Chapter 4

E2F Transcription Factors Control the Roller Coaster Ride of Cell Cycle Gene Expression

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Abstract

Initially, the E2F transcription factor was discovered as a factor able to bind the adenovirus E2 promoter and activate viral genes. Afterwards it was shown that E2F also binds to promoters of nonviral genes such as *C-MYC* and *DHFR*, which were already known at that time to be important for cell growth and DNA metabolism, respectively. These findings provided the first clues that the E2F transcription factor might be an important regulator of the cell cycle. Since this initial discovery in 1987, several additional E2F family members have been identified, and more than 100 targets genes have been shown to be directly regulated by E2Fs, the majority of these are important for controlling the cell cycle.

The progression of a cell through the cell cycle is accompanied with the increased expression of a specific set of genes during one phase of the cell cycle and the decrease of the same set of genes during a later phase of the cell cycle. This roller coaster ride, or oscillation, of gene expression is essential for the proper progression through the cell cycle to allow accurate DNA replication and cell division. The E2F transcription factors have been shown to be critical for the temporal expression of the oscillating cell cycle genes.

This review will focus on how the oscillation of E2Fs and their targets is regulated by transcriptional, post-transcriptional and post-translational mechanism in mammals, yeast, flies, and worms. Furthermore, we will discuss the functional impact of E2Fs on the cell cycle progression and outline the consequences when E2F expression is disturbed.

Key words E2F transcription factors, Cell cycle, Oscillation, Gene expression, RB/E2F pathway

1 Introduction

Quiescent cells are able to enter the cell cycle in G1-phase upon proper mitogenic stimulation (Fig. 1). Growth factors activate the RB/E2F-pathway, an important pathway for cell cycle progression, by stimulating G1-Cyclins and Cyclin-dependent kinases (CDKs) to form complexes. Activated Cyclin-CDK complexes will phosphorylate Rb-family members (RB, p107, p130), which are bound to E2Fs. Hyperphosphorylation of Rb-family members is the point of no return in the cell cycle, committing the cell to a full cycle of DNA replication and cell division. It leads to a conformational change of RB, releasing E2Fs from the complex.

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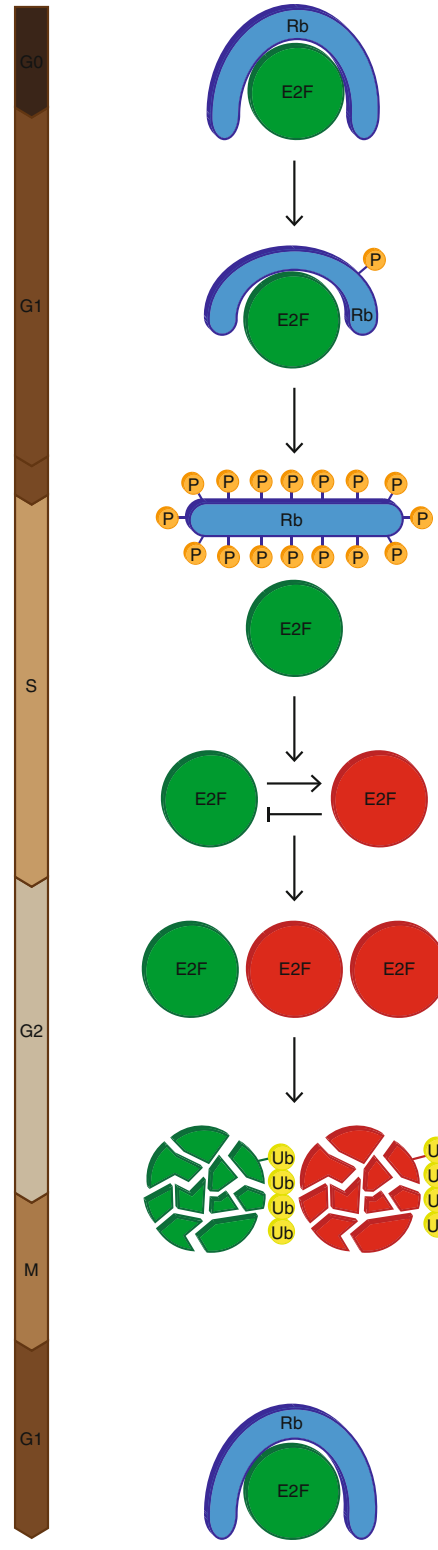

Fig. 1 The pRb/E2F pathway is activated by growth signals when a cell enters a new round of the cell cycle. Cyclin D-CDK4/6-complexes phosphorylate Rb-family members, leading to release of activator E2Fs and progression into S-phase. Activated repressor E2Fs bring down the levels of activator E2Fs, guiding the cells to G2-phase and mitosis, when the transcription factors are degraded

The accumulation of free activating E2Fs leads to increased expression of their target genes. Activating E2Fs mainly bind to promoters of genes that are important for S-phase progression leading to the initiation of DNA replication [1–4]. This critical upswing of S-phase gene expression is followed by a downswing and is induced by the action of atypical E2Fs, the newest members of the mammalian family of E2F transcription factors, which are induced by activator E2Fs [5–11]. This mechanism of transcriptional repression to shut down S-phase gene expression during late S- and G2-phase is most likely required for the timely progression through G2–M; however, experimental evidence is still lacking.

In addition to downregulating S-phase gene expression, atypical E2Fs repress classical E2F activators during G2, creating a direct negative feedback-loop to control the oscillating expression pattern of E2F target genes. Interestingly, atypical E2Fs also repress their own transcriptional activity [10, 11], providing a second negative feedback-loop, most likely to guarantee the repressive activity has been shut down before a cell starts the next cell cycle. A posttranslational mechanism has been identified on top of the transcriptional mechanism to induce degradation of S-phase proteins during G2–M, for example through the SCF-SKP2 complex in G2-phase, or anaphase-promoting complex or cyclosome (APC/C) mediated degradation from anaphase onwards [12–14]. Together these findings demonstrate that a complex mechanism exists to control the roller coast ride of E2F target gene expression during the cell cycle.

2 Regulation of Oscillating E2F Target Gene Expression in Mammals

Proper oscillating E2F target gene expression is vital for cell cycle progression. Their altered expression can have detrimental consequences for the cell, such as speeding up or slowing down the cell cycle. In some occasions, it can even result in a cell cycle arrest or apoptosis, for example when the expression of specific members of the E2F family is altered.

The mammalian E2F family consists of eight members, which are divided into activators (E2F1–3) and repressors (E2F4–8). E2F1–6 are classical E2Fs, with one DNA-binding domain, and are required to heterodimerize with DP1/DP2 proteins before they can bind target gene promoters and activate or repress their expression. E2F7 and E2F8 are considered as atypical E2Fs. They have two DNA-binding domains, and they can repress target genes independent of DP heterodimerization. Instead, they can form homodimers and heterodimers with each other [10, 15, 16].

The protein expression of the activator E2F1–3 increases in G1, peaks during S, and decreases in G2-phase [17]. RB blocks the

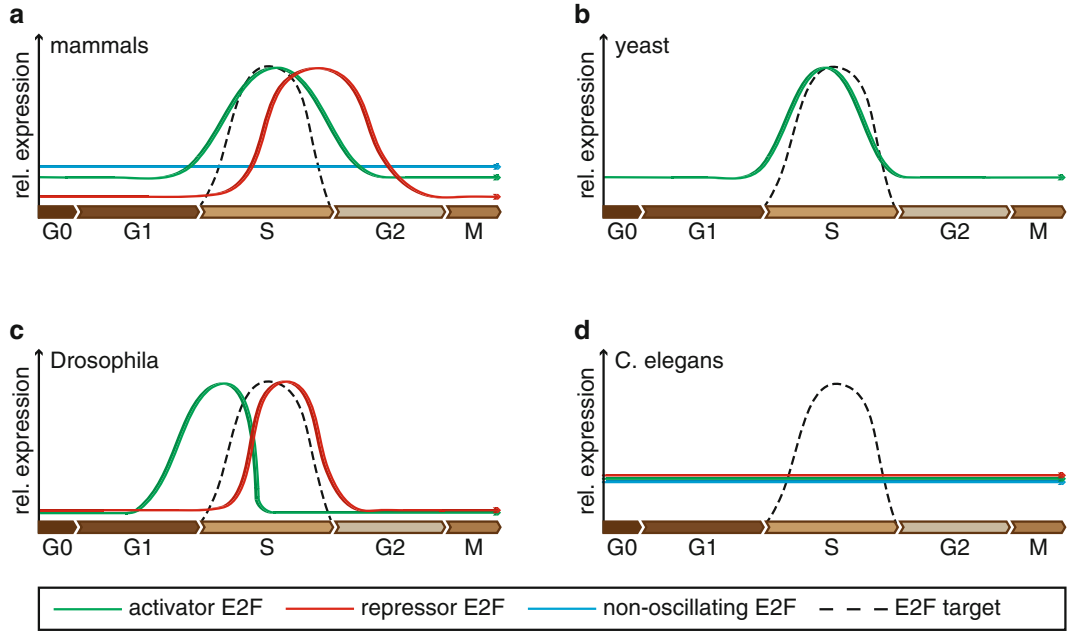

Fig. 2 E2F expression patterns. (**a**) The mammalian E2F family consists of activator, repressor, and non-oscillating E2Fs. (**b**) Yeast contains one oscillating, activator E2F. (**c**) The *Drosophila* E2F family consists of an activator and a repressor E2F, both E2Fs are oscillating. (**d**) The *C. elegans* E2F family consists of three members, all of which are non-oscillating

transcriptional activity of the activator E2Fs in G1 through the occupation of their transactivation domain. The RB-E2F complex dissociates upon RB hyperphosphorylation, and activation of E2F target gene expression starts, followed by entry into S-phase (Fig. 2a) [2, 18, 19].

The individual loss of E2F1, E2F2, or E2F3 has minor effects on the target gene expression and the cell cycle progression. Cells have a lengthened S-phase, but keep proliferating (*see* Table 1) [20–26]. However, the combined loss of E2F1–3 abolishes the possibility for mouse embryonic fibroblasts to enter S-phase and proliferate (*see* Table 1). This loss triggers a p53-p21^{Cip1} response and leads to a G1-phase arrest. However, as long as there is still one functional allele of one of the three activators, cells are able to continue through the cell cycle. These findings demonstrate a clear redundancy between the activating E2Fs [27, 28]. Remarkably, the requirement of E2F1–3 for cell cycle progression appears to be cell type specific, as deletion of the activator E2Fs has no effect on cell cycle progression in epithelial stem cells and lens progenitor cells. Nevertheless, the lens progenitor cells deficient for E2F1–3 display increased expression of cell cycle regulated genes, high levels of DNA damage and an activated p53-pathway, leading to massive apoptosis later in development, suggesting that E2F1–3 function as transcriptional repressors in stem cells most likely via

Table 1
Impact of altering E2F expression: deletion

	Oscillation	Level	Cell cycle progression	Cell fate	Reference
E2F1	No effect	No effect	Lengthened S-phase	No effect	[20–22]
E2F2	Earlier upswing	Increased	Enhanced S-phase	Hyper-proliferation	[23–25]
E2F3	Delayed upswing	Decreased	Lengthened S-phase	Apoptosis	[21, 26]
E2F1–3	No upswing	Decreased	G1-phase arrest	Apoptosis, hyperploidy	[27, 28, 66]
E2F6	No effect	No effect	No effect	No effect	[37]
E2F7	No effect	No effect	No effect	No effect	[10]
E2F8	No effect	No effect	No effect	No effect	[10]
E2F7–8	No downswing	Increased	No effect	Apoptosis	[10]
SBF	Unknown	Decreased	G1-phase arrest	Apoptosis	[39, 41, 43–45]
MBF	Unknown	Decreased	G1-phase arrest	No effect	[39, 41, 45]
dE2F1	No upswing	Decreased	G1-phase arrest	Unknown	[19, 53]
dE2F2	Unknown	Increased	No effect	No effect	[19, 56]
EFL-1	Unknown	Increased	Enhanced S phase	Reduced apoptosis, endoduplication	[58, 59, 61]

their interaction with Rb [18]. The ectopic expression of activator E2Fs is sufficient to bypass upstream signals and drive cells into S-phase (*see* Table 2). When the activator E2F levels remain high after S-phase entry, cells will undergo apoptosis [2, 7, 29]. High levels of activator E2Fs lead to the induction of apoptotic target genes, such as APAF1 and p73, especially in response to DNA damage [2, 30].

E2F4 and E2F5 play important roles in keeping cells in quiescence, the resting phase of the cell cycle (G0). During this time, E2F4 and E2F5 form repressing complexes with the Rb-family members p107 and p130. Their binding to promoters of E2F target genes leads to inhibition of their expression and results in blockage of cell cycle progression. E2F4 and E2F5 are constitutively expressed throughout the cell cycle (Fig. 2a), but their subcellular localization changes during cell cycle progression to regulate their repressing transcriptional activity [31, 32]. In G0 and early G1, E2F4/5 are present in the nucleus to inhibit E2F target gene expression, but upon p107/p130 hyperphosphorylation through enhanced CDK activity during G1, E2F4/5 can be relocated to

Table 2
Impact of altering E2F expression: overexpression

	Oscillation	Level	Cell cycle progression	Cell fate	Reference
E2F1	Unknown	Increased	Enhanced G1-phase	Apoptosis	[2, 7, 29]
E2F2	Unknown	Increased	Induced S-phase entry	No effect	[2, 95]
E2F3	Earlier upswing	Increased	Induced S-phase entry	Hyper-proliferation	[2, 96]
E2F6	Earlier downswing	Decreased	S-phase arrest	Unknown	[37, 97]
E2F7	Earlier downswing	Decreased	During G1: S-phase arrest	Apoptosis	[5, 6, 11]
E2F8	Unknown	Decreased	S-phase arrest	Unknown	[8, 9, 98]
SBF	Unknown	Increased	Enhanced G1/S	Toxic	[46]
MBF	Unknown	No effect	No effect	Toxic	[46]
dE2F1	Unknown	Increased	During G1: induction S phase During S: G1-arrest	Apoptosis	[54]
dE2F2	Unknown	Decreased	No effect	No effect	[19]
EFL-1	Unknown	Unknown	Unknown	Sterility	[61, 99]

the cytoplasm [33]. Experiments in synchronized cell populations have shown that E2F4 is able to repress and activate target genes during the cell cycle, suggesting a more versatile role for this transcription factor than its known function during quiescence [34].

E2F6–8 are transcriptional repressors, and are important for inhibiting the expression of target genes in S/G2-phase, most likely to ensure proper cell cycle progression. Like E2F1–3, the expression of E2F6–8 oscillates during the cell cycle. Since E2F1–3 induce the expression of E2F6–8, the upswing of E2F6–8 expression occurs a couple of hours later compared to the upswing of E2F1–3 expression. E2F6–8 expression peaks at S-G2 and declines during G2–M (Fig. 2a). They appear to function independent of Rb-family members, because they lack the classical pocket protein binding domain [6, 8, 11, 33, 35–37].

The exact role of E2F6 in cell cycle regulation and the effects of the loss or gain of this transcription factor are still unclear (Tables 1 and 2). It has been shown that E2F6 binds to promoters of target genes that are important for G1/S-phase progression during G2/M phase, repressing their expression so the cells can continue the cell cycle [37]. A loss of E2F6 can be compensated by E2F4, since this transcription factor is also able to bind to the same target gene promoters, and co-deletion of both E2F4 and E2F6 leads to a derepression of their target genes during S-phase [35, 37].

Cells lacking both E2F7 and E2F8 continue cycling as well, even though the RNA levels of the E2F target genes are strongly derepressed during S/G2-phase (Table 1). One possible explanation for the continuation of the cell cycle is that other E2F repressors, such as E2F4 or E2F6, can compensate for the loss of E2F7/8 to partially repress E2F target genes [10]. In addition, enhanced production of E2F target protein in response to loss of E2F7/8 will be most likely compensated through enhanced degradation mechanisms during G2/M.

Remarkably, the effects on cell cycle progression of ectopic expression of E2F7 depend on the phase of the cell cycle (Table 2). The induction of E2F7 during G1-phase leads to a strong repression of its target genes involved in DNA replication, metabolism and repair, and to an early S-phase arrest. However, when E2F7 is induced later, during S-G2, cell cycle progression is not disturbed. Prolonged ectopic expression of E2F7 results in DNA damage and apoptosis [11]. Overexpression of E2F8 has been shown to reduce the proliferation rate [9]. However, overexpression studies where E2F8 is induced at different phases of the cell cycle are still missing.

2.1 Regulation of Oscillating E2F Target Gene Expression in Yeast, Flies, and Worms

E2Fs are highly conserved through evolution. This strong conservation of the E2Fs throughout different species allows us to use less complex systems to learn more about the general mechanisms that regulate the oscillating E2F target gene expression pattern.

2.1.1 E2Fs in Yeast

There is a robust functional similarity between the mammalian and yeast E2F proteins, but there is no detectable sequence homology throughout the protein [38]. The budding yeast E2F family consists of two activating proteins, SBF and MBF, which overlap in function. As in mammals, SBF and MBF are present in G1-phase, but they are bound to Whi5, the yeast protein functionally comparable to RB. SBF and MBF are released from its binding partner Whi5 by CDK-dependent hyperphosphorylation of Whi5, and become active in late G1-phase to promote target gene expression and subsequent cell cycle progression into S-phase (Fig. 2b) [38, 39]. SBF and MBF are inactivated in S-G2/M by B-type cyclins. SBF and MBF promote expression of B-type cyclins in late G1, which in turn inhibit the expression of SBF and MBF by phosphorylation [38, 40]. This phosphorylation leads to nuclear export of SBF and MBF [41]. A second level of regulation of MBF target genes is via Nrm1, a G1-target of MBF. This factor can bind MBF and together they form an inhibitory complex, repressing MBF target gene expression [42].

Deletion of SBF or MBF leads to a G1-phase arrest accompanied with a decreased expression of the target genes (Table 1), which can lead to apoptosis [39, 41, 43–45]. Overexpression of SBF or MBF leads to an enhanced G1–S-phase transition and is toxic to the cells (Table 2) [46].

2.1.2 E2Fs in Flies

Compared to mammals, *Drosophila melanogaster* has E2F family members that are both functionally and sequentially more conserved than in yeast. *Drosophila* has two E2F proteins, an activator E2F (dE2F1), with over 65 % sequence homology to human E2F1 in the DNA binding domain and 50 % homology in the RB-interacting domain [47], and a repressor E2F (dE2F2), with a similar level of homology as dE2F1 [48]. The Rb homologue RBF1 is a strong regulator of dE2F1, and is important to limit dE2F1-regulated activation of target genes in G1-phase, similar to its function in mammals [49–51]. dE2F1 accumulates in G1-phase, when RBF1 is phosphorylated and dissociates from the transcription factor. Once the cells progress into S-phase, dE2F1 is rapidly degraded by the $Cul4^{Cdt2}$ E3 ubiquitin ligase, via a PCNA-interacting-protein motif (Fig. 2c) [52].

The loss of dE2F1 leads to a G1-phase arrest, as there is no upswing of the activator target genes to push the cells forward into S-phase (Table 1) [19, 53]. Ectopic expression of dE2F1 in S-phase has effects on the cell in the following cell cycle (Table 2). Cells are unable to enter the next S-phase with continued expression of dE2F1. However, if the ectopic expression is limited to G1, there will be a strong induction of S-phase due to high levels of target genes. A subset of these genes regulates apoptosis, leading cells with high dE2F1 levels to their fate [54].

An important function of dE2F2 is to antagonize the function of dE2F1 through repression of their common E2F target genes. This competition between the activator and repressor E2F is important for cell cycle progression, as the cell cycle progression phenotypes caused by deletion of dE2F1 can be rescued by deletion of dE2F2 [19, 54, 55].

The deletion or overexpression of dE2F2 has no clear effects on the cell cycle (Tables 1 and 2). There is an increase in its target genes when dE2F2 is absent, but without any resulting phenotypes. Ectopic expression of dE2F2 leads to a decrease in target gene expression, but surprisingly also without any effect on cell cycle progression or cell fate [19, 56].

2.1.3 E2Fs in Worms

C. elegans has three E2F transcription factors, namely EFL-1, EFL-2, and the recently identified EFL-3. None of their expression levels appear to be cell cycle regulated (Fig. 2d). E2F proteins in *C. elegans* are important during development, regulating tightly controlled cell divisions redundantly with several regulatory pathways, including the RAS/MAP kinase cascade [57]. EFL-1 shares its structure with mammalian E2F4 and E2F5, its DNA binding domain is highly conserved and the dimerization domains have 38 % homology [58]. This transcription factor acts as a transcriptional repressor and thereby inhibits S-phase entry [59]. It forms a repressor complex with LIN-35 (most homologous to p107 and p130, overall 19 % and 20 % amino acid homology, respectively) in

G1, repressing G1/S genes [57, 58]. In G1-phase, the Cyclin D1 (CYD1)/CDK-4 complex phosphorylates the LIN-35/EFL-1 complex, relieving the inhibitory effect on target genes like Cyclin E (CYE1), pushing the cells into S-phase [57, 60].

The loss of EFL-1 in *C. elegans* leads to enhanced S-phase entry and hyperplasia, as the negative regulation is gone and target genes are derepressed, similar to the loss of LIN-35 (Table 1). There is not much known about the effects of ectopic expression of EFL-1 on the cell cycle, only that *C. elegans* overexpression mutants are sterile (Table 2) [61].

EFL-2 is most similar to mammalian E2F3 and E2F6 (the dimerization domain homology is 37 %) and is proposed to act as an E2F activator during the cell cycle. However, current data only supports a transcriptional activator role during apoptosis [58, 61]. EFL-3 is a novel homologue of the mammalian E2F7 and E2F8, and it does not appear to be essential for regulating cell cycle progression. However, it has been shown that EFL-3 acts as a repressor in cooperation with Hox to regulate apoptosis [62, 63].

The function of the activator E2Fs to stimulate target gene expression and moving the cell into S-phase is highly conserved from yeast to mammals. Furthermore, the inhibitory effect of RB interaction with E2F activators is strongly conserved as well. In contrast, the evolution of E2F repressors in different species is quite diverse. In line with this observation, different mechanisms have evolved in mammals, yeast, flies, and worms to regulate the downswing of E2F target gene expression. The relevance of down-regulation of E2F target genes for cell cycle progression remains obscure, since inactivation of E2F repressors has no major impact on cell cycle progression and cells continue to proliferate even in the absence of E2F repressors. Recent studies provide evidence that loss of E2F repressors such as E2F7/8 can lead to hyperproliferation or inhibition of abortive cell cycles [64, 65]. Future studies are necessary to determine what the long-term effects are of deleting E2F repressors to understand functional impact on tissue homeostasis, aging, and tumorigenesis.

3 Mechanisms That Regulate the Oscillation of the E2F Transcription Factors

There are several mechanisms that tightly regulate the oscillating E2F expression during the cell cycle to prevent aberrant cell cycle progression. These mechanisms are on transcriptional, post-transcriptional, and post-translational levels. Transcriptionally, the most common regulation of E2Fs in proliferating cells is via a feedback-loop by E2Fs themselves [1, 66]. Activator E2Fs promote the expression of repressor E2Fs by binding to their promoter sites. Repressor E2Fs can bind to the promoters of activator E2Fs and inhibit their transcription, creating a negative feedback within the system [1].

E2Fs are also strongly regulated by c-Myc [67, 68]. c-Myc binds to the promoter of activator E2Fs after cells are triggered to start proliferating, leading to increasing levels of E2F mRNA and consequently E2F protein, and activation of E2F target genes [69]. It has also been shown that c-Myc is essential for the loading of E2F1 on activator E2F promoters. Immunoprecipitation experiments for c-Myc and E2F1 on endogenous E2F2 promoters after serum stimulation show that c-Myc binds from 4 h after stimulation onwards, while E2F1 does not bind until 16 h after serum stimulation. Importantly, mutations in E box elements in the promoter abolishes both c-Myc and E2F1 binding to the promoter [69].

In recent years, it has been shown that E2Fs are also under post-transcriptional regulation by microRNAs (miRNAs); short noncoding RNAs that are involved in many biological processes such as cell proliferation, differentiation, and oncogenesis. For instance, c-Myc activates a cluster of six miRNAs. Two of these miRNAs can inhibit the expression of E2F1, namely miR-17-5p and miR-20a. The inhibition of E2F1 by these miRNAs seems to be a mechanism to control E2F activation by c-Myc via a negative feedback loop in G1-phase, preventing uncontrolled activation of E2F1 [70]. Another example of miRNA regulation is the repression of E2F7 via miR-26a in acute myeloid leukemia (AML) cells. The E2F7 3′-UTR contains two putative binding sites for miR-26a, and E2F7 levels are increased after knockdown of this miRNA [71].

E2Fs are also regulated through post-translational regulation. Chk1 is an important kinase for the regulation of cell cycle progression after replication stress and DNA damage. During replication stress, E2F6 replaces the activator E2Fs on the target gene promoters, repressing their expression. Once the DNA damage caused by replication stress is repaired and the DNA replication checkpoint is satisfied, Chk1 phosphorylates E2F6. This leads to the dissociation of E2F6 from the promoters, and freeing them for activator E2Fs to bind again and promote cell cycle progression [35].

Transcriptional repression of transcription factors is an important step in limiting target gene expression, but this no longer has any effect on the already synthesized pool of proteins. Cyclin-CDK complexes are vital in E2F regulation. These complexes phosphorylate and inactivate Rb and E2Fs during different phases of the cell cycle [3, 72].

The regulation of protein turnover is another way to control the activity of E2Fs. The decrease of E2F1–3 in late S-G2 phase is caused via the SKP2-CUL1 complex, which targets the proteins for degradation [12]. A second degradation mechanism acts primarily in mitosis, namely via APC/C^{Cdh1}. The levels of E2F3 are slowly decreased upon cell cycle exit through APC/C^{CDh1}. E2F3 interacts with both Cdh1 and Cdc20 in vitro, but it seems to be a predominant target of Cdh1 in vivo [73]. Another substrate for APC/C is E2F1. E2F1 interacts with Cdh1 and Cdc20 in vitro,

similar to E2F3, but is mainly degraded by Cdc20 in vivo [13]. There are currently no reports on APC/C-mediated degradation of repressor E2Fs, but their oscillating expression pattern during the cell cycle suggests that these transcription factors are degraded as well [6, 8].

4 Mechanism of Controlling Target Gene Transcription by E2Fs

Activator and repressor E2Fs work together to control the oscillating expression pattern of E2F target genes. It is known that activator and repressor E2Fs can bind to a common set of promoters to balance target gene expression in vivo, for instance in the liver and placenta [64–66]. Loss of activator E2F1–3 in the mouse liver results in downregulation of target genes, while loss of repressor E2F7/8 leads to upregulation of the same target genes [65, 66]. However, it is still unknown how different E2Fs regulate the expression of target genes at the promoter levels.

All E2Fs can bind to the E2F consensus sequence TTTSSCGC, but the different E2F factors can also bind to non-consensus motifs [11, 34, 74]. One possible mechanism is that activating and repressing E2Fs compete for the same E2F binding sites, and the E2F factor with the highest DNA-binding affinity or the highest expression levels has a stronger effect on the transcriptional outcome (Fig. 3) [11, 75]. Another possibility is that binding to certain promoters or low-affinity sites is stabilized in cooperation with other transcription factors, for instance the binding of E2F7/8 to hypoxia-induced factor (HIF) during hypoxia, as HIF is essential for inhibiting cell cycle proliferation under this condition [76–78].

Since many promoters contain multiple consensus and non-consensus E2F binding sites, an alternative mechanism would be that different E2Fs bind to different sites on the same promoter

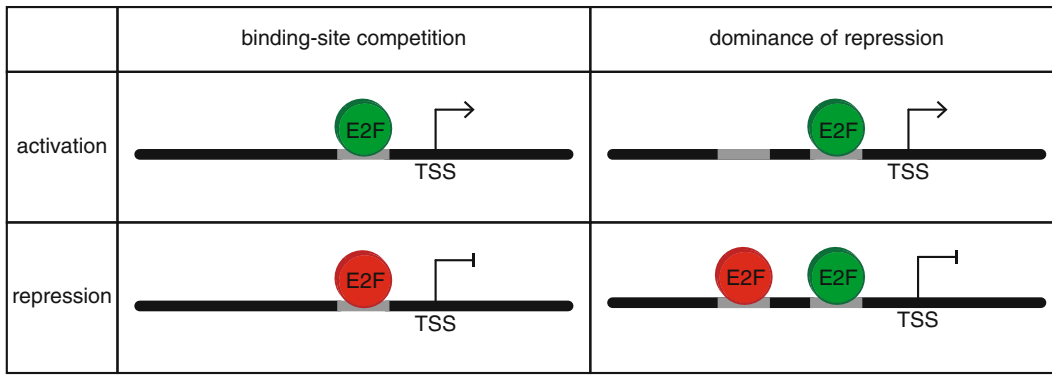

Fig. 3 There are two main models of E2F target gene regulation. The first shows competition between activator and repressor E2Fs for the same binding site, the second shows simultaneous binding and a dominant effect of repressor binding

and the combinatorial activating and repressing transcriptional activity of each individual E2F factor determines the transcriptional outcome. Previous studies provide evidence for the presence of positive- and negative-acting E2F promoter elements, for example in the *CDK1* and *CCNB1* promoter, whereby E2F1–3 bind to the positive acting site and E2F4 to negative acting sites [74, 79]. The expression patterns of E2F1–3 and E2F6–8 show a strong overlap especially during S-phase, so it is likely that they have overlapping binding sites as well. Currently it is unclear whether the competitor model or the activator/repressor specific-site model is critical to determine the transcriptional outcome of E2F target genes during S-phase.

Recently, it has been shown that E2F1 and E2F7 can bind to the same binding sites in the E2F1 promoter utilizing the gel shift assay [80]. Interestingly, it was also demonstrated that E2F1 and E2F7 can form a complex, and that the binding of E2F1-E2F7 heterodimer towards the promoter required the presence of two adjacent E2F binding sites [80]. Together, these findings show that activator and repressor E2Fs can bind to the same binding site, but also to different E2F sites in the same promoter, providing support for both models outlined above. However, it still unclear how the different binding options for activator and repressor E2Fs regulate the transcriptional activity of the target gene. For example, does induced expression of repressor E2F lead to disappearance of activator E2Fs from the promoter and vice versa? Does the number of consensus and non-consensus E2F sites in a promoter influence the transcriptional activity of a promoter? Do activator and repressor E2Fs bind to the same promoter in vivo during S-phase? Does the distance between E2F binding sites and the transcriptional start site influence the transcription rate? Future studies will be necessary to unravel the mechanism of how activator and repressor E2Fs regulate the expression of target genes at the promoter level.

4.1 Altered Regulation of E2F Target Gene Expression

The majority of human cancers show enhanced expression of E2Fs and E2F target genes (reviewed in [81]), providing evidence that proper control of E2F target expression is critical to avoid uncontrolled proliferation. Altered expression of E2F target genes is often caused by specific mutations in upstream regulators of the RB/E2F pathway, such as Cyclin D amplification [82], or by mutations of the RB/E2F pathway itself (reviewed in [81]). Transgenic mouse models for E2F activators demonstrated that enhanced expression of E2F1–3 leads to enhanced E2F target gene expression and spontaneous tumor formation (reviewed in [81]). Moreover, deletion of activator E2Fs in mouse models of cancer can reduce tumorigenesis, such as inactivation of E2F3 in a mouse model of mammary cancer [83]. Importantly, E2F activators have oncogenic potential, but can also function as tumor suppressors in specific tissues. E2F1 plays an important role in repressing skin

carcinogenesis by inducing DNA repair and apoptosis in response to DNA damage [84, 85].

The role of repressor E2Fs (E2F6–8) in controlling tumorigenesis is still undetermined, and it is unclear whether the timely downswing of oscillating E2F target gene expression is critical to suppress tumorigenesis. Preliminary data from our group show that inactivation of atypical E2Fs leads to spontaneous tumor formation in mice (unpublished data), providing strong evidence that enhanced expression of E2F target genes can lead to uncontrolled proliferation and cancer. Further support that repressor E2Fs can function as tumor suppressors is provided by studies showing that E2F7 is involved in cellular senescence and DNA damage. During oncogene-induced senescence or DNA damage, E2F7 is a direct transcriptional target of p53 and represses target genes that are involved in cell cycle progression to promote a strong cell cycle arrest [86–89].

Tight control of E2F target expression is not only important to prevent tumorigenesis but also for development. Inactivation of E2Fs in mice and zebrafish and the subsequent deregulation of E2F target gene expression results in many developmental defects of the placenta [64, 66] and the embryo [10, 78, 81]. Studies performed in the mammalian placenta and liver, as well as in flies and plants, revealed that E2Fs are not only important for regulating the normal cell cycle, but are also critical for the control of abortive cell cycles (reviewed in [90]). Abortion of the cell cycle can occur before entering mitosis (endocycle), during mitosis (endomitosis) or during cytokinesis (incomplete cytokinesis) and leads to the formation of polyploid cells, cells with increased numbers of chromosome sets. Remarkably, inactivation of the repressors E2F7/8 prevents the formation of polyploid cells in hepatocytes and giant trophoblast cells, while inactivation of activator E2F1 enhances polyploidization [65, 66]. These findings demonstrate that low levels of E2F target gene expression promote an abortive cell cycle leading to the formation of polyploid cells in the placenta and liver. In contrast, when the levels of E2F target gene expression were increased through inactivation of the repressors E2F7/8, cells completed a normal cell cycle and polyploidization was blocked. This suggests that the levels of E2F target gene expression determine whether a hepatocyte or trophoblast cell enters a normal or an abortive cell cycle.

5 Outstanding Questions

There have been great advancements in understanding how E2Fs function since their discovery in the late 1980s [91]. However, there are still many open questions concerning the regulation of the oscillating E2F target expression. Since many cancers are characterized by the deregulation of E2F target expression, it will

be critical to understand the mechanism that influences E2F target gene expression in more detail. For example, what are the dynamics of expression of E2Fs and its targets in single cells in response to growth signals or DNA damage? What is the expression profile of all E2Fs in different tissue and cell types during cell cycle progression? What is the direct impact of the acute deletion or overexpression of E2Fs on the length of each cell cycle phase? A bottleneck in answering these questions has been the redundancy in the mammalian E2F family members, and the limitations of the techniques by looking at cell populations. Recent developments in single cell analysis techniques have made it possible to take the next step. It is now feasible to analyze the gene expression pattern of a single cell by single cell transcriptomics, which can provide more detailed knowledge about the regulation of E2Fs and their target genes [92, 93]. This will allow one to study whether each cell within a particular tissue has the same expression pattern, or whether there are subsets of cells within a tissue that have distinct expression profiles.

Another strong tool to help elucidate questions about E2F functions during the cell cycle is live time lapse microscopy of cells expressing fluorescent cell cycle indicators, such as the ubiquitination-based cell cycle indicator (FUCCI) system [68, 94]. Thus, cell cycle progression of single cells over time can be visualized, and the effects of manipulating the activity of specific E2Fs can be monitored. Combining these two single cell techniques makes it possible to analyze in detail the entire network of E2F target genes during specific phases of the cell cycle.

Utilizing these novel single cell analysis technologies will help to provide answers to the burning fundamental questions about the dynamics and mechanism of expression of E2Fs and its targets. Moreover, it will help to design novel strategies to avoid or inhibit altered E2F target gene expression patterns in diseases such as cancer.

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