Chapter 2

To Grow, Stop or Die? – Novel Tumor-Suppressive Mechanism Regulated by the Transcription Factor E2F

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Additional information is available at the end of the chapter

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1. Introduction

Proliferation of mammalian cells is strictly regulated by growth stimulation. Cell proliferation is stimulated not only by normal growth stimulation but also by abnormal growth stimulation originated from oncogenic changes. Such abnormal growth stimulation leads to tumorigenesis, if not properly guarded by appropriate cellular response. Cells are endowed with intrinsic tumor suppressor pathways to protect cells from tumorigenesis upon such oncogenic threat [1]. The tumor suppressor pathways halt cell proliferation either by restraining cell cycle progression or by inducing apoptosis (programmed cell death) in case of being unable to stop aberrant cell cycle progression. Consequently, the cell-fate, whether to grow, stop growing or die, is dependent on the balance between growth-promoting effects originated from oncogenic changes and growth-suppressive effects mediated by the tumor suppressor pathways upon oncogenic changes (Figure 1). When the tumor suppressor pathways are disabled by further oncogenic changes, the balance of cell-fate determination shifts from growth suppression to proliferation, and cells start deregulated proliferation, leading to tumorigenesis. Among the intrinsic tumor suppressor pathways, two major pathways are the RB pathway and the p53 pathway. Both pathways are important for induction of cell cycle arrest or apoptosis [2]. In addition, accumulating evidence indicates that the tumor suppressor TAp73, a member of the p53 family, also plays crucial roles in tumor suppression by inducing apoptosis independent of p53 [3, 4].

The transcription factor E2F, the main target of the RB pathway, plays crucial roles in cell cycle progression by activating growth-promoting genes [5]. In this regard, E2F is thought to mediate growth-promoting effects originating from normal growth stimulation and oncogenic changes. Supporting this notion, E2F could be an oncoprotein [6]. On the other hand, recent studies indicate that E2F also plays crucial roles in activation of the major intrinsic tumor suppressor path-

ways by sensing oncogenic changes, halting cell proliferation by inducing cell cycle arrest or apoptosis. Supporting this notion, E2F could also be a tumor suppressor [7]. Taken together, these observations indicate that E2F is located at the center of the balance between cell proliferation and cell cycle arrest or apoptosis, determining the cell-fate upon oncogenic changes (Figure 1). E2F can be regarded as a double-edged sword in cell growth control. In this chapter, we will describe the major intrinsic tumor suppressor pathways and activation of the tumor suppressor pathways by E2F upon oncogenic changes. We will focus on how E2F differentially regulates expression of target genes upon normal growth stimulation and oncogenic changes that have completely opposite roles in cell-fate determination, to grow, stop or die.

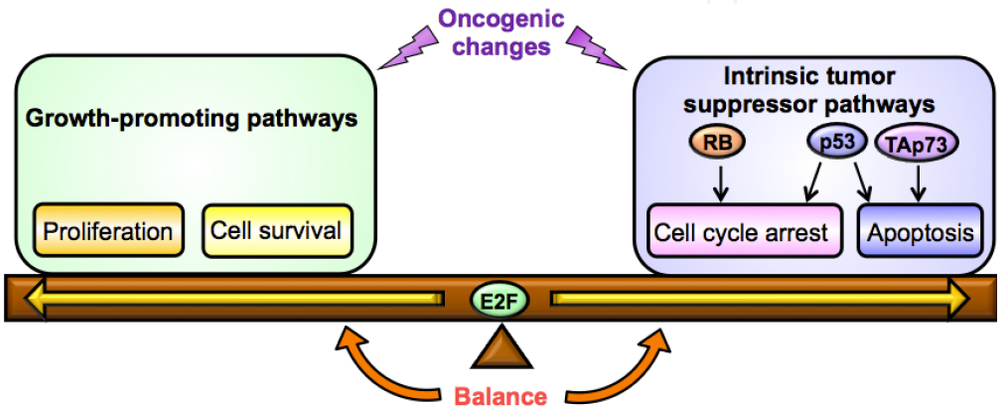

Figure 1. To grow, stop or die? The balance between the activity of growth-promoting pathways and tumor suppressor pathways determines the cell fate upon oncogenic changes. E2F is located at the center of the balance (see also Figure 6).

2. Major tumor-suppressor pathways

2.1. The RB pathway (CDK inhibitor–Cyc/CDK–RB)

The retinoblastoma gene (*RB1*) is the first identified tumor suppressor gene [8]. Individuals with heterozygous deletion or mutation of the *RB1* gene are susceptible to retinoblastoma in early life by additional deletion or mutation of the other allele. The *RB1* gene product pRB, together with its relatives, p107 and p130, comprises a family of pocket proteins [9, 10] (Hereafter we refer all pocket proteins to RB, and the *RB1* gene product to pRB). The main target of RB is the transcription factor E2F, which plays central role in cell proliferation by activating growth-promoting genes including those required for DNA replication and cell cycle progression [9]. In quiescent phase, RB binds to E2F and suppresses transcriptional activity of E2F. Moreover, RB recruits various chromatin-modifying factors, such as histone deacetylases (HDACs) [11] and histone methyl transferase SUV39H1 [12] to actively suppress expression of E2F target genes. Hence the main role of RB in tumor suppression is thought to be suppression of cell proliferation through suppression of E2F target gene expression. Growth stimulation induces expression of cyclins and activates cyclin dependent kinases (CDKs), which are called accelerators and engines in cell cycle progression, respectively. CDKs, in turn, inactivate RB by phosphorylation,

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leading to expression of E2F target genes by releasing them from suppression by RB during G1 to S phase cell cycle progression (Figure 2) [13]. In contrast, growth-suppressive signals such as contact inhibition and DNA damage induce expression of CDK inhibitors, which are called brakes in cell cycle progression owing to their ability to inhibit activity of CDKs. Suppression of CDKs keeps RB in hypo-phosphorylated form, which binds to and inhibits E2F. Consequently, the activity of E2F to activate growth-promoting genes is controlled by the activity of RB, which is regulated by CDKs and CDK inhibitors. The pathway converging to RB, including CDKs and CDK inhibitors, is referred to the RB pathway.

Whether a cell progresses one round of the cell cycle or not is determined at the restriction point, which is located at late G1 phase of the cell cycle. Once a cell passed through the restriction point, it is programmed that the cell cycle automatically proceeds to the end of M phase. Thus, whether a cell proliferates or not is determined by whether the cell passes through the restriction point or not. Two major determinants whether a cell passes through the restriction point or not are E2F activity and cyclin dependent kinase activity, which is induced by E2F through activation of the *Cyclin E* (*CycE*) gene [14]. Since E2F plays essential roles in passing through the restriction point, RB can be regarded as a gatekeeper in cell cycle progression by controlling E2F activity. Hence disruption of the RB pathway and consequent activation of E2F is thought to be an essential event for tumorigenesis [2]. Actually, deletion or mutation of the *RB1* gene is observed in about 30% of cancers. Moreover, defects in the RB pathway upstream of RB such as overexpression of CycD and dysfunction of CDK inhibitors such as $p16^{INK4a}$ are frequently observed in other cancers retaining *RB1* [2]. It is predicted that all cancers have at least some defect in the RB pathway.

The RB pathway plays essential roles in tumor suppression by inducing cell cycle arrest or cellular senescence through suppression of the activity of E2F. As described below, disruption of the RB pathway reinforces cell cycle arrest by induction of CDK inhibitor $p21^{Cip1}$ expression through the p53 pathway or $p27^{Kip1}$ expression. When cells failed to induce cell cycle arrest, apoptosis is triggered through the p53 pathway or TAp73, a p53 family member, which activates various pro-apoptotic genes.

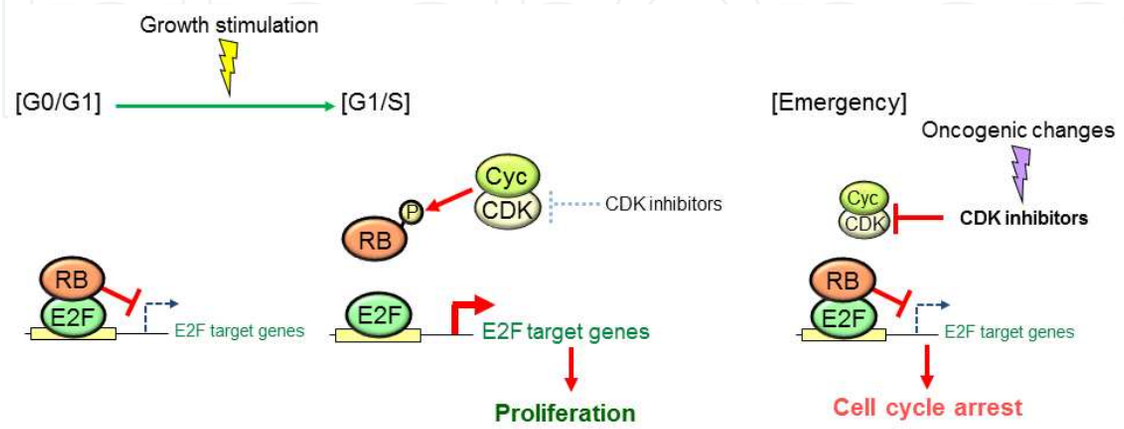

Figure 2. The regulatory mechanism of the transcription factor E2F by the RB pathway.

2.2. The p53 pathway (ARF–p53–cell cycle arrest or apoptosis related effectors)

The tumor suppressor p53 is a transcription factor that is activated by a variety of stress signals, including DNA damage, hypoxia and various oncogenic changes including aberrant activation of E2F [15]. In response to such stress signals, p53 induces either cell cycle arrest or apoptosis. Cell cycle arrest is mainly mediated through activation of the CDK inhibitor $p21^{Cip1}$ gene [16], whose product suppresses wide range of CDKs. Apoptosis is mainly mediated through activation of the *Bax* and BH3 only family genes, whose products destabilize mitochondrial membrane to facilitate cytochrome c release, which triggers apoptotic cascades of caspase activation.

Since p53 plays crucial roles in induction of cell cycle arrest or apoptosis, the expression level of p53 is kept low by rapid ubiquitin/proteasome-dependent degradation, mainly caused by Mdm2 (mouse double minute 2, Hdm2 in humans), which is often overexpressed in many cancers [15]. Mdm2 is E3 ubiquitin ligase, which directly binds to p53 and promotes p53 degradation. Mdm2 also inhibits *TP53* mRNA translation [17]. The *Mdm2* gene is a target of p53, forming a negative-feedback loop to control the level of p53 [18]. Another negative regulator of p53 is MdmX, also known as Mdm4. MdmX has recently emerged as a discrete critical negative regulator of p53 [19]. Though MdmX is not a direct target of p53, structure of MdmX is significantly similar to that of Mdm2. MdmX is reported to enhance p53 ubiquitination by altering the substrate preference of the Mdm2, thereby indirectly regulating p53 [20].

Regarding response to oncogenic stresses, a potent activator of p53 is the tumor suppressor ARF (alternative reading frame, known as p14 in humans and as p19 in rodents) [2]. ARF directly binds to and sequesters Mdm2 to nucleoli, stabilizing p53 that leads to the expression of its target genes [21]. The pathway including upstream and downstream of p53 is referred to the p53 pathway. Of note, the *ARF* gene is a direct target of E2F [22] and this E2F-ARF interaction connects the RB pathway and the p53 pathway, enabling efficient tumor-suppressive response. When the RB pathway is disrupted by oncogenic changes, E2F is activated to induce ARF gene expression, leading to activation of the p53 pathway, which inhibits oncogenic cell growth by inducing cell cycle arrest or apoptosis (Figure 3). Therefore, disruption of both of the tumor suppressor pathways powerfully shifts the cell-fate determination balance to proliferation (Figure 1) and is thought to be essential to induce deregulated cell proliferation that leads to tumorigenesis. Indeed, about 50% of cancers carry *TP53* mutations or deletion and most cancers have defects in the p53 pathway including those in upstream and downstream of p53.

2.3. The TAp73 pathway (E2F–TAp73–pro-apoptotic targets)

The tumor suppressor TAp73 is a homologue of p53 and can induce apoptosis independently of p53 [3, 4, 23]. The *TP73* gene encodes two isoforms, TAp73 and DNp73, which are driven by different promoters. DNp73 lacks the transactivation (TA) domain and counteracts TAp73 and p53 [24]. Thus DNp73 is anti-apoptotic. The *TAp73* gene is thought to be a tumor suppressor gene and is known to be a target of E2F1 [3, 4, 25]. Similarly to p53, TAp73 is activated by both oncogenic changes and DNA damage [26, 27] and TAp73 target genes

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partly overlap with those of p53, such as the *PUMA* and *NOXA* genes [28, 29] that are crucial for induction of apoptosis. Moreover, TAp73 can induce apoptosis in the absence of p53 [3, 4]. Therefore, TAp73 seems to back-up the important tumor suppressive function of p53.

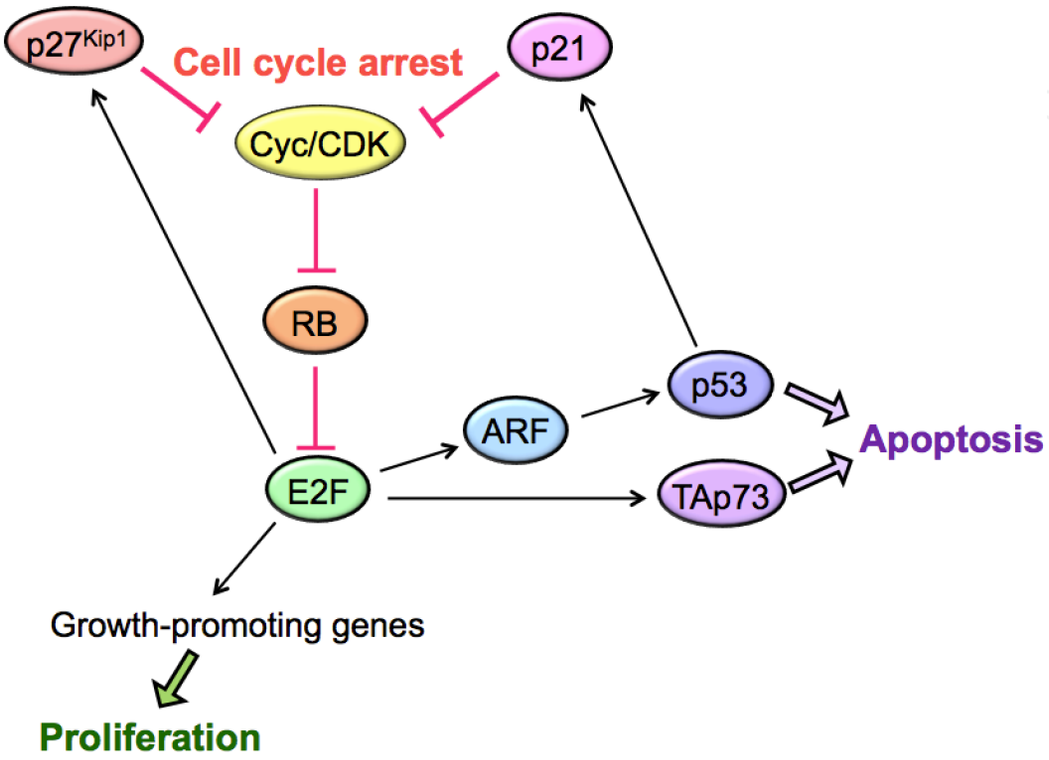

Figure 3. E2F activates major intrinsic tumor suppressor pathways.

3. The transcription factor E2F

The transcription factor E2F was originally identified as a cellular DNA-binding protein, which mediate E1A-dependent activation of the adenovirus E2 promoter [30]. Members of E2F family are downstream targets of the tumor suppressor RB and make repressor complexes with RB that keep cells in quiescent state [9, 10]. Though E2F plays central roles in cell proliferation by activating growth-promoting genes, certain members of E2F can induce apoptosis [15]. In this paragraph, we will describe three points as follows (1) E2F family members, the role of them in cell cycle progression, cell cycle arrest and apoptosis. (2) Regulatory mechanism of E2F. (3) E2F target genes, to better understand the regulatory mechanism of cell-fate determination mediated through E2F.

3.1. E2F family members

E2F consists of eight family members (E2F1-E2F8). E2F1-E2F5 are bound by their repressor RB family proteins through RB binding domain. E2F1-E2F5 activate transcription when free

from RB and repress transcription when bound by RB. E2F1-E2F3a are induced at G1/S boundary and activate transcription free from RB. In contrast, E2F3b-E2F5 are expressed all through the cell cycle and play main roles in transcriptional repression in G0/G1 bound by RB. E2F6-E2F8 repress transcription independently of RB. Hence E2F family members are divided into two groups: activator E2Fs (E2F1-E2F3a) and repressor E2Fs (E2F3b-E2F8). Activator E2Fs play major roles in activation of target genes involved in growth promotion, growth suppression and induction of apoptosis. Repressor E2Fs are roughly divided into two groups; E2F3b-E2F5, which make repressor complex together with RB, and recently identified E2F6-E2F8, which function independently of RB.

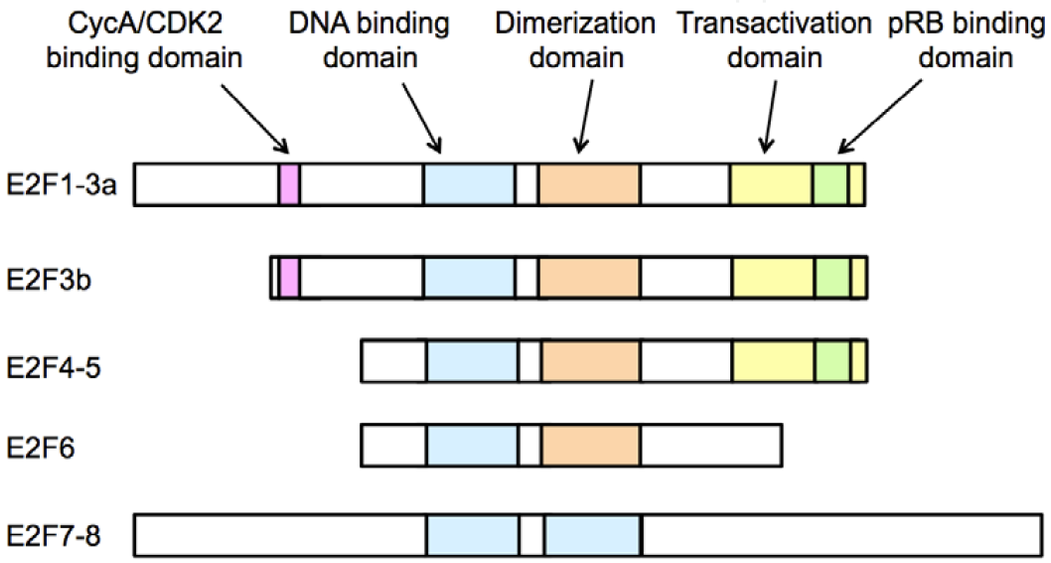

Figure 4. Structure of E2F family members. E2F1-E2F6 bind to their target promoter with binding partner DP proteins through dimerization domain. E2F7 and E2F8 have two DNA binding domains and make homodimers or E2F7/E2F8 heterodimer.

A structural characteristic of activator E2Fs (E2F1-E2F3a) is longer N terminal region, which does not exist in repressor E2Fs (E2F3b-E2F6). Expression of E2F1-E2F3a is induced by E2Fs themselves [31]. When growth stimulation inactivates p130 by phosphorylation through activation of CycD/Cdk4 or CycD/Cdk6, repressor E2Fs (E2F4 and E2F5) are released from p130 and activates the *E2F1-E2F3a* genes. E2F1-E2F3a in turn replace E2F4 and E2F5, and activate growth-promoting genes including E2F1-E2F3a themselves. Each activator E2F has preferential roles in cell cycle progression and induction of apoptosis. p107 preferentially make complexes with E2F4-E2F5 in G1/S to S phases.

E2F1 is generally thought to be the most powerful transcriptional activator of pro-apoptotic genes among activator E2Fs. Overexpression of E2F1 in tissue culture cells alone can induce cell cycle progression in otherwise quiescent fibroblasts [32]. Overexpression of E2F1 can be oncogenic *in vitro* [33, 34] and *in vivo* [6]. In contrast, E2F1 is dispensable for cell cycle progression, since E2F1 knockout mice are viable. On the other hand, over expression of E2F1 in cancer cell lines leads to apoptosis [35]. E2F1 also plays a key role in induction of cellular

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senescence by activating the ARF-p53 pathway, when overexpressed in human normal fibroblasts [36]. Moreover, E2F1 null mice resulted in tumorigenesis [7]. Taken together, E2F1 seems to play the most important roles in tumor suppression among activator E2Fs by activating genes involved in apoptosis and cell cycle arrest.

E2F2 has 46% overall amino acid sequence similarity to E2F1 [37] and is thought to play roles in both cell proliferation and tumor suppression. E2F2 null mice exhibit increased proliferation of hematopoietic cells and frequently develop autoimmunity and tumors [38, 39]. In tumor suppression, E2F2 plays major roles in suppression of Myc-induced T cell lymphomagenesis. Inactivation of neither E2F1 nor E2F3 had no effect on tumor progression in T cells, and only loss of E2F2 accelerated lymphomagenesis [40].

E2F3 is thought to be the most important activator E2F in cell proliferation. Although E2F1 or E2F2 null mice are viable and tumor-prone, E2F3 null mice are typically embryonic lethal in pure background [41] or show partially penetrant embryonic lethality in mixed background [42]. Although mouse embryonic fibroblasts (MEFs) with combined knockout of E2F1 and E2F2 proliferate, those with combined knockout of E2F1, E2F2 and E2F3 fail to proliferate and can not re-enter the cell cycle [5]. The E2F3 locus encodes two isoforms, E2F3a and E2F3b, a truncated variant of E2F3 in its N-terminus [43, 44]. Although both E2F3s partly overlaps their roles in cell cycle progression [45], E2F3a is expressed in G1/S to S phases and is thought to play crucial roles on cell proliferation, while E2F3b is expressed equivalently in quiescent and proliferating cells, and associates with pRB, representing the predominant E2F-pRB complex in quiescent cells [44, 46]. E2F3b also plays roles in myogenic differentiation by promoting gene expression related to differentiation [47].

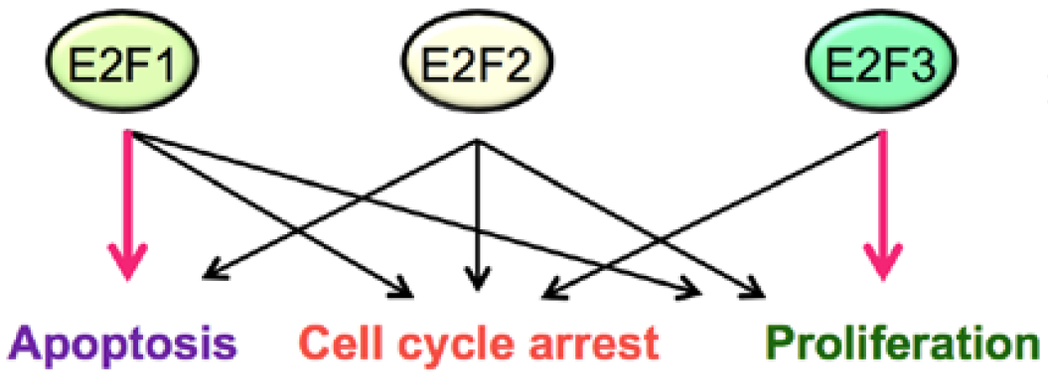

Figure 5. Roles of activator E2Fs in cell-fate determination. E2F1 plays crucial roles in induction of apoptosis. E2F3 is thought be essential for cell proliferation. It is predicted that the character of E2F2 is in the middle of E2F1 and E2F3.

E2F4 and E2F5 were cloned by their association with p107 and p130, and are significantly detected in quiescent cells [48, 49]. Knockout mice of either *E2F4* or E2F5 are viable [50-52]. E2F4 knockout mice are runted and display defects in late stage of maturation. In addition, these mice present reduced thickness of the gut epithelium and developmental craniofacial defects. E2F5 knockout mice develop hydrocephalus after birth apparently due to increased secretion of cerebrospinal fluid by the choroid plexus. E2F4 and E2F5 double knockout mice die before birth because of developmental defects, suggesting that E2F4 and E2F5 have some redundant functions during development [53]. Cells lacking E2F4 and E2F5 are unable to stop cell cycling upon growth-suppressive signals such as TGF-β treatment, suggesting that E2F4 and E2F5 are major repressor E2F in restraining cell cycle progression. These two E2Fs are thought to be important for cell cycle exit and terminal differentiation.

E2F6 does not possess RB binding domain. As predicted from the structure, E2F6 contributes gene silencing independent of RB [54] and is predicted to make repressor complex with polycomb proteins [55]. Consistent with this, *E2f6*-/- animals display overt homeotic transformations of the axial skeleton that are strikingly similar to the skeletal transformations observed in polycomb deficient mice [56]. E2F6 is reported to bind the same E2F-repressor site as E2F4, suggesting E2F6 may partly overlaps its function as transcriptional repressor with E2F4 in S phase [57].

E2F7 and E2F8 are thought to function as transcriptional repressors independently of RB proteins. An important target of E2F7 and E2F8 is the *E2F1* gene. E2F7 and E2F8 knockout mice are embryonic lethal, at least in part, due to apoptosis caused by inability to down regulate expression of E2F1, leading to activation of p53 [58]. Recent report showed that E2F7 and E2F8 promote angiogenesis through transcriptional activation of the VEGFA promoter with hypoxia inducible factor 1 (HIF1) [59].

3.2. Regulatory mechanism of E2F

E2F1 through E2F6 associate with DP family proteins (DP1 or DP2) to form heterodimeric complexes that bind to DNA in a sequence-specific manner (consensus sequence: TTT$^{C}/_{G}{}^{G}$/$_{C}$CGC). E2F7 and E2F8 have two DNA binding domains and do not require DP proteins for binding to DNA. E2F7 and E2F8 make homodimer or E2F7/E2F8 heterodimer to bind to the target [58].

Transcriptional activity of E2F1 through E2F5 is suppressed by binding of RB family proteins. pRB preferentially binds E2F1 through E2F3, whereas p107 and p130 preferentially bind E2F4 and E2F5. However, pRB can also bind E2F4, depending on the cellular circumstances. E2F6 through E2F8 can repress transcription independently of pRB family proteins [10, 60]. In quiescent phase, E2F3b/pRB, E2F4/p130 and E2F5/p130 repress promoters of E2F target genes. Upon growth stimulation, activated CycD/Cdk4 and CycD/Cdk6 phosphorylate pRB and p130, inhibiting their binding to the E2Fs and allowing accumulation of the free E2Fs [31]. This release from repression conferred by the pRB family proteins is the primary activation step for induction of E2F target genes. In this context, E2F3b, E2F4 and E2F5 mainly act as repressors together with the pRB family proteins during G0/G1 phases. Expression of E2F1, E2F2 and E2F3a is induced at the G1/S boundary by E2F itself, and activate

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the growth related genes, including the *CycE* gene. CycE activates Cdk2, whose activity is essential for initiating DNA replication, and drive cells into S phase [31]. In late S phase, CycA/Cdk2 complex represses the transcriptional activity of E2F/DP complex by phosphorylation, which releases the complex from the binding element [61].

3.3. E2F target genes

Classical E2F targets are genes involved in DNA replication and cell cycle progression. In addition to these, recent studies with DNA microarray and chromatin immunoprecipitation (ChIP) identified a variety of E2F targets. These include genes involved in DNA repair, checkpoint, differentiation, development, metabolism, micro RNAs, apoptosis, cell cycle arrest and others.

DNA replication

E2F regulates expression of most of the genes involved in initiation of DNA replication: the *ORC1* (*origin recognition complex1*), *CDC6*, *MCM* (*maintenance of minichromosome*) *2-7*, *ASK* and *CDC45* genes. These factors are assembled into pre-replication complex, which is activated by CycE/Cdk2 to initiate DNA replication [31]. Cdt1 also plays crucial roles in initiation of DNA replication and is negatively regulated by geminin. The *Cdt1* and *geminin* genes are both E2F targets [62]. Genes, which code for machineries responsible for DNA replication, are classical E2F targets. These include the *DHFR* (*dihydrofolate reductase*), *DNA polymerase α*, *thymidine kinase, thymidylate synthase* and *PCNA (proliferating cell nuclear antigen)* [53].

DNA repair, checkpoint

E2F target genes related to DNA repair are the *Rad51*, *MSH2* and *MLH1* genes, which are involved in homologous recombination repair and mismatch repair [63]. E2F target genes related to checkpoint are the *ATM*, *Chk1*, *Mad3*, *Bub1*, *Claspin* and *RanBP1* genes [53, 64-67]. It is expected that E2F regulates these DNA repair and checkpoint genes to prepare machineries to quickly respond in case of emergency.

Cell cycle progression

E2F induces expression of genes, which play major roles in induction of S phase, such as the *CycE* and *activator E2F* (*E2F1-E2F3a*) genes themselves [31]. Also, the upstream negative regulators of pRB, the *Emi1* and *Skp2* genes are E2F targets [68, 69]. The genes, which encode CycA, Cdc2 (CDK1), CycB and B-myb that are important for S and G2/M phase progression, are also E2F targets [53]. Repressor E2Fs, *E2F7* and *E2F8* genes are reported to be targets of E2F1 that promote embryonic development by suppressing E2F1-p53 induced apoptosis, forming a negative feedback loop. Although c-Myc is reported as a target of E2F [70], E2F may play a role in suppression of c-Myc expression upon negative growth signals [71].

Development and differentiation

E2F also regulates expression of genes involved in development and differentiation. The *Firizzled homologs1-3*, *Homeobox* and *TGF* genes are shown to be targets of E2F

[53]. It is reported that overexpression of E2F1-3 induced various genes involved in development and differentiation [72]. Interestingly, although E2F7 and E2F8 are generally regarded as transcriptional repressors, a recent study reported the roles of E2F7 and E2F8 in transcriptional activation of genes such as VEGFA as written before [59].

Cellular metabolism

Recent work reported that E2F1 and pRB are required for repression of genes implicated in oxidative metabolism [73]. E2F1 repressed key genes that regulated energy homeostasis and mitochondrial functions in muscle and brown adipose tissue, and E2F1 null mice had a marked oxidative phenotype. Their work suggests a metabolic switch from oxidative to glycolytic metabolism that responds to stressful conditions.

Micro RNAs

Accumulating evidence indicates that expression of E2F is regulated by microRNAs and that E2F also induces expression of microRNAs [74]. One of the major microRNAs regulated by E2F is miR-17~92. miR-17~92 is a negative regulator of E2F1-E2F3 and is also a target of E2F1-E2F3, constructing a fail-safe mechanism to regulate E2F activity. E2F seems to be rigorously controlled by many miRNAs, suggesting that E2F activity must be strictly controlled for appropriate cell cycle progression.

Apoptosis and cell cycle arrest

E2F can activate genes involved in apoptosis and cell cycle arrest, which are inconvenient for cell proliferation. Regulatory mechanism of these growth-suppressive genes by E2F is yet to be elucidated, especially regarding regulation of cell growth versus tumor suppression in response to normal growth stimulation and oncogenic changes. The *caspase3*, *caspase7* and *Apaf1* genes, which code for apoptotic machineries, are direct targets of E2F [53]. Caspases are expressed as inactive precursors (procaspases), and expression of procaspases and Apaf-1 alone does not necessarily induce apoptosis. These apoptotic machineries require upstream signals to be activated to induce apoptosis. Expression of these two pro-apoptotic E2F targets and all E2F targets mentioned above is induced by growth stimulation. Thus, expression of the pro-apoptotic machinery by growth stimulation is thought to be fail-safe mechanism to induce apoptosis in case of emergency. We refer these E2F target genes, whose expression is induced by growth stimulation, to 'typical E2F targets'. Typical E2F targets are activated by growth stimulation, which physiologically inactivates RB by phosphorylation and activates E2F by release from repression.

In contrast to typical E2F targets, we identified three E2F targets, which are not activated by growth stimulation. These include the tumor suppressor *ARF*, *TAp73* and CDK inhibitor $p27^{Kip1}$ genes. As described below, these genes are critically important in tumor suppression. We describe the regulatory mechanism of the tumor suppressor *ARF*, *TAp73* and CDK inhibitor $p27^{Kip1}$ genes by E2F in next paragraph.

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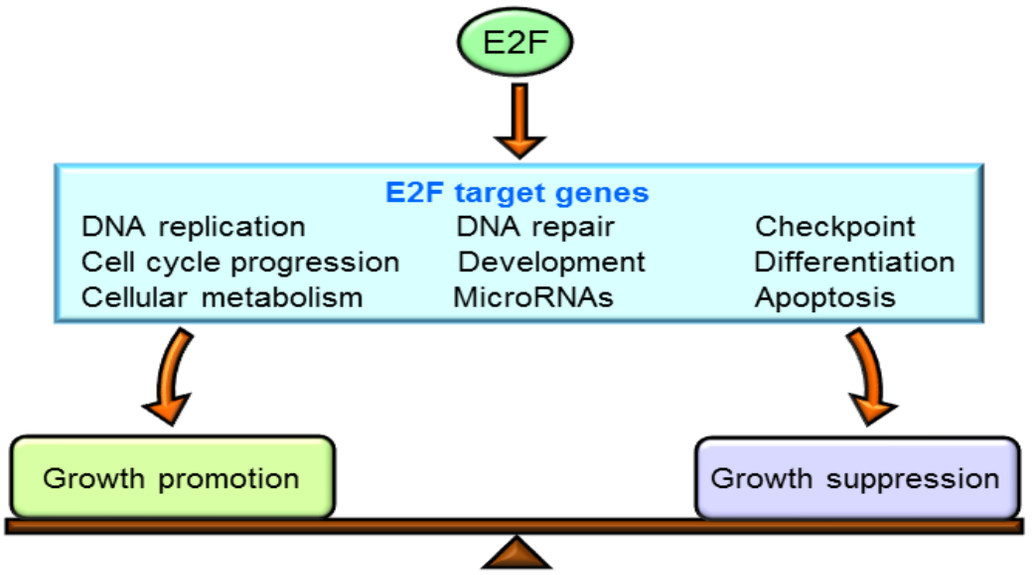

Figure 6. E2F activates both growth-promoting genes and growth- suppressive genes, including E2F itself. It is predicted that the expression levels of E2F targets, related to growth- promotion or growth- suppression, decide the balance of cell-fate determination.

4. Deregulated E2F

Since E2F1-E2F3a are activated by growth stimulation, it is generally thought that their target genes are all activated by growth stimulation. However, E2F1-E2F3a also activate genes involved in cell cycle arrest or apoptotic, which are inconvenient for cell growth. It has yet to be elucidated how E2F regulates genes involved in cell cycle arrest or apoptosis, regarding normal cell growth and tumor suppression.

Since all E2F targets are thought to be activated by growth stimulation, it is surprising that our previous work identified three growth suppressive E2F targets, which were not activated by growth stimulation at all in human normal fibroblasts [75-77]. In contrast, these E2F targets were activated by deregulated E2F activity induced by overexpression of E2F1 or forced inactivation of pRB. Overexpression of E2F1 generates exceeding amount of exogenously introduced E2F1, which becomes out control by RB proteins. Forced inactivation of pRB induces endogenous deregulated E2F activity out control by pRB. We refer these growth suppressive E2F targets, which are not activated by growth stimulation but are activated by deregulated E2F, to 'atypical E2F targets'. These atypical E2F targets include the tumor suppressor *ARF* and *TAp73* genes and the CDK inhibitor $p27^{Kip1}$ gene These three atypical E2F target genes play major roles in tumor suppression. ARF is an upstream activator of the tumor suppressor p53. CDK inhibitor $p27^{Kip1}$ activates the RB pathway by inhibiting CDKs. TAp73 is the tumor suppressor, which can induce apoptosis in dependently of p53. Our observations suggest that E2F activity induced by RB dysfunction, one of major on-

cogenic changes, has distinct function from that induced by growth stimulation in activating target genes (Figure 7).

E2F activity induced by RB dysfunction activates both typical and atypical E2F targets. In contrast, E2F activity induced by growth stimulation activates only typical E2F targets and interestingly, not atypical E2F targets. Both of the E2Fs are similar in the sense that they are released from repression by RB. However, it is shown that growth stimulation does not totally inactivate pRB and some portion of activator E2Fs is still in complex with pRB [78]. Moreover, the *RB1* gene is an E2F target [79] and expression of pRB is increased in G1/S to S phases [80]. Indeed, the amount of activator E2Fs/pRB complex rather increases in G1/S to S phases, as examined by gel mobility shift assay [78]. This observation indicates that E2F activity induced by growth stimulation is still under control of RB. In contrast, E2F activity induced by dysfunction of RB is thought to be out of control by RB. Here, we refer E2F activated by growth stimulation to 'physiological E2F' and that by dysfunction of RB to 'deregulated E2F'. Our findings indicate that deregulated E2F is functionally different from physiological E2F.

In this paragraph, we describe the regulatory mechanism of atypical E2F targets by deregulated E2F and discuss about the characteristics of deregulated E2F activity regarding its role in tumor suppression.

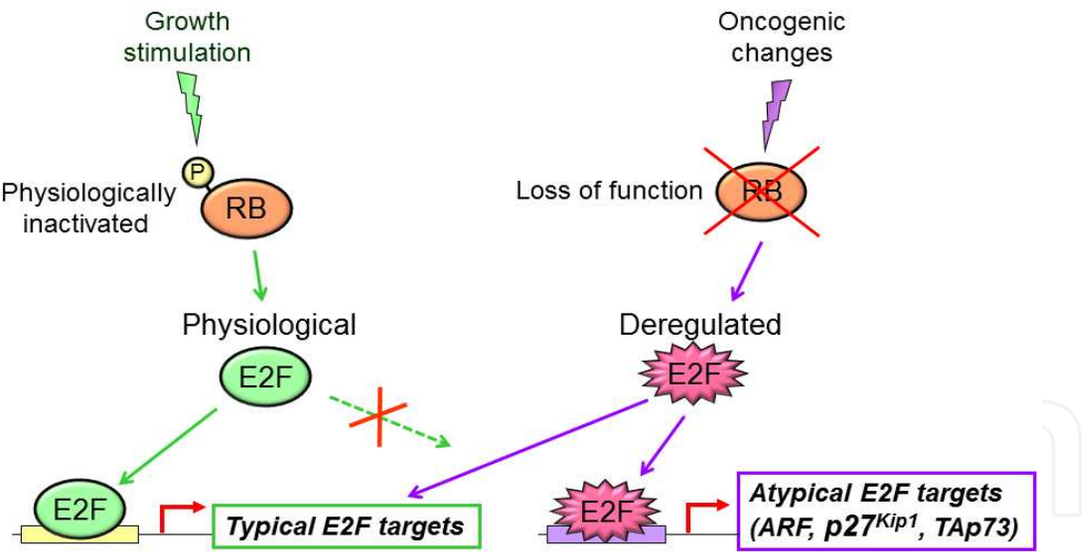

Figure 7. Atypical E2F target genes are specifically activated by deregulated E2F activity through specific E2F responsive elements.

4.1. Atypical E2F targets

The first atypical E2F target identified was the *ARF* gene [75]. ARF is the major activator of p53 pathway and links the RB and p53 tumor suppressor pathways [22], playing crucial roles in tumor suppression. Consistent with this notion, deletion, mutation or silencing of

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the *ARF* gene is frequently observed in cancers. Moreover, *Arf* null mice are highly prone to tumorigenesis [81]. Various oncogenic signals are able to elicit the activation of the *ARF* gene. Overexpression of adenovirus E1a or E2F1 in primary mouse embryonic fibroblasts (MEFs) rapidly induces ARF gene expression and p53-dependent apoptosis [16]. Myc overexpression and oncogenic mutant Ras are also strong activators of the *ARF* gene and combination of absence of Arf in mice severely impaired the tumor suppressive activity of p53 [82]. Arf promoter seems to monitor these oncogenic signals as shown by ARF promoter-*GFP* transgenic model, in which GFP expression was observed in tumors induced by Myc or Ras but not in normal growing tissues [83]. Other studies elucidated that ARF also restrains cell growth independently of p53, interacting with other factors [84]. Taken together, the *ARF* gene plays crucial roles in tumor suppression through p53-dependent and independent pathways.

The CDK inhibitor $p27^{Kip1}$ is an upstream regulator of the RB pathway and known to contribute to the ability of pRB to induce cell-cycle arrest, differentiation and senescence [85-87]. There is cross regulation between $p27^{Kip1}$ and pRB. $p27^{Kip1}$ enhances pRB growth suppressive function by inhibiting Cyc/CDK, keeping pRB in hypo-phosphorylated form. pRB increases the amount of $p27^{Kip1}$ by sequestrating Skp2, a component of E3 ubiquitin ligase complex, which promotes degradation of $p27^{Kip1}$ [86]. pRB also known to cooperate with APC/C^{cdh1}, another E3 ubiquitin ligase, to induces Skp2 degradation, stabilizing $p27^{Kip1}$ [88]. Taken together, pRB and $p27^{Kip1}$ seem to keep close relationship in the RB pathway to efficiently suppress aberrant cell proliferation. Indeed, our previous study showed that inactivation of RB by adenovirus E1a increased BrdU (bromodeoxyuridine)-positive cells much earlier in *$p27^{Kip1}$*-/- MEFs than in wild type MEFs [66]. These results support the notion that $p27^{Kip1}$ plays important roles in the RB pathway to suppress cell cycle progression induced by oncogenic changes.

The tumor suppressor *TAp73* gene had been identified as a direct target of E2F using cancer cell lines [4]. We found that the *TAp73* gene was activated by deregulated E2F but not by physiological E2F in human normal fibroblasts [77]. TAp73 is a p53 family member and plays important roles in tumor suppression with its other family member p53 and p63. All of the three genes express differentially spliced isoforms [89, 90]. Two major isoforms are TA isoforms, which retain transactivation (TA) domain, and delta N (DN) isoforms, which lack TA domain. Since these family members activate their targets as tetramers and DN isoforms lack transactivation (TA) domain, DN isoforms have dominant-negative properties [24]. Although p53 is deleted or mutated in half of all human cancers, deletion or mutations of p73 and p63 occur rarely [91, 92]. Rather over expression of DN form of p73 and p63 are commonly observed in many cancers, such as over expression of DNp73 isoform in gliomas and carcinomas of the breast and the colon [93, 94], and that of DNp63 isoform in bladder carcinomas [95]. Each DN isoform can suppress all three types of TA forms [92, 96, 97]. Therefore, it is expected that tumor-suppressive TA isoforms are suppressed by DN isoforms in many cancers. This could explain why deletion or mutations of the *TP73* and *TP63* genes are rare. *TAp73* can induce apoptosis independently of p53. Moreover, TAp73 knockout mice are tumor prone,

infertile, sensitive for carcinogen-induced tumorigenesis and defective for maintenance of genomic stability [98]. The *TAp73* gene is activated by various stress signals including deregulated E2F and DNA damage. These observations suggest that TAp73 contributes to tumor suppression in addition to the p53 pathway in response to various oncogenic changes.

Accumulating evidence indicates that the three atypical E2F targets explained above play crucial roles in tumor suppression, by activating the RB pathway, the p53 pathway and the p53 independent pathway. Taken together, deregulated E2F seems to be critically important for activating major intrinsic tumor-suppressor pathways in responding to oncogenic changes to suppress tumorigenesis (Figure 8).

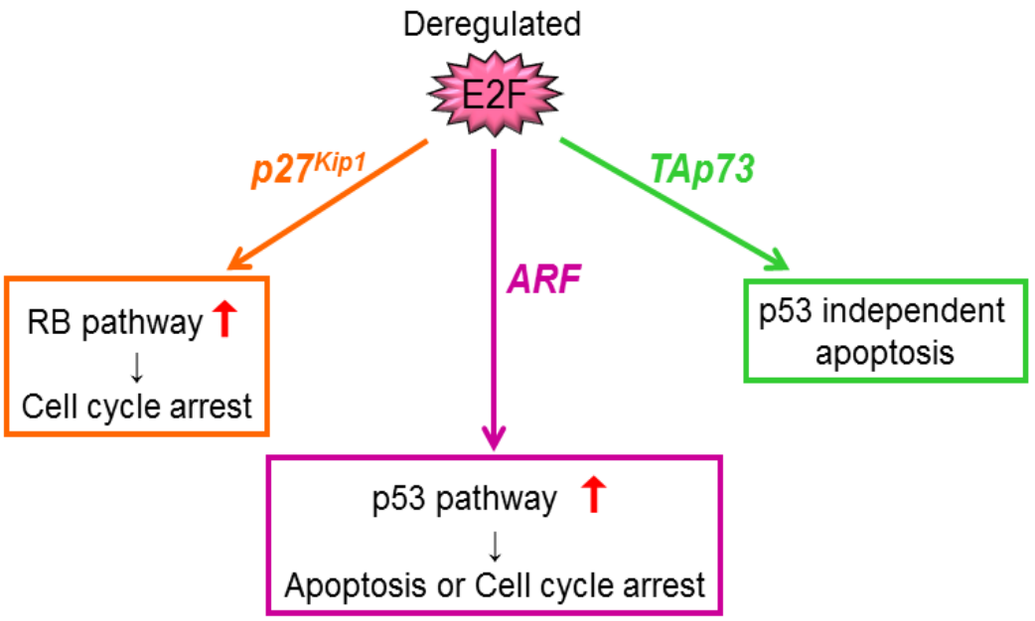

Figure 8. Deregulated E2F plays important roles in activating major tumor suppressor pathways (RB, p53 and TAp73 pathways) by activating the *ARF*, *p27*^{Kip1} and *TAp73* genes.

4.2. Distinct transcriptional regulatory mechanism mediated by deregulated E2F

ARF, p27^{Kip1} and TAp73 exert its effects when expressed unlike pro-apoptotic targets, which are expressed as inactive precursors such as pro-caspases. Thus the regulation of expression of these genes is critically important for tumor suppression. The finding that these genes are specifically activated by deregulated E2F but not by physiologically activated E2F indicates that there is a mechanism to specifically respond to oncogenic changes to suppress tumorigenesis, while allowing normal cell growth upon normal growth stimulation.

We first identified the tumor suppressor *ARF* gene as an atypical E2F target [75]. In our studies, we used human normal fibroblasts (HFFs or WI-38) to examine the responsiveness of each growth-suppressive E2F target to physiological and deregulated E2F. This is because most of previous studies used cancer cell lines and were unable to examine responsiveness

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of E2F target genes to normal growth stimulation. To induce physiological E2F, we used serum stimulation, common growth stimulation for fibroblasts. To induce deregulated E2F activity, we used ectopically expressed E2F1 or forced inactivation of RB either by adenovirus E1a, which binds to and inactivates all RB family proteins, or shRNA against *RB1* (shRB), which represses the expression of pRB. The latter (adenovirus E1a and shRB) is expected to induce endogenous deregulated E2F activity.

Ectopically expressed E2F1, adenovirus E1a and shRB induced ARF gene expression in RT-PCR, and activated ARF promoter in reporter assay. However, serum stimulation, which physiologically activates E2F, did not induce ARF gene expression or activate ARF promoter under the condition that CDC6 gene (one of typical E2F targets involved in DNA replication) expression was significantly induced and CDC6 promoter was clearly activated. These results indicate that the *ARF* gene is specifically activated by deregulated E2F but not by physiological E2F. Promoter analyses identified the E2F responsive element of ARF promoter (EREA), which specifically responds to deregulated E2F activity. Interestingly, the sequence of EREA was composed of only GC repeat and lacked T stretch. This is in contrast to that of consensus E2F binding motif, which is composed of T stretch and GC repeat ($TTT^{G}/_{C}{}^{C}/_{G}CGC$) in typical E2F targets. In addition, the location of EREA was far upstream from transcription start site compared to that of typical E2F sites, which is within 100 bp from transcription start site in most cases. Moreover, our gel mobility shift assay and ChIP assay showed that EREA specifically binds ectopically expressed E2F1 but not physiological E2F1 induced by serum stimulation, both *in vitro* and *in vivo*, respectively. Taken together, these observations suggest that the *ARF* gene is specifically activated through EREA by deregulated E2F activity, triggered by ectopically expressed E2F1 or forced inactivation of RB, but not by physiological E2F activity, induced by serum stimulation (Figure 7).

A later study showed that ARF promoter lacking EREA was still activated by overexpression of E2F1 [99]. Our further analyses of ARF promoter identified multiples of EREA-like elements in ARF promoter. It seems that, although EREA is the major E2F responsive element in ARF promoter, it is not the sole responsive element and multiple EREA-like elements co-operate to specifically respond to deregulated E2F activity (manuscript in preparation).

Defects in the RB pathway activate E2F out of control by RB, promoting abnormal cell growth. In response to such oncogenic insults, deregulated E2F activates the *ARF* gene, leading to activation of p53 to protect cell from tumorigenesis. For induction of tumorigenesis, the p53 pathway must be disabled by further oncogenic changes. Indeed, defects in the p53 pathway are observed in almost all cancers. It is expected that the presence of deregulated E2F be tolerated in cancer cells by further inactivation of the p53 pathway. We thus examined the existence of deregulated E2F activity in *RB1* deficient cancer cell lines and in normal growing fibroblasts. When we introduced constitutively active form of pRB into *RB1* deficient cancer cell lines (5637, Saos-2 and C-33 A) and normal growing fibroblasts (WI-38 and HFF), activity of EREA and ARF promoter were decreased in *RB1* deficient cancer cell lines, but not in normal growing fibroblasts. These results showed that deregulated E2F activity specifically exists in *RB1* deficient cancer cell lines but not in normal growing fibro-

blasts. The presence of deregulated E2F activity may serve as a useful marker to discriminate cancer cells from normal growing cells.

Our search for new E2F targets with subtraction method identified the CDK inhibitor *p27^{Kip1}* gene as an atypical E2F target [66]. p27^{Kip1} plays important roles in cell cycle arrest by inhibiting Cyc/CDKs. Using reporter assay, we showed that EREK (E2F responsive element of p27^{Kip1}) was responsible for specifically sensing deregulated E2F activity in human normal fibroblasts and that EREK was specifically activated in the *RB1* deficient cancer cell lines [76]. Consistent with EREA, the location of EREK was far upstream compared to typical E2F binding sites. Interestingly, the sequence of EREK contained T in addition to GC repeat and is rather similar to that of typical E2F binging site. However, EREK bound deregulated E2F1 but not physiological E2F1 in ChIP assay, showing that its character was similar to EREA. These results suggest that not only the sequence of E2F responsive elements, but also the sequence around the responsive elements may be important for discriminating deregulated E2F activity from physiological E2F activity. There is also a possibility that structure of the whole promoter might also affect the discrimination.

Third atypical E2F target is the tumor suppressor *TAp73* gene [77]. TAp73 promoter specifically responded to deregulated E2F activity through four ERE73s (E2F responsive elements of TAp73), which were specifically activated by deregulated E2F activity. The sequences of ERE73s contained T stretch and were similar to that of typical E2F binding sites. Importantly, our ChIP assay showed that bindings of ectopically expressed 'exogenous' E2F1 and deregulated 'endogenous' E2F1 induced by adenovirus E1a were detected on ERE73s, but not that of physiological E2F1 induced by serum stimulation. Thus, although the sequences of ERE73s were similar to or almost same as that of typical E2F binding sites, the characters of both were completely different. ERE73s were specifically activated by deregulated E2F activity and specifically bound to both 'exogenous' and 'endogenous' deregulated E2F1. These results support the notion that both sequences of the E2F binding site and its flanking region may be important for discriminating deregulated E2F activity from physiological E2F activity. Consistent with EREA and EREK, reporter assay showed that ERE73s were also activated in the *RB1* deficient cancer cell lines and not in normal fibroblasts. Moreover, reintroduction of the constitutive active from of pRB by recombinant adenovirus reduced the expression of the *TAp73* gene in all the cancer cell lines in RT-PCR, indicating that the cancer cell lines harbor deregulated E2F activity that activates the endogenous *TAp73* gene.

Interestingly, our unpublished data suggest that not only the *RB1* deficient cancer cell lines but also cancer cell lines retaining pRB harbor deregulated E2F activity. Activity of ERE73s and expression of the *TAp73* gene were suppressed by introduction of the constitutive active form of pRB in cancer cell lines retaining pRB. These results suggest the possibility that E2F-mediated transcriptional program can sense defects in the RB pathway, not only pRB itself but also upstream regulators of pRB. Taken together, deregulated E2F activity might become a universal means to discriminate cancer cells (may be regardless of the presence of pRB) from normal growing cells.

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4.3. Difference between deregulated E2F and physiologically activated E2F

Deregulated E2F and physiologically activated E2F are similar in a sense that both are 'released from RB'. However, there is a functional difference between deregulated E2F and physiologically activated E2F. Deregulated E2F activates both typical and atypical E2F targets. In contrast, physiologically activated E2F activates typical E2F targets but not atypical E2F targets. Why atypical E2F targets are activated by deregulated E2F and not by physiologically activated E2F? What is the difference between deregulated E2F and physiologically activated E2F? Deregulated E2F is totally 'out of control' by RB due to dysfunction of the RB pathway. In contrast, physiologically activated E2F is temporarily released from RB, predicted to be 'under control' of RB. During normal cell growth, activator E2Fs are induced at G1/S boundary of the cell cycle. At the point, it is generally believed that pRB is phosphorylated and inactivated. However, pRB is not totally inactivated at the point. Previous studies indicate that the *RB1* gene is an E2F target [79] and expression of pRB is increased in G1/S to S phases [80]. Moreover, the amount of activator E2Fs/pRB complex rather increases at G1/S boundary as shown by gel mobility shift assay [78], indicating that some portion of pRB is still active and is regulating the activity of activator E2Fs. Thus, physiologically activated E2F is still under control by pRB. It is likely that activity of activator E2Fs is strictly controlled by degree of phosphorylation of pRB dependent on the activity of CDKs, reflecting the strength of growth stimulation. There must be difference between activation of E2F out of control by pRB and activation of E2F under control by pRB. Our studies of regulatory mechanism of atypical target promoters by E2F elucidated the four different points between regulation of typical E2F target promoter and that of atypical E2F target promoter.

1. Although sequences of EREK and ERE73s resemble that of typical E2F binding sites, sequence of EREA is different from that of typical E2F sites. The sequence of consensus E2F binding motif of typical E2F targets is composed of T stretch and GC repeat ($TTT^{G}/_{C}{}^{C}/_{G}CGC$). EREA is composed of only GC repeat and lacks T stretch. Difference in binding sequence suggests the possibility that there may be a difference in factors, which recognize the sequence and bind.
2. In all three cases, location of E2F responsive elements of atypical E2F targets is far from the transcriptional start site compared to that of typical E2F binding sites in typical E2F targets. In the case of typical E2F targets, location of E2F binding sites is within 100 bp from transcriptional start site in most cases. Typical E2F targets are under repression by E2F/RB complex in quiescent phase and are released from repression upon growth stimulation. Close proximity of E2F binding sites to transcriptional start site may be required for this mode of regulation. In contrast, atypical E2F targets are not under repression by E2F/RB complex and literally activated by deregulated E2F. E2F responsive elements of atypical E2F targets behave as enhancer elements, which can function from distance.
3. Deregulated E2F1 bound to EREA, EREK and ERE73s and physiologically activated E2F1 did not bind to these elements as shown by ChIP assay. In the case of EREA, it is also shown that repressor type E2F4 does not bind to EREA. This observation is compatible with the observation that these atypical E2F targets are not under repression by

E2F/RB. The fact that deregulated E2F bind to atypical E2F targets, while physiological E2F does not bind to atypical E2F targets, suggest that there is difference in binding behavior between deregulated E2F and physiologically activated E2F.

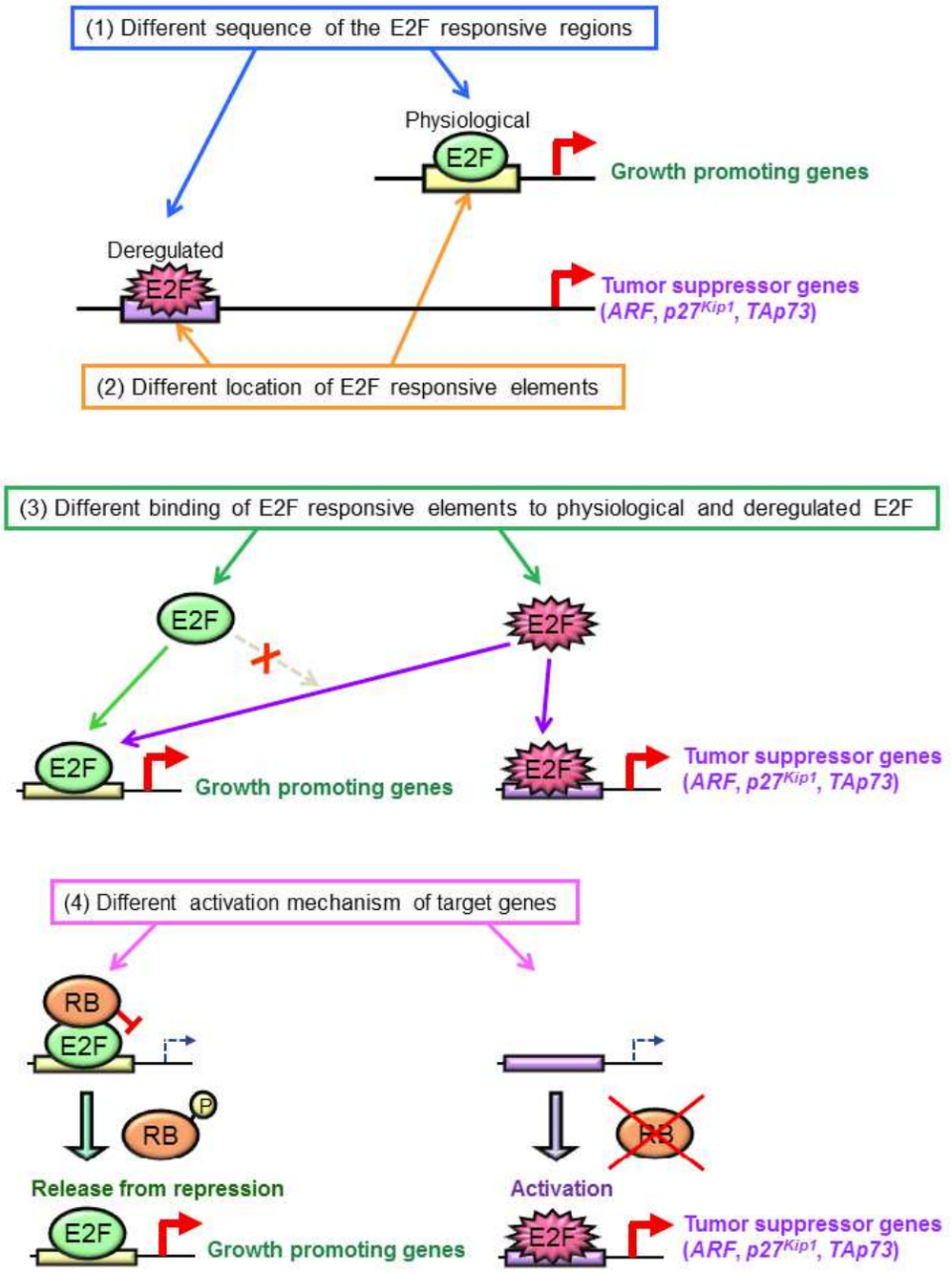

Figure 9. Differences in E2F regulation of target promoters between deregulated E2F and physiological E2F. (1) Sequence of the atypical E2F responsive elements and surrounding regions are different from that of typical E2F targets. (2) E2F responsive elements of the atypical E2F targets locate far upstream from transcriptional start sites compared to that of typical E2F targets. (3) E2F responsive elements of atypical E2F targets specifically bind deregulated E2F and not physiologically activated E2F. (4) Unlike typical E2F targets, promoters of atypical E2F targets are not under repression of RB and are specifically activated by deregulated E2F activity.

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4. Regulatory mechanism of promoters is different between typical E2F targets and atypical E2F targets. Promoters of typical E2F targets are repressed by E2F/RB complex. Growth stimulation inactivates RB and releases promoters from the repression by RB. Thus, so-called activation of typical E2F targets by physiological E2F is 'release from repression by RB'. In contrast, activation of atypical E2F targets by deregulated E2F is literally 'activation'. Mutation of EREA, EREK or ERE73s in corresponding full-length promoter did not enhance basal promoter activities, indicating that these three promoters are not under repression through the E2F responsive elements. This is consistent with the observation that binding of physiological E2F to promoters of atypical E2F targets was not observed in ChIP assay, including repressor type E2F4 (Figure 9).

It is generally accepted that the amount of free E2F is important for differential regulation of E2F targets, which have opposite roles in cell-fate determination, as proposed as threshold model [100]. According to this model, when the amount of free E2F (released from repression of RB) is below the threshold, E2F activates only growth-related target genes. When the amount of free E2F exceeds the threshold, E2F activates not only growth-related targets but also pro-apoptotic targets. However, molecular mechanism of how different amount of free E2F differentially regulates target genes is not yet elucidated. The 'quantitative' difference of free E2F seems not sufficient to explain the four differences between deregulated E2F and physiologically activated E2F. The above-mentioned differences strongly suggest the presence of qualitative difference between physiologically activated E2F by 'temporal release' from RB and deregulated E2F induced by 'dysfunction' of the RB pathway [75-77] (Figure 9). The 'qualitative' difference between both of the E2Fs seems to be a useful cue to elucidate how cells discriminate oncogenic growth stimulation from physiological growth stimulation.

5. Conclusion and further research

Accumulating evidence indicates that E2F plays essential roles in cell-fate determination, to grow, stop or die. Together with G1/S gatekeeper RB, E2F governs control of the restriction point, deciding whether to grow or not. Upon normal growth stimulation, physiologically activated E2F facilitates cell proliferation. Upon dysfunction of the RB pathway, deregulated E2F suppresses cell growth by inducing $p27^{Kip1}$ to restrain cells from aberrant cell growth. $p21^{Cip1}$, induced by p53 through activation of the *ARF* gene, may also contribute to suppression of cell growth. When the arrest mechanism failed to stop the aberrant cell cycle progression, E2F induces apoptosis through activation of p53 and TAp73 to protect cells from tumorigenesis. E2F seems to sense and discriminate between normal growth signals and abnormal growth signals originating from various oncogenic changes, asking cells whether to grow, stop or die.

Deregulated E2F activity specifically exists in cancer cell lines but not in normal growing fibroblasts, suggesting that deregulated E2F activity may be a useful means to discriminate abnormally growing cancer cells from physiologically growing normal cells. Since the generation of deregulated E2F activity is expected to be based on the mechanism of oncogenesis, deregulated E2F activity could be a universal marker to discriminate cancer cells from

normal growing cells. Analyses of atypical E2F targets suggest that deregulated E2F might be qualitatively different from physiologically activated E2F. One of the most intriguing issues in the future studies would be the molecular nature of deregulated E2F. By elucidating the molecular nature of deregulated E2F, we might be able to specifically approach cancer cells without affecting normal growing cells. For this purpose, qualitative difference between deregulated E2F and physiological E2F is eager to be elucidated.

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