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Chapter

Distinct E2F-Mediated Transcriptional Mechanisms in Cell Proliferation, Endoreplication and Apoptosis

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Abstract

E2F and DP family proteins are evolutionarily conserved transcription factors among higher eukaryotes. E2F and DP proteins typically form a heterodimeric complex, which controls cell proliferation by regulating expression of growth-related genes. In addition, E2F family proteins have roles in various cellular events that require the expression of context-specific genes. E2F proteins use distinct mechanisms to regulate context-specific genes in different circumstances. The primary goal of this chapter is to compare three distinct mechanisms of mammalian E2F-mediated transcriptional regulation that control cell proliferation, endoreplication and apoptosis. Briefly, E2F7 and E2F8 control endoreplication by suppressing the expression of their target genes. They do not require DP or pRb. In control of apoptosis, E2F1 regulates the expression of the tumor suppressor gene *Arf* by binding to a non-canonical E2F binding site, within the *Arf* promoter, in a DP-independent manner. Furthermore, we examine the functions of E2F and DP in *Drosophila melanogaster* (fruit fly) to identify those mechanisms of E2F-mediated transcriptional regulation that have been evolutionarily conserved. The detailed mechanisms of how E2F protein regulates the expression of context-specific target genes will be instrumental in understanding how a single family of transcription factor regulates diverse pleiotropic cellular processes in an organism.

Keywords: E2F, DP, pRb, Arf, p53, cell cycle, endoreplication, apoptosis

1. Introduction

The temporal control of gene expression is essential to the execution of cellular events such as proliferation, growth, self-renewal, differentiation and death. For example, a proliferating cell performs the sequential processes of the cell cycle by the orderly expression of genes involved in DNA replication, DNA repair, mitosis and cytokinesis [1, 2]. During the cell cycle, *E2* promoter binding *f*actor (E2F) and *D*RTF1-*p*olypeptide (DP) form a heterodimeric complex E2F/DP, which functions with retinoblastoma protein (pRb) to regulate the timing of expression of growth-related genes at the level of transcription [1–5]. In quiescent cells, the E2F/DP complexes interact with pRb family proteins to prevent cell cycle re-entry by actively

repressing the expression of growth-related genes (**Figure 1A** and **B**). Mitogenic signals promote the assembly and activation of the Cyclin D/cyclin-dependent kinase (cdk) 4 complex in the cell nucleus. Phosphorylation of pRb family proteins by the Cyclin D-cdk4 complex results in their dissociation from the E2F/DP complex (**Figure 1A**), and consequently growth-related genes are de-repressed. The free E2F/DP complexes also promote transcription of their target genes (**Figure 1A** and **B**). Primary E2F target genes in the cell cycle encode DNA-replication factors. In addition, the E2F/DP complex induces the expression of cyclins (E, A, and B) and genes involved in DNA repair, mitosis and cytokinesis. Thus, the pRb/E2F/DP pathway controls not only the G1/S transition, but also influences other processes of the cell cycle. Once the level of mitogen signals is reduced, cdk activity is down-regulated, under-phosphorylated pRb family proteins accumulate, E2F activity is repressed, and cells exit from the cell cycle.

Genome wide gene expression profiles and analysis of the function of individual E2F family proteins have revealed that E2F family members have roles in various cellular processes including endoreplication [6], cell death [7], autophagy [8] and differentiation [9]. Since it is unlikely that E2F simultaneously induces genes involved in these distinct, often mutually exclusive cellular processes, cells must have multiple mechanisms, by which specific E2F target genes are expressed to function in line with intracellular circumstances. Indeed, the mechanism of E2F1 regulation of tumor suppressor genes, *Alternative reading frame of cdkn2a* (*Arf*), *p27* and *p73* is distinct from that of growth-related genes [10–12]. Surprisingly, the requirement for DP is different between E2F1 regulation of *Arf* and growth-related genes such as *cell division cycle 6* (*cdc6)*, implying that DP protein is not involved in all E2F-mediated transcriptional regulation [13]. In this chapter, we describe mechanisms of E2F-mediated transcriptional regulation in three different cellular functions, cell proliferation, endoreplication and apoptosis, and discuss the requirement for DP in these processes. In addition, we compare and contrast these mechanisms in mammals and flies, to identify those that have been conserved or emerged during the process of evolution.
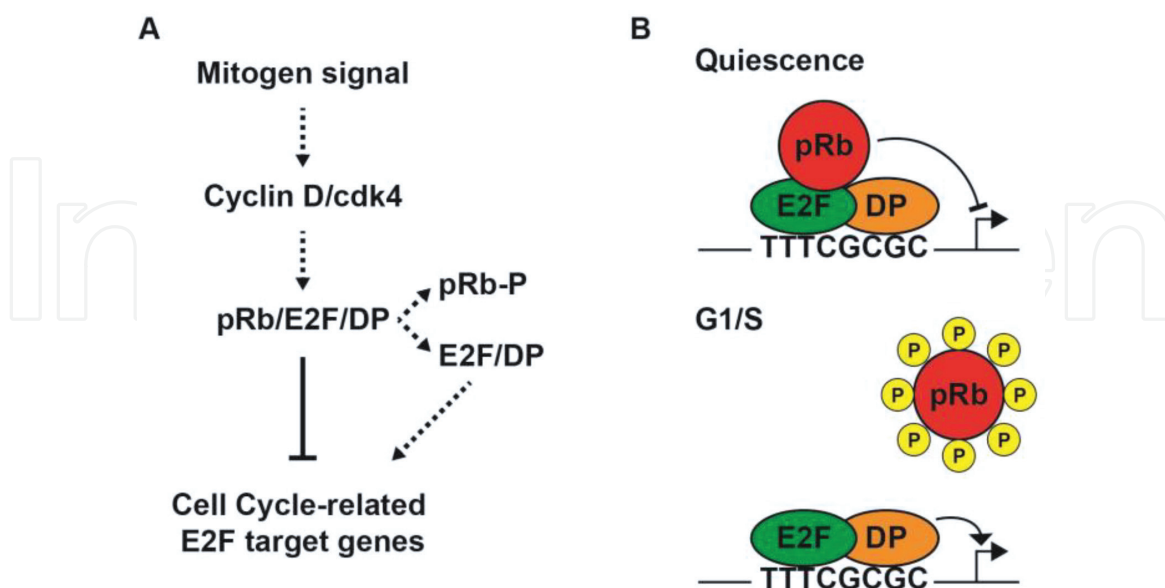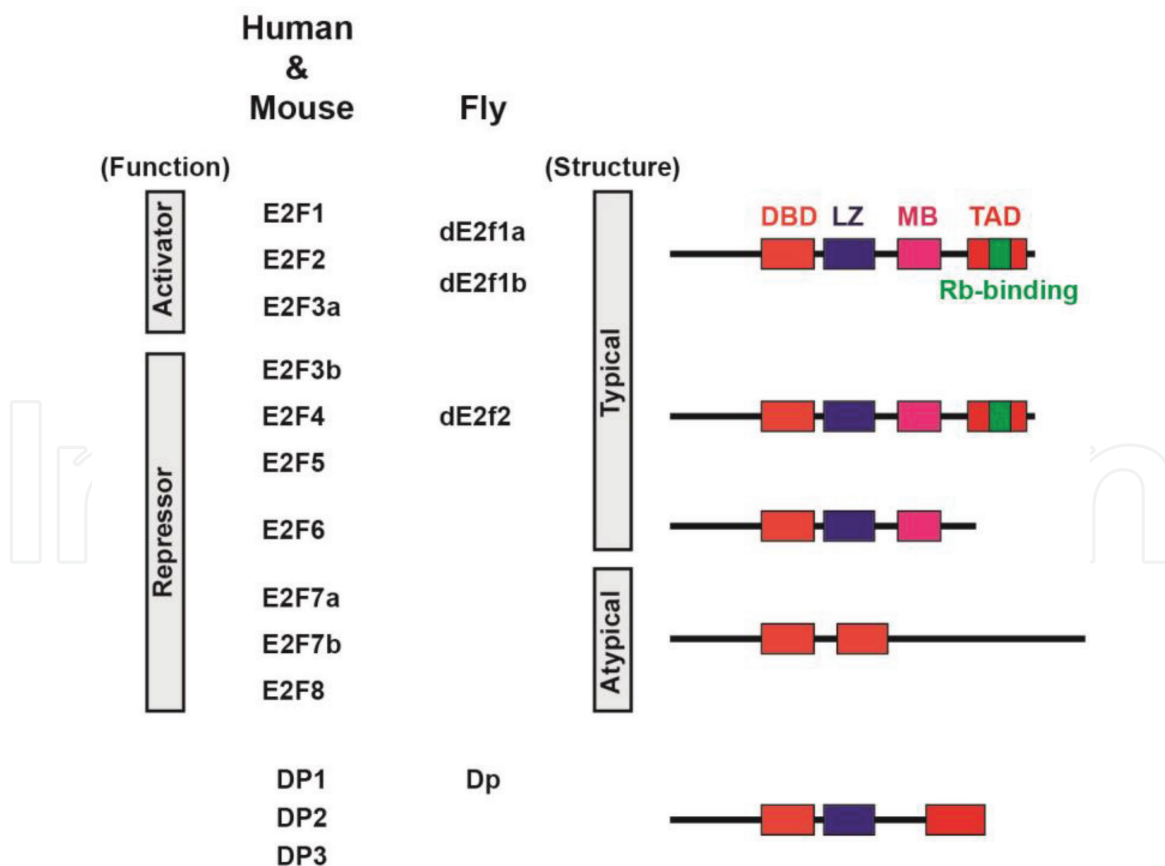

Figure 1.
The E2F/DP complex controls cell cycle progression with pRB. (A) Schematic view of the pRb/E2F/DP pathway. Solid line indicates the function of pRb/E2F/DP complex to repress the expression of growth-related E2F-target genes in quiescence. Dashed arrows indicate the signal cascade following activation by mitogenic signals. pRb-P indicates phosphorylated pRb. (B) Regulation of growth-related E2F target genes by the pRB/E2F/DP complex during the cell cycle. The pRb/E2F/DP complex actively represses the transcription of growth-related E2F target genes in quiescence, while free activator E2F/DP complex promotes transcription of the target genes when pRb family proteins are phosphorylated by G1-cyclin/cdk.

2. Structures of mammalian and fly E2F and DP family members

The human and mouse genomes contain eight E2F family genes and three DP family genes (**Figure 2**) [3]. E2F family proteins can be distinguished as "activator" or "repressor" by their functions in transcription, or "typical" or "atypical" based on their structure. E2F1–5 genes encode proteins composed of a winged-helix *D*NA-*b*inding *d*omain (DBD), *L*eucine *z*ipper (LZ) domain, *M*arked-*b*ox (MB) domain and *trans*activation *d*omain (TAD) that includes pRb-binding motif (**Figure 2**). E2F6 protein lacks the TAD and pRb-binding motif (**Figure 2**). E2F1–3a are categorized as activator E2Fs because they are essential to activate transcription of target genes in cell culture [3]. E2F3b-5 are designated repressor E2Fs since their main function is to suppress the expression of target genes by interacting with pRb family proteins in resting states [14–16]. E2F6 is also a repressor E2F, but functions without interacting pRb family proteins [17–19]. E2F1–6 proteins interact with one of the DP proteins through LZ and MB domains (dimerization domain: DD). All DP family members possess a DBD and DD [3]. In addition, the C-terminus of DP1 can interact with TFIIH, suggesting that DP1 directly contributes to activation of target gene transcription [20]. DP1 and DP2 support the transcriptional activation of E2F target genes while DP3 functions to inhibit E2F-dependent transcription [21, 22]. The E2F/DP complex typically recognizes specific DNA sequences TTT^C/$_G$^{G}/$_C$CG^C/$_G$ (hereafter referred to as "canonical E2F binding site" in this chapter) [23]. E2F1 is also able to

Figure 2.
E2F and DP family members in human, mouse and fly. E2F families can be divided into two groups by functional or structural properties. Activator E2Fs are required to induce expression of growth-related E2F target genes at G1/S phase transition. E2F3a-5 are expressed in quiescence (G0) and G1 phase to repress target gene transcription by pRb-dependent mechanisms. E2F6–8 have the ability to repress target gene transcription by pRb-independent mechanisms. Typical E2Fs possess DBD, LZ, MB and TAD, while atypical E2Fs possess two DBD without a TAD. DP family proteins share DBD and LZ. TAD is found in mammalian DP1.

bind to a non-canonical E2F binding site called *E*2F-*r*esponsive *e*lement of *A*rf (EREA), comprised of the sequence CGCGCGCGCGCCTCC [10].

After completion of the human and mouse genome sequencing projects, searches for homologous sequences to the E2F-DBD identified atypical E2F family members, E2F7 and E2F8 that are structurally distinct from E2F1 to 6 (**Figure 2**) [24–29]. E2F7 and 8 contain two DBDs but lack a transactivation domain. These atypical E2Fs recognize canonical E2F binding sites in a DP-independent manner and function to repress transcription of E2F target genes by a pRb-independent mechanism.

The fly genome contains two *e2f* genes and a single *dp* gene [3, 4, 30]. *de2f1* encodes two isoforms, which function as transcriptional activators, while dE2f2 acts as a repressor in transcription (**Figure 2**) [31, 32]. Because all other higher eukaryotes also possess functional homolog of activator E2F, repressor E2F and DP, the mechanism of transcriptional control by the E2F/DP complex has been evolutionally conserved [33]. Atypical E2Fs are shared in several model organisms including mammals, worm and plant, but not in fly (**Figure 2**) [33]. DP-independent transcriptional regulation by atypical E2Fs seems to have been emerged and been lost during the process of evolution.

## 3. The role of the E2F/DP complex in cell proliferation

3.1 DP is essential in transcriptional regulation of growth-related E2F target genes in cultured cells

The concept of the pRb/E2F/DP pathway is based primarily on evidence obtained using rodent fibroblasts in culture [1–5]. The advantage of this system is the ease of manipulating the proliferative capacity of cells without losing cell viability by the absence or presence of fetal bovine serum in the culture medium [34, 35]. Fibroblasts continuously proliferate in culture medium that contains abundant serum. Withdrawing serum from the media causes cells to exit from the cell cycle, while re-introduction of serum leads to re-entry into the cell cycle. Thus, this system enables control of endogenous E2F activity by manipulating the amount of serum in culture media. In addition, using expression vectors and recombinant adenovirus containing various E2F genes, we can examine the specific activity of individual E2F proteins in quiescent fibroblast without other confounding proliferating signals [2]. The functional analysis of individual E2F proteins in quiescent fibroblasts revealed that activator E2Fs are sufficient to promote the G1/S transition in the cell cycle [34, 35]. Conversely, loss of activator E2Fs causes cell cycle arrest at G1 phase [36]. Accordingly, DP1 is required for G1/S transition and cell proliferation in human fibroblasts [13]. These observations indicate that the activator E2F/DP complexes promote and regulate the G1/S transition in cultured cells. However, subsequent studies revealed that the cell cycle arrest caused by concomitant loss of activator E2Fs in cultured fibroblasts is mediated through the p53-p21 axis, which reduces cdk activity [37]. Inactivation of p53 restores the proliferative ability of *E2f1–3* triple mutant fibroblasts [38]. These results suggest that cells have permissive factor(s), which promote G1/S transition in the absence of activator E2Fs, and raise the question of how activator E2Fs suppress p53 activity in proliferating cells. A strong candidate of E2F target genes that induce activation of p53 in this context was *Arf*, since permanent loss of *E2f3* induces *Arf* expression [38, 39]. However, acute inactivation of E2F activators does not increase *Arf* expression and activation of p53 does not require *Arf* in *E2f1–3* triple mutant fibroblasts [38]. Therefore, induction of *Arf* gene expression by loss of *E2f3* is presumably an indirect effect

and activator E2Fs suppress activation of p53 by Arf-independent mechanisms in fibroblasts. Elucidating the mechanisms, by which activator E2Fs suppress p53 activity in proliferating cells, will provide deeper understanding of how activator E2Fs regulate cell proliferation.

Cell culture systems and *in vitro* biochemical experiments have also revealed molecular mechanisms underlying transcriptional regulation of target genes by the E2F/DP complex [1, 2, 5]. Expression levels of growth-related E2F target genes are very low in quiescent fibroblasts, while serum stimulation increases the expression of these genes concomitant with phosphorylation of pRb family proteins by G1-cyclin/cdks [1–5]. Indeed, loss of all *Rb* family genes increases the level of growth-related E2F target gene expression in quiescent mouse fibroblasts [40]. The concept that pRb/E2F/DP complex actively represses the transcription of growth-related E2F target genes in quiescence was conceived based on results from reporter assays, in which the promoter region of an E2F target gene is isolated and fused upstream of a reporter gene such as *chloramphenicol acetyltransferase* or *luciferase* to monitor promoter activity [34, 41]. Analysis of such reporter activity during the cell cycle revealed that the wild-type promoter recapitulates endogenous gene expression patterns throughout the cell cycle, while mutations in canonical E2F binding sites de-represses the promoter activity in quiescence and does not allow further up-regulation at the G1/S transition (**Figure 3**). E2F4 and p130 occupied canonical E2F binding sites on promoters of growth-related E2F target genes in quiescence [14, 16]. DP1 is required for E2F4 binding on the promoter of *cdc6* in quiescent human fibroblasts and for repression of the *cdc6* promoter activity [13] (Komori & Ohtani unpublished data). pRb family proteins repress E2F target promoter activity by recruiting histone modifier proteins and chromatin remodeling complexes [42–47]. Thus, the repressor E2F/DP complex acts as a platform on the target promoters to recruit pRb family proteins, which induce a dynamic change of chromatin structure, robustly shutting down the transcription of growth-related E2F target genes (**Figure 1B**).

Activator E2Fs induce stronger activation of E2F target gene transcription compared to repressor E2Fs in reporter assays. Co-overexpression of DPs induces further activation of reporter transcription while knockdown of DP1 reduces promoter activity induced by activator E2Fs [13, 48, 49]. Thus, DP is essential for both active repression and activation of growth-related E2F target gene transcription
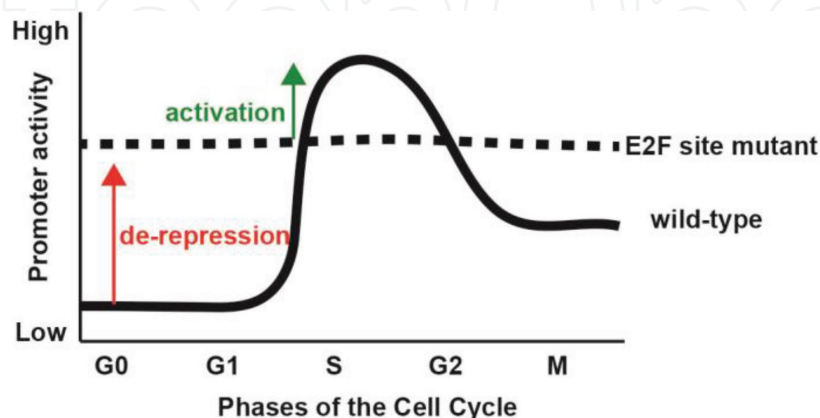
Figure 3.
The promoter activity of growth-related E2F target genes during the cell cycle. Solid line indicates the activity of wild-type promoter of growth-related E2F target genes. Dashed line indicates the activity of canonical E2F binding site mutant promoter of growth-related E2F target genes. The difference in promoter activity between wild-type and E2F binding site mutant is due to pRB/E2F/DP complex-dependent repression. Activator E2Fs increase the wild-type promoter activity to a higher level than the mutant promoter activity at the G1/S phase transition.

(**Figure 1B**). The main role of DP in the regulation of growth-related E2F target gene expression is in DNA binding of the E2F/DP complex [3, 20]. A monomer of E2F or DP is able to bind to DNA *in vitro*, whereas the heterodimer complex shows much stronger DNA binding affinity [48, 49]. Structural analysis of the complex of E2F4/DP2 bound to a canonical E2F binding site revealed that the E2F/DP complex holds DNA by DBDs of both E2F and DP [50]. Thus, DP protein ensures a stable binding of the E2F/DP complex to precisely control expression of growth-related E2F target genes during the cell cycle.

3.2 The role of the E2F/DP complex in mouse development

In order to form functional tissues and organs, stem cells and progenitor cells continuously proliferate to generate progeny cells, which must exit from the cell cycle at the onset of commitment to a differentiated state [51]. Because loss of all activator E2Fs or loss of DP1 induces cell cycle arrest of cells in culture [13, 36, 52], *E2f1–3* triple mutant was expected to show a premature exhaustion of cell proliferation during development. However, the *E2f1–3* triple mutant mouse embryo grows to mid-gestation stage without severe defects of cell proliferation [53, 54]. In the absence of activator E2Fs, the Myc family transcription factors compensate for their function to promote the G1/S transition [54, 55]. n-Myc can functionally substitute for activator E2Fs in retinal progenitors, while c-Myc complements activator E2Fs in intestinal stem cells. *E2f1–3* triple mutant embryos also exhibit an apoptotic phenotype in multiple tissues, in which activation of p53 is observed. This suggests that activator E2Fs suppress the activation of p53 during development *in vivo*, in addition to cells in culture. *E2f1–3* triple knock out analysis revealed another aspect of the function of activator E2Fs [55]. The expression of growth-related E2F target genes is increased by inactivation of E2f1–3 in differentiated cells while loss of E2F1–3 reduces the expression of growth-related E2F target genes in proliferating progenitor cells, suggesting that E2F1–3 contribute to repress target gene expression in differentiated cells. However, loss of E2F1–3 does not induce differentiated cells to re-enter into the cell cycle, implying that, in addition to de-repression of growth-related E2F target genes, other factors are required to cause re-entry of terminally differentiated cells into the cell cycle.

Removing all E2f genes from a single mouse is technically impossible at this stage. The DP family has three members and DP1 is highly expressed in many tissues. Thus, it is anticipated that loss of DP1 would mimic the phenotype expected due to elimination of all E2F/DP complexes in the mouse. *DP1* mutant mice die *in utero* due to defects in placenta development, while their somatic cells proliferate without severe defects [56, 57]. There is a possibility that other DP family proteins complement the function of DP1 in *DP1* mutant animals. However, the levels of DP2 expression are very low in the wild-type and its expression is not significantly changed in *DP1* mutant embryos. The expression of DP3 has not been investigated in *DP1* mutant mice. However, since DP3 inhibits activation of growth-related target genes by E2F1 in cultured cells [21, 22], it is not likely that DP3 compensates for DP1 function. In conclusion, the E2F/DP complex function is required for normal development, but the viability seems to be determined by functions that are independent from cell cycle control.

3.3 The role of DP in fly development

Mammalian genomes contain several E2F and DP family members, and their functional relationships are very complicated. Because the fly genome contains two E2Fs, only one DP and no atypical E2Fs, the combinatorial interactions and

possibility of compensation by other family members are more limited [4, 33]. In drosophila, loss of *de2f1* reduces cell proliferation during development [58]. The decline in cell proliferation induced by loss of *de2f1* is restored by removing *de2f2* gene function, indicating that dE2f1 and dE2f2 have opposite functions in control of cell proliferation [31, 32]. *dDP* mutant larvae phenocopy the *de2f1 and de2f2* double mutant with respect to cell proliferation and lethality at the late pupal stage [31, 32]. The viability of the *dDP* mutant fly is rescued by restoring the defects in muscle development and/or fat body cell growth [59, 60], implying that the lethality is due to non-cell cycle function of dDP. These indicate that the E2F/DP complexes are not necessary for cell proliferation during fly development. The possibility that dMyc may compensate for the function of the dE2f/dDP complex in cell proliferation has yet to be examined.
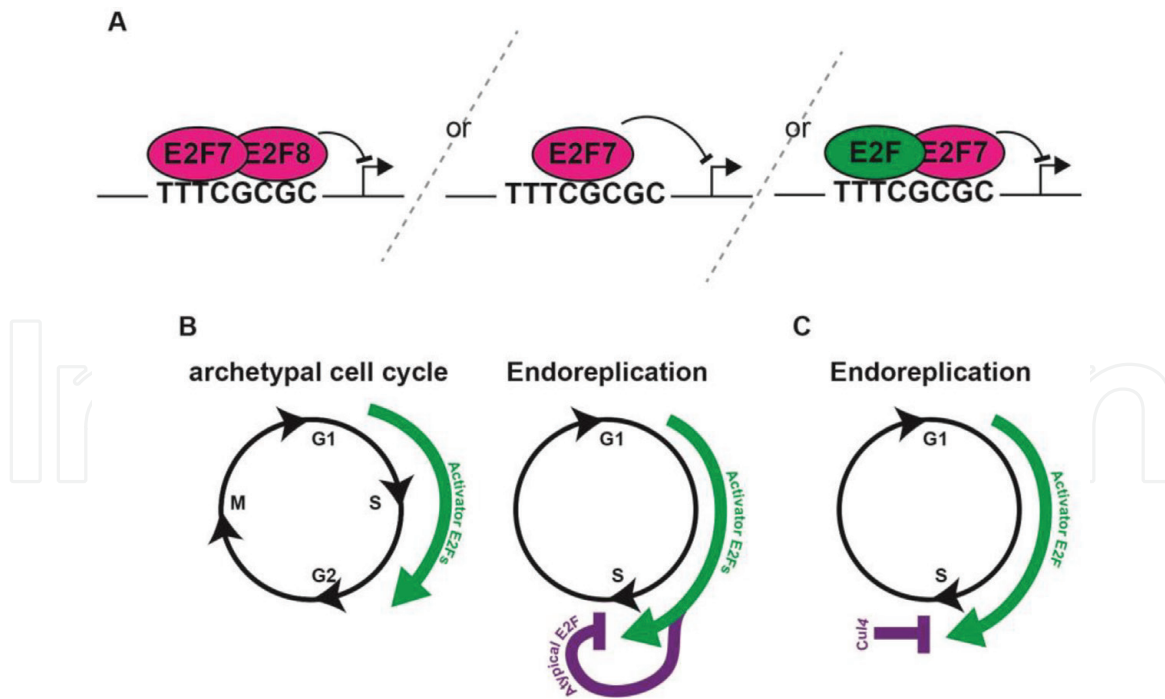
4. The role of atypical E2Fs in endoreplication

4.1 Atypical E2Fs inhibit cell cycle progression in cultured cell

E2F7 and E2F8 are atypical E2F proteins, which are composed of two DBDs lacking a transactivation domain or pRb binding motif (**Figure 2**) [3, 24–29, 33]. They do not interact with DP, and thus atypical E2Fs function independently of DP protein. Since overexpression of atypical E2Fs represses the promoter activity of growth-related E2F target genes, they are designated repressor E2Fs. They function via a pRb-independent mechanism. E2F7 recruits the transcriptional co-repressor CtBP to repress the activity of its target promoter in fibroblasts [61]. Details of molecular mechanisms of how atypical E2Fs regulate E2F target genes have been controversial. DBD1 of E2F7 is similar to the DBD of the E2F family, while DBD2 of E2F7 is homologous to that of the DP family. Both DBDs are necessary for the DNA binding ability of atypical E2Fs. Crystal structure analysis revealed that DBD1 and DBD2 bind to a typical E2F binding site in a manner similar to the E2F4/DP2 heterodimeric complex [62]. This suggests the possibility that atypical E2Fs are able to function as a monomer (**Figure 4A**). However, they form a homodimer or a heterodimer of E2F7 and E2F8 in cells (**Figure 4A**) [24–29]. How this dimeric complex of atypical E2Fs binds to DNA remains to be addressed. In addition, it is reported that E2F7 inhibits the function of E2F1 by direct interaction (**Figure 4A**) [61]. How then does an E2F1/E2F7 heterodimer bind to DNA and how does E2F7 dominate E2F1 activity on their target promoter? Overexpression of atypical E2Fs inhibits proliferation of fibroblasts in culture [24–29]. Expression of atypical E2Fs is upregulated in G1/S transition of fibroblasts because their expression is under the control of typical E2Fs. These suggest that atypical E2Fs may function as a negative feedback loop to antagonize the function of activator E2Fs in S-phase of the cell cycle. However, cells can proliferate in the presence of atypical E2Fs. If they are dispensable in the control of cell proliferation, what is the role of atypical E2Fs in the physiological setting?

4.2 Atypical E2Fs control endoreplication during mouse development

Mouse atypical E2Fs play crucial roles in endoreplication of placental *t*ropho-blast *g*iant *c*ells (TGCs) and liver hepatocytes [3, 33, 63]. While the archetypal cell cycle proceeds to G2 and M phases after completing DNA replication, endoreplica-tion is a variant cell cycle, which repeats the cycle of G1 and S phase in the absence of intervening mitoses (**Figure 4B**) [64]. Consequently, endoreplication produces mononucleated polyploid cells. Since endoreplication does not permit cells to enter

Figure 4.
Atypical E2Fs regulate growth-related E2F target genes by DP-independent mechanism(s) and control endoreplication. (A) Possible mechanisms to repress target promoters by atypical E2Fs. Atypical E2Fs repress the transcription of their target genes by forming a dimer of atypical E2Fs, or monomer, or binding to an activator E2F. (B) Archetypal cell cycle and endoreplication. Four phases (G1, S, G2, M) are proceeded in the archetypal cell cycle, while endoreplication repeats G1 and S phases. Atypical E2Fs are induced at G1/S phase and antagonize activator E2Fs at S phase in an endoreplication cycle. (C) Endoreplication in fly. Cul4 induces an acute degradation of dE2f1 to skip G2 and M phases.

into mitosis, precise control of mitotic cyclin/cdk activity is important to determine the initiation and termination of endoreplication.

Loss of *E2f1–3* genes results in continuous excess rounds of endoreplication in TGCs, while double knock out of *E2f7* and *E2f8* genes induces a defect in endoreplication [3, 63]. Thus, the activator E2F/DP complex promotes exit from endoreplication, while atypical E2Fs maintain it. The reduced number of endoreplication cycles in *E2f7* and *E2f8* double mutant TGCs is partially restored by eliminating the function of *E2f1*. These results indicate that DP-independent E2F regulation by atypical E2Fs competitively antagonizes the function of DP-dependent E2F regulation by typical E2Fs during endoreplication (**Figure 4B**). Atypical E2Fs suppresses the expression of Cyclin A as well as cdc2, preventing the entry into M phase during endoreplication. The transient up-regulation of atypical E2F expression during S phase is important to suppress the expression of target genes immediately after DNA replication [24–29]. However, the transient accumulation of atypical E2Fs does not instruct the timing of initiation and termination of endoreplication. The basal level of their expression is changed during tissue development [63]. An increase in the basal level of atypical E2Fs expression allows cells to initiate endoreplication, while a reduction in basal levels of atypical E2Fs induces cells to exit from endoreplication. Thus, a combination of developmental and cell cycle cues determines the timing and duration of endoreplication during tissue development.

4.3 Endoreplication in fly development

Fly is a model organism that lacks atypical *E2f* genes in its genome [4, 33]. Yet, endoreplication is conducted in cells of multiple tissues including secretory cells of

salivary glands, subperineural glia of the brain, and ovarian nurse and follicle cells [64]. How is cdk activity downregulated immediately after DNA replication in these cell types without atypical E2Fs? Instead of atypical E2F-mediated suppression of E2F target gene expression, ubiquitin ligase Cul4 induces a rapid degradation of dE2f1 during S phase, reducing the expression of E2F target genes including Cyclin E and mitotic cyclins (**Figure 4C**) [65]. Downregulation of Cyclin E/cdk2 activity during S phase does not allow cell cycle to progress through the G2/M phase transition in fly, thereby returning the cell cycle back into G1 phase, resulting in endoreplication [66, 67]. These observations imply that organisms lacking atypical E2Fs have mechanism(s) to complement their function.

5. The role of E2F1 in apoptosis and tumor suppression

5.1 E2F1 induces apoptosis in a DP-independent manner in mammalian fibroblasts

Cultured cell systems also demonstrated that E2F1 can function to induce apoptosis [1, 2, 7]. Overexpression of E2F1 induces p53-dependent and p53-dependent apoptosis in cultured cells in the absence of survival signals provided by serum (**Figure 5A**). Overexpression of E2F1 induces the expression of Arf, which activates p53 through suppression of MDM2 [1, 2, 5, 10, 68]. E2F1 bypasses p53-dependent apoptosis by inducing the expression of the p53 homolog, p73 [69, 70]. In contrast to control of cell proliferation, DP1 and DP2 are not necessary for E2F1-induced apoptosis [13], indicating that E2F1-induced apoptosis is the second model of DP-independent E2F function. The function of E2F1 to induce apoptosis is intuitively contradictory to its role in promoting cell proliferation. Why does E2F1 protein have these opposing roles? Since endogenous pRb family proteins are unable to suppress the activity of E2F generated by overproduction of E2F, exogenous overexpression of E2F1 is supposed to recapitulate deregulated E2F activity that results from functional defects in pRb family proteins. Consistent with this theory, adenovirus E1A protein, which directly binds to pRb and inhibits its function, also induces apoptosis through the Arf-MDM2-p53 pathway in fibroblasts [71–73]. Therefore, the role of E2F1-mediated apoptosis has been interpreted as a defensive mechanism to protect against and/or counter oncogenic activation of E2F1 that induces abnormal proliferation in the context of pRb dysfunction.

5.2 DP-independent regulation of Arf expression by E2F1 in mammalian fibroblast

The transcription of the *Arf* and *p73* genes is not increased by endogenous E2F activated by serum stimulation in fibroblasts, while the expression of growth-related E2F target genes is induced (**Figure 5B**) [10, 12]. One possible explanation of the inability of endogenously activated E2F to increase *Arf* and *p73* expression is that serum stimulation activates survival signals that specifically counteract the induction of apoptotic genes by E2F [74]. Phosphatidylinositol-3 kinase (PI3K) signaling inhibits expression of some apoptotic genes, induced by overexpression of E2F1, such as AMPKa2, which is involved in metabolism and is not a typical apoptotic gene [75]. However, it is not clear whether the PI3K signaling regulates these genes at the transcriptional level or post-transcriptional level. In addition, PI3K signaling does not suppress the induction of *Arf* gene expression by overexpression of E2F1 [76]. Therefore, mechanisms must exist to allow the *Arf* gene to specifically sense and respond to deregulated E2F activity.
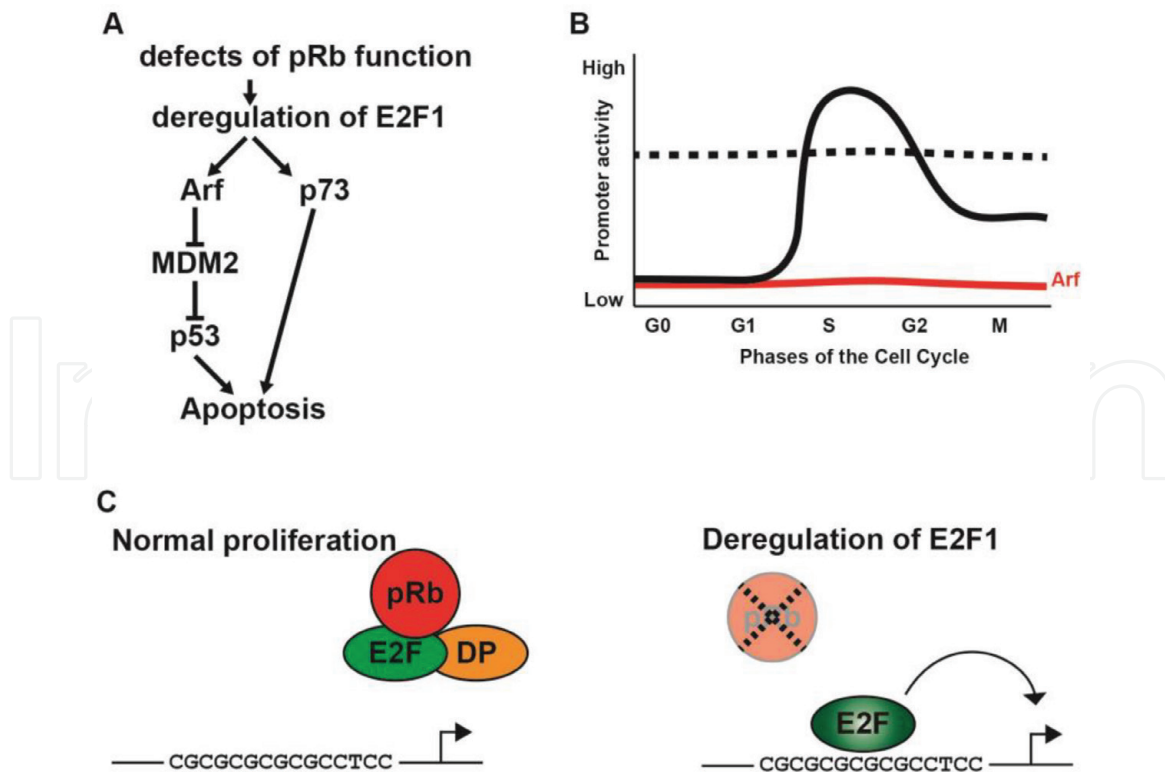
Figure 5.
E2F1 regulation of Arf gene transcription and apoptosis in Rb-deficiency. (A) Schematic view of E2F1-induced apoptosis. E2F1 induces apoptosis through p53-dependent and -independent mechanisms. (B) Arf promoter activity during the cell cycle. Black line indicates the activity of the wild-type promoter of growth-related E2F target genes. Dashed black line indicates the activity of canonical E2F binding site mutant promoter in growth-related E2F target genes. Red line indicates the activity of the Arf promoter. (C) E2F regulation of the Arf promoter in normal cell proliferation and in the context of deregulated E2F activity in response to dysfunction of pRb.

We have approached this issue by investigating mechanisms of how E2F regulates *Arf* gene expression in human fibroblast [10, 13]. Analysis of *Arf* promoter regulation has revealed the following points. (i) the pRb/E2F/DP complex does not actively suppress the *Arf* promoter during the cell cycle (**Figure 5C**). (ii) The *Arf* promoter is very sensitive to deregulated E2F activity, but not to E2F activity induced by serum stimulation. (iii) E2F1 regulation of the *Arf* promoter does not require DP. (iv) The regulation is mediated through a non-canonical E2F binding site, EREA (**Figure 5C**). The sequence of EREA is highly conserved in the *Arf* promoters in human and mouse and an isolated EREA reporter construct from the mouse *Arf* promoter showed similar activity in response to serum stimulation and overexpression of E2F1. Thus, E2F regulation of EREA may be shared between mouse and human [10]. Our finding raised a new question of how E2F1 binds to EREA. *In vitro* studies showed that, while E2F1 alone is able to bind to a canonical E2F binding site, the presence of DP1 drastically enhances E2F1 binding affinity to this DNA element. In contrast, the presence of DP does not impact E2F1 binding to EREA. Since the presence of DP does not interfere with E2F1 binding to the EREA, the E2F/DP complex perhaps binds to EREA utilizing only the E2F DBD. In general, a transcription factor works in combination or acts synergistically with a functional partner protein. Thus, there may be specific factor(s) that cooperatively function with E2F1 to regulate EREA.

In parallel to our study, Hallstrom et al. reported different mechanisms of regulation of E2F1-induced apoptosis [74, 77]. They found that (i) The MB domain determines specificity of E2F1 to induce apoptosis. A chimeric E2F1 mutant containing E2F3 MB loses the ability to induce apoptosis, while insertion of the E2F1 MB domain into E2F3 confers the ability to induce apoptosis. (ii) E2F1 binds

to Jab1, a subunit of the COP9 signalosome (CSN), through its MB domain. The E2F1-Jab1 interaction is specific to E2F1 because the amino acid sequence of MB domain varies among individual E2F proteins. (iii) Jab1 enhances E2F1-indued activation of p53 and apoptosis. (iv) Jab1 enhances induction of *Arf* gene expression by overexpression of E2F1. (v) Jab1 binds to the *Arf* promoter [78]. These are indirect evidence, but suggest that Jab1 contributes to activation of *Arf* gene transcription by E2F1, leading us to postulate that the EREA plays a role in Jab1 binding to the *Arf* promoter. Further investigation will be required to reveal details of mechanisms underlying E2F regulation of *Arf* gene expression and apoptosis.

5.3 Arf suppresses tumorigenesis in the Rb mutant mouse

Homozygous mutation of the *Rb* gene induces ectopic proliferation and apoptosis in multiple tissues of mouse embryos [3, 79]. These phenotypes are rescued by removing *E2f1* gene function [80], suggesting that pRb regulates E2F activity and deregulated E2F1 induces apoptosis *in vivo*. Ablation of the *Arf* gene does not suppress the apoptotic phenotype in *Rb* homozygous mutant embryos [81], indicating that E2F1 induces apoptosis through Arf-independent mechanisms in particular cell types. The *Rb* gene is one of the most prominent tumor suppressor genes and loss of pRb function is observed in wide variety of human cancers [5]. Heterozygous mutation predicts retinoblastoma and osteoblastoma in human [5]. These observations suggest that E2F1-induced *Arf* may counteract tumorigenesis in *Rb* mutant cells. In a mouse model, heterozygous mutation of *Rb* typically produced pituitary and thyroid tumors, which is used in study of tumor suppressive function of *Rb in vivo* [79]. In these tumors, wild-type *Rb* allele is also lost during tumor formation and loss of *E2f1* suppresses tumorigenesis in heterozygous *Rb* mutant mice [82], indicating that E2F1 is deregulated to induce abnormal proliferation in these tumor cells. In addition, because the tumor phenotype in pituitary and thyroid of *Rb* heterozygous mutant mouse is exacerbated by loss of *Arf* [81], it is likely that deregulated E2F1 activity counteracts tumor formation through inducing *Arf* in these tumors. $Rb^{-/+}; Arf^{-/-}$ compound mutant mice develop pituitary gland lesions earlier than $Rb^{-/+}$ and $Rb^{-/+}; p53^{-/-}$ [81]. Therefore, Arf should function independently of p53 to suppress tumor formation in the pituitary and thyroid glands of *Rb* mutant mice.

5.4 E2F-induced apoptosis in fly requires DP

Overexpression of *de2f1* or homozygous mutation of *rbf1* induces apoptosis in multiple tissues in the fly [4, 83], indicating that consistent with vertebrates, deregulation of dE2f1 induces cell death in fly. However, there are two inconsistent observations. Firstly, dE2f1 requires dDP to induce apoptosis [4, 84, 85]. Secondly, dE2f1-induced apoptosis is primarily independent of p53 [86]. The role of fly p53 in apoptosis is not as predominant as that of mammalian p53 and the *Arf* gene does not exist in the fly genome. Mechanisms of both E2F1-induced and p53-dependent apoptosis, and interactions between them, may have been modified and adapted during the process of evolution.

6. Conclusion and perspective

In this chapter, we considered multiple roles of E2F transcription factor by introducing three distinct mechanisms of E2F-mediated transcription. The classic model of the E2F/DP complex explains how the heterodimeric complex regulates growth-related E2F target genes by collaborating with pRb family proteins during the cell

cycle [1–5]. There is no doubt that the pRb/E2F/DP pathway instructs both entry into and exit from the cell cycle. However, recent studies revealed that cells have complementary mechanisms in control of cell cycle progression at the G1/S phase transition. Future studies should address the mechanisms, by which cells can control the exit from the cell cycle in the absence of the pRB/E2F/DP complex. The classic E2F/DP model is not sufficient to explain all function of E2F family proteins. Two additional mechanisms, described above, at least partly fill that knowledge gap. Atypical E2Fs play a crucial role in endoreplication by antagonizing the function of activator E2Fs in DP-independent and Rb-independent manners [3, 33, 63]. In addition to their role in endoreplication, atypical E2Fs may also mediate tumor suppression in multiple tissues including liver and skin [87, 88]. How they function in suppressing tumorigenesis is an important question for future studies. E2F1 also functions via DP-independent mechanisms, which mediate tumor suppression via regulation of apoptotic genes including *Arf* [13]. In addition to modulating tumor suppression, these E2F1 pathways may have a role in restricting the plasticity of cell fate in differentiated cells. Overexpression of four transcription factors (Oct4, Sox2, c-Myc and klf4) reverts the competence of fibroblast to the level of pluripotent embryonic stem cells [89]. The efficiency of this reprograming is restricted by the pRb-p53 pathway [90, 91]. E2F-dependent transcription of *Arf*, or regulation of p53, may be involved in the mechanism to restrict the plasticity of cell fate in fibroblasts. In addition, *Rb* and *Arf* contribute to limit the reversion of myocytes to myoblasts in regeneration of muscle [92]. These possibilities merit further investigation, and discoveries of new mechanisms of E2F regulation of its target genes will open a new paradigm to understand the diverse roles of E2F transcription factor families.

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Conflict of interest

Authors have no conflict of interest.

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