

NEW PERSPECTIVES ON RETINOBLASTOMA FAMILY FUNCTIONS IN DIFFERENTIATION

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Received 5/10/98 Accepted 5/29/98

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1. ABSTRACT

Cell differentiation is a coordinated process that includes cell cycle exit and the expression of unique genes to specify tissue identity. The focus of this review is the recent progress in understanding the functions of the RB family (RB, p130, p107) in cell differentiation. Much work has focused on the functions of RB in G1 regulation. However, much evidence now suggests a diverse function in differentiation. For discussion, differentiation will be divided into three general steps: cell cycle exit, apoptosis protection, and tissue-specific gene expression. These processes are coordinated to provide the final and unique tissue characteristics. The RB family and targets such as E2F and HBP1 have functions in each step. While there is much knowledge on each separate step of differentiation, the mechanisms that coordinate cell cycle and tissue-specific events are still not known. New evidence suggests that this coordination contains both positive and negative regulation of tissue-specific gene expression. RB, p130, HBP1, and other proteins appear to have unexpected functions in regulating tissue-specific gene expression. The ubiquitous expressions of these proteins suggest

membership in a new and general pathway to coordinate cell cycle events with tissue-specific gene expression during differentiation. The collective observations hypothesize the existence of a differentiation checkpoint to insure fidelity.

2. INTRODUCTION

2.1 General Background of the RB Family

Cell differentiation is a fundamental process that imparts unique identity through a coordinated tissue-specific gene expression program. For example, the muscle and adipocyte differentiation programs consist of genes that specify contractile proteins and fat mobilization, respectively. Despite the unique differences in tissue-specific gene expression, a general feature of many tissues is a notable lack of proliferation and the maintenance of an irreversible cell cycle exit. During differentiation, the expression of tissue-specific genes is tightly coordinated with cell cycle exit. Many studies have provided knowledge on cell cycle arrest and on key regulatory transcription factors that give tissue specific gene expression. However, little is known on the mechanisms

RB family and Differentiation

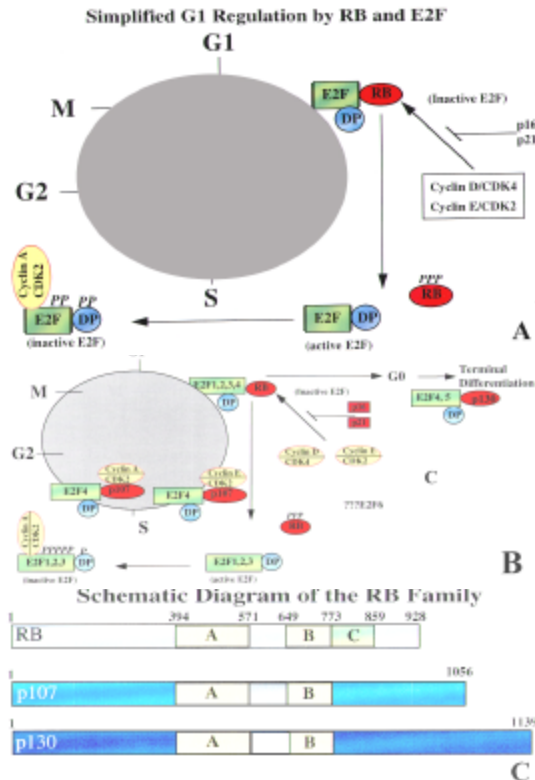


Figure 1. Summary of the E2F and RB family regulation in the cell cycle. The E2F activity is composed of a heterodimer of E2F and DP proteins to achieve the maximal DNA binding activity. The paradigm of E2F and RB regulation is diagrammed in simple (A) and realistic (B) view. The simplified view highlights the basic features of E2F and RB family members. A detailed discussion can be found in the text and within (5,7, 8). Figure 1C is a schematic diagram of the RB family. The RB family consists of RB, p107 and p130. A common feature is the pocket, which is the region of tumorigenic mutations, viral oncoprotein binding and E2F interactions. The A and B pocket domains are denoted in yellow. The C domain is unique to RB and is denoted in green. The spacer regions between the A and B are similar in p107 and p130, denoted in a grid pattern. The spacer region is different in RB, denoted in a plain pattern. All other regions are largely divergent, denoted in varying shades of blue. Please see text for more details (reviewed in (17)).

that insure tight coordination of cell cycle exit and tissue-specific gene expression. Yet, these are necessary features in which lapses in coordination have severe consequences. For example, the abrogation of the cell cycle exit allows re-entry of otherwise differentiated tissues into the cell cycle. In disease, this aberrant proliferation is a characteristic of early changes that may lead to cancer in some tissues. In normal functions, the proliferation of differentiated tissues allows regeneration. A notable case is the regeneration of liver in response to injury. Thus, the

regulation of cell cycle exit in the context of differentiation has important implications for both cancer and normal tissue biogenesis. Recent studies with the Retinoblastoma (RB, p107, p130) family have provided new insights into the coordination of cell cycle and tissue-specific events during differentiation.

Studies from cancer biology, cell biology, and virology have converged to establish RB and E2F in one paradigm for G1 regulation. RB was first discovered as a tumor suppresser gene with frequent mutation in human cancer. The expression of RB led to suppression of the G1 phase of the cell cycle. As summarized in figure 1A, a key discovery is the identification of the E2F transcription factor as the first nuclear target for RB. E2F regulates the expression of numerous genes necessary for S-phase of the cell cycle, such as thymidine kinase, DHFR, DNA Pol α , cdc6. For example, the direct link of cdc6 to DNA replication provides a satisfying example of E2F coordination of S-phase (1). RB inhibits E2F-dependent transcription of these essential genes and provides one mechanism for the observed G1 arrest by RB. The under-phosphorylated form of RB is associated with inhibition of E2F. In G1, sequential RB phosphorylations by Cyclin D/CDK4 and by Cyclin E/CDK2 lead to dissociation of RB and E2F. The net result is free E2F and the activation of genes for S-phase entry and progression. In S-phase, E2F is inactivated through phosphorylation by Cyclin A/CDK2 and thereby allowing exit from S-phase (2-4). Thus, the phosphorylation of RB and E2F by CDKs contribute to regulation of cell cycle progression in normal cells. This fundamental pathway has been subverted by the viral oncoproteins of the DNA tumor virus (E1A, Large T, E7) through the sequestration of RB and release of E2F to give S-phase (reviewed in (5, 6) and references within).

While the inhibition of E2F by RB was a satisfying explanation, the rapidly emerging knowledge suggests that this paradigm is far too simple (see figure 1B and reviewed in (7, 8)). There are three RB family members (RB, p107, and p130), 6 E2Fs (E2F1-6) and 3 DP(1,2,3). A summary of the various E2F complexes is provided in figure 1B. The challenge remains the functional delineation of each RB and E2F family member. The overall E2F levels are further regulated on the protein level through ubiquitin mediated degradation (9-11). While the studies of RB have been dominated by E2F, there is growing appreciation that RB has a diverse role in cellular regulation and that functionally important non-E2F targets of RB must exist. A simple argument is that the concentration of RB family members is much greater than that of E2F. Thus, the beautiful work with E2F may be just a glimpse of regulation by RB family members.

Figure 1C depicts a schematic diagram of RB, p107, and p130. RB is the first and best

characterized member in which most studies have focused on the pocket domain. This region is the site for interactions with E2F and other proteins. In the case of E2F, disruption of the pocket:E2F interaction leads to the release of inhibition and to subsequent activation of E2F. Disruption could be achieved by mutation, viral oncoprotein binding, or CDK phosphorylation. The observation that numerous cancers are correlated with RB pocket domain mutations remains a seminal discovery in tumor biology. Despite extensive efforts, there has been no correlation of pocket mutations in p107 and p130 with cancer. Loss of p130 expression has been observed in small cell lung carcinoma (12). A possibility is the inactivation of p130 and p107 functions may still lead to cancer. Unlike RB, this neutralization may not result from genetic mutation, but could arise through disruptive protein interactions or phosphorylation. However, this remains to be proven.

Numerous viral and cellular proteins use a conserved LXCXE motif for pocket interaction with RB, p107, and p130. Notably, E2Fs use a different motif (13). RB also contains a C-pocket domain that is necessary for interaction with E2F1-3 and with c-abl (for review, see (14)). Notably, The RB C-pocket is not found in p107 or p130. Additionally, the p107 and p130 proteins contain a spacer region between the A and B pockets that directs the interaction and apparent inhibition of Cyclin A/CDK2 or Cyclin E/CDK2 ((15, 16)). The p107 and p130 spacer regions differ from RB, which does not interact with CDK complexes. The N-terminal regions of RB, p107, and p130 are divergent and may provide the specificity within the family (reviewed in (17)).

This review will highlight the RB family in three distinct aspects of cell differentiation: cell cycle exit, tissue-specific gene expression, and apoptosis protection. The multiple functions of RB suggests a role in global coordination to insure fidelity during differentiation. The primary focus will be on the muscle and adipocyte differentiation models. The elegant and extensive work on MyoD and c/EBP families of transcriptional regulators has provided a backdrop for elucidating how cell cycle regulation coordinates with tissue specific gene expression. Recent observations in several labs suggest the existence of both positive and negative regulation to control the progression of differentiation. We hypothesize the existence of a differentiation checkpoint that coordinates cell cycle and tissue-specific events in a full differentiation pathway.

3. DISCUSSION: FUNCTIONS IN DIFFERENTIATION

3.1. Summary of Knockout Mice

RB, p130 and p107 are expressed in all tissues and suggest general differentiation and developmental functions. The insights of functions

beyond G1 came from the analysis of mice deficient in RB, p107, and/or p130 (reviewed in (18)). These studies also demonstrated extensive functional redundancy and compensation, providing a significant challenge to the delineation of specific p107, RB, and p130 functions.

Mice deficient for RB exhibited unique differentiation defects (19-21) ; reviewed in (22)). The embryonic lethality did not occur until day 14-15 and suggests that either p107 or p130 was able to compensate for RB in early embryonic development. The extensive apoptosis and defective differentiation was observed in the nervous system and liver hematopoietic cells. While grossly normal, the complete muscle status could not be fully evaluated because of the embryonic lethality of the RB^{-/-} mice. Later studies revealed that a threshold level of RB was necessary for proper cell cycle exit and apoptosis protection in muscle differentiation (23). Thus, the observations from the RB^{-/-} mice suggest specific RB functions in apoptosis protection and in the maintenance of cell cycle exit. Strikingly, only limited tissues were affected by loss of RB. A probable explanation for the restricted tissue effects in the RB^{-/-} mice was functional compensation by the wild-type p107 or p130 in the unaffected tissues.

What are the functions of p107 and p130 in differentiation? P107 and p130 share the most amino acid homology of the family members, but opposite expression in differentiation (see below). In murine development, mice deficient for either p130 or p107 have no overt defects, again suggesting functional compensation by existing wild-type RB family members (24, 25). In the absence of p130, p107 can substitute for p130 functions in E2F regulation (24, 26, 27). Mice defective for both p130 and p107 exhibited neonatal lethality with deregulated chondrocyte growth and defective bone development (24, 25). While RB could not compensate in cartilage and bone, many other tissues were grossly normal.

The take-home message from the knockout mice is that RB, p130, p107 mutations gave rise to abnormalities late in embryonic development and to defects associated with tissue differentiation. While some tissues were uniquely affected, the redundancy and functional compensation of RB, p107 and p130 illustrate the difficulty in defining specific, non-overlapping roles. Nonetheless, a comprehensive analysis of the gene expression profiles in cells deficient in p107/p130 or in RB reveals de-regulation of different genes and highlights the functional differences between p107, p130 (26). Realizing that RB, p107, and p130 exert their activities through protein-protein interactions, specificity may be achieved through the differential use of target proteins, such as E2Fs and HBP1. The E2F family can distinguish RB, p130, and p107 (see reviews in (5). Recently, a new transcriptional repressor HBP1 can distinguish RB and p130 from p107 (28). Thus, these

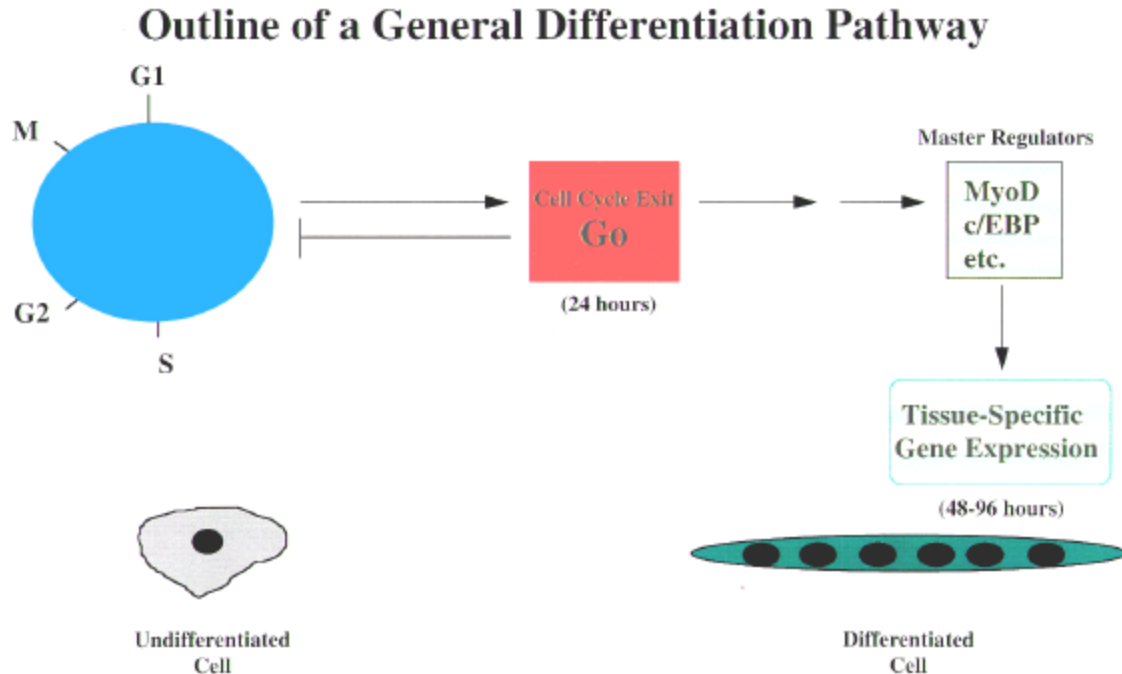


Figure 2. Outline of a General Differentiation Pathway. A general feature of differentiation is irreversible cell cycle exit followed by tissue-specific gene expression and morphological changes. The temporal progression is listed for the C2 muscle cell line in which MyoD family members control the tissue-specific phase. Please see text for more details.

and other specific targets may ultimately provide insights into the distinct roles of p130, p107, and RB in cell differentiation.

3.2. Differentiation Models

The work in mice have provided compelling evidence for functions of RB in differentiation. Because of the availability of cell lines that reproduce early events, much work in differentiation has used the muscle and adipocyte models to uncover general principles. The major strength of the adipocyte and muscle models has been the identification of the key transcription factors that trigger the global expression of tissue-specific genes. These include the MyoD and c/EBP family of transcription factors that activate the expression of muscle-specific and fat-specific genes, respectively. The mechanics of the MyoD and c/EBP transcription factors have provided a beautiful picture for the expression of genes necessary for the differentiated phenotype. However, the regulation of the activity of c/EBP and MyoD in the context of a full differentiation pathway is not well understood.

In both models, the expression of tissue-specific genes follows cell cycle exit. Distinct changes in cell morphology also accompany the expression of tissue-specific genes. In the C2 muscle cell line, cell cycle exit occurs within 24-36 hours after differentiation induction by serum withdrawal. However, the expression of tissue-specific genes does not begin until 48-72 hours of differentiation (See figure 2). In C2 cells, a morphological fusion of individual myoblasts into multi-nucleated myotubes

accompanies tissue-specific gene expression (e.g. myosin heavy chain, muscle creatine kinase, etc). A parallel muscle model is the rat L6 line, but the kinetics of differentiation is markedly slower (8-9 days). A similar progression occurs in adipocytes, except that expression of fat-specific genes occurs over 7-8 days. Lipid deposition is a characteristic feature of differentiated adipocytes. The temporal regulation in both models insures that full cell cycle exit and apoptosis protection precede the tissue-defining gene expression. While the specific mechanisms are not understood, the precise coupling of cell cycle exit and tissue-specific gene expression does provide fidelity in tissue biogenesis.

A glimpse into the complexity of RB family regulation may be found in both differentiation models. In the C2 muscle model, cell cycle exit occurs within 24 hours of differentiation, but full tissue specific gene expression does not begin until 48-72 hours. In C2 cells, the levels of p107 are high in undifferentiated and proliferating cells and decline with differentiation. In contrast, the levels of RB and p130 are induced 5-8 fold, but with differing kinetics. Expression of p130 is rapidly induced within 24 hours and remains high throughout differentiation. In contrast, the maximal accumulation of the under-phosphorylated RB form occurs at 48-72 hours. In the scheme of differentiation events, induction of p130 expression correlates with cell cycle exit. The maximal induction of RB and of its under-phosphorylated "active" form correlate with tissue specific gene expression (28-31).

Recent work suggests that the RB family and selected target proteins may contribute to the fidelity and progression of all aspects in differentiation. For discussions on the molecular functions of the RB family, we will emphasize three major characteristics of differentiation: a) initiation and maintenance of cell cycle arrest; b) apoptosis protection; and c) activation of tissue-specific genes. The cell cycle and apoptosis regulation provide the characteristic irreversible cell cycle exit and increased survivability associated with most tissues. Furthermore, cell cycle exit is necessary, but not sufficient for the expression of tissue-specific genes. Other mechanisms must exist for activation of MyoD-like regulators in response to completion of cell cycle arrest and apoptosis inhibition. Because of the extensive knowledge, the muscle and adipocyte differentiation models do provide ideal systems to link general cell cycle and apoptosis regulation with activation of tissue-specific genes in a full differentiation pathway.

3.3. Role in Cell Cycle Exit

The initiation and maintenance of cell cycle exit are necessary features of muscle, adipocyte and many other differentiation systems. Numerous studies in muscle have established that preventing cell cycle exit blocks differentiation. The expression of oncogenes such as RAS, mdm2, Cyclin D1 and others block differentiation by enhancing proliferation and thus preventing cell cycle exit (reviewed in (32)). Cell and tissue differentiation are further characterized by an irreversible and dominant cell cycle exit, despite optimal growth conditions. This situation is in sharp contrast to non-differentiating cell lines in which the cell cycle exit is reversible. In this section, we will summarize the role of RB and p130 in the mechanisms that initiate and maintain cell cycle exit during differentiation.

The relative expression patterns imply that RB and p130 could be important for differentiation. A major aspect of the cell cycle exit mechanisms is transcriptional repression by p130 and RB mediated through specific transcription factors. The RB- and p130 regulation of E2Fs and HBP1 exemplify the transcriptional repression of cell cycle genes in differentiation.

3.3.1. E2F

Numerous observations link p130 with general cell cycle exit and to cell differentiation (for recent review, see (7)). In quiescent and differentiating cells, the E2F sites are now repressor elements, and a single E2F4-p130 complex is predominant. This conversion of the E2F sites from transcriptional activation to transcriptional repression is a convenient mechanism for inactivating the cell cycle program to give cell cycle exit. The phosphorylation of p130 may also regulate the formation of the E2F4-p130 complex (30, 33-36). In adipocyte differentiation, an additional mechanism may be the inactivation of E2F function by phosphorylation of the DP partner (37).

The situation with E2F in proliferating cells is quite different. Numerous critical cell growth control genes do utilize E2F sites for transcriptional activation in proliferating cells. There exists several E2F complexes, including one with p107. While the p107 expression pattern provides a link with undifferentiated cells, the precise transcriptional functions of p107 are still not known.

What of RB? Many studies of RB and E2F have also shown that this complex is an efficient transcriptional repressor and RB is still present in cells during the early stages of differentiation. Yet, a consistent observation has been that the major E2F complex in G0 and differentiation contained p130; E2F-RB complex was largely undetectable (33-35). In both G1 and G0, E2F-dependent repression appears critical, but apparently with different complexes of E2F and RB family members. In G0, a complex of p130 and E2F4,5 contribute to transcriptional repression of cell cycle genes in differentiation and in G0. In G1, RB and E2F1-3 may be the transcriptional repressor (34, 35). The differential appearance of E2F complexes with RB and with p130 could be a concrete distinction between G1 and G0, respectively. Clearly, there must exist other differences between RB and p130 in cellular regulation, as they do appear to regulate different subsets of genes involved in proliferation (26).

The best characterized example of RB- and p130-mediated repression lies with the E2F and occurs through a dual mechanism (for review, see (5, 38)). RB and p130 bind within the E2F activation region in the C-terminal (e.g. (39)). This physical interaction not only neutralizes transcriptional activation, but allows the recruitment of an intrinsic repression domain within the RB or p130 pocket (40, 41). Several recent studies have demonstrated that the RB-mediated repression occurs through histone-deacetylase recruitment to the promoter (42-44). Acetylation of chromatin has been associated with actively transcribing genes and with increased promoter accessibility to transcription factors. Deacetylation would then prevent transcription factor accessibility. Thus, the recruitment of a deacetylase by E2F and RB would "tighten" the local chromatin structure. This would restrict access by RNA polymerase II and the associated basal transcription factors (reviewed in (45)).

3.3.2. HBP1

While an E2F-p130 complex can support transcriptional repression and provides a satisfying model for cell cycle exit, E2F may not be the only RB- or p130- targeted transcription factor in cell cycle exit and differentiation. First, the relative levels of RB and p130 vastly exceeded E2Fs, which are exceedingly rare proteins (e.g.(46)). Indeed, there is now a growing list of non-E2F proteins that interact with RB (Cyclin D1, BRG1, c-abl, elf-1 etc. (47-53)). Second, while p130-E2F complexes were easily

detected in differentiated cells, E2F-RB complexes were difficult to detect. The clear presence of the RB protein in differentiated cells may suggest that E2F is not the major target for repression. Third, E2F sites are simply one of many control elements in numerous promoters that regulate the expression of growth control genes. These collective observations raise the possibility that other RB may target other proteins in differentiation.

To address the functions of p130 and RB in cell differentiation, we screened a differentiated C2 library to isolate new targets that govern cell cycle exit and other aspects of differentiation. We and Kouzarides independently isolated HBP1 in a two hybrid screen with p130 (28, 54). HBP1 had been previously cloned as a cDNA whose expression complemented a potassium channel defect (55). However, HBP1 is clearly a transcription factor by homology to the sequence-specific HMG factor family. The signature motif is an HMG box that specifies binding to DNA ((56). Several members (e.g. LEF1, TCF, SRY) are linked to development and differentiation (reviewed in (57) and (58)). Consistently, HBP1 shares extensive homology in the HMG box, but is otherwise divergent. In contrast to the tissue-restricted expression of LEF1 and other members, HBP1 exhibited ubiquitous tissue expression with the earliest detectable expression at day 8 of murine embryogenesis (Tevosian, Yee, unpublished). Despite ubiquitous expression, HBP1 was up-regulated in the C2 muscle and in adipocyte differentiation models and suggested a unique role in establishing differentiation (28, 55).

A major feature of HBP1 is selective interaction with RB and p130, but not p107. Thus, HBP1 joins E2F as the another target that can exhibit specificity within the RB family. In contrast to E2F, HBP1 utilizes two LXCXE motifs. Significantly, the expression of RB, p130, and HBP1 is up-regulated in the course of differentiation and suggests a functional relevance. One HBP1 DNA binding site was (A/T)(C/G) AATGGG (see figure 3). We have now identified two cell cycle-regulated promoters with co-existing E2F and HBP1 sites. Our initial work demonstrated that HBP1 was a transcriptional repressor for the promoter of the N-MYC oncogene (28). The N-MYC promoter was amongst the first described E2F-containing cellular promoters (e.g. (59)). Recent work has now identified the Cyclin D1 promoter as another HBP1 target (Sampson, Tevosian, Pestell, Yee, in preparation). Like N-MYC, the Cyclin D1 promoter also contains E2F sites. The Cyclin D1 promoter is sensitive to signals that dictates the G0 to G1 transition (reviewed in (60)). In the context of differentiation, the expression of the N-MYC and Cyclin D1 genes declined, whereas HBP1 levels were increased. Together, this suggests that HBP1 may be a differentiation-induced repressor of certain cell cycle genes and contribute to cell cycle exit.

3.3.3. Irreversibility of cell cycle exit

A major difference between NIH 3T3 and differentiating cells is the reversibility of cell cycle exit. In 3T3 cells, cell cycle exit is fully reversible. By contrast, in the C2 and other differentiating lines, cell cycle exit is irreversible. Thus, a key question for differentiation are the mechanisms that enforce an irreversible cell cycle exit. Many differentiated tissues fail to re-enter the cell cycle, despite a rich growth environment. Thus, during differentiation, cells must acquire this dominant cell cycle exit.

The general mechanisms of irreversible cell cycle exit are not known, but both RB and HBP1 may have potential functions. In muscle, suboptimal levels of RB resulted in differentiated muscle cells that can now re-enter the cell cycle ((23, 31, 61). Consistent with the transcriptional regulation, the expression of HBP1 also inhibited S-phase and effectively rendered the cell refractory to growth signals (28). It is possible that RB may elicit the irreversible cell cycle exit through HBP1 in normal differentiation. In animal systems, we have recently demonstrated that HBP1 may be a critical component of maintaining Go. The liver is an excellent system for examining cell transitions, as the Go to G1 transition can be induced by injury. Transgenic mice expressing low levels of HBP1 in the liver were defective in cell cycle reentry upon injury (Shih, Leiter, Paulson, Yee, in preparation). Thus, in two distinct tissues, HBP1 has the qualities of a dominant cell cycle inhibitor necessary for differentiation. By analogy with RB, a key question will be whether HBP1-/- muscle cells can now re-enter the cell cycle. Generation of HBP1-/- mice is underway.

3.4. Role In Apoptosis Protection

A universal feature of differentiated tissues is decreased apoptosis. Fully differentiated muscle cells exhibit a marked decrease in apoptosis. In the early stages of differentiation and upon serum withdrawal, the myoblasts undergo extensive apoptosis. In contrast, the fully differentiated muscle myotube is refractory to apoptosis. Thus, the acquisition of mechanisms that protect against apoptosis is a necessary feature of differentiation and tissue biogenesis.

Recent studies have implicated RB in apoptosis protection. In non differentiating cellular models, there now exists several examples of RB's ability to block apoptosis (reviewed in (62). RB expression blocked apoptosis induced by chemical inducers of DNA damage (63, 64). In other apoptosis models (TNF and FAS), degradation of RB occurred at a conserved site in the C-terminal of RB, and this site matched a consensus caspase 3 site. When the caspase 3 site was mutated, the mutant RB was now resistant to cleavage during apoptosis. This caspase-resistant RB was more effective than wild-type in apoptosis protection (65, 66). These observations suggest caspase-dependent cleavage of RB may

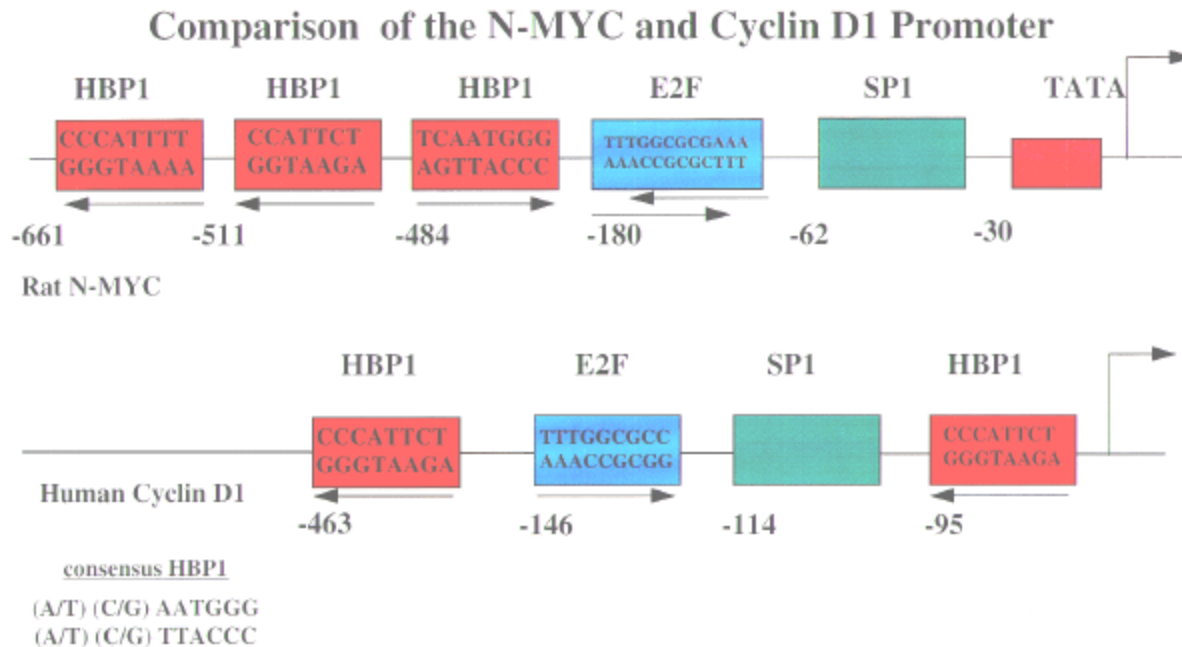


Figure 3. Comparison of the N-MYC and Cyclin D1 Promoters. The HBP1 (red), E2F (blue), and other sites in the promoters for rat N-MYC [accession number X63281; (99)] and for human Cyclin D1 (accession number L09054; gift of RG Pestell (100)). The consensus HBP1 site was determined by the alignment of DNA sequences that bound recombinant HBP1 (28). The repression by HBP1 in both promoters has been demonstrated (28) and Sampson, Tevosian, Pestell, Yee, in preparation).

be a natural consequence of apoptosis to remove inhibition (reviewed in (67)).

In cell differentiation, three independent lines of evidence suggest that RB is involved in the protection of apoptosis (reviewed in (68)). First, the RB knockout mice exhibited excessive cell death in certain tissues (19-21, 69, 70). Second, the transgenic expression of low levels of RB in the RB-/- background resulted in muscle with highly increased apoptosis (23). Third, in C2 cells, the early stages of differentiation are marked by high levels of apoptosis, yet the fully differentiated cell exhibits little to no apoptosis. p21 has been implicated in apoptosis protection during differentiation (71). The induction of p21 is a frequent event in muscle and other differentiating systems (72-75). However, ectopic p21 expression could only block apoptosis in RB+/+, but not in RB-/- cells (76). Taken together, these data all suggest that RB may be necessary for apoptosis protection in differentiated cells. However, these studies only provide a first glimpse of RB and apoptotic protection in differentiation and much remains to be understood.

A possible target for RB-mediated apoptosis protection may be HBP1. Preliminary results from our laboratory indicate that HBP1 conferred survival in an RB-dependent manner. We generated cell lines expressing low levels of HBP1 and found that these lines conferred enhanced survival to distinct apoptotic signals, such as serum withdrawal and DNA damaging agents. HBP1 levels are induced rapidly upon C2 differentiation. A plausible function is that HBP1 may also provide apoptosis

protection during differentiation (Shih and Yee, in preparation). If HBP1 is a key target for RB-mediated apoptotic protection, a prediction for future work is that an HBP1-/- cell should exhibit increased susceptibility to apoptosis..

3.5. Role In Tissue-Specific Gene Expression

The defining event in terminal differentiation is the expression of specific genes that determine the tissue phenotype. The best characterized system remains muscle with the landmark discovery of the MyoD family (MyoD, myf 5, myogenin, mrf 4). This important transcription factor family is involved in the determination and differentiation of muscle tissue. The primary function is the coordinated activation of muscle specific genes. In concert with ubiquitous E-box transcription factors (E12/47), MyoD family members are basic helix-loop helix transcription factors that activate transcription of numerous muscle specific genes through a common E-box DNA element (CANNTG). The physical interaction of MyoD family members with the constitutive E-proteins is regulated in part by the Id proteins, which prevent interaction in undifferentiated cells. Some Id members are down-regulated upon differentiation, thereby allowing the formation of a productive complex of MyoD and E12/E47. Additionally, the Mef2 and p300 families can additionally modulate the efficiency of MyoD family transcriptional activation (reviewed in (77-79)).

Functional redundancy and extensive autoactivation of promoters are an essential feature of the MyoD family. Either MyoD and Myf5 are required for full muscle

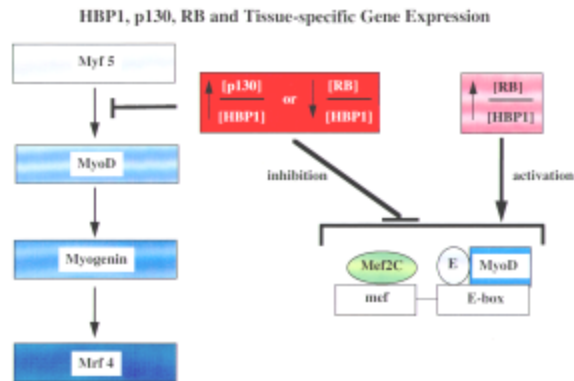


Figure 4. Schematic of HBP, p130, RB in Muscle Differentiation. The summary of our result in the HBP1-mediated block in differentiation is diagrammed (90). Briefly, the expression of MyoD and myogenin was inhibited in HBP1-expressing C2 cells, but myf 5 expression was normal. The hierarchy of MyoD family members was determined by the embryonic expression, and its autoactivation. For example, myf 5 can activate the MyoD promoter. Consistent with the differentiation inhibition, HBP1 expression also inhibited MyoD family transcriptional activation. The inhibition by HBP1 was fully dependent on the LXCXE motifs, suggesting that a complex with RB or p130 was required. Increasing RB restored both differentiation and MyoD family transcriptional activation. Together, we hypothesize that the ratios of [HBP1] with [RB] or [p130] determine whether there is inhibition or activation of MyoD family members in differentiation.

formation. Both are expressed in C2 cells and determine the muscle lineage, but Myf 5 expression does precede MyoD in development. Myogenin is required for full differentiation since undifferentiated myoblasts accumulate in the knockout mice. Myogenin is amongst the first induced genes in C2 muscle differentiation. Mrf4 is linked to myofiber maturation and is not expressed in the tissue culture models. The expression of each MyoD family member is dependent on the E-box elements in the respective promoters (reviewed in (80)). For example, myf 5 can directly activate the MyoD promoter through the E-box elements. In the adipocyte models PPAR and c/ebp family of transcription factors have equally critical roles for tissue-specific gene expression during differentiation (reviewed in (81)).

The major questions for differentiation are the mechanisms by which the MyoD family and other “master” regulatory factors are activated in a full differentiation pathway. In muscle and fat, cell cycle exit precedes the expression of MyoD-regulated muscle specific genes and suggests that these general and tissue-specific events are tightly coordinated in the full differentiation pathways. While necessary, cell cycle exit is not sufficient to give full terminal differentiation. Mutant cell lines of C2 cells fail to express tissue specific genes (NFB), but still exit the cell cycle (35, 82). Additionally, the appearance of the E2F4-p130 complex was also not sufficient for differentiation (83). These studies predict additional mechanisms that

couple cell cycle exit to the activation of the MyoD family and its resulting tissue-specific gene expression.

The current work suggests that RB and possibly p130 can coordinate cell cycle exit with tissue-specific gene expression in fat and muscle differentiation. Several new studies have implicated p130, HBP1, and other proteins in cell cycle exit, but with unexpected inhibition of tissue-specific gene expression. Several studies imply a unique role for RB in the “activation” of MyoD family members. Because of these unusual functions, we hypothesize that these proteins may be part of a “differentiation checkpoint” that consist of both positive and negative regulation. The net result is a tight coordination to insure that complete cell cycle exit precedes the expression of genes that terminally define the tissue phenotype.

3.5.1. Positive Regulation

While RB was expressed in all tissues, the surprising finding was that RB contributes to the activation of tissue-specific gene expression in differentiation. RB regulated the activity of MyoD and of c/EBP, which are key activators of tissue-specific genes in muscle and adipocyte differentiation, respectively. While MyoD expression can drive the muscle lineage in several pluripotent cells, RB^{-/-} cells are refractory to MyoD-mediated muscle determination and differentiation. Re-expression of RB in the RB^{-/-} pluripotent fibroblast was sufficient to restore both MyoD-mediated transcriptional activation and muscle conversion. Both functions of MyoD were still manifested in p130^{-/-} or p107^{-/-} fibroblasts, suggesting that the positive activation of MyoD may be unique to RB (61). The precise mechanism has been controversial, as there were early reports of a direct physical interaction (84). Yet later studies have failed to detect specific physical interactions (61). Clearly, RB can regulate MyoD for transcriptional activation and differentiation, but probably not through a direct physical interaction.

A similar requirement for RB is manifested in the adipocyte differentiation model. The MyoD “equivalent” in the adipocyte system is the c/EBP transcription factor family (reviewed in (85)). Like MyoD, c/ebp expression could convert an RB^{+/+}, but not an RB^{-/-}, pluripotent cell to an adipocyte. Again, re-expression of RB restored the ability of c/EBP to specify the adipocyte lineage. Like MyoD, RB is a positive co-factor for transcriptional activation by c/EBPs. While the RB requirement for MyoD activation is probably indirect, a direct physical interaction between RB and c/EBP was demonstrated, providing a satisfactory mechanism for adipocyte conversion and transcriptional activation by c/EBPα (86).

Thus, RB is an excellent candidate in coupling cell cycle exit to tissue-specific gene expression in differentiation. The functions of RB in cell cycle control are well established and are necessary for irreversible cell cycle exit. Two new studies have provided genetic evidence that argues strongly for distinct RB functions in G1 control and in tissue-specific gene expression. Kaelin and colleagues have now characterized new RB mutants in the pocket domain (e.g., pm 661W) that are defective in

E2F binding, but not in activation of differentiation (87). While used extensively as an RB-negative line in studies on G1 control, the SAOS 2 osteosarcoma cells also provide a model for bone differentiation in which restoration of RB gave expression of bone markers. Additionally, these RB mutants were functional in MyoD activation and in muscle differentiation. p130 was less effective than RB in triggering full differentiation, although p130 was clearly functional in triggering cell cycle arrest. In two distinct models, an “E2F-defective” RB still activated differentiation.

Lee and colleagues have demonstrated that the N-terminal of RB is required for full muscle formation in animals. These investigators used transgenic expression to introduce an RB mutant lacking the N-terminal region in an attempt to complement the RB-/- mice. This RB mutant retains a functional pocket domain (located in the C-terminal region). Despite increased survival, the transgenic mice still exhibited defects in muscle differentiation (88).

The studies by Kaelin and Lee provide the first evidence that G1 and differentiation regulation by RB could be genetically uncoupled. Both the N-terminal region and pocket may be involved in triggering tissue-specific genes. These studies should not be interpreted as cell cycle exit is dispensable for differentiation, but rather that RB has an additional function in triggering tissue-specific activation during differentiation. In normal circumstances, cell cycle exit might be accomplished through p130, but RB may uniquely activate MyoD or c/EBP α to give full differentiation. This requires further investigation.

3.5.2. Negative Regulation

Several new studies have suggested the existence of an inhibitory pathway in differentiation without preventing cell cycle exit. Three distinct differentiation inhibitors (HBP1, CHOP, p202) have been described (89-91). Each protein is induced with differentiation of adipocytes or muscle cells and elicits cell cycle exit. Despite efficient cell cycle exit, each protein unexpectedly blocks differentiation through the inhibition of the tissue-specific master regulators (MyoD or c/EBP). Additionally, HBP1 and p202 are also targets of the RB family. Finally, Classon and Harlow have recently provided evidence for unexpected inhibitory functions for p130 and/or p107 in differentiation (92).

These observations provide new insights into the direct coupling of cell cycle exit and tissue-specific gene expression during differentiation. p202, HBP1 and CHOP are excellent candidates for proteins that elicit cell cycle arrest, but block tissue-specific gene expression. A potential outcome is a transient inhibition of differentiation to allow completion of cell cycle exit before triggering tissue-specific genes. Positive and negative regulation by RB family members could then regulate the fidelity of terminal differentiation. The recent observations with HBP1, p202, CHOP, and p130 are summarized below.

3.5.2.1. HBP1

Our unexpected observations with HBP1 and muscle differentiation provide evidence for an RB family-

mediated negative pathway in differentiation (90). We had thoroughly characterized HBP1 as a transcriptional repressor and cell cycle inhibitor. Unexpectedly, the expression of HBP1 in C2 cells completely blocked full muscle differentiation, yet cell cycle exit was completely normal. This phenotype was manifested in both transient and stable expression assays arguing against trivial cell line artifacts. The ability of HBP1 to block differentiation but not affect cell cycle exit is unique. Most oncogenes block differentiation by preventing cell cycle exit (for recent review, see (32)). The inhibition of differentiation by HBP1 is clearly distinct from the well-characterized proliferative functions of oncogenes.

As diagrammed in figure 4, the block in differentiation by HBP1 is unusual in the relative expression of MyoD family members. Myogenin and MyoD expression was completely absent, providing a plausible reason for the inhibition of differentiation. Yet, Myf 5 expression was normal. A key feature of the MyoD family is the differing expression patterns in development and autoactivation due to E-boxes in each of the promoters. Thus, Myf 5 probably activates MyoD and then myogenin to achieve full differentiation in normal cells. Consistently, HBP1 expression could inhibit transcriptional activation by Myf 5. The regulation by HBP1 required binding to RB or p130, as the LXCXE motifs were necessary. Consistently, re-expression of MyoD or myogenin restored differentiation and suggested that both MyoD and myogenin may lie downstream of HBP1 and Myf 5. Thus, HBP1 probably blocks differentiation by inhibiting Myf 5 function and thereby preventing the expression of MyoD and Myogenin.

Our studies further reveal that the relative ratio of [RB] to [HBP1] may be a major determinant for full MyoD transcriptional activation and differentiation. At a low [RB] to [HBP1] ratio, cell cycle exit existed, but there was no differentiation and transcriptional activation MyoD was inhibited. At increased [RB] to [HBP1] ratio, cell cycle exit was still intact, but both differentiation and transcriptional activation by MyoD were functional. It is important to note that a model in which HBP1 simply sequesters RB in an E1A-like fashion is not sufficient to explain the results. A simple neutralization of RB functions cannot explain selective involvement of HBP1 in tissue-specific activation without interfering with cell cycle exit.

While the results are entirely consistent in our experimental system, does the ratio of [RB] to [HBP1] occur in natural muscle differentiation? The answer is yes (28, 30). In muscle differentiation, [p130], [RB], and [HBP1] are all increased about 5-fold at the end of differentiation, which is marked by the expression of muscle-specific genes at about 72-96 hours. However, [HBP1] and [p130] are rapidly increased within 12-24 hours of C2 differentiation and remain high throughout the entire differentiation period. Notably, in this early period, there is no tissue-specific gene expression. The overall [RB] levels and the critical under-phosphorylated form do accumulate more slowly and reach a maximum at 48 hrs. This time period is just before the activation of MyoD family members and of muscle-specific gene expression. In

terms of relative ratios, the [RB] to [HBP1] ratio is low in the early period of endogenous C2 differentiation when cell cycle exit is predominant and there is no tissue-specific gene expression. The [RB] to [HBP1] ratio is ~5-fold higher just before full tissue-specific gene expression. Thus, the endogenous expression patterns and our experimental results argue that the relative ratios of [RB] to [HBP1] regulate the activation of MyoD family members and tissue specific gene expression. In this way, the relative ratio of [RB] to [HBP1] could serve as a barometer for completion of cell cycle exit to insure coordination with tissue-specific gene expression.

3.5.2.2. p202

The p202 protein was first identified as a murine 52 kD protein that was induced by interferon treatment. Because interferon treatment led to cell growth inhibition, Lengyel and colleagues discovered that p202 could inhibit E2F-dependent transcriptional activation. Intriguingly, p202 could bind both RB and E2F1 (93, 94). Similar to our work with HBP1, the direct expression of p202 also led to cell cycle arrest (91). These studies demonstrated that p202 could function as a cell cycle inhibitor by targeting the RB/E2F pathways.

Strikingly like HBP1, the p202 protein was induced with C2 differentiation. But, the expression of p202 fully blocked muscle differentiation in C2 cells. While HBP1-expressing cells had no MyoD or myogenin expression, p202-expressing C2 cells had normal Myogenin expression, but no MyoD. Myf 5 expression was not tested for p202. Like HBP1, p202 also blocked transcriptional activation by MyoD family members. In contrast to HBP1, p202 blocked DNA binding by MyoD to inhibit tissue-specific gene expression (91). This data suggests that HBP1 and p202 are both proteins that are induced upon differentiation, but block tissue-specific gene expression by inhibiting MyoD family members differently.

3.5.2.3. CHOP

CHOP is a member of the C/EBP transcription factor family and an inhibitor of adipogenesis (95). CHOP contains the dimerization region, but lacks an activation domain. Thus, CHOP inhibits C/EBP function through a dominant-negative blockade of C/EBP transcriptional activation. CHOP was originally isolated as GADD153 and as a gene that was induced with growth arrest and DNA damage. A CHOP translocation has been associated with liposarcoma (96).

CHOP shares striking functional similarity to HBP1 and p202. CHOP expression is normally induced in adipocyte differentiation, but ectopic expression paradoxically blocks adipocyte differentiation. The expression of C/EBP α restores differentiation in CHOP-expressing cells, suggesting that CHOP also inhibits differentiation by blocking the "master regulator" (89). The direct expression of CHOP also leads to cell cycle arrest (97). CHOP also has ubiquitous tissue distribution and suggests general negative regulation of differentiation

(Tevosian *et al.*, unpublished; (55, 97)). Unlike HBP1 and p202, there are no reports of any CHOP and RB interactions. However, CHOP regulates c/EBP α which is targeted by RB (86).

3.5.2.4. p130

A recent study has revealed unexpected functions for p107 and/or p130 in differentiation, but support the notion of both positive and negative regulation by RB family members during differentiation. Classon and Harlow investigated the requirement of RB family members in adipocyte differentiation using cells that were RB-/- or p107-/-p130-/- (92). As expected, the loss of RB prevented adipocyte differentiation. The surprising result was that the loss of both p107 and p130 gave increased differentiation and suggested that a negative pathway might be compromised. Because of the extensive functional compensation, the relative contribution of p107 and p130 cannot be cleanly delineated and must be addressed in future studies. However, in differentiation, p130, not p107 is the predominant member. In our lab, we have also shown that expression of p130 does not support C2 differentiation, consistent with the notion that p130 may be a negative inhibitor of differentiation (Sheppard, unpublished). Together with our work on HBP1, an exciting possibility is that HBP1 and p130 may mediate a negative pathway.

4. FUTURE PERSPECTIVES

4.1. Working Model: A Differentiation Checkpoint??

Differentiation is a highly coordinated process of cell cycle exit with the expression of tissue specific genes. How do we rationalize the recent results addressing positive and negative regulation in differentiation? In the working model of figure 5, we hypothesize a "differentiation checkpoint" to insure orderly progression and fidelity in differentiation. This differentiation checkpoint model is a balance of positive and negative regulation to insure fidelity. Negative regulation (through p130, HBP1, CHOP, and/or p202) transiently suspends the differentiation pathway between cell cycle exit and tissue-specific gene expression. This would prevent inappropriate expression of tissue-specific genes until completion of cell cycle exit. The positive signals can now activate RB and subsequently MyoD (or other global regulators) to trigger the final activation of tissue-specific genes. A differentiation checkpoint involving RB family members provides tight coordination and temporal progression of cell cycle exit and tissue-specific gene expression.

The unexpected functional similarities of three distinct proteins (HBP1, CHOP, and p202) suggest the existence of a regulatory pathway that may insure that complete cell cycle exit. The completion then "signals" the positive activation of MyoD- or C/EBP-like transcription factors to trigger tissue-specific gene expression. The recent demonstration that p130 and/or p107 was inhibitory to differentiation may suggest a new function for RB family members. Additional RB targets with paradoxical functions in differentiation have been isolated (Kaelin, personal communication).

Positive and Negative Regulation In Differentiation

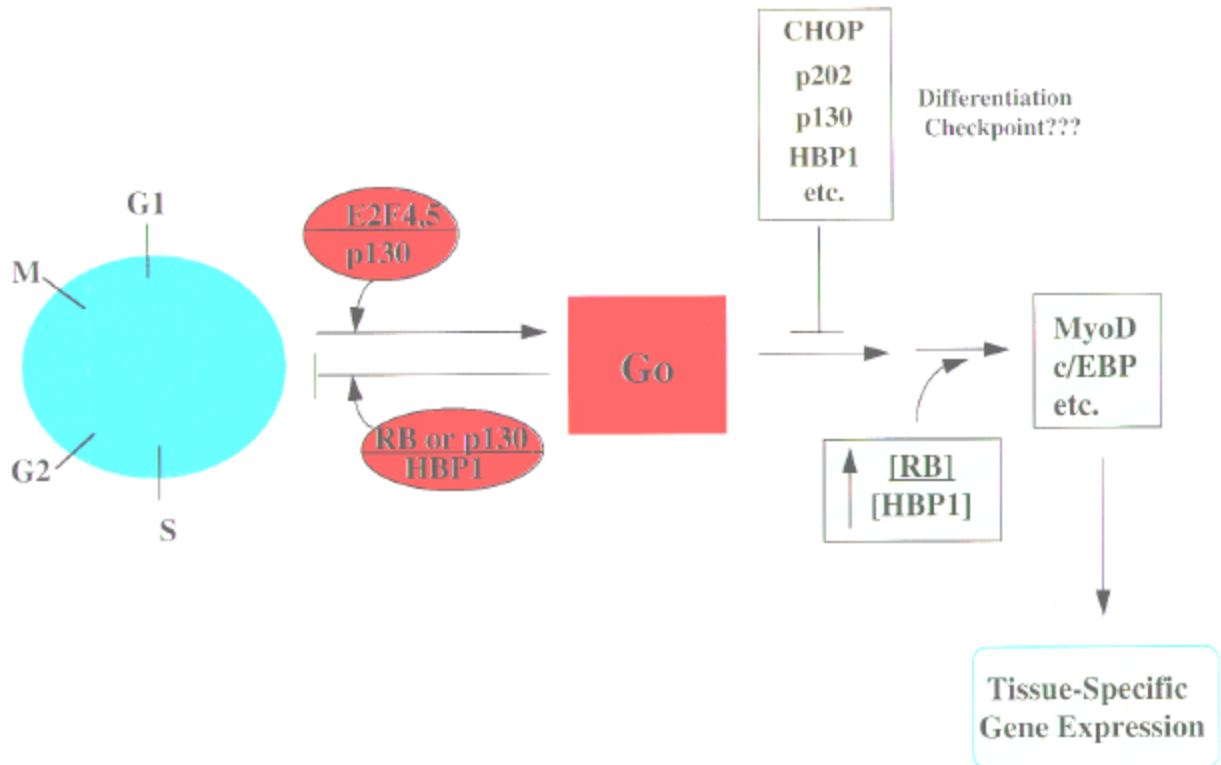


Figure 5. Positive and Negative Regulation in Differentiation. The cell cycle exit and tissue-specific aspects of differentiation are summarized in this diagram. In the cell cycle exit phase, the coordinate actions of E2F4,5, p130 and HBP1 contribute to the repression of cell cycle genes. Additionally, RB and HBP1 may contribute to the irreversibility of cell cycle exit that is manifested in differentiation. We hypothesize a differentiation checkpoint consisting of positive and negative factors that insure precise coupling of cell cycle and tissue-specific regulation. RB contributes to positive activation of MyoD and other global regulators in an ill-defined mechanism. CHOP, HBP1, and p202 all contribute to negative regulation of MyoD-like factors, although each protein elicits cell cycle exit. Thus the ratio of positive and negative signals (denoted by RB/HBP1) may be cellular “barometer” for the appropriate environment for differentiation. A low [RB]/[HBP1] ratio would favor cell cycle exit, but not tissue-specific gene expression. A high [RB]/[HBP1] ratio reflects an increase in RB and would favor the activation of MyoD-like factors and of tissue-specific gene expression.

4.1.1. The Early Phase: Cell Cycle Exit and Apoptosis Protection

Figure 5 depicts a working model that integrates the recent observations on the relative functions of RB, p107, and p130 into the framework of a full differentiation pathway. We have divided differentiation into two general steps: cell cycle exit and tissue-specific gene expression. Considering existing data, E2F, HBP1, p130, RB collaborate to give general transcriptional repression of cell cycle genes. Whether HBP1 and p130 collaborate with E2F is currently unknown, but separately, each constitutes an efficient repression complexes. Regardless of the precise molecular interactions, the net effect is cell cycle exit. RB and HBP1 may also function in establishing the irreversibility of cell cycle arrest.

The initial phase of differentiation is characterized by extensive apoptosis, and RB may have

some role in protection of apoptosis protection. Numerous studies in knockout mice and cellular systems now support a role for RB in apoptosis protection. While the specific mechanisms still require much work, a potential role for RB in the early phase of differentiation may be apoptosis protection, possibly with p21. The induction of p21 occurs in numerous differentiation models.

Figure 4 and 5 focus on the inhibition of the master regulators by HBP1 and p130 or RB. We speculate that two related mechanisms may work to insure fidelity and progression during differentiation. First, HBP1 and p130 may be an active inhibitor of MyoD and other master regulators of tissue-specific genes. Second, the relative ratio of overall HBP1 and RB concentrations may constitute a barometer for cell cycle exit or full differentiation. At low [RB] to [HBP1] ratios during the early phase of differentiation, cell cycle exit predominated,

but tissue-specific gene expression was blocked. Future investigations must directly test these possibilities.

4.1.2. The Late Phase: Activation of Tissue-specific Gene Expression

The defining feature of differentiation is the expression of tissue-specific genes. The prevailing evidence suggests that RB has a positive role in the activation of tissue-specific genes in both fat and muscle. In either model, RB^{-/-} cells fail to differentiate. Restoration of RB allows full differentiation through transcriptional activation by MyoD or c/EBP. The time course for the accumulation of the under-phosphorylated RB is also consistent with triggering MyoD and the activation of tissue-specific genes. Despite ubiquitous tissue expression, RB may be a necessary co-factor for activation of tissue-specific genes through MyoD and similar regulators. The simultaneous involvement of RB in cell cycle and tissue-specific regulation provides a convenient means to coordinate these processes in a full differentiation pathway. Additionally, a high overall [RB] to [HBP1] ratio in this phase may signal an appropriate environment for the final expression of tissue specific genes.

4.2. Future Perspectives

The past few years have provided new appreciation for the complexities in RB family function during cell differentiation. Numerous studies have established that cell cycle exit is clearly necessary for differentiation, since preventing exit by expression of oncogenes or growth factors block differentiation. While cell cycle exit is necessary, it is clearly not sufficient for full differentiation. Mechanisms must clearly exist to couple cell cycle exit with tissue-specific gene expression. Several recent studies now document proteins that elicit cell cycle exit, but also prevent differentiation. Recent studies on distinct functions of RB, p130 and p107 in differentiation also provide further evidence of a pathway that coordinates general and tissue-specific events. Collectively, we hypothesize a differentiation checkpoint that insures that appropriately arrested and viable cells can initiate tissue-specific gene expression.

Clearly, the studies of these novel coordination mechanisms are still in their infancy, but the early evidence does suggest an exciting, but complex view of differentiation. Based on the relative expression and on the tantalizing data from Classon and Harlow, a clear prediction is that p130 may have an inhibitory role in MyoD transcriptional activation. This putative inhibitory role for p130 must still be clearly demonstrated, but may be difficult with the existing functional redundancy in the RB family. Additionally, this model also predicts that complexes of HBP1 with RB or with p130 may have different functions through differentiation. For example, as RB levels increase, does RB displace p130 in a complex with HBP1? These questions require answers, but the low levels of HBP1 will surely hamper definitive results. Mice deficient in HBP1 and eventually crosses with mice deficient in RB, p107 and p130 may be needed to resolve these questions.

More work is necessary to establish the involvement of RB, p130, HBP1, and other proteins in coordinating cell cycle exit and tissue-specific gene expression in differentiation. The initial observations do suggest differential functions for RB, p107 and p130, and further experiments are necessary to solidify the differences. These important investigations will be difficult with the extensive functional redundancy. Furthermore, the precise mechanisms for positive activation of MyoD by RB are still unclear. If the [RB]/[HBP1] ratio is a “sensor” of differentiation, then how is this signal transduced into concrete molecular changes at the level of MyoD family transcriptional activation? Is there a direct phosphorylation event to alter transcriptional activation or DNA binding by MyoD? Or are there changes in MyoD’s physical interactions with p300 or MEF2c? Is RB “activated” to give overall enhanced MyoD transcriptional activation? If so, what are the signalling pathways?

For completion, we have documented apoptosis protection and the role of RB in differentiation. However, much work still remains in establishing how RB participate in general apoptosis protection during differentiation. An open question is the role of p21. To add complexity, a recent study in keratinocyte differentiation now suggest that p21 has functional similarities to HBP1, p202, and CHOP. While induced with differentiation and involved in cell cycle arrest, keratinocyte differentiation was blocked upon p21 expression (98). Again, cell cycle arrest and probably apoptosis protection by p21 was not sufficient to give keratinocyte differentiation.

An exciting future question will be how tissues acquire the fundamental features of cell cycle exit and apoptosis protection and trigger the necessary gene expression. We and others have provided only a glimpse of the complex positive and negative mechanisms that coordinate differentiation. Cell cycle exit must be a potent regulatory signal for the activation of MyoD and other factors to complete differentiation. An important future investigation will be the signal transduction pathways that intersect general cell cycle regulation with tissue-specific gene expression in differentiation.

5. ACKNOWLEDGMENTS

We thank Drs. Marie Classon, Ed Harlow, and Bill Kaelin for providing information prior to publication. We apologize to many colleagues whose work could not be cited due to space limitation. The work was recently supported by grants to A.S.Y. from the American Heart Association, the NIH (GM44634), and from the GRASP-Digestive Disease Center at New England Medical Center (NIDDK, P30 DK-34928). A.S.Y. is an Established Investigator of the American Heart Association.

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Key Words: Retinoblastoma, E2F, HBP1, p130, p107, Differentiation

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