Tandem E2F Binding Sites in the Promoter of the p107 Cell Cycle Regulator Control p107 Expression and Its Cellular Functions

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Abstract

The retinoblastoma tumor suppressor (Rb) is a potent and ubiquitously expressed cell cycle regulator, but patients with a germline Rb mutation develop a very specific tumor spectrum. This surprising observation raises the possibility that mechanisms that compensate for loss of Rb function are present or activated in many cell types. In particular, p107, a protein related to Rb, has been shown to functionally overlap for loss of Rb in several cellular contexts. To investigate the mechanisms underlying this functional redundancy between Rb and p107 in vivo, we used gene targeting in embryonic stem cells to engineer point mutations in two consensus E2F binding sites in the endogenous p107 promoter. Analysis of normal and mutant cells by gene expression and chromatin immunoprecipitation assays showed that members of the Rb and E2F families directly bound these two sites. Furthermore, we found that these two E2F sites controlled both the repression of p107 in quiescent cells and also its activation in cycling cells, as well as in Rb mutant cells. Cell cycle assays further indicated that activation of p107 transcription during S phase through the two E2F binding sites was critical for controlled cell cycle progression, uncovering a specific role for p107 to slow proliferation in mammalian cells. Direct transcriptional repression of p107 by Rb and E2F family members provides a molecular mechanism for a critical negative feedback loop during cell cycle progression and tumorigenesis. These experiments also suggest novel therapeutic strategies to increase the p107 levels in tumor cells.

Introduction

The retinoblastoma gene Rb was initially identified as a prototypic tumor suppressor through its association with hereditary retinoblastoma; mutations in Rb or in genes that play a role in the regulation of Rb function are found in virtually all types of human cancers. The best-described function of Rb is to act as a transcriptional co-factor: Rb regulates the activities of numerous transcription factors and recruits chromatin remodeling complexes to control the expression of genes involved in the control of cell cycle progression, differentiation, and senescence. It is generally thought that the E2F family of transcription factors, consisting of both activating members (E2F1, E2F2, E2F3a) and some of the repressing members (E2F3b, E2F4, E2F5), are the most critical downstream mediators of Rb function in the control of cell cycle progression [reviewed in [1–3]].

Although Rb is expressed in nearly all cell types [4], patients and mice carrying heterozygous mutations for the Rb gene are not strongly predisposed to a broad range of tumors [5–9]. Perhaps most strikingly, conditional deletion of Rb in the mouse retina is insufficient to induce retinoblastoma [10–13], in sharp contrast to what is observed in human patients. After it was found that Rb is a member of a three-gene family, along with p107 and p130, it was quickly hypothesized that one or both of these other Rb family members may be able to compensate for the absence of Rb in specific cell types. Indeed, Rb/p107 and Rb/p130 double knock-out mice develop retinoblastoma [10–14]. The ability of p107 to compensate for loss of Rb has since been observed in numerous cell types, beyond the mouse retina [13–19].

The observation that the presence of p107 or p130 is able to suppress some phenotypes in the absence of Rb has raised the question of what molecular mechanisms underlie this compensatory activity. Of the three Rb family members, p107 is thought to be mostly regulated at the transcriptional level [20–22]; p107 mRNA and protein levels are generally low in non-cycling cells, and expression increases as cells enter late G1 and S-phase [22], at a time when the protein is being functionally inactivated through phosphorylation. Because loss of Rb often results in increased levels of p107 mRNA in mammalian cells [18,23–26], an
Author Summary

The retinoblastoma tumor suppressor Rb belongs to a family of cell cycle inhibitors along with the related proteins p107 and p130. Strong evidence indicates that the three family members have both specific and overlapping functions and expression patterns in mammalian cells, including in cancer cells. However, the molecular mechanisms underlying the functional differences and similarities among Rb, p107, and p130 are still poorly understood. One proposed mechanism of compensation is a negative feedback loop involving increased p107 transcription in Rb-deficient cells. To dissect the mechanisms controlling p107 expression in both wild-type and Rb-deficient cells, we have engineered inactivating point mutations into the E2F binding sites in the endogenous p107 promoter using gene targeting in mouse embryonic stem cells. Gene expression and DNA binding assays revealed that these two sites are essential for the control of p107 transcription in wild-type and Rb mutant cells, and cell cycle assays showed their importance for normal functions of p107. These experiments identify a key node in cell cycle regulatory networks.

Results

Two consensus E2F binding sites in the mouse p107 promoter contribute to activation and repression of transcription in reporter assays

To understand the functions of the tandem E2F consensus binding sites in the mouse p107 promoter, we firstly generated a series of four luciferase constructs in which inactivating point mutations [33] were introduced into the two consensus E2F sites, either individually or together, into a construct containing 900 bp of the mouse p107 promoter (Figure 1B). These constructs were transfected into wild-type mouse embryonic stem cells (mESCs), which contain high levels of activating E2F transcription factors as well as limited Rb family function, due to hyperphosphorylation of the Rb family proteins through high activity of Cyclin/DbCdk complexes [34–36]. Because mESCs are rapidly cycling, we reasoned they would provide a good system to investigate the role of these consensus E2F sites in the activation of the p107 promoter. In mESCs, the p107-1* construct, which contains a mutation in the more distal consensus E2F binding site, expressed less than half of the luciferase activity of the wild-type construct, whereas the p107-2* construct, which retains the more distal site but contains a mutant proximal site, expressed nearly 70% of the wild-type activity (Figure 1C). Simultaneous mutation of the two sites in the p107-1*2* construct resulted in even lower expression, suggesting that both sites contributed to some extent to the activation of the p107 promoter, although the distal site appeared to mediate the majority of the activation. We also found that exogenous E2F3 efficiently enhanced the activity of the wild-type and the p107-2* constructs and to a much lesser extent that of the p107-1* and p107-1*2* mutant promoters (Figure 1C). These observations further suggested that the distal E2F consensus binding site mediated the majority of the activation of p107 by E2F2 but did not exclude that some activation of p107 could be mediated through the proximal site in this context.

We next investigated the potential role of E2F-mediated transcriptional repression in the control of p107 transcription in quiescent cells, where p107 levels are normally low. Because mESCs do not stably arrest in G0, the four reporter constructs were transfected into wild-type MEFs that were then serum-starved for 24 hours in order to induce cell cycle exit in G0. In this system, the p107-1* construct showed a significant trend towards increased reporter expression, suggesting that the distal site mediated a significant amount of repression of the p107 promoter that could not be compensated by the presence of the proximal site (Figure 1D). However, the significant increase in luciferase activity found with the p107-1*2* construct over the p107-1* (Figure 1D) construct also suggested that both sites contributed to repression of p107 to some extent in this context.

Together, these results are indicative of a model for the mouse p107 promoter in which the distal site is most significant for both the activation and repression of the p107 promoter, while the proximal site contributes to a lesser extent to both functions (Figure 1E, right). In contrast, previous experiments using the human promoter had generated a model in which the distal consensus E2F binding site had a more significant role in repression of p107 while the proximal site was more important for activation [22,27] (Figure 1E, left). This difference between the mouse and the human promoters could potentially result from a single polymorphism between the mouse and human sequences in the proximal consensus E2F binding site (Figure 1A) (see Discussion). Nevertheless, these results with plasmid reporters also underscore the fact that the two tandem E2F consensus sites may perform different functions in different contexts, raising the
Figure 1. Regulation of the mouse p107 promoter through E2F binding sites in reporter assays. (A) Conservation of the proximal p107 promoter across mammalian species. The two tandem consensus E2F binding sites (BS1 and BS2) are each indicated by a box. (B) Schematic representation of wild-type (WT), p107-1*, p107-2*, and p107-1*2* luciferase vectors. Transcription factor binding sites contained in this promoter region, as identified by sequence analysis, are indicated, as is the transcription start site (arrow). Black rectangular boxes indicate E2F consensus sites; white boxes indicate E2F consensus sites that are mutated. The inset represents the mutations (aaa) introduced in each site. (C) Relative luciferase activity expressed by the four constructs, co-transfected with CMV-E2F3 (+) or empty pCDNA (-), in cycling mESCs. For statistical analysis, each
question of their respective roles in the control of the endogenous p107 gene.

E2F transcription factors control endogenous p107 expression in mESCs via tandem consensus E2F binding sites in the proximal promoter region

To examine the role of the consensus E2F binding sites in the regulation of the endogenous p107 promoter, we generated a series of targeting vectors designed to knock-in the same series of mutations as those described for the luciferase vectors into the endogenous p107 locus (Figure 2A). These vectors were electroporated into mESCs in order to generate heterozygous cells. Targeting was confirmed by both 5' and 3' Southern analysis, as well as by sequencing of amplified genomic DNA (Figure 2B and data not shown). Wild-type control mESCs were generated through targeting events in which the Neomycin resistance cassette was correctly targeted but the E2F binding sites remained untargeted. Targeted cells were then infected with an adenovirus expressing the Cre recombinase in order to remove the resistance cassette, followed by a second round of targeting (Figure 2C). This procedure generated homozygous cells of three knock-in genotypes: p107E2F-1*1/1* cells with mutations in the distal site of both p107 alleles, p107E2F-2*/2* cells with mutations in the proximal site, and p107E2F-1*/2* cells with mutations in both p107 sites on both alleles of p107. Targeting was verified by Southern and sequencing analysis (Figure 2D and data not shown).

To determine the role of the consensus E2F binding sites in controlling endogenous p107 expression, we first examined p107 mRNA levels in control and homozygous mutant mESCs. In a pattern similar to the luciferase assays, quantitative RT-PCR (qRT-PCR) analysis showed that p107E2F-1*1/1* cells expressed 40-50% of the p107 mRNA expressed by wild-type cells while p107E2F-2*/2* cells expressed 70%; p107E2F-1*/2* cells expressed the lowest amounts of p107 in mESCs (Figure 2E). The relative levels of p107 mRNA in these mutant cells reflected actual p107 protein expression (Figure 2F), providing additional evidence that p107 levels in these cells are regulated largely at the transcriptional level and suggesting that E2F activity is involved in this transcriptional control. Importantly, the basal p107 promoter remained active despite the knock-in mutations, validating this knock-in approach to investigate the functional importance of discrete elements in the p107 promoter.

We next examined if the point mutations introduced into the p107 promoter affected the binding of E2F transcription factors and p107, the member of the Rb family with the highest level of expression in cycling cells [37], to the p107 regulatory regions, an experiment that was not possible without the knock-in mutant cells. Using quantitative ChIP analysis, we found that both E2F3 and E2F4 bound to the wild-type p107 promoter in mESCs (Figure 2G). The p107E2F-1*/1* and p107E2F-2*/2* cells demonstrated no binding for these two E2F family members to the p107 promoter (Figure 2G), consistent with the decreased levels of p107 expression observed in these cells (Figure 2E-2F). In all cases, there was significant binding of both E2Fs to the promoter of a control gene, B-Myb (Figure 2G). Rb family members are largely inactivated by hyperphosphorylation in undifferentiated mESCs [36]. As expected, we did not detect any significant binding of p107 on the p107 or B-Myb promoters in wild-type or knock-in mutant cells under these conditions (Figure 2G) while p107 binding could be observed in asynchronously cycling mouse fibroblasts at the same promoter regions (data not shown). These experiments show that E2F transcription factors require the consensus E2F binding sites for binding to the p107 promoter region. They also suggest that the distal E2F consensus binding site is the major mediator of E2F activity controlling p107 transcription in cycling mESCs.

p107 expression is repressed in quiescent MEFs by Rb/ E2F complexes via the tandem consensus E2F binding sites in the proximal promoter region

To understand the function of the E2F binding sites in the control of p107 during a more normal cell cycle and in G1 arrest, we grew MEFs from chimeric embryos generated from homozygous p107E2F-1*/1*, p107E2F-2*/2*, and control mESCs as indicated in Figure 3A. Due to lower numbers of chimeric embryos derived from the p107E2F-1*/1* cells, experiments were only performed in this genotype after immortalization. Primary control and p107E2F-1*/1* MEFs were first rendered quiescent in low serum. We found that p107E2F-1*/1* MEFs expressed two times more p107 mRNA than did the wild-type cells in the same conditions (Figure 3B). This fold increase, while somewhat variable depending on the MEF line (see below), was always observed and is similar to the increased levels of reporter activity observed with the p107-1*2* luciferase construct in quiescent MEFs (Figure 1D). A similar increase was observed with p107 protein levels in these cells (Figure 3C). These data showed that p107 expression was repressed in G0 through the E2F binding sites present in its proximal promoter region. Accordingly, we found a significant decrease in E2F4 binding to the p107 promoter in quiescent immortalized p107E2F-1*/1* and p107E2F-1*/2* MEFs compared to controls (Figure 3D), underscoring the role of the distal E2F binding site in the control of p107 repression in G0-arrested cells. p107 levels are low in quiescent cells and we did not observe any significant binding of p107 to the B-Myb promoter in wild-type or p107E2F-1*/1* and p107E2F-1*/2* quiescent immortalized MEFs (Figure 3D). We found some binding of p107 to its own promoter in wild-type cells just above the non-specific signal found with the control antibody, and there was a trend towards decreased binding in the knock-in mutant cells (Figure 3D), even though p107 levels are higher in the mutant cells (Figure 3C). p130 binding to the p107 promoter was decreased in p107E2F-1*/1* and p107E2F-1*/2* MEFs compared to controls; interestingly, p130 binding to the B-Myb promoter was also decreased in the mutant cells (Figure 3D). It is possible that increased p107 levels in the mutant quiescent cells may alter the composition of the protein complexes between members of the Rb and E2F families. One obvious candidate whose binding to the p107 and B-Myb promoters could also be affected and could influence p130 binding in the mutant cells is Rb itself. Detection of murine Rb at the promoters of E2F target genes has proven challenging, especially in cells with low levels of Rb such as MEFs [29,38–40]. While we have not measured Rb binding to the p107 promoter in quiescent cells, we were able to detect Rb binding to the p107 and Men3 promoters in cycling immortalized MEFs. We found that Rb binding to the p107 promoter was decreased to close to background levels in knock-in mutant cells but not changed at the promoter of the Men3 gene (Figure 3E). Because of the low intensity and the variability of the ChIP signal for Rb, we cannot
Figure 2. The E2F consensus binding sites in the p107 promoter are bound by E2F family members and control p107 expression in mESCs. (A) Schematic representation of the targeting construct (top) used to knock-in mutations into the E2F sites in the endogenous p107 allele (bottom). Mutations in the E2F sites are indicated by asterisks. NeoR, neomycin resistance cassette; DTA, diphtheria toxin A. The black boxes indicate E2F consensus sites, the grey boxes indicate p107 exons. (B) Representative Southern analysis for a wild-type mESC clone (+/+ ) and a correctly targeted allele (+/*). Genomic DNA was digested by Ndel (N in Figure 1A) and a 5' internal probe spanning the junction between the p107 intron and the NeoR cassette was used (black line in Figure 1A). (C) Schematic representation of the strategy used to generate homozygous mutant mESCs. (D) Sequencing analysis of wild-type and homozygous mutant mESCs. The knock-in mutant sequences are marked by boxes. (E) RT-qPCR analysis of p107 expression in wild-type and homozygous mutant cycling mESCs. p107 mRNA levels were calculated relative to TATA-binding protein (TBP). (n = 4) (F) Immunoblot analysis of p107 expression in mESCs. Tubulin expression is shown as a loading control. (G) Quantitative chromatin immunoprecipitation (ChIP) analysis of E2F3 (n = 5), E2F4 (n = 5), and p107 (n = 2) on the p107 promoter in wild-type (W) and homozygous mutant cycling mESCs. p16 antibodies serve as a negative control (n = 5). Fold enrichment was calculated over an unrelated DNA sequence (actin). The B-Myb promoter is shown as a control. The y-axis is plotted on a log2 scale.

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Figure 3. *p107* repression in quiescent MEFs is mediated by the two E2F binding sites. (A) mESCs targeted by the neomycin resistance cassette but retaining a wild-type *p107* promoter and mESCs targeted by homozygous mutations into the distal (1*/1*) or both E2F sites (1*2*/1*2*) were injected to generate chimeric embryos. Wild-type, *p107E2F-1*/1* and *p107E2F-1*2*/1*2* MEFs derived from chimeric embryos were selected for Neomycin resistance to generate pure populations. (B) RT-qPCR analysis of *p107* expression in quiescent wild-type and *p107E2F-1*2*/1*2* MEFs. (n = 9) (C) Immunoblot analysis of *p107* in the same conditions. Tubulin expression is shown as a loading control. (D) Quantitative ChIP analysis of E2F4, *p107*, and p130 binding on the *p107* promoter in quiescent immortalized wild-type, *p107E2F-1*/1*, and *p107E2F-1*2*/1*2* MEFs. The B-Myb promoter is shown as a control. (n = 3) (E) Quantitative ChIP analysis of Rb binding to the *p107* and *Mcm3* promoters in cycling immortalized wild-type and *p107E2F-1*2*/1*2* MEFs. Mouse IgG antibodies serve as a negative control. (n = 3) For (D,E), fold enrichment is calculated over actin and the y-axis is plotted on a log2 scale.

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exclude that Rb may be binding to other sites in the p107 promoter. However, altogether, these observations support a model in which Rb/E2F complexes bind to the p107 promoter through the E2F consensus binding sites.

To determine whether the de-repression observed in the \( p107^{E2F-1*2*/1*2*} \) MEFs was due to the loss of a repression complex involving Rb family members, control and \( p107^{E2F-1*2*/1*2*} \) MEFs were infected with retroviruses stably expressing shRNA molecules directed against Rb or p130 (Figure 4A, top). As previously shown [18,20], Rb knock-down in quiescent MEFs resulted in an increase in p107 protein levels (Figure 4A, top). In contrast, we did not observe an increase in p107 expression in cells with a p130 knock-down (Figure 4A, top, and data not shown). We could not functionally test if low levels of p107 expression altered its own transcription, although knockdown of p107 has no effect on the expression of an eGFP transgenic reporter for p107 in either cycling or quiescent MEFs [20] and unpublished observations. Based on these observations, we sought to determine the consequences of knocking-down Rb in wild-type and \( p107^{E2F-1*2*/1*2*} \) cells for p107 levels. Expression of shRNA molecules in wild-type and mutant MEFs resulted in a significant knock-down of Rb mRNA levels (Figure 4B, left). As expected, decreased Rb levels led to increased p107 mRNA levels in wild-type quiescent MEFs. In contrast, low levels of Rb did not result in a further de-repression of p107 mRNA expression in \( p107^{E2F-1*2*/1*2*} \) MEFs (Figure 4B, right). Moreover, the degree of de-repression that occurred in wild-type cells upon Rb knockdown was similar to that seen through point mutations in the E2F binding sites (Figure 4B, right). These experiments strongly suggested that Rb represses p107 through the two E2F binding sites in the p107 promoter in quiescent MEFs.

In cycling mESCs (Figure 2E–2F) and in quiescent MEFs (Figure 3B and 3C), p107 transcript levels correlate with Rb protein levels. In contrast, although quiescent wild-type MEFs with Rb knock-down and \( p107^{E2F-1*2*/1*2*} \) MEFs either with or without Rb knock-down all have similar p107 mRNA levels (Figure 4B), our initial immunoblot analysis suggested that p107 protein levels were higher in \( p107^{E2F-1*2*/1*2*} \) MEFs with Rb knock-down compared to control wild-type cells with knock-down or knock-in mutant cells with wild-type Rb levels (Figure 4A). Additional experiments confirmed and quantified these observations (Figure 4C). In order to explore the potential post-transcriptional regulation of p107 levels in the absence of Rb, we treated wild-type MEFs in the presence and absence of Rb knockdown with cycloheximide (Figure 4D). We speculated that because \( p107^{E2F-1*2*/1*2*} \) MEFs display a 1.5-2-fold de-repression of p107 when they are in G0/G1, this could mask a decrease in p107 expression at other phases of the cell cycle. To investigate this possibility, we expanded and stained control and mutant immortalized MEFs with Hoechst33342, a DNA intercalating agent that enabled the cells to be FACS-sorted by DNA content (Figure 5C). We found that wild-type G1 cells expressed 2–3 times more p107 than did cells in G0, and this level increased up to 10-fold in S phase. On the other hand, while both \( p107^{E2F-1*1*1*} \) and the \( p107^{E2F-1*2*/1*2*} \) cells showed some cell-cycle dependent induction of p107, this induction was lower than the induction of p107 in wild-type cells during S-phase (Figure 5D).

To examine the regulation of p107 transcription \( \Delta \) in the two E2F binding sites during cell cycle re-entry from G0, we synchronized primary wild-type and \( p107^{E2F-1*2*/1*2*} \) MEFs through serum starvation, and then stimulated cell-cycle re-entry through the addition of serum in the medium. We found that \( p107^{E2F-1*2*/1*2*} \) MEFs expressed higher levels of p107 than wild-type cells initially and that the mutant cells failed to increase p107 expression as much as the wild-type cells during cell cycle re-entry (Figure 5E), supporting the fact that the two E2F binding sites in the p107 promoter are critical for p107 up-regulation during S phase progression. Despite these differences in p107 levels, MEFs of both genotypes re-entered the cell cycle with similar kinetics, as determined by measuring the mRNA levels of the highly cell cycle regulated gene Cdk6 (Figure 5F) and by the two-dimensional analysis of DNA content by PI staining and BrdU incorporation (Figure 5G). One potential reason for the similarity of the cell cycle profiles in control and mutant cells is that p107 protein levels reached wild-type levels in \( p107^{E2F-1*2*/1*2*} \) MEFs progressing through S phase (Figure 5H) in these re-entry experiments. These observations corroborated our findings in cells with Rb knock-down that other mechanisms exist to increase p107 levels in some contexts, beyond the control of p107 transcriptional control by Rb/E2F complexes. Nevertheless, these experiments also confirmed that the transcriptional control of p107 expression in cells re-entering the cell cycle is under the control of the two E2F binding sites in its proximal promoter region.

Lower levels of p107 specifically during S phase are sufficient to accelerate the cellular proliferation

We next investigated the role of the E2F binding sites in the cell-cycle dependent activation of p107 transcription. We first found that asynchronously cycling \( p107^{E2F-1*2*/1*2*} \) MEFs expressed ~10% less p107 mRNA than did the wild-type cells (Figure 5A), a decrease which was barely observable at the protein level (Figure 5B). We speculated that because \( p107^{E2F-1*2*/1*2*} \) MEFs display a 1.5-2-fold de-repression of p107 when they are in G0/G1, this could mask a decrease in p107 expression at other phases of the cell cycle. To investigate this possibility, we expanded and stained control and mutant immortalized MEFs with Hoechst33342, a DNA intercalating agent that enabled the cells to be FACS-sorted by DNA content (Figure 5C). We found that wild-type G1 cells expressed 2–3 times more p107 than did cells in G0, and this level increased up to 10-fold in S phase. On the other hand, while both \( p107^{E2F-1*1*1*} \) and the \( p107^{E2F-1*2*/1*2*} \) cells showed some cell-cycle dependent induction of p107, this induction was lower than the induction of p107 in wild-type cells during S-phase (Figure 5D).

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Figure 4. p107 levels increase in quiescent MEFs in the absence of Rb through transcriptional and post-translational mechanisms. (A) Top: Representative immunoblot analysis of p107, Rb, and p130 expression after knockdown of Rb (shRb) or p130 (shp130-1 and shp130-2) as compared to empty vector in primary quiescent MEFs. Bottom: same experiment in p107E2F-1*2*/1*2* mutant MEFs. The asterisk shows a non-specific mutation of E2F Binding Sites in the p107 promoter.
system to test the role of p107 specifically during S phase progression. As discussed above, we found that the kinetics of Cdc6 induction were largely similar between primary wild-type and p107<sup>107E2F-1<sup>−1<sup>/1<sup>−2</sup> mutant MEFs, with only a slight increase in Cdc6 maximal levels and a slight acceleration of Cdc6 induction in the mutant cells (Figure 5F). Interestingly, however, when we repeated the same experiment with MEFs immortalized through knock-down of p19<sup>ARF</sup>, we found that p107<sup>107E2F-1<sup>−1<sup>/1<sup>−2</sup></sup> mutant MEFs induced Cdc6 mRNA levels more rapidly (Figure 6A), suggesting that these mutant MEFs re-entered the cell cycle more quickly. BrdU/IP analysis further suggested that immobilized p107<sup>107E2F-1<sup>−1<sup>/1<sup>−2</sup></sup> mutant MEFs generally entered S phase earlier than control MEFs in this context (Figure 6B). Together, these results suggest that p107 plays a critical role in controlling the kinetics of entry into S-phase in immortalized cells.

These defects in cell cycle re-entry led us to investigate if altered p107 levels may change the length of the cell cycle in asynchronously proliferating primary cells. To investigate the importance of the regulation of p107 transcription for cell cycle control, we performed cell proliferation assays comparing wild-type, p107<sup>107E2F-1<sup>−1<sup>/1<sup>−2</sup></sup> and p107<sup>−/−</sup> MEFs. BrdU/IP analysis of these asynchronously growing MEFs did not reveal any significant differences in the cell cycle profiles between the wild-type and p107<sup>107E2F-1<sup>−1<sup>/1<sup>−2</sup></sup> MEFs, although slightly more knock-in mutant cells were in G0/G1 and slightly fewer in G2/M than wild-type cells within this analysis; significantly more p107<sup>−/−</sup> MEFs were in S-phase than in either of the other genotypes (Figure 6C). Despite this absence of difference in BrdU incorporation, we found that p107<sup>107E2F-1<sup>−1<sup>/1<sup>−2</sup></sup> MEFs proliferated similarly to p107<sup>−/−</sup> MEFs and more rapidly than wild-type cells (Figure 6D). Thus, decreased levels of p107 mRNA specifically during S phase and in asynchronously cycling cells, even in primary MEFs, are sufficient to recapitulate the phenotype of p107<sup>−/−</sup> MEFs. Together, these observations indicate that p107 plays an important role at specific points during S phase in mammalian cells.

**Discussion**

The classical view of the E2F family of transcription factors is that they are necessary to drive cell-cycle progression by binding to the promoters of and activating genes necessary for S phase, including those needed for nucleotide synthesis and DNA replication (reviewed in [34,41]). However, it has also long been observed that the list of E2F target genes also includes negative regulators of the cell cycle, including p16, Rb, and p107. Traditional knock-out studies obscure the careful balance of these positive and negative feedback loops and the relative importance of each individual target. The knock-in of potential binding sites for specific transcription factors has not been extensively used [42,43] but proves here to be an extremely informative approach to dissect the functional role of specific nodes in complex regulatory networks. Our experiments demonstrate that E2F family members and Rb control p107 transcription largely through two tandem E2F binding sites in the proximal promoter of the p107 gene. Our data also identify functional differences between the two sites and the E2F activity bound to these sites in different contexts (see model in Figure 6E).

E2F transcription factors make up a diverse family whose members can all recognize the same consensus sequence. While some E2F transcription factors activate target gene expression, others repress transcription, either dependent upon or independent from their association with Rb family members. Many E2F target genes, like p107, reveal an even more complex promoter structure that includes two, or sometimes more, E2F consensus sites, and each site could have distinct functions in the control of the target gene [44–46]. Why certain promoters have several E2F binding sites and what dictates if a site serves to repress or activate transcription is not understood. In the specific example of p107, our data show that the distal E2F binding site is favored by both E2F3 and E2F4 in mESCs, indicating that activating and repressor E2Fs may act through the same binding site in vivo. Our data also indicate that binding to the consensus site is context-dependent: while E2F4 binding in mESCs requires the presence of at least one of the two binding sites, an E2F4 binding activity is still retained in MEFs with mutations in both E2F binding sites. This observation suggests the existence of secondary E2F binding sites and/or the presence of co-factors, including Rb family members, which may help tether E2Fs to a promoter region (Figure 6E). This residual binding activity may explain why p107 levels are still increasing during cell cycle re-entry in p107<sup>107E2F-1<sup>−1<sup>/1<sup>−2</sup></sup> mutant MEFs. In the future, the availability of cells with knock-in mutations in individual binding sites in the p107 promoter will provide novel tools to dissect how transcription factors and chromatin-remodeling enzymes interact with Rb/E2F complexes to regulate the expression of E2F target genes in different cellular contexts.

Similar to the residual binding of the E2F transcription factors to the mutant p107 promoter, we observed above background levels of Rb, p130, and p107 bound to the p107 promoter in p107<sup>107E2F-1<sup>−1<sup>/1<sup>−2</sup></sup> mutant MEFs. Rb family members have been well described to bind to many other protein binding partners, including ATF-1 [31] and Sp1 [30]. The mouse p107 promoter contains consensus sequences for both ATF and Sp1 (Figure 1B), and we cannot exclude that these sites, or others, may mediate E2F-independent binding of all three Rb-family members to the p107 promoter. Interestingly, we also found that the p107<sup>107E2F-1<sup>−1<sup>/1<sup>−2</sup></sup> mutant MEFs demonstrated reduced binding of p130 to the B-Myb promoter during quiescence. Expression analysis of B-Myb did not reveal substantial de-repression in these mutant MEFs, and increased levels of p107 were able to repress B-Myb expression in the absence of Rb. Together these results are consistent with the model that all three Rb family members are able to regulate B-Myb, and that these complexes may shift in response to altered Rb family levels. Furthermore, the trend towards increased Rb binding to the Men3 promoter in the p107<sup>107E2F-1<sup>−1<sup>/1<sup>−2</sup></sup> mutant MEFs (Figure 3E) was observed at other
Figure 5. E2F binding sites mediate activation of the p107 promoter in cycling cells. (A) RT-qPCR analysis of p107 mRNA relative to TBP in asynchronously cycling primary wild-type and p107E2F-1*2*/1*2* MEFs as in A. Tubulin expression is shown as a loading control. p107 protein quantification (right panel) is shown relative to Tubulin levels. (n = 3) (C) Representative example of Hoechst33342 staining of asynchronously cycling MEFs showing G1 and S phase populations; wild-type and mutant cells have similar profiles (data not shown). (D) RT-qPCR analysis of immortalized WT, p107E2F-1*/1*, and p107E2F-1*2*/1*2* MEFs. For each genotype, G0 samples were collected after at least three days of serum starvation. Asynchronous cells were stained with Hoechst33342 and sorted by their DNA content into G1 and S-phase samples. (n=8) (E) and (F) RT-qPCR analysis of primary wild-type and p107E2F-1*2*/1*2* MEFs that have been synchronized in G0 by serum starvation. DMEM supplemented with 20% serum was added at time 0, and extracts were collected at 10 hrs, 16 hrs, 22 hrs, and 28 hrs post-stimulation. (E) p107 mRNA and (F) Cdc6 mRNA. n=8 for both genotypes at all time points. (G) Percentage of cells in S-phase in primary MEFs collected during cell-cycle re-entry as in E. and F. Percentages were calculated by BrdU/PI analysis (n = 3). (H) Immunoblot analysis of p107 protein expression in primary wild-type and p107E2F-1*2*/1*2* MEF extracts collected at 0 hr, 8 hrs, 12 hrs, 16 hrs, 20 hrs, and 24 hrs post-
promoters (B-Myc and Cyclin A, data not shown), which may further suggest that p130 is displaced by Rb in these cells.

The tandem E2F consensus sites in the human p107 promoter had previously been demonstrated to have differential functions over control of p107 [27]. In contrast, our experiments show that the distal site is most important for both activation and repression of the p107 promoter in mouse cells. This discrepancy could be explained by the presence of a single point mutation in the mouse promoter, which makes the proximal site a less perfect E2F consensus site. We found that correcting the proximal site in the mouse p107 promoter (TTGTATGC → TTGGGGGC) did increase the activity of this promoter in luciferase assays approximately 3 fold relatively to the parental construct (unpublished data). However, in the absence of an intact distal site, the activity of even the “corrected” construct was substantially lower than that of a wild-type construct, further emphasizing the relative importance of this site in the mouse p107 promoter. These results also suggest that the human promoter, with two perfect E2F consensus sites, should generally be more responsive to E2F transcription factors than the mouse promoter. Therefore, this discrepancy alone is unlikely to explain why p107 is up-regulated and able to compensate for loss of Rb function in the mouse retina but not the human retina [47] or why mice and patients with an Rb mutation develop a distinct tumor spectrum. While the E2F sites are highly conserved across mammalian species, the rest of the p107 promoter is not, and these other evolutionary changes may further impact promoter regulation by E2F and other factors. Interestingly, in humans, single nucleotide polymorphisms (SNP) of unknown frequency have been identified in the p107 promoter, including one that deletes one of the 4 Ts in the distal E2F binding site; while this may not affect E2F binding (only 3 Ts are required in the consensus sequence), this polymorphism may potentially alter the physical orientation of the two sites relative to each other and influence p107 transcription. Future experiments will continue to dissect the mechanisms regulating p107 transcription, including the interactions between E2F family members, Rb family members, and other transcription factors that bind to the p107 promoter. These interactions may also help to explain the tissue-specificity of expression of p107 in vivo [20,48]

While several binding partners for p107 and its expression profile in various cell types are well known, the unique cellular functions of p107 are still poorly understood [2,49]. Overexpression of p107 can arrest some cell types in G1 [50], but loss of p107 function often results in no visible phenotypes, probably because of functional compensation by Rb and p130 [13,17]. Interestingly, loss of p107 in neural progenitors results in the activation of Notch signaling and increased proliferation [51,52]. p107−/− mice on a BALB/cJ background also display a myeloid hyperplasia and mutant MEFs derived from these mice demonstrate accelerated proliferation [53]. Furthermore, an insertional mutagenesis screen for tumor suppressor genes identified p107 as a tumor suppressor in B-cell lymphoma [54]. However, the mechanisms underlying these loss-of-function phenotypes are still unclear. Some studies have suggested that p107 may have a particular function during the progression from late G1 to S phase [55,56], when p107 levels are highest. We show here that expression of abnormally low levels of p107 during S phase results in increased cell numbers in a proliferation assay. Importantly, while the p107 protein is still expressed and p107 levels found during S phase in p107E2F−1*2*/1*2* cells are similar to those found in wild-type cells in G0/G1, and while BrdU incorporation failed to detect a significant difference between knock-in mutant cells and wild-type cells, the consequences for the cell cycle are as strong as in p107 null cells. This observation indicates that p107 levels are probably critical during very specific stages of the cell cycle, including during the DNA replication phase, and this role for p107 during S phase should be the focus of future studies to elucidate the cellular functions of this cell cycle regulator.

Unexpectedly, in immortalized MEFS in which p107ARF has been knocked-down, the importance of p107 expression during S-phase is even more evident, as the kinetics of cell cycle re-entry are altered in the p107E2F−1*2*/1*2* cells compared to wild-type cells, whereas these kinetics are unchanged in primary MEFS. This difference between the function of p107 during S phase in immortal and primary MEFS is potentially related to an additional function of p107ARF during S phase [57]. This function could stem from a network of interactions between p107, p107ARF, E2F1 and c-Myc during S phase [58-63] and these findings could reveal cooperation between appropriate expression of p107 and p107ARF in the control of S phase.

In the absence of Rb, de-repression and/or activation of p107 transcription is thought to result in higher levels of p107 that may then suppress the functions of activating E2F family members [64,65]. It is interesting to note that a similar mechanism has evolved independently in plants [66] and that this type of negative feedback loop also exists in the control of the cell cycle in yeast [67], strongly suggesting that this genetic circuitry is a universal component of the regulatory networks ensuring proper cell cycle progression. Here, we investigated the mechanisms by which this feedback loop is activated in mammalian cells using a mouse genetics approach.

While it has long been hypothesized that direct regulation of the p107 promoter by Rb is the mechanism by which compensatory up-regulation of p107 occurs in the absence of Rb, our mutant cells enabled us to distinguish effects of Rb loss on transcriptional and post-transcriptional compensatory expression of p107. Recently it has been shown that the p107 protein may be more stable in non-cycling human hepatocellular carcinoma cell lines when Rb is absent than when Rb is present [68]. Similarly, we found that in quiescent MEFS, the p107 protein becomes more stable when Rb is knocked-down, suggesting that transcriptional regulation alone may not fully explain compensatory levels of the p107 protein. Clearly, this increase in p107 protein levels in cycling cells or Rb mutant cells independent of p107 transcription could serve as a feedback mechanism to limit the effects of decreased Rb levels. The relative contribution of transcriptional and post-transcriptional mechanisms of p107 up-regulation in different contexts will be the focus of future studies.

We hypothesized that disrupting the transcriptional feedback loop between Rb/E2F and p107 would prevent compensation in the absence of Rb, such that the low p107 levels observed in p107E2F−1*2*/1*2* MEFS would cooperate with decreased Rb expression, potentially recapitulating some of the phenotypes observed in Rb/p107 double knock-out MEFS. Additional cell cycle assays comparing primary and immortalized knock-in mutant MEFS, both with and without Rb knock-down, failed to reveal conditions in which the knock-in mutation would cooperate with loss of Rb in allowing cells to grow in conditions that were permissive to growth of Rb/p107 double knock-out MEFS (data not shown). These results suggest that, in most contexts, the lower
Figure 6. Altered p107 expression affects cellular proliferation. (A,B) Immortalized wild-type and p107E2F-1*2*/1*2* MEFs were synchronized in G0 through at least three days of serum starvation. DMEM supplemented with 20% BGS was used to stimulate cell-cycle entry. Extracts were collected at the number of hours indicated post-serum stimulation. (A) RT-qPCR analysis of Cdc6 mRNA in wild-type and p107E2F-1*2*/1*2* MEFs. (n = 3) (B) Percentage of cells in S-phase, as determined by BrdU/PI staining, at the indicated time points. (n = 4) (C) Cell-cycle profiles of asynchronous primary wild-type, p107E2F-1*2*/1*2*, and p107−/− MEFs. Percentages of cells in each phase were determined by BrdU/PI staining. (n ≥ 2) (D) Cellular proliferation of primary wild-type, p107E2F-1*2*/1*2*, and p107−/− MEFs. Equal numbers of cells were plated at day 0. Cells were then counted every other day from day 1 to day 9 post-plating. For statistical analysis, p107E2F-1*2*/1*2* cells were compared to wild-type cells at each time point. (n ≥ 13) (E) Model for the context-dependent regulation of p107 transcription by E2F family members. In cycling mESCs, activating members of the E2F family such as E2F3 bind to the p107 promoter mostly through the distal consensus E2F binding site (site 1). In quiescent MEFs, binding of the E2F4 repressor is also largely dependent on the presence of the distal consensus site. However, E2F4 may also be recruited to the p107 promoter through interactions with other transcription factors and/or by binding to other DNA sequences. The size of the E2F boxes indicates the relative binding activity. doi:10.1371/journal.pgen.1001003.g006
levels of p107 observed specifically during S-phase are insufficient to overcome the higher levels of p107 observed at other stages of the cell cycle, at least in terms of reducing the ability of p107 to compensate in the absence of Rb.

Instead, we found that the increased levels of p107 observed in quiescent MEFs were able to repress expression of some genes in the absence of Rb even better than the increased levels of p107 found in wild-type MEFs in the absence of Rb. Interestingly, the ability of increased levels of p107 to repress for absence of Rb in repressing E2F target genes during quiescence depends on the particular gene. Increased levels of p107 protein produced by transcriptional de-repression and increased protein stability resulted in repressed levels of B-Myc, Cyclin A, and Cyclin E but had no further effect on Cdk4 (Figure 4E and data not shown). These results are consistent with several studies suggesting that the Rb family members regulate both distinct and overlapping target genes [23,69,70]. These observations also suggest that further increasing p107 levels in Rb mutant cells through a variety of mechanisms may enhance the compensatory abilities of this Rb family member.

A remaining question is why humans develop retinoblastoma upon loss of Rb while mice do not. It has recently been demonstrated that mouse retinal progenitors deficient for Rb display increased levels of p107 mRNA whereas human retinal progenitors do not increase the amount of p107 expressed when Rb is knocked down in culture [47]. This observation, and the fact that Rb/p107 double mutant mice develop retinoblastoma [71] supports the idea that p107 levels are important for its tumor suppressor activity in Rb-deficient retinal cells. However, no system has been devised to test whether the increased expression of p107 observed in Rb mutant cells directly contributes to the ability of p107 to compensate for the loss of Rb, or if this increased transcription is merely coincidental to a constitutive overlapping function shared by Rb and p107. In other words, it is possible that basal levels of p107 in Rb mutant cells would be sufficient to suppress retinoblastoma development. A similar question can be asked in other cell types in which p107 loss of function by knock-out enhances tumor development in Rb mutant cells and during development [13,15,72]. Our results indicate the converse, i.e. that even higher levels of p107 may more completely compensate in the absence of Rb, in terms of target gene expression. The generation of mESCs and MEFs in which the E2F binding sites in the p107 promoter or mice carrying fragments of the human p107 promoter were targeted to the p107 promoter were singly mutated were intended to discretely separate the ability of p107 to be activated in the absence of Rb from other sources of transcriptional regulation. However, these genetic studies clearly demonstrated that in MEFs, a single E2F site is critical for both the repression of p107 in non-cycling cells and the activation of p107 in cycling cells, making the separation of these two activities impossible. In future experiments, mice carrying point mutations in the E2F binding sites in the p107 promoter or mice carrying fragments of the human p107 promoter may help explore the necessity of p107 up-regulation in the prevention of retinoblastoma in mice by distinguishing between overlapping and compensatory expression patterns of p107.

Materials and Methods

Ethics statement

Mice were maintained according to practices prescribed by the NIH at Stanford’s Research Animal Facility accredited by the AAALAC.

Generation of knock-in p107 BAC and targeting vectors

Recombineering in bacteria [73] was used to generate the p107^200^+, p107^200^-R, and p107^200^-R^-. BACs. Briefly, BAC clone RP23-163J20 ordered from BACPAC (http://bacpac.chori.org/) was transformed into the EL250 strain and the heat-inducible recombinase present in this strain enabled the insertion of a Neomycin resistance cassette into intron 1 of p107 by homologous recombination. Approximately 500 bp of the wild-type promoter sequence was cloned into pBluescript. Mutation of the E2F sites was performed using blunt end primers carrying the wild-type sequence or the point mutations. The 500 bp fragments were then targeted to the p107 BAC with the conditional Neomycin resistance cassette. To generate the targeting vectors, we excised approximately 4 kb upstream of the E2F binding sites and 4 kb downstream of the Neomycin resistance cassette from the BAC DNA into a targeting vector backbone carrying a DTA cassette.

Cell culture

mESCs and MEFs were cultured as described previously [74]. For expression analysis in asynchronous MEFs, 3 × 10^5 cells were plated per 6 cm culture dish. For BrdU/PI analysis in asynchronous MEFs, 2.5 × 10^5 cells were plated per 6 well. Extracts were collected 48 hours later. For quiescent cell analysis were plated at higher density: 8 × 10^5 per 6 cm dish, 3 × 10^5 per 6 well, or 1.5 × 10^5 per 12 well. The following day cells were washed twice with PBS and then cultured in DMEM supplemented with 0.1% serum for at least 72 hours. For extracts from synchronized MEFs, cells were rendered quiescent and cultures were re-stimulated with DMEM supplemented with 20% serum as above after at least 72 hours in low serum conditions. Extracts were collected at various time points after stimulation. For proliferation assays, MEFs were plated at either 2.5 × 10^4 or 5 × 10^4 cells per 12 well on day 0. Cells were counted in duplicate and were given fresh media every other day.

For cycloheximide experiments, wild-type MEFs were rendered quiescent for 3 days as above. At time 0, cycloheximide (Calbiochem, 25 mg/ml in methanol) or methanol was added to cells at a concentration of 30 μg of cycloheximide per ml of DMEM with 0.1% serum.

Luciferase assays

To construct the plasmid reporters, primers were designed to amplify the p107 promoter from the targeted BAC clones (wild-type and mutants) to enable cloning directly into the multiple cloning site of PG3-basic (Promega). The reverse primer was positioned to include all sequence upstream of the translation start site. 1.4 × 10^5 mESCs (V6.5) and 1.25 × 10^5 MEFs were plated in wells of 48-well plates and transacted one day later. For mESCs, luciferase activity was read two days after transfection following the manufacturer’s instructions (Promega). For quiescent MEFs, luciferase activity was read 24 hours after the withdrawal of serum. In all luciferase assays, 500 ng of each p107 construct was co-transfected with 125 ng of a Renilla luciferase vector. For exogenous E2F3 experiments, 100 ng of DP1 (a gift from the Dyson lab) was co-transfected with either 100 ng of pCDNA empty vector or 100 ng of CMV-E2F3. Transfections were carried out using the Fugene6 Reagent (Roche).

Lentiviral and retroviral infections

Rb knock-down was achieved using the pSicoR lentivirus [75], as described [18]. The sequence in the Rb cDNA that is targeted by the shRNA molecules is 5’T-GAGAGGAAGATGCTCTCA-3’, p15^46^ and p130 knock-down was achieved using the pSiripp retrovirus [18], as above. The sequence in the mouse p19 cDNA that is targeted by the shRNA molecules is: 5’-CAGCGG-GAAATGCAGAAG-3’. The sequences in the mouse p130 cDNA that is targeted by the shRNA molecules are: 5’-
Mutation of E2F Binding Sites in the p107 Promoter

Thomas H. H. Bradburn, T. K. Wilson, Richard S. Farnham

Abstract

We report here the characterization of transcriptional enhancers in the promoters of p107 and p130, the genes encoding the retinoblastoma-associated proteins p107 and p130. Our studies demonstrate that these enhancers are regulated by E2F transcription factors and are required for the expression of p107 and p130 in vivo. We also show that E2F binding sites in the p107 promoter are required for the expression of p107 in vivo. These findings suggest that E2F transcription factors play an important role in the regulation of the expression of p107 and p130 in vivo.

Introduction

The retinoblastoma gene (p107) is located on chromosome 11 and encodes a protein that is involved in the regulation of the cell cycle. The p107 gene is expressed in a variety of tissues and is involved in the regulation of cell proliferation, differentiation, and apoptosis. The p107 gene is also involved in the regulation of the expression of other genes, including the p130 gene.

Materials and Methods

We used a combination of in vitro and in vivo methods to study the regulation of the expression of p107 and p130. We used a variety of cell lines and animal models to study the regulation of the expression of p107 and p130. We used a variety of techniques, including ChIP and DNA arrays, to study the regulation of the expression of p107 and p130.

Results

We found that E2F transcription factors play an important role in the regulation of the expression of p107 and p130. We found that E2F binding sites in the p107 promoter are required for the expression of p107 in vivo. We also found that E2F binding sites in the p130 promoter are required for the expression of p130 in vivo.

Conclusion

Our findings suggest that E2F transcription factors play an important role in the regulation of the expression of p107 and p130 in vivo. These findings provide a new perspective on the regulation of the expression of p107 and p130 and may have implications for the understanding of the regulation of cell proliferation, differentiation, and apoptosis.

Author Contributions

The authors confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. All authors agree that they meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship.

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