Cell

Re-Evaluating Cell-Cycle Regulation by E2Fs

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DOI 10.1016/j.cell.2006.11.019

Activation of E2F transcription factors is thought to drive the expression of genes essential for the transition of cells from G1 to S phase and for the initiation of DNA replication. However, this textbook view of E2Fs is increasingly under challenge. Here we discuss an alternative model for how E2Fs may work.

The retinoblastoma (*RB*) tumor suppressor pathway is frequently deregulated in cancer. Characterizing its downstream effectors could provide insight into the mechanisms driving the proliferation of cancer cells. The identification of E2F transcription factors as downstream effectors of the RB protein has ignited research efforts resulting in more than 3000 publications to date (according to PubMed). Target genes of E2Fs were initially implicated in the regulation of the G1 to S phase transition of the cell cycle and in DNA replication. More recently, microarray and chromatin immunoprecipitation (ChIP) assays have shown that genes involved in differentiation, apoptosis, and DNA-damage signaling pathways are also targets of E2F activity. Despite the wealth of data about E2Fs, there is still debate as to the actual role of these transcription factors: Do E2Fs drive the cell cycle or do they inhibit it, and is this action due to E2F-mediated transcriptional activation or repression?

Class Differences within the Family

So far, eight mammalian E2F family members have been identified. The expression of various E2Fs is differentially regulated throughout the cell cycle, and some E2Fs are expressed in a cell type-specific manner. Five E2F family members have the ability to interact with the RB family of proteins (pRB, p107, and p130, also known as pocket proteins). These E2Fs harbor both transactivation domains and repression domains (see Figure 1A). When bound to pocket proteins, E2Fs mediate transcriptional repression; when released by pocket proteins, these E2Fs can act as transactivators, and target genes are expressed. For transcriptional activation, E2Fs form complexes with histone acetyl transferases (HATs) such as p300, CBP, P/CAF, and Tip60. For transcriptional repression, E2F forms complexes (via pocket proteins) with histone deacetylases (HDACs), chromatin-remodeling proteins such as BRG-1, and histone methyl transferases like SUV39H1.

To bind to DNA, E2Fs (1 through 6) need to form heterodimers with a dimerization partner (DP-1 or DP-2) protein. E2Fs 1 to 5 all contain both activation and repression domains, yet E2Fs 1, 2, and 3 are frequently classified as E2F activators, whereas E2Fs 4, 5, and 6 are classified as repressors. Recently identified members, E2F7 and E2F8, are atypical in that they can bind to DNA without having to form dimers with DP-1 or DP-2. These new E2F members appear to be transcriptional repressors that act in a growth-inhibitory manner.

There are clear structural differences between the two better-characterized subgroups: E2Fs 1 to 3 contain their own nuclear localization signals, whereas E2Fs 4 and 5 carry nuclear export signals (see Figure 1A). E2Fs 4 and 5 are localized primarily in the nucleus when bound to pocket proteins and function in repressor complexes; when "free" (that is, not bound to pocket proteins), they are excluded efficiently from the nucleus (Attwooll et al., 2004; Frolov and Dyson, 2004).

The view of E2Fs 1, 2, and 3 as activators is an oversimplification because these E2Fs also have the potential to form complexes with RB, allowing transcriptional repression to occur. In fact, E2F1 was originally identified because of its ability to associate with RB. In addition, unbound E2Fs 1, 2, and 3 are not necessarily always found in complexes with HAT proteins. So, even though the division of E2Fs into activators and repressors has a molecular basis, we should not be too dogmatic about this classification.

The Activation Model

What is the available data supporting the view that E2Fs are transactivators required for asynchronous cell-cycle progression? Researchers have generated mouse embryonic fibroblasts (MEFs) that lack one or more members of the E2F family (*E2f1* to *E2f6* individually, or both *E2f1* and *2*, or *E2fs 1*, *2*, and *3*, or *E2f4* and *5*). With the exception of the loss of *E2f3*, lack of any other E2F does not result in impairment of cell proliferation (Attwooll et al., 2004). MEFs lacking *E2f3* display a slow growth phenotype (Humbert et al., 2000), suggesting that E2F3 is required to activate target genes and drive progression through the cell cycle. The most compelling data in support of this notion comes from

the conditional ablation of *E2f3* in mice lacking *E2f1* and *E2f2*, which results in MEFs that are unable to proliferate at all (Wu et al., 2001).

E2Fs can also be inactivated by targeting their dimerization partner, DP-1. One study used a dominant-negative mutant of DP-1, which retains its dimerization domain but lacks a DNA-binding domain (Wu et al., 1996) (see Figure 1B). This mutant sequesters DP-dependent E2Fs (E2F1 to 6) so that they cannot bind to E2F sites in the DNA. This mutant DP-1 induces G1 cell-cycle arrest. Likewise, in another study, depletion of DP-1 using small hairpin RNAs induced cell-cycle arrest (Maehara et al., 2005). These data suggest that E2Fs must bind to DNA in order to stimulate cellular proliferation. Based on these findings, it seems that E2F-mediated transactivation is crucial for the expression of target genes, which drive cell-cycle progression (see Figure 2A). This activation model has become the textbook view of how E2Fs contribute to cell-cycle regulation.

Repressing the Activation Model

These data, however, are in contrast to data from other studies where E2Fs are inactivated in different ways. Such studies use a mutant E2F (called E2F-DB) that lacks a carboxyl terminus, resulting in a dominant-negative E2F that is neither an activator nor a repressor but still retains its DNA binding domain (see Figure 1B). This mutant binds to DNA, displacing both activator and repressor E2Fs from E2F-responsive promoters. Most of these studies use an E2F1 mutant (Gonzalo et al., 2005; Krek et al., 1995; Maehara et al., 2005; Rowland et al., 2002; Zhang et al., 1999), but one study uses an E2F2 mutant (Bargou et al., 1996). If the activation model is correct, E2F-DB should inhibit the expression of target genes resulting in cell-cycle arrest. However, the exact opposite is observed: E2F-DB stimulates cellular proliferation, transforms cells, and makes them refractory to antiproliferative signaling by the $p16^{INK4a}$-pRB, TGFβ, and $p19^{ARF}$-p53 tumor-suppressor pathways. In addition, the expression of E2F target genes is not inhibited by E2F-DB but in most cases is switched on (Bargou et al., 1996; Gonzalo et al., 2005; Krek et al., 1995; Maehara et al., 2005; Rowland et al., 2002; Zhang et al., 1999). Thus, these data support a model where E2F-mediated transactivation is not required for cell-cycle progression, and E2Fs regulate their target genes at the level of repression.

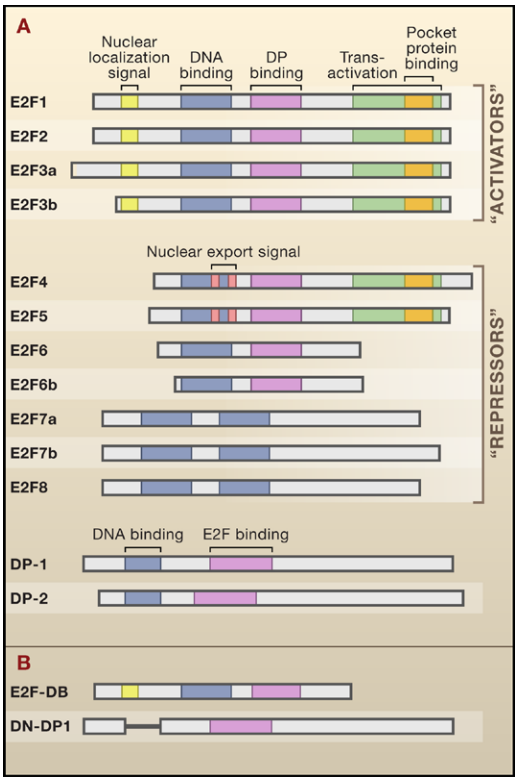

Figure 1. The Family of E2F Transcription Factors

(A) Mammalian cells have at least eight different E2F transcription factors, some of which have multiple isoforms. E2Fs 1 to 5 can be either activators or repressors of transcription. If these E2Fs are bound by a pocket protein (pRB, p107, or p130), they form transcriptional repressor complexes. When not bound to pocket proteins, these E2Fs have the potential to act as transactivators by binding to histone acetyl transferases (HATs). E2Fs 1 to 3 are frequently referred to as the activator E2Fs. E2Fs 4 and 5 primarily act as repressors as they are exported from the nucleus when not bound to pocket proteins. E2Fs 6 to 8 do not have transactivation domains and are seen strictly as repressors. E2F6 forms repressor complexes by interacting with polycomb group proteins (Pc-G); E2Fs 7 and 8 act as repressors through an unknown mechanism. E2Fs 1 to 6 bind to E2F sites on target gene promoters by forming dimers with DP-1 and DP-2; E2Fs 7 and 8 bind to DNA independently of DP proteins.

(B) Molecular tools for studying E2F functions. The E2F-DB dominant-negative mutant (also called dn-E2F or E2F-1 [–TA] or ΔE2F-2) is a carboxy-terminal deletion mutant of E2F1 (or E2F2). This mutant lacks a transactivation domain and pocket protein-binding domains but retains its *D*NA-*b*inding domain. This mutant displaces endogenous E2Fs from E2F-regulated target gene promoters. DN-DP1 is a deletion mutant of DP-1 that lacks its DNA-binding domain but retains its E2F-binding domain. This mutant binds to DP-dependent E2Fs and prevents them from binding to E2F-responsive promoters. These mutants differ because E2F-DB targets all E2Fs, whereas DN-DP1 targets only the DP-dependent E2Fs.

Activating the Repression Model

How can we reconcile these opposing conclusions? Are the data actually in conflict or is there an alternative model that incorporates all of these data? Although E2F1, 2, and 3 can be activators of transcription, they do not necessarily always act this way. It is equally possible that these E2Fs function primarily as "antirepressors": that is, they could occupy E2F sites on promoters to prevent the binding of repressor E2Fs (see Figure 2A).

Recent data show that the proliferation defect observed in *E2f3*-deficient MEFs is dependent on $p19^{ARF}$ and that E2F3 is not strictly required for proliferation. Apparently, E2F3 represses $p19^{ARF}$, such that loss of *E2f3* in MEFs that lack *p19ARF* no longer results in a proliferation defect (Aslanian et al., 2004). Thus, the proliferation defect exhibited by *E2f3*-deficient MEFs is not a function of loss of target gene activation but rather reflects a loss of target gene repression. This corresponds with the earlier observation that the dominant-negative mutant E2F-DB induces expression of $p19^{ARF}$. Together, these data demonstrate that $p19^{ARF}$ is indeed a target for repression by E2Fs. Illustrating the complexity of interactions among E2F target genes,

E2F-mediated transcriptional repression is also required downstream of $p19^{ARF}$. Indeed, inactivation of E2Fs with E2F-DB allows MEFs to proliferate even as $p19^{ARF}$ levels increase (Rowland et al., 2002). It is currently unknown which E2Fs are critical players downstream of $p19^{ARF}$ in signaling pathways that block cellular proliferation.

What about the more pronounced proliferation defect seen in MEFs that lack *E2fs 1*, *2*, and *3*? Are these cells defective in proliferation due to the absence of E2F-mediated transactivation? Or do the remaining E2Fs 4 through 8 repress promoters regulated by E2Fs in the absence of competing E2Fs 1, 2, and 3? We argue in favor of the latter model—that is, the remaining E2Fs that normally repress transcription in a regulated manner replace the absent E2Fs 1, 2, and 3 at sites in target gene promoters (see Figure 2A). The biological effect would be similar in both cases—cell-cycle arrest due to loss of expression of E2F target genes—but the underlying mechanism would be different.

Consider the opposing results obtained with the E2F-DB mutant protein (which promotes proliferation) and inactivation of E2Fs by targeting DP-1 (which inhibits proliferation). One explanation could be that either approach may have nonspecific side effects. A more appealing explanation is that DP-1 ablation prevents E2Fs 1 through 6 from binding to DNA (binding is DP dependent) but does not prevent E2Fs 7 and 8 (atypical members that act as repressors and cause cell-cycle arrest) from binding to DNA (binding is not DP dependent). It seems feasible that in the absence of DNA binding of E2Fs 1 to 6, the antiproliferative effects of the remaining two E2Fs become dominant. This would then resemble the model for proliferation deficiency in MEFs lacking *E2fs1*, *2*, and *3* (see Figure 2A). In both cases, removal of a subset of E2Fs leaves the target gene promoter sites free for repression by the remaining E2Fs.

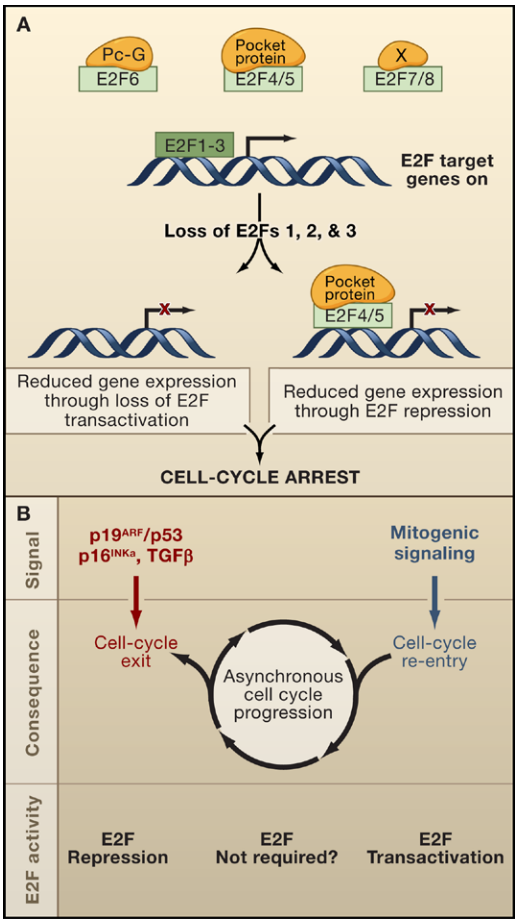

Figure 2. E2F Activation versus Repression

(A) Loss of E2Fs 1 to 3 prevents expression of E2F target genes resulting in cell-cycle arrest. Either of two mechanisms could be at work. (1) E2Fs 1 to 3 transactivate expression of E2F target genes, and when lost, E2F target gene expression decreases resulting in cell-cycle arrest. (2) Alternatively, E2Fs 1 to 3 may prevent downregulation of target genes by E2Fs that act as repressors. These include E2Fs 4 and 5 bound to pocket proteins, E2F6 bound to Polycomb group proteins (PcG), and E2Fs 7 and 8 bound to unknown repressive proteins (X). When E2Fs 1, 2, and 3 are lost, the repressor E2Fs are free to repress the expression of target genes resulting in cell-cycle arrest. This repression could be mediated by any of the remaining E2Fs (alone or in combination).

(B) E2F effects on the cell cycle. During asynchronous cell proliferation, E2F-mediated transactivation is dispensable, and E2Fs regulate the expression of their target genes by means of limited transcriptional repression. However, during cell-cycle exit—in response to contact inhibition or signaling via $p19^{ARF}$/p53, TGFβ, or $p16^{INK4a}$—E2F-mediated transcriptional repression is required. E2F-mediated transactivation is only needed under specific circumstances, such as during cell-cycle re-entry from quiescence.

Flying ahead

Mammalian cells have at least eight different E2Fs, five of which can regulate both transcriptional activation and repression. In contrast, the fruit fly *Drosophila* has only two E2Fs with clear-cut opposing effects: dE2F1 is strictly an activator, whereas dE2F2 is strictly a repressor. When *Drosophila de2f1* was deleted, E2F target genes were expressed at much lower levels resulting in serious proliferation defects (Duronio et al., 1995). The logical conclusion was that dE2F1 is required to activate expression of its target genes. Without E2F-driven expression of these target genes, proliferation cannot take place.

The defect imposed by loss of activating *de2f1*, however, could be restored by the simultaneous loss of repressing *de2f2*, resulting in proliferation in the absence of E2Fs altogether (Frolov et al., 2001). These results indicate that in *Drosophila* the activating and repressing E2Fs act antagonistically and suggest that in the absence of the activating E2F, the repressing E2F can exert its function in a dominant manner resulting in cell-cycle arrest. Furthermore, fly E2Fs apparently control the expression of E2F target genes but are not essential for proliferation per se. In support of this view, expression of a fly dE2F1 mutant called $dE2F^{i2}$ (which is the *Drosophila* version of mammalian E2F-DB) rescues the proliferation defect in fly larvae lacking *de2f1* (Royzman et al., 1999). This again suggests that the key function of dE2F1 is not related to transactivation and that dE2F1 may be required to prevent the recruitment of dE2F2 to E2F sites on target gene promoters.

Extrapolating these results to mammalian cells, this would argue in favor of a model where loss of activating E2Fs (through deletion of *E2fs1*, *2*, and *3* or inactivation of DP) may be com-

pensated by loss of repressing E2Fs. If so, complete loss of all E2Fs may result in deregulation of the cell cycle-controlled expression of E2F target genes, yielding cells that proliferate proficiently but are refractory to pocket protein-mediated growth-inhibitory signals. By inference, loss of E2Fs should have the same effect as loss of pocket proteins. This is the case in the worm *Caenorhabditis elegans* where loss of the E2F homolog *efl1* results in the same developmental defect as loss of the pRB worm homolog *lin-35* (Ceol and Horvitz, 2001). Similarly, loss of all three pocket proteins in mammalian cells yields the same phenotype as expression of E2F-DB, that is, immortalization (Dannenberg et al., 2000; Rowland et al., 2002).

Notably, although fly larvae lacking both *de2f1* and *de2f2* develop relatively normally, they cannot mature to become viable flies (Frolov et al., 2001). Likewise, many of the knockout mice lacking one or more members of the E2F family exhibit impaired embryonic development (Attwooll et al., 2004). It is possible that in these developmental settings, where there are periods of quiescence during embryonic development, E2F-mediated transactivation is required (see below). However, equally, these developmental phenotypes may be a mere consequence of dysregulated transcriptional repression.

Why Have a Transactivation Domain at All?

Why do E2Fs 1 to 5 all have transactivation domains? Surely, this domain must have a function? And where did the activation model come from in the first place? In fact, the activation model was derived primarily from studies on cell-cycle re-entry, rather than studies on asynchronous proliferation of cells. Most of the work on endogenous E2F transactivation and associated HATs has been performed in cells that were synchronized by serum starvation and then analyzed during serum re-stimulation. Recently, ChIP experiments have shown that during cell-cycle re-entry, E2F4/p130 complexes and HDACs are replaced on E2F-responsive promoters by E2Fs 1 to 3 and HATs. The timing of this change corresponds with the increase in acetylation of histones and the expression of E2F target genes (Frolov and Dyson, 2004). Furthermore, a transactivation-deficient E2F mutant blocks cell-cycle re-entry in response to serum re-stimulation (Rowland et al., 2002), suggesting that for this function E2Fs do need their transactivation domain. Correspondingly, upon overexpression, multiple E2F family members have the potential to drive immortal cells from quiescence into the cell cycle (DeGregori et al., 1997; Lukas et al., 1996), for which ectopic E2F requires its transactivation domain (Johnson et al., 1993; Lukas et al., 1996).

Thus, although asynchronously proliferating cells may not require E2F-mediated transactivation, cells that are exiting quiescence and re-entering the cell cycle do require the transactivation domain. Cell-cycle re-entry appears to be a specific circumstance in which E2F-mediated transactivation is required.

Conclusions and Implications

We propose that cell-cycle progression is not driven by E2F transcription factors, but rather that E2Fs impose a level of regulation on key components of the cell cycle. In this way, E2Fs establish a link between the cell cycle and either cell-extrinsic or cell-intrinsic factors involved in cell-cycle regulation that may be either pro- or antimitogenic. Quiescent cells are driven into the cell cycle by mitogens in an E2F transactivation-dependent manner. In contrast, proliferating cells can be arrested in an E2F repression-dependent manner by various signaling pathways including those of $p16^{INK4a}$/pRB, TGFβ, and $p19^{ARF}$/p53 (see Figure 2B).

One implication of this model is that design of anticancer therapies that inactivate the total pool of E2Fs may be unwise as this could promote proliferation of tumor cells. However, for cancers where cells do not proliferate exponentially but rather pass through stages of quiescence, targeting E2Fs would make sense as such tumor cells may be impaired in their potential to re-enter the cell cycle.

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