

Combinatorial gene control involving E2F and E Box family members

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Various studies point to the potential role of combinatorial action of transcription factors as a mechanism to achieve the complexity of eukaryotic gene control with a finite number of regulatory proteins. Our previous work has focused on interactions involving the E2F family of transcription factors as an example of combinatorial gene control, leading to the identification of TFE3 and YY1 as transcription partners for several E2F proteins. We now show that additional E2F target genes share a common promoter architecture and are also regulated by the combined action of TFE3 and E2F3. In contrast, the thymidine kinase (TK-1) promoter is also regulated by E2F3 but independent of TFE3. Other promoters exhibit distinct specificity in the interaction with E2F proteins that includes a role for E2F1 but not E2F3, examples where both E2F1 and E2F3 are seen to interact, and promoters that are regulated by TFE3 but independent of an E2F. We propose that these examples of combinatorial interactions involving E2F proteins provide a basis for the specificity of transcription control in the Rb/E2F pathway.

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Introduction

The massive complexity of transcription control for the large number of protein-coding genes in a eukaryotic cell presents a fundamental challenge in achieving specificity of transcription control with a limited number of transcription factors. A solution to this problem has been proposed based on a combinatorial mechanism of transcription control whereby a finite number of transcription factors yield a substantial level of complexity by working in combination (Yamamoto *et al.*, 1998). Various studies have now provided evidence for such combinatorial specificity, involving both upstream binding transcription factors and components of the basal transcription machinery (Pilpel *et al.*, 2001; Smale, 2001). For

example, the interaction of the herpesvirus VP16 transcriptional activator protein with the cellular factor HCF-1 and the cellular Oct-1 transcription factor redirects Oct 1 to herpesvirus immediate early promoters by virtue of an expanded DNA sequence recognition (Babb *et al.*, 2001). A further example of combinatorial specificity, as a mechanism for specificity of transcription factor function, is the pancreatic islet factor STF-1, which interacts with Pbx in a cooperative fashion and targets Pbx to a subset of promoters containing STF-1-binding sites (Peers *et al.*, 1995).

The various activities exhibited by the E2F and retinoblastoma (Rb) families of proteins have been shown to be critical for proper regulation of cell growth (Dyson, 1998; Nevins, 1998), and disruptions in their signaling pathways are a leading cause of many human cancers (Sherr, 1996; Nevins, 2001). The E2F family is composed of seven distinct gene products that are divided into three subfamilies based on their sequence homology—the E2F1–3 genes, the E2F4, 5 genes, and E2F6 and E2F7 genes. This division, based primarily on sequence, is also reflected in their functional properties. For example, the E2F1–3 genes are tightly regulated by cell growth and during cell cycle, whereas E2F4–6 are constitutively expressed. Furthermore, E2F1–3 act as positive regulators of transcription, whereas E2F4 and E2F5, when bound to p130 or Rb, act as transcriptional repressors (Dyson, 1998; Nevins, 1998). E2F6 has also been reported to act as a transcriptional repressor, but in a manner independent of Rb (Cartwright *et al.*, 1998; Gaubatz *et al.*, 1998; Trimarchi *et al.*, 1998, 2001). Further functional division can be seen in the E2F1–3 subgroup. While the E2F1, E2F2, and E2F3 proteins can each induce S phase when overexpressed in cells, the ablation of E2F3 activity, but not E2F1 activity, blocks cell proliferation. Conversely, E2F1 is a more potent inducer of apoptosis (DeGregori *et al.*, 1997; Bates *et al.*, 1998; Kowalik *et al.*, 1998; Moroni *et al.*, 2001; Lin *et al.*, 2001; Leone *et al.*, 2001).

These functional distinctions might best be explained by distinct specificities in target gene control. Nevertheless, assays of promoter DNA sequence recognition by E2F family members have not provided compelling evidence for distinct DNA sequence recognition (Zheng *et al.*, 1999). Thus, we reasoned that an alternative mechanism for promoter specificity could involve characteristic protein–protein interactions (Gstaiger *et al.*, 1995; Peers *et al.*, 1995; Strubin *et al.*, 1995; Luo *et al.*, 1998). Possibly, sequences within E2F3 allow an interaction with a subset of cellular proteins that provide a basis for promoter specificity and that are distinct from the proteins that can interact with other E2F family members. To this effect, we have previously identified the TFE3 transcription factor as a specific partner for E2F3 and showed that the Marked Box domain of E2F3, but not that of E2F1, is both necessary and sufficient for this interaction (Giangrande *et al.*, 2003). In addition, TFE3 and E2F3 can act synergistically to activate the p68 subunit gene of DNA polymerase α , dependent on the ability of the two proteins to interact physically.

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Importantly, the interaction of TFE3 and E2F3 with specific DNA sequences is mutually dependent and requires the Marked Box of E2F3. Moreover, a functional link between TFE3 and the Rb/E2F pathway is provided by recent experiments that show that TFE3 can rescue an Rb-mediated growth arrest (M Nijman, S Hijmans, and R Bernards, personal communication).

We now provide *in vivo* evidence for a selective role of TFE3 and E2F3 in the regulation of a subset of E2F target genes (i.e. ribonucleotide reductase 1 and 2 as well as DNA pol α p68) and show that loss of TFE3 can be compensated by USF1, but not USF2 or c-myc, two other E Box-binding proteins. These results predict that a promoter containing an E Box element paired with an E2F element is a potential E2F3-specific target. However, it is important to note that this model does not limit the action of E2F3 to TFE3 nor does it limit the action of TFE3 to E2F3. Indeed, we show that the promoters for thymidine kinase (TK)-1, the p180 subunit of DNA polymerase α (p180), dihydrofolate reductase (DHFR), and cyclin E1 are E2F3 targets, but not TFE3 targets; in contrast, the promoters for SMAD7 and tyrosinase-related protein 1 (Tyrp1) are bound by TFE3, but not by E2Fs. More importantly, we show that a subset of E2F target genes (i.e. E2F1, E2F2, and p19ARF) are bound by E2F1 but not E2F3 and consequently are deregulated in the E2F1 null mouse embryo fibroblasts (MEFs) but not in MEFs lacking E2F3. This is the first example, to date, of selective

gene control by two closely related members of the E2F family.

Results

Cooperative action of E2F3 and TFE3

Our previous work has shown that the activation of the DNA polymerase α p68 gene requires the combined action of the E2F3 and TFE3 transcription factors that bind to E2F elements and E Box elements in the promoter, respectively (Giangrande *et al*, 2003). Specifically, using chromatin immunoprecipitation (ChIP) assays we have shown that the ability of E2F3 or TFE3 to interact with the p68 promoter is dependent on the presence of both E2F3 and TFE3 proteins (Giangrande *et al*, 2003).

While the absence of E2F3 eliminates the interaction of TFE3, further study reveals that the absence of TFE3 impairs but does not eliminate the interaction of E2F3 with the p68 promoter; in particular, there is a delayed interaction of E2F3 with the p68 promoter in the absence of TFE3. As shown in Figure 1A (top panel), E2F3 and TFE3, but not E2F1 nor E2F2, were seen to interact with the p68 promoter in the wild-type (WT) cells at 15, 18, and 21 h post serum addition. Neither protein was bound to the promoter in the quiescent cell sample. The absence of E2F3 dramatically impaired the ability of TFE3 to bind to the p68 promoter. In contrast, the absence of TFE3 delayed the binding of E2F3 to the p68

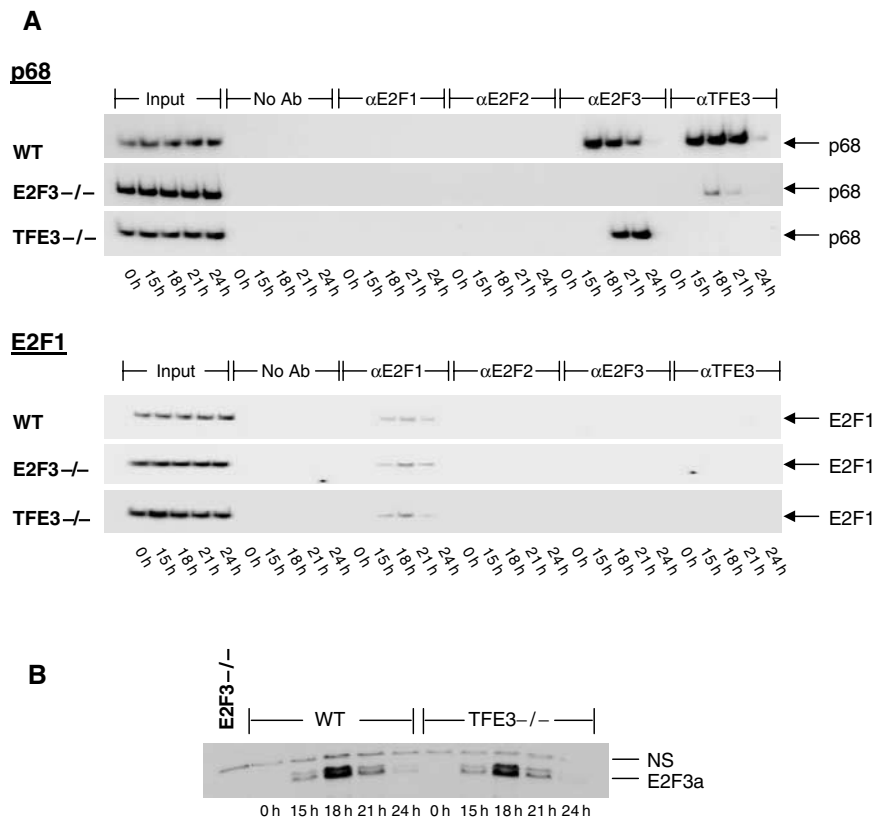


Figure 1 Mutually dependent binding of E2F3 and TFE3 to the murine p68 promoter. **(A)** ChIP assays for interaction of E2F1, E2F2, E2F3, or TFE3 with the p68 promoter (top panel) and E2F1 promoter (bottom panel) as cells progress through the cell cycle. MEFs (WT, E2F3 $^{-/-}$, or TFE3 $^{-/-}$) were harvested either at 0, 15, 18, 21, and 24 h following serum stimulation and crosslinked with the addition of formaldehyde as described previously (Takahashi *et al*, 2000). The arrows indicate the position of the p68 and E2F1 promoter PCR products. **(B)** Western blot for E2F3 (SC-879) assessing E3F3a protein levels in WT and TFE3 $^{-/-}$ MEFs at the indicated times following serum addition.

promoter by approximately 3 h compared to that of WT cells. Importantly, the kinetics of chromatin interaction of E2F3 with the p68 promoter reflects the kinetics of gene expression of the p68 gene in the TFE3 null cells (see Figure 6). In contrast, E2F3 protein levels were not altered in the TFE3^{-/-} cells (Figure 1B) confirming that the delay in binding of E2F3 to the p68 protein is not simply a consequence of slower growth properties of these cells. Likewise, there was no delay in binding of the E2F1 protein to the E2F1 promoter in TFE3 null cells.

Role of E Box-binding proteins in the regulation of p68 gene transcription

The ChIP experiments in Figure 1 confirm that in the absence of TFE3, E2F3 is still capable of binding to the p68 gene, but does so with delayed kinetics. One possible explanation for this delay is that other TFE3-related E Box-binding factors (Hodgkinson *et al*, 1993; Siritto *et al*, 1994) might compensate for the loss of TFE3, but do so less efficiently than TFE3. To address this possibility, we carried out ChIP assays to assess the interactions using p68 WT (WT) and mutant reporter constructs harboring mutations either within the E2F sites (E2Fm) or the E Box-binding elements (E Boxm) (Figure 2A). We reasoned that, if a member of the E Box family of transcription factors was compensating for loss of TFE3, this factor would not be able to bind to an E Box mutant reporter construct and thus would fail to recruit E2F3 to DNA. Asynchronous WT MEF cultures were transfected with either WT, E2F mutant, or E Box mutant p68 reporter constructs. Cells were harvested 24 h post-transfection and crosslinked with formaldehyde as described above. As shown in Figure 2B, both E2F3 and TFE3 proteins, but not the E2F1 protein, were bound to the WT p68 reporter. In contrast, there was no E2F3 protein bound to either the E2F mutant or the E Box mutant reporters, suggesting that both sites are necessary for E2F3 to interact with the p68 promoter. Binding of TFE3 to the E2F mutant reporter was markedly reduced compared to the binding observed with the WT reporter and there was no detectable TFE3 on the E Box mutant

reporter. These data are consistent with previous observations (Giangrande *et al*, 2003). Together, these data highlight a role for E Box-binding factors in E2F3 transcriptional regulation of the p68 gene.

The above data suggest that in the absence of TFE3, E2F3 is still capable of binding to the p68 gene and that mutation of the E Box element, within the p68 promoter, completely abrogates binding of E2F3 to DNA. These results thus implicate another E Box-binding protein(s) in promoting E2F3 binding to the p68 promoter. To address directly this possibility, we carried out ChIP assays for E2F3, TFE3, as well as, other E Box-binding proteins (i.e. USF1 and c-myc) (Figure 3A). WT and TFE3^{-/-} MEF cultures were brought to quiescence and then stimulated to grow by the addition of serum to a 20% final concentration. Cells were harvested at either quiescence, 15, 18, 21, or 24 h following growth stimulation and then crosslinked with formaldehyde as described above. As shown in Figure 3A, the p68 promoter was detected in the E2F3 and TFE3 immunoprecipitates of WT cells at 15, 18, and 21 h post serum addition. As observed previously, neither protein was bound to the promoter in the quiescent cell sample. Consistent with the ChIP data in Figure 1, the absence of TFE3 delayed the binding of E2F3 to the p68 promoter by approximately 3 h compared to that of WT cells. E2F3 protein levels were not altered in the TFE3^{-/-} cells, indicating that the observed delay in recruitment of E2F3 to the p68 promoter is not due to a delay in expression of E2F3 but, more directly, to loss of TFE3 (Figure 1B).

Assays for other E Box-binding proteins revealed that the USF1 transcription factor, but not c-Myc, interacts with the p68 promoter. Although USF1 only weakly bound to the p68 promoter in WT cells, there was a substantial interaction in the TFE3 null cells. Importantly, the kinetics of USF1 interaction mirrored that of E2F3 despite the constant presence of USF1 throughout the cell growth cycle, suggesting that USF1 binding to the promoter might be E2F3 dependent and/or cell cycle regulated (Figure 3B).

Taken together, these data suggest that USF1 may compensate for loss of TFE3 to recruit E2F3 to the p68 promoter. To determine whether USF1 was the key E Box-binding factor responsible for recruiting E2F3 to DNA in the absence of TFE3, we performed ChIP assays using TFE3 null cells that had been depleted of USF1 protein using siRNAs specific to mouse USF1 (Figure 3C). Consistent with the data in Figure 3A, E2F3 and USF1 bound to the p68 promoter with similar kinetics (scrambled). In contrast, binding of E2F3 to the promoter was drastically reduced in the absence of both TFE3 and USF1 proteins (compare siUSF1 to scrambled). Transfection of TFE3 null cells with the siRNA pool against mouse USF1 resulted in an 85% reduction of USF1 protein levels when compared to control (mock-transfected cells) or cells transfected with a pool of four nonspecific siRNAs (scrambled) (Figure 3D). Importantly, binding of E2F3 to the p68 promoter was reconstituted by cotransfection of a human USF1 expression plasmid (pCI-USF1) along with the siUSF1 pool (Figure 3C, bottom panel). Under these conditions, USF1 protein levels were restored to those of endogenous cells (Figure 3D, compare last lane with first two lanes). The siRNA pool against mouse USF1 did not result in the depletion of human USF1 protein due to differences between the mouse and human USF1 mRNAs.

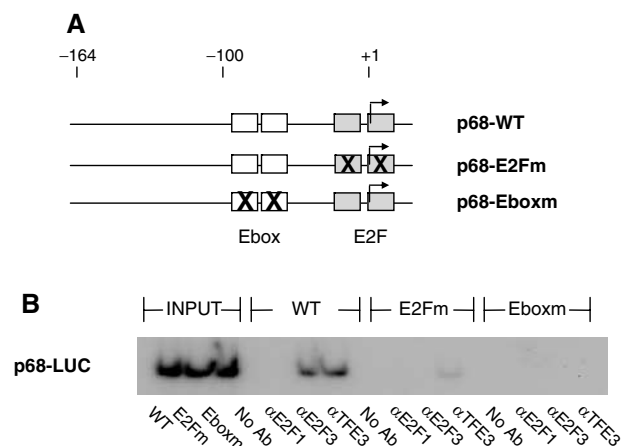


Figure 2 The interaction of E2F3 with the p68 promoter is dependent on E Box-binding proteins. (A) Schematic of p68 luciferase reporter constructs assayed in the ChIP assay. (B) ChIP assay for interactions of E2F1, E2F3, and TFE3 with the p68 WT (WT) and mutant reporter constructs harboring mutations either within the E2F sites (E2Fm) or the E Box-binding elements (E Boxm).

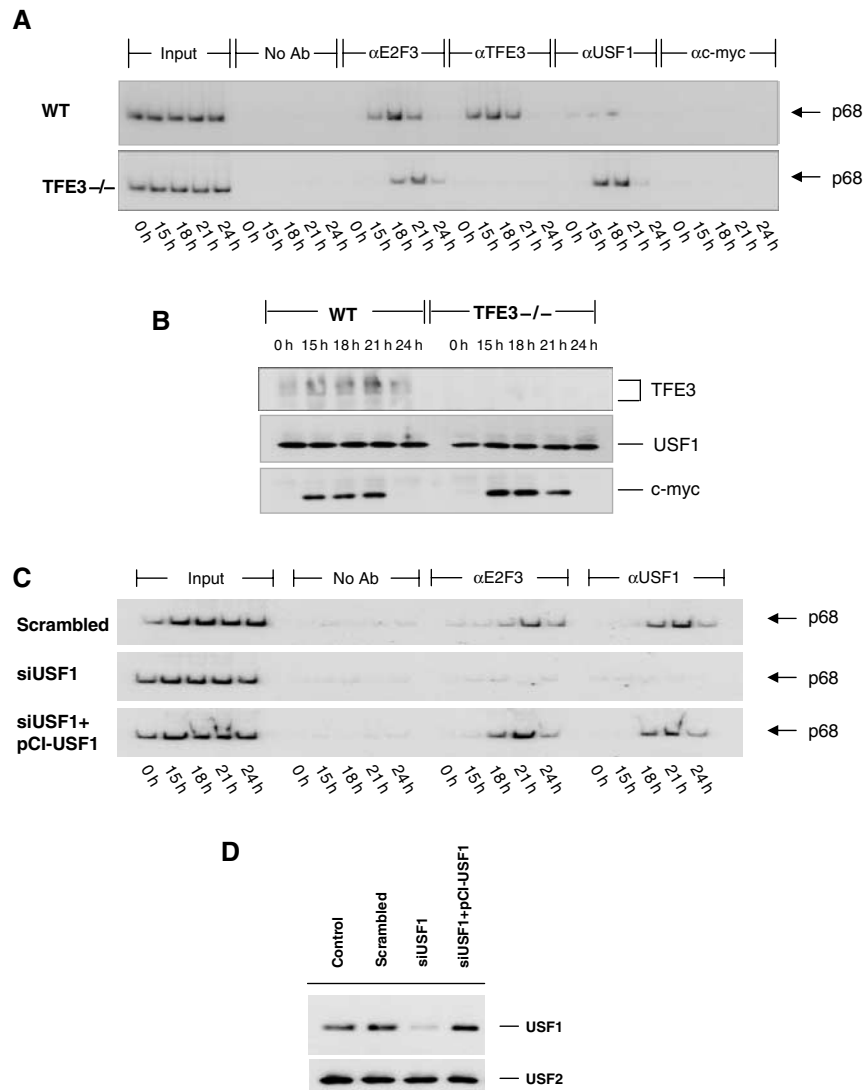


Figure 3 A role of USF1 in recruiting E2F3 to the p68 promoter in TFE3 null MEFs. **(A)** ChIP assays for interaction of E2F3, TFE3, USF1, or c-myc with the p68 promoter as cells progress through the cell cycle. MEFs (WT or TFE3^{-/-}) were harvested either at 0, 15, 18, 21, and 24 h following serum stimulation and crosslinked with the addition of formaldehyde (see Materials and methods). The arrows indicate the position of the p68 promoter PCR product. **(B)** Aliquots of the input material from panel A were resolved in an SDS acrylamide gel and assayed for the presence of TFE3, USF1, or c-myc by Western blotting with specific antibodies (see Materials and methods). **(C)** ChIP assays for the interaction of E2F3 and USF1 with the p68 promoter in TFE3^{-/-} cells transfected with either a pool of four nonspecific siRNAs (scrambled), a pool of four independent siRNAs specific to mouse USF1 (siUSF1), or siUSF1 plus an expression plasmid for human USF1 (pCI-USF1). Cells were harvested as in (A) and immunoprecipitates were analyzed for p68 promoter sequences. **(D)** Extracts of TFE3^{-/-} cells transfected with either the scrambled siRNAs, siUSF1, or siUSF1 along with pCI-USF1 were resolved on SDS acrylamide gels and assessed for presence of USF1 and USF2 by Western blotting with specific antibodies.

Taken together, these data show that USF1 is the key E Box-binding factor that compensates for loss of TFE3 to recruit E2F3 to the p68 promoter. Furthermore, it suggests that USF1 and E2F3 may interact *in vivo*. To address this possibility, we carried out co-immunoprecipitation assays for endogenous E2F3 and USF1 proteins in WT MEFs (Figure 4A). WT MEFs were lysed in IP lysis buffer, and lysates were incubated with antibodies specific to E2F3, TFE3, USF1, USF2, or c-myc. The levels of endogenous co-immunoprecipitated proteins were measured by Western blotting with antibodies specific to the proteins of interest (Figure 4A). As observed previously, E2F3 co-immunoprecipitated with TFE3 and vice versa (Giangrande *et al*, 2003). Importantly, the E2F3 immunoprecipitate also contained USF1, and the

USF1 immunoprecipitates revealed the presence of E2F3 in addition to USF2, a heterodimeric partner of USF1, suggesting that E2F3 and USF1 interact *in vivo*. In contrast, the USF1 immunoprecipitate from TFE3^{-/-} MEFs did not contain E2F1, suggesting that USF1, like TFE3, is an E2F3-specific partner (Figure 4B). Furthermore, c-Myc immunoprecipitates did not reveal binding of E2F3 to c-myc (Figure 4A). These data suggest that, in the absence of TFE3, the E Box-binding factor, USF1, binds to E2F3, and recruits E2F3 to the p68 promoter to promote the G1/S phase transition.

We next addressed the role of the Marked Box domain of E2F3 in mediating the interaction between E2F3 and USF1 as well as transcriptional activation together with USF1. TFE3^{-/-} MEFs were transfected with HA-E2F1, HA-E2F3,

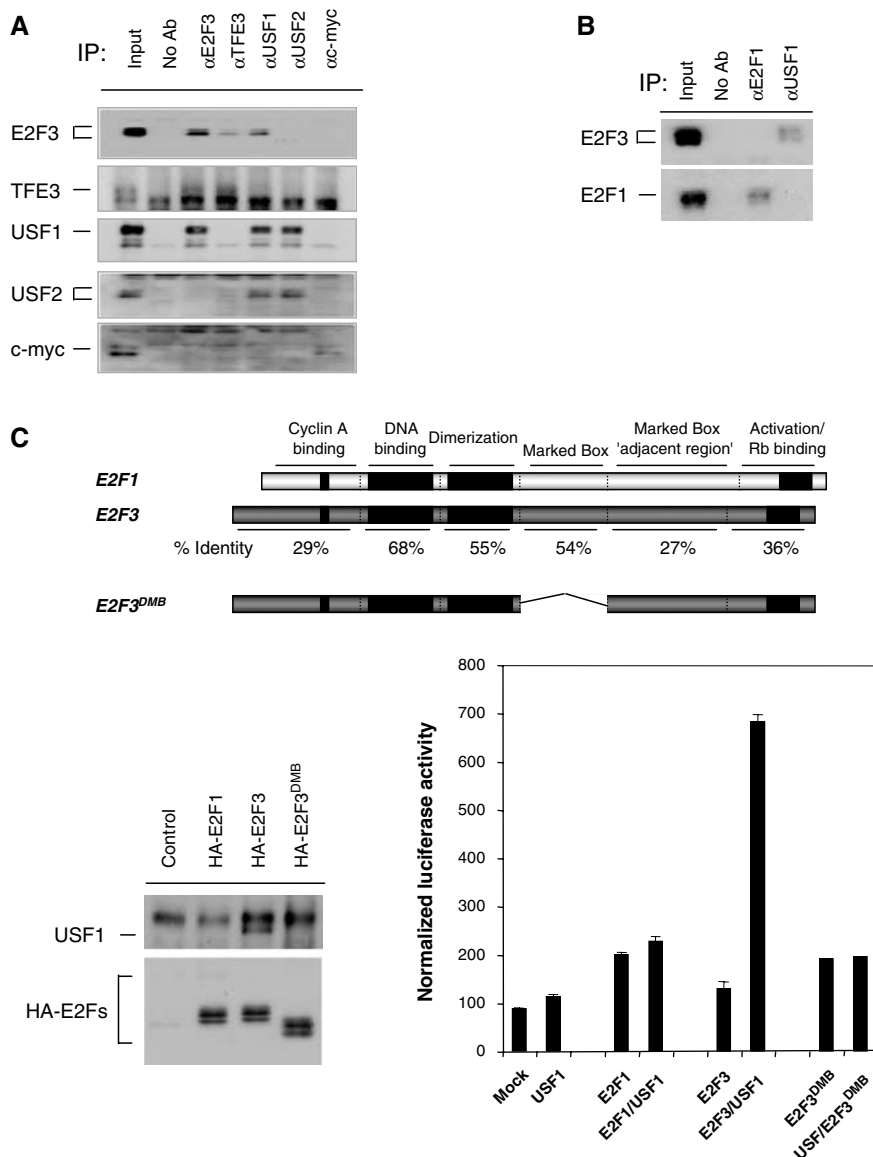


Figure 4 USF1 binds specifically to E2F3 via the E2F3 Marked Box. (A) WT MEFs were lysed in IP lysis buffer and lysates were incubated with antibodies to E2F3, TFE3, USF1, USF2, or c-myc immobilized onto Protein A Plus/Protein G Agarose beads (Oncogene). The levels of endogenous co-immunoprecipitated proteins were measured by Western blotting with antibodies specific to the proteins of interest. (B) TFE3^{-/-} MEFs were lysed in IP lysis buffer and lysates were incubated with antibodies to E2F1 or USF1 immobilized onto Protein A Plus/Protein G Agarose beads (Oncogene). The levels of endogenous co-immunoprecipitated proteins were measured by Western blotting with antibodies specific to the proteins of interest. (C) Schematic of HA-tagged E2F fusion constructs used in the binding and transcription assays. (Left panel) The HA-tagged E2F fusions have been described previously (Giangrande *et al*, 2003). TFE3^{-/-} MEFs were transfected with the HA-tagged E2F proteins and cells were lysed 24 h after transfection as described previously. HA antibody immobilized on Sepharose beads (Covance) was used to immunoprecipitate the HA-Tagged E2Fs. Endogenous USF1 that co-immunoprecipitated with the E2Fs was detected by Western blotting with antibodies specific to USF1. The amounts of the E2Fs that were recovered in the immunoprecipitates were determined by Western blotting with HA antibody. (Right panel) TFE3^{-/-} cells were transfected with the p68 promoter and 10 ng of either empty vector (Mock), expression plasmids for E2F1, E2F3, and E2F3^{AMB} proteins alone or in combination with 10 ng of the USF1 expression plasmid. USF1 alone was used as a control. Cells were harvested for determination of *Renilla* and *Luciferase* activity as detailed in the manufacturer's protocol (Promega).

or the HA-E2F3^{AMB} mutant as described previously (Giangrande *et al*, 2003). Consistent with the binding data in Figure 4B, USF1 co-immunoprecipitated with HA-E2F3 but not HA-E2F1 (Figure 4C, left panel). Furthermore, deletion of the E2F3 Marked Box (HA-E2F3^{AMB}) resulted in loss of USF1 binding.

To address the ability of the HA-E2Fs to mediate transcriptional synergy of the p68 promoter in the presence of USF1, TFE3^{-/-} MEFs were transfected with a p68 luciferase reporter construct together with plasmids encoding HA-E2F1,

HA-E2F3, or HA-E2F3^{AMB}. As observed for the TFE3/E2F3 interaction (see Giangrande *et al*, 2003), E2F3, but not E2F1, was able to activate synergistically the p68 promoter together with USF1 (Figure 4C, right panel). In contrast, the HA-E2F3^{AMB} mutant did not lead to synergistic activation of the p68 promoter together with USF1. These data provide strong evidence for a role of the Marked Box of E2F3 in mediating a physical interaction with USF1 that coincides with the ability to mediate cooperative transcription activation.

Specificity in E2F chromatin interactions and control of transcription

The E2F3–TFE3 interaction provides a potential mechanism for specificity by directing E2F3 to promoters that share E2F elements with E Box elements. To extend the analysis beyond the p68 example, we searched a collection of promoter sequences of known E2F target genes for the presence of both E2F elements and E Box elements. Several additional genes were identified that contained E2F elements and E Box elements including p68, RR2, RR1, thymidylate synthase (TS), E2F1, and E2F2; in each case, the sequences were within proximal promoter sequences (–320 to +20 bp) (Figure 5A). Like the p68 promoter, the mouse RR1, RR2, and TS promoter sequences reveal the presence of both E2F sites and E Box elements that cluster around the transcription start site (TSS) (Figure 5A; Group I).

To further explore the interaction of TFE3 and either E2F1 or E2F3 with these E2F target promoters *in vivo*, we performed ChIP assays with WT, E2F3–/–, and TFE3–/– cells. As shown in Figure 5B, E2F3 and TFE3 were both found to interact with the RR1, RR2, and TS promoters *in vivo*. The interaction of TFE3 and E2F3 with the RR1 and RR2 promoter sequences reflected the interactions observed with the p68 promoter since there was a mutually dependent interaction of E2F3 and TFE3 with each of these promoters. Furthermore, E2F1 did not interact with either the RR1 or the RR2 promoter under these conditions, suggesting that, like the p68 promoter, these promoters are E2F3-specific targets.

Both E2F3 and TFE3 were found to interact with the TS promoter but, in contrast to the co-dependency seen for p68, RR1, and RR2, the interaction of E2F3 and TFE3 with the TS promoter was not dependent on the presence of both proteins although the interaction of TFE3 was reduced in the absence of E2F3 (Figure 5B). Moreover, the interaction of E2F3 with the TS promoter was specific since E2F1 did not interact with this promoter.

Analysis of additional promoters provided evidence for interactions that are independent of TFE3. ChIP assays for TK-1, p180, cyclin E, and DHFR revealed an interaction of E2F3 but no evidence for a TFE3 interaction (Figure 5C), consistent with an absence of E Box elements in these promoters (Figure 5A). In some cases, we also observed that E2F1 was bound to the p180 and DHFR promoters along with E2F3. In contrast, we also identified promoter interactions that were specific for E2F1, not involving E2F3. As shown in Figure 5D, E2F1 is seen to interact with the E2F1, E2F2, and p19^{ARF} promoters, independent of TFE3 and E2F3. Taken together, these results would suggest both E2F1 and E2F3 promoter-specific interactions, some of which are dependent on the TFE3 factor, while some are independent of TFE3.

Finally, additional assays revealed evidence for TFE3 interactions independent of E2F3. The Smad7 and Tyrp1 promoters have previously been shown to be regulated by TFE3 and Smad proteins (Hua *et al*, 2000). In each case, E Box elements cluster with SMAD-binding elements (Figure 5A). As shown in Figure 5E, TFE3 was found to interact with the SMAD7 and Tyrp1 promoters during quiescence and S phase in both WT and E2F3 null cells, but not in TFE3 null cells. Thus, in this context, the interaction of TFE3 is E2F3 independent and likely entirely E2F independent given the apparent absence of E2F sites in the mouse SMAD7 and Tyrp1 promoters.

A role for E2F3 and TFE3 in growth-regulated gene activation

The ChIP assays demonstrate specificity in the interaction of E2F1 and E2F3 proteins with various E2F-regulated promoters. To explore the extent to which the specificity of promoter interactions reflects specificity of transcription control of these genes, we compared the expression of a subset of these E2F-responsive genes in WT MEFs and cells deficient for E2F1, E2F3, and TFE3 (Figure 6). Quiescent primary MEFs were stimulated to grow by serum addition and samples were taken at the indicated times to generate RNA for Northern blot analysis (Figure 6A), which was then normalized and quantitated (Figure 6B). The patterns of expression of these E2F-regulated genes were compared to the levels of GAPDH, a gene whose expression does not vary dramatically in quiescent or cycling cells. FACS analysis of propidium iodide-stained cells from the same experiment demonstrated that the cell cycle progression of the E2F3 null cells displayed a 3 h delay and that only <30% of the cells progressed through S phase when compared to the WT counterparts (data not shown). Each of these genes exhibited a cell cycle-regulated pattern of expression, with peak accumulation occurring at 12–15 h following growth stimulation. The expression of the p68, RR1, RR2, TK-1, TS, DHFR, and p180 genes was substantially reduced in the E2F3–/– cells, demonstrating a role of E2F3 in the control of expression of these genes and consistent with other recent work (Humbert *et al*, 2000). In contrast, expression of these genes was unaffected by the absence of E2F1. The expression of the E2F1 and E2F2 genes, both of which have been shown to be E2F regulated (Johnson *et al*, 1994; Hsiao *et al*, 1994; Neuman *et al*, 1994; Sears *et al*, 1997), was not affected by the loss of E2F3, consistent with previous work (Humbert *et al*, 2000) and with the ChIP analysis in Figure 5, whereas the expression of these genes was severely impaired in the E2F1 null cells. Of course, this is expected for E2F1 because of the gene deletion, but the result provides clear evidence for a specific role for E2F1 in the control of E2F2 expression. Interestingly, the expression of the p180 gene was also slightly delayed in E2F1 null cells when compared to p180 gene expression in WT cells. Indeed, expression of this gene is consistent with the ChIP data in Figure 5, which shows binding of both E2F1 and E2F3 proteins to the p180 promoter.

To assess the role of TFE3 in the control of these E2F-regulated genes, we made use of fibroblasts derived from TFE3 null embryos (Steigrimsson *et al*, 2002). Although the absence of TFE3 did not have as dramatic an effect on the expression of the p68, RR1, and RR2 genes as seen in the E2F3 null cells, it was nevertheless clear that the activation was delayed in TFE3–/– cells, consistent with the ChIP data (Figure 6A, bottom panel). In contrast, expression of E2F1, E2F2, TK-1, and DHFR was not altered in the TFE3–/– cells, consistent with previous ChIP data and indicating that the delay in p68, RR1, and RR2 expression was not simply a nonspecific consequence of slowed growth.

Discussion

Networks of transcriptional control depend on the specificity of action of individual, trans-acting transcription factors. Although some of this specificity can be attributed to the selectivity in DNA sequence recognition by these transcription

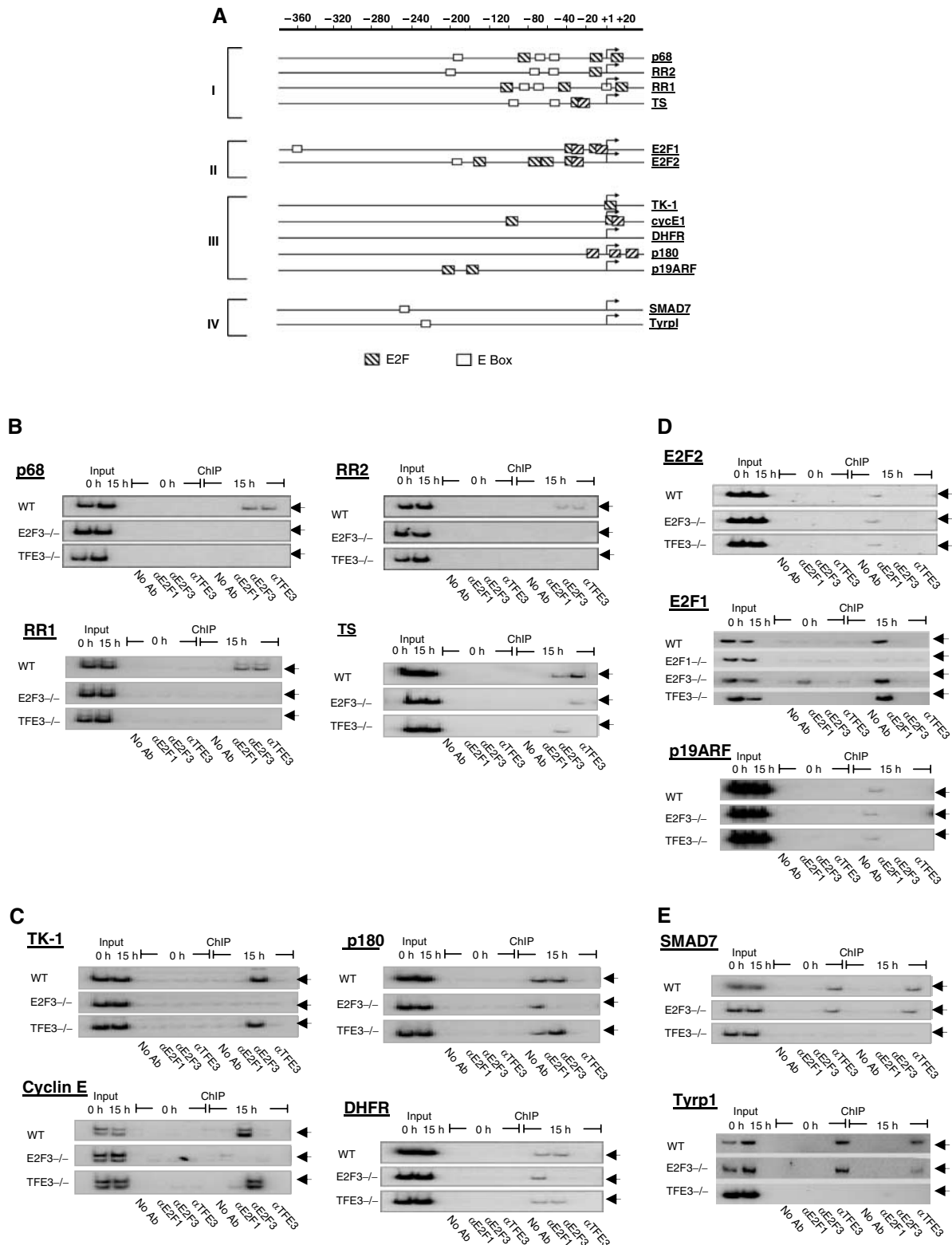


Figure 5 Evidence for selective and combinatorial gene control of a subset of E2F target genes. (A) Schematic of E2F and/or TFE3 target promoters assayed in the ChIP assays. I: promoter sequences containing both E2F elements and E Box elements that cluster around the TSS (designated with an arrow); II: promoter sequences containing E2F elements and E Box elements that do not group around the TSS; III: E2F target promoter sequences that contain E2F elements but lack E Box elements; IV: promoter sequences that contain only E Box elements but not E2F elements. (B) ChIP assays for interactions of E2F1, E2F3, or TFE3 with mouse p68, RR1, RR2, and TS promoters. MEFs (WT, E2F3^{-/-}, or TFE3^{-/-}) were harvested either at 0 or 15 h following serum stimulation, and crosslinked with the addition of formaldehyde as described previously (Takahashi *et al*, 2000). Chromatin was immunoprecipitated with antibodies to either E2F1, E2F3, or TFE3, decrosslinked, and DNA released from the immunoprecipitates was used for PCR analysis to measure the presence of target promoter sequences as discussed above. The arrows indicate the position of the promoter PCR product. (C) ChIP assays for interactions of E2F1, E2F3, or TFE3 with mouse TK-1, p180, DHFR, and cyclin E promoters. (D) ChIP assays for interactions of E2F1, E2F3, or TFE3 with mouse E2F1, E2F2, and p19ARF promoters *in vivo*. (E) ChIP assays for interactions of E2F1, E2F3, or TFE3 with mouse SMAD7 and Tyrp1 promoters *in vivo*.

factors, a number of observations suggest that this alone is not sufficient. Specificity dependent on a single transcription factor that recognizes a simple DNA sequence, often no more than 5 bp in length, would require an enormous number of such proteins to generate the gene regulatory networks known to exist. Moreover, the frequency with which a 5 bp sequence might randomly occur within the genome, unrelated to a transcriptional control element, is sufficiently high to suggest that such a sequence alone is unlikely to function independently. A role for protein–protein interactions in promoter recognition and transcription control has the potential to increase the complexity of the DNA recognition and thus add specificity to the process. One of the first such examples can be seen in the context of herpes simplex virus (HSV) infection where the combination of the viral VP16 protein with the Oct-1 and HCF-1 transcription factors creates a complex that recognizes a unique 9 bp DNA sequence (TAATGARAT) found in HSV immediate early promoters (Wysocka and Herr, 2003). Importantly, this increase in the complexity of sequence recognition due to the VP16–Oct1 interaction also provides a mechanism to increase the complexity of action of these two transcription factors if they should also interact with other sequence-specific proteins. It is the potential for multiple such interactions that provides the basis for the concept of combinatorial specificity in which a limited number of transcription factors can achieve regulation of a large number of target genes (Yamamoto *et al*, 1998).

The evolution of the E2F family can be clearly seen as a series of gene duplications that have allowed divergence of sequence and function. Functional distinctions can be observed for the E2F proteins, with perhaps the most striking being the ability of E2F1 to signal apoptosis and induce p53 accumulation (DeGregori *et al*, 1997; Kowalik *et al*, 1998) versus the prominent role for E2F3 in the control of cell proliferation (Leone *et al*, 1998; Humbert *et al*, 2000). Certainly, it is also evident that there is overlap of function within the E2F family such that the ability to trigger apoptosis

can also be linked to proteins other than E2F1 (Hateboer *et al*, 1998; Ziebold *et al*, 2001), and additional E2F family members besides E2F3 appear also to contribute to cell proliferation. For instance, while the loss of E2F3 does impair cell proliferation, the combined loss of E2F3 with that of additional E2F activities more severely reduces proliferative capacity (Wu *et al*, 2001). Nevertheless, despite the evidence for overlapping function, possibly a reflection of a relatively recent evolutionary divergence of these genes, there are clear indications for distinct functional roles for the E2F1 and E2F3 products. It seems most likely that the distinctions in the function of E2F1 and E2F3 are due to differential gene activation events. For example, loss of E2F3 (in the E2F3 null fibroblasts), but not E2F1, resulted in deregulated expression of a number of S-phase genes (i.e. p68, RR1, RR2, TS, and DHFR) (Figure 6, top panel). Whereas, loss of E2F1 protein, but not E2F3 protein, resulted in deregulated expression of E2F2, clearly implicating E2F1 in the regulation of this gene (Figure 6, middle panel).

The fact that E2F3 can interact with TFE3 resulting in a synergistic activation of the p68 promoter, whereas E2F1 does not interact with TFE3 and thus does not synergize in transcriptional activation, provides a basis for the E2F3-specific activation of transcription. In this particular instance, a promoter jointly containing a TFE3 site (E Box element) together with an E2F site would be a potential E2F3 target. It is important to note that this model does not limit the action of E2F3 to TFE3 nor does it limit the action of TFE3 to E2F3. Indeed, previous work has demonstrated a role for TFE3 in synergistic activation of the PAI-1 promoter with the Smad3 protein (Hua *et al*, 1998) and TFE3 has been shown to function synergistically with PU.1 and Ets-1 (Dang *et al*, 1998). Together, these data suggest that TFE3 could also participate in other promoter activation events independent of E2F3 (Rao *et al*, 1997; Tian *et al*, 1999).

Importantly, the above results also predict that a promoter containing an E Box element paired with an E2F element is a

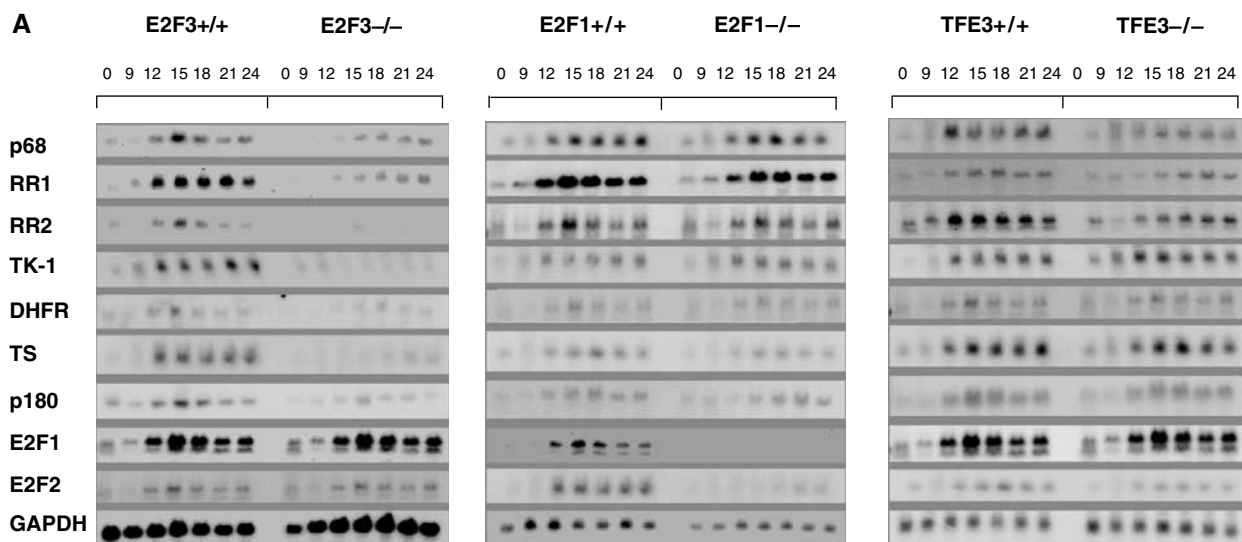


Figure 6 A role for E2F3 and TFE3 in the transcriptional activation of the p68 gene. (A) WT, E2F3, E2F1, and TFE3 mutant MEFs were synchronized by serum starvation and then stimulated by the addition of serum. RNA was isolated at each time point. Equal amounts of total RNA were subjected to Northern blot analysis to determine the pattern of expression of p68 and various E2F-responsive genes. (B) The expression level of the genes shown in (A) was quantitated by PhosphorImager analysis and normalized to the GAPDH control. WT (◆); KO (■).

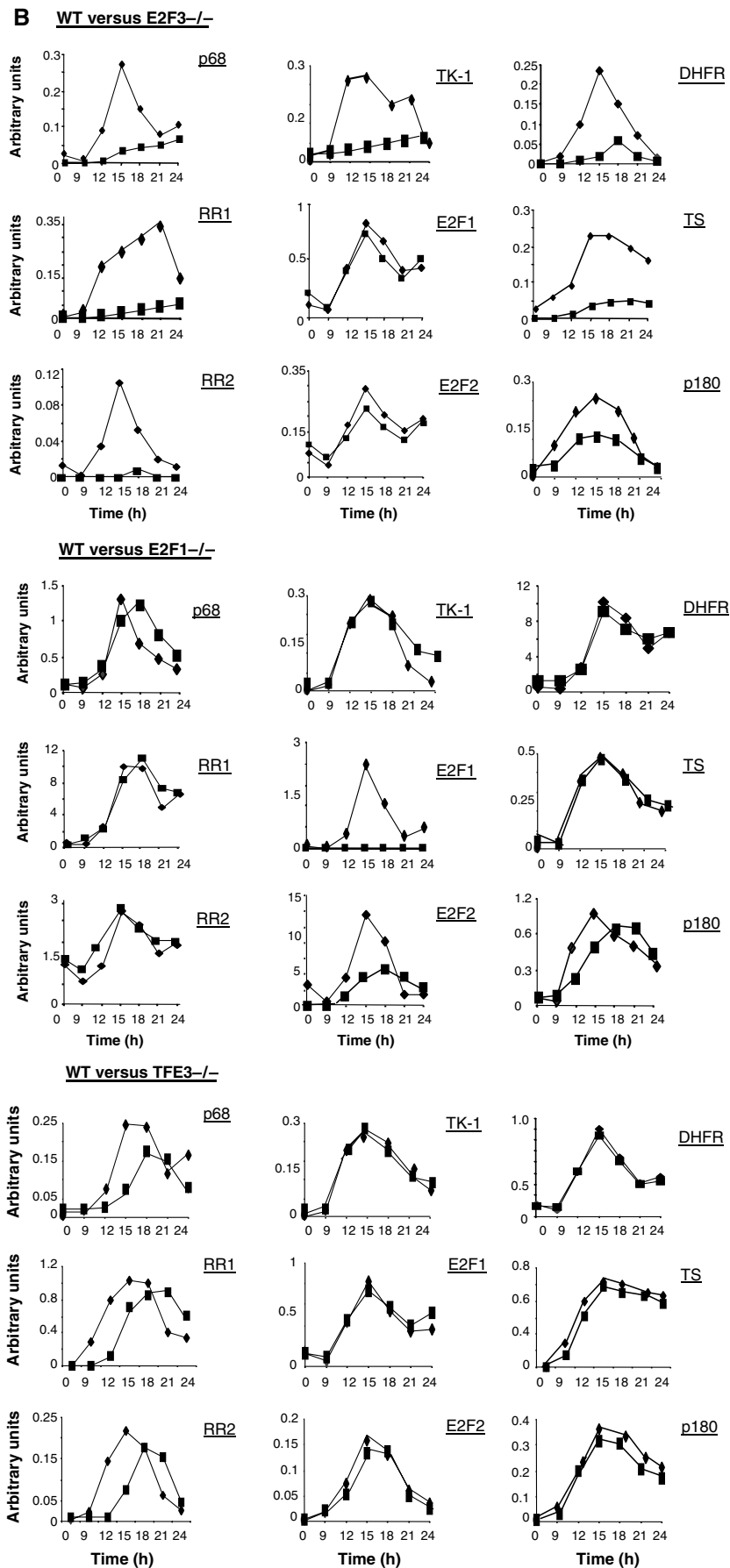


Figure 6 Continued

potential E2F3-specific target. In this model, specificity of function is achieved by the ability of transcription factors to interact, coupled with the joint presence of binding sites for the factors in a given promoter. Likewise, we also expect that E2F3 likely makes use of other partners besides TFE3 to achieve a specificity of function. For instance, the interaction of E2F3 with the DHFR, TK-1, p180, and cyclin E1 promoters would appear to be TFE3 independent. Indeed, it is the potential for multiple such interactions that would represent the basis for combinatorial specificity. The results from the ChIP assays would suggest that part of the mechanism by which E2F3 and TFE3 cooperate to activate transcription involves the physical recruitment of both proteins to the promoter. One might envision that the interaction of either factor alone (E2F3 or TFE3), while specific, is nevertheless weak and not sufficiently stable to promote the formation of a functional transcription complex. In this model, it is only when the two proteins jointly interact that they bind stably to a promoter and can then recruit other co-activators and ultimately the general transcription machinery. Of course, this model does not preclude an additional role for the E2F3–TFE3 complex in creating a unique surface that recruits other transcriptional activating proteins.

The ability of other E Box-binding factors, such as USF1, to compensate for the loss of TFE3, in binding to and recruiting E2F3 to the p68 promoter, provides another level of combinatorial transcriptional control. In this case, the specificity lies within the E Box-binding transcription factor family, in that not all E Box factors can substitute for each other. Indeed, here we provide clear evidence for the role of USF1 in recruiting E2F3 to the p68 promoter in the absence of TFE3 (Figure 3). In addition, unlike USF2 or c-myc, USF1 is capable of binding to E2F3, again suggesting that a physical interaction between these factors is necessary for proper binding to DNA (Figure 4A). Importantly, as seen with TFE3, USF1 binds specifically to E2F3, but not to E2F1, in a manner dependent on the Marked Box of E2F3 (Figure 4B and C). The functional role of this interaction is seen in the ability of E2F3, but not E2F1, to activate synergistically transcription from the p68 promoter. These data imply that in the absence of TFE3, USF1 is responsible for binding to and targeting E2F3 to target promoter regions in order to promote proper S-phase transition.

The functional significance of this role of USF1 is unclear. In one scenario, the evolution of the E Box family of transcription factors may have left USF1 with some degree of functional equivalence to TFE3, a property only evident when TFE3 is not present. If this situation never normally occurs, then this is only a vestige of evolution of the E Box family of transcription factors. Alternatively, it is also possible that the ability of TFE3 as well as USF1 to partner with E2F3 is physiologically relevant—perhaps some cellular condition or tissue-specific circumstance creates a role for USF1 over TFE3.

In conclusion, the E2F3–TFE3 interaction could provide a mechanism for selective gene control within the E2F family. Indeed, we have initiated two hybrid screens with other E2F family members and have identified interactions that are specific for other E2F proteins and that are dependent on the Marked Box domain (Schlisio *et al*, 2002; Hallstrom and Nevins, 2003). If true then, the variation in function that has been observed for the E2Fs could reflect specificity of protein

interaction and thus specificity of transcription control. This need not be limited to transcription activation, since other E2F family members, in association with Rb family proteins, have been shown to function in transcription repression by recruiting activities such as histone deacetylase and CtBP. We would propose that specificity of transcription repression could also reflect specificity of protein–protein interaction at target promoters.

Materials and methods

Cell culture and transient transfections

Primary and established (MEFs) were maintained in 15% heat inactivated fetal bovine serum (FBS). Null MEFs, of a given genotype, were compared to MEFs generate from WT 13.5E-day-old littermates. Transient transfection assays were performed using Superfect Reagent (Qiagen) as described previously (Giangrande *et al*, 2003). All transient transfections were carried out in triplicate.

siRNA transfection assays

Passage 19 TFE3^{−/−} MEFs were plated at a density of 6×10^5 on 60 mm plates and 1×10^7 on 150 mm plates 24 h before transfection with the siRNAs. siRNAs specific to mouse USF1 were purchased from Dharmacon (Louisville, CO) as a pool of four independent siRNAs (M-006368-01). The nonspecific siRNA pool (M-006368-00) was used as a negative control for these experiments. The pCI-USF1 expression plasmid for human USF1 (a generous gift from Dr Emerson, UPenn, Philadelphia, PA) was cotransfected along with the siRNA pool to mouse USF1 to reconstitute USF1 protein levels. Transfection assays were performed with Superfect Reagent as detailed above.

Immunoprecipitations

Immunoprecipitations of endogenous E Box proteins and E2F3 were carried out as follows. WT MEFs were lysed in IP lysis buffer (0.5% NP-40, 50 mM Tris (pH 8), 25% glycerol, 0.1 mM EDTA, 150 mM NaCl, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM PMSF or Pefabloc (Boehringer Mannheim)), and the lysates were precleared two times with 20 μl of Protein A Plus/Protein G Sepharose beads (Oncogene) at 4°C for 4 h. The precleared lysates were incubated with antibodies specific to E2F3 (Santa Cruz; SC-879), TFE3 (Pharmin; 554263), USF1 (Santa Cruz; SC-229), USF2 (Santa Cruz; SC-862), or c-myc (Santa Cruz; SC-764) immobilized onto Protein A Plus/Protein G Agarose beads. The bound proteins were resolved by SDS–PAGE. Endogenous proteins were detected by Western blotting using specific antibodies as described below.

Western blot assay

Nuclear extracts of passage 18 WT or TFE3 null MEFs containing equal amounts of protein were boiled for 5 min in protein sample buffer and subjected to SDS–PAGE on 8.5% polyacrylamide gels. Proteins were subsequently transferred onto a PVDF membrane and blocked in PBS containing 5% skim milk for 2 h. Blots were then incubated with primary antibodies (1:1000 dilution) in PBS containing 5% milk overnight at 4°C, washed with PBS plus 0.05% NP-40, and incubated in PBS plus 0.05% NP-40 and secondary antibodies (1:5000 dilution) for 1 h at room temperature. Blots were processed with Amersham's ECL system as described by the manufacturer. The following primary antibodies were employed for immunoblotting: αE2F3 (Santa Cruz; SC-879); αTFE3 (Pharmin; 554263); αUSF1 (Santa Cruz; SC-229); αUSF2 (Santa Cruz; SC-862); αc-myc (Santa Cruz; SC-42).

Northern blot analysis

Passage 4 WT, E2F1, E2F3, and TFE3 null MEFs, derived from 13.5E-day-old littermates, were contact inhibited for 24 h and then plated onto 15 cm dishes at 5×10^6 cells/dish in DMEM containing 0.1% HIFBS for 24 h. Starved cells were released by adding serum to a final concentration of 20% HIFBS and cultured at 37°C for 0, 9, 12, 15, 18, 21, or 24 h. Total RNA was isolated using Trizol Reagent (Gibco Brl). Total RNA (30 ng) was separated by agarose gel electrophoresis under denaturing conditions, transferred to nylon membranes, and probed under conditions of high stringency with

various probes labeled with ³²P as described previously (DeGregori *et al*, 1995). Probes included the following: p68 fragment (1–937 bp; IMAGE:3708884); RR1, mouse *Eco*R1 fragment; RR2, 600 bp mouse *Eco*R1 fragment; TK-1, human *Bam*HI fragment; E2F1, 1.3 kb mouse *Eco*R1/*Xho*I fragment; E2F2, human *Scal*/*Pst*I fragment; DHFR, hamster *Bam*HI fragment; p180, human *Nco*I fragment; TS, human *Pst*I fragment; GAPDH, 780 bp *Pst*I/*Xba*I fragment.

Chromatin immunoprecipitation assays

We performed chromatin and reporter plasmid immunoprecipitations using a modification of a previously published method (Takahashi *et al*, 2000). Immunoprecipitates were incubated with 1 μg each of antibodies against E2F1, E2F3, or TFE3 (E2F1, sc-251; E2F3, sc-879; TFE3, Pharmingen 15451A) at 4°C overnight. We found that the polyclonal E2F1 antibody, sc-193, crossreacts with mouse E2F3. Therefore, we used the monoclonal E2F1 antibody, sc-251, for addressing promoter specificity in our ChIP assays. Decrosslinking of chromatin and/or input reporter plasmid was performed as described, and samples were analyzed by semiquantitative PCR (Takahashi *et al*, 2000). A total of 28 cycles of PCR were performed in 25 μl with 5 μl of immunoprecipitated material, 50 pmol of each primer set, 0.5 U *Taq* Gold DNA polymerase (Applied Biosystems), and 0.01 μCi of ³²P-dGTP or ³²P-dCTP. We used EZ Retrieve (<http://sirusb.umdj.edu:18080/EZRetrieve/>) to access mouse promoter sequences as well as information in the TRANSFAC database for identification of E2F and E Box elements. To amplify E2F- and/or TFE3-responsive promoter regions, the following primer sets were designed to promoter sequences between –500 to +50 for the following genes: p68 (Unigene ID: Mm.320), RR1 (Unigene ID: Mm.656), RR2 (Unigene ID: Mm.99),

E2F2 (Unigene ID: Mm.100478), E2F1 (Unigene ID: Mm.18036), p19ARF (Unigene ID: Mm.4733), TK-1 (Unigene ID: Mm.2661), p180 (Unigene ID: Mm.1923), DHFR (Unigene ID: Mm.23695), SMAD7 (Unigene ID: Mm.34407), cyclin E (Unigene ID: Mm.16110), and TS (Unigene ID: Mm.5879). Primer sequences for the above promoter regions are available upon request. The p68-luciferase reporter plasmids pGV-B, pKL12(–164), pKL12E2FAB, and pKL12M3-Pall used for the reporter ChIP assays were kindly provided by Masako Izumi (RIKEN, Japan) (Nishikawa *et al*, 2000). The following primers were used to amplify p68-luciferase reporter plasmids present in the immunoprecipitates: forward primer, 5'-cgggaggaccggcgctgc; reverse primer, 5'-cttcataccttatcagttgct. The identification of E2F- and TFE3-binding sites on each promoter was based on previously published reports (Johnson *et al*, 1994) as well as on outputs from the TRANSFAC database (Brodin *et al*, 2000; Hua *et al*, 2000). PCR products were electrophoresed on 6% polyacrylamide gels. Each experiment was performed at least three independent times, and representative data are shown.

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