacting protein (Shibuya et al. 1996; Yamaguchi et al. 1995) almost concomitantly with the discovery of the Smad proteins (Heldin et al. 1997). TAK1 has been proposed to be involved in TGF-β-induced cell death through its ability to activate p38 in a Smad-independent manner (Sano et al. 1999). Until recently, the biochemical link between the TβRI-TAK1 complex and p38 activation remained unidentified (Sorrentino et al. 2008; Yamashita et al. 2008). The E3-ligase TRAF6 can bind constitutively to TβRI. Upon ligand binding and TGF-β receptor TβRI-TβRII hetero-oligomerization, TRAF6 becomes auto-ubiquitinated by polyubiquitin chains linked via their Lys63. TRAF6 then catalyzes Lys63-mediated polyubiquitination of TAK1, and thus activates it. It is important to stress that unlike Lys48-linked polyubiquitination, Lys63-linked polyubiquitination is not a signal targeting proteins for degradation by the proteasome complex. Although its role in the TAK1 activation process is not fully understood, TAK1 polyubiquitination seems to facilitate TAK1 juxtaposition-induced auto-phosphorylation. Alternatively, it might favor a conformational change allowing exposure of the TAK1 catalytic domain. In turn, TAK1 phosphorylates and activates the MAP kinase kinases: MKK3 and MKK6, which ultimately phosphorylate MAPK p38. In this context, Smad7 might act as a docking protein facilitating the phosphorylation cascade, having previously been shown (*1*) to interact with the TAK1, MKK3, and p38 proteins and (*2*) to be required for TGF-β-induced apoptosis in prostate cancer cells (Edlund et al. 2003) (Fig. 5.3).

Interestingly, activation of TAK1 and p38 does not require TβRI kinase activity (Sorrentino et al. 2008; Yamashita et al. 2008), but the presence of TRAF6 and its E3-ubiquitin ligase activity are indispensable to TGF-β-mediated apoptosis in prostate cancer cells (Sorrentino et al. 2008) and hepatocytes (Yamashita et al. 2008). Artificial down-regulation of *TRAF6* in prostate cancer cells or hepatocytes abolishes TGF-β-triggered apoptosis, and similar observations have been made on TRAF6-deficient mouse embryonic fibroblasts (Sorrentino et al. 2008; Yamashita et al. 2008). Moreover, overexpression of a deletion mutant of TRAF6 lacking E3-ubiquitin ligase activity inhibits TGF-β-mediated TAK1 ubiquitination and subsequent activation and also p38 phosphorylation (Sorrentino et al. 2008).

Another adaptor protein has recently been implicated in this noncanonical TRAF6-TAK1 pathway initiated by TGF-β: TTRAP (TRAF and TNF receptor associated protein). TTRAP forms a ternary complex with TAK1 and TRAF6, which can be recruited to TβRI in the presence of the ligand. The presence of TTRAP potentiates the E3-ubiquitin ligase activity of TRAF6, thereby promoting TAK1 ubiquitination. Artificial down-regulation of TTRAP impairs TGF-β-induced activation of p38 (Varady et al. 2011).

5.4 Conclusion

In conclusion, to the extent that the tumor-suppressive mechanisms orchestrated by TGF-β have been deciphered, it is obvious that the cytostatic and apoptotic responses of a cell to TGF-β involve totally different regulatory events and processes. The cytostatic program, on the one hand, involves regulation of a limited number of genes by Smad proteins, with few additional effectors intervening to block the cell cycle before the G1 restriction point. The apoptotic program, on the other hand, involves many modifying enzymes, notably kinases, and also requires Smad-mediated transcriptional regulation in order to favor pro-apoptotic instead of pro-survival pathways. The mechanism by which a cell switches from one program to the other depends on an intricate dialogue with its microenvironment and is still largely unknown.

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