HHS Public Access

Author manuscript

Bone. Author manuscript; available in PMC 2017 January 09.

Published in final edited form as:

Bone. 2014 December ; 69: 47–54. doi:10.1016/j.bone.2014.08.009.

p107-dependent recruitment of SWI/SNF to the alkaline phosphatase promoter during osteoblast differentiation

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Abstract

The retinoblastoma protein family is intimately involved in the regulation of tissue specific gene expression during mesenchymal stem cell differentiation. The role of these proteins, pRB, p107 and p130, is particularly significant in differentiation to the osteoblast lineage, as human germ-line mutations of *RB1* greatly increase susceptibility to osteosarcoma. During differentiation, pRB directly targets certain osteogenic genes for activation, including the alkaline phosphatase-encoding gene *Alpl*. Chromatin immunoprecipitation (ChIP) assays indicate *Alpl* is targeted by p107 in differentiating osteoblasts selectively during activation with the same dynamics as pRB, which suggests that p107 helps promote *Alpl* activation. Mouse models indicate overlapping roles for pRB and p107 in bone and cartilage formation, but very little is known about direct tissue-specific gene targets of p107, or the consequences of targeting by p107. Here, the roles of p107 and pRB were compared using shRNA-mediated knockdown genetics in an osteoblast progenitor model, MC3T3-E1 cells. The results show that p107 has a distinct role along with pRB in induction of *Alpl*. Deficiency of p107 does not impede recruitment of transcription factors recognized as pRB co-activation partners at the promoter; however, p107 is required for efficient recruitment of an activating SWI/SNF chromatin-remodeling complex, an essential event in *Alpl* induction.

Keywords

pRB; p107; SWI/SNF; *Alpl*; osteoblasts

Introduction

The retinoblastoma protein, pRB and its related family members p107 and p130 are important mediators of various cell processes including cell cycle progression, apoptosis and

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Conflict of interest statement

The authors declare they have no competing financial interests in relation to the work described.

differentiation (1–3). In particular, the retinoblastoma family is intimately involved in the regulation of tissue specific gene expression during mesenchymal stem cell differentiation. pRB is implicated in the differentiation of chondrocytes (4), myoblasts (5–8) adipocyctes (9–11) and osteoblasts (12–17). The osteoblast differentiation function has particular relevance for the tumor suppressor role of pRB, as human germ-line mutations of the pRB-encoding gene *RB1* greatly increase susceptibility to osteosarcoma (18).

The molecular mechanisms by which pRB influences osteoblast differentiation are still emerging, but it is clear that certain osteogenic promoters are targeted directly by pRB for activation. The best studied is the *Bglap* gene, encoding osteocalcin. During late differentiation, pRB occupies the activated osteocalcin promoter. pRB forms a binding partnership with the osteogenic transcriptional regulator RUNX2 (13, 19–20). Another pRB activity involves releasing repression at promoters of certain osteogenic genes through displacement of the repressor KDM5A demethylase (syn.: RBP2). pRB physically associates with KDM5A, and after displacement of KDM5A, pRB returns to the target promoter in the company of activating factors including E2F1 (17, 21–22). The gene encoding the major early marker of osteoblast differentiation, alkaline phosphatase (*Alpl*) is another direct activation target of pRB (22). Chromatin immunoprecipitation (ChIP) probes also revealed a converse p130-dependent repression of both *Alpl* and *Bglap* (23). Regulation of *Alpl* and *Bglap* requires pRB and p130 respectively acting in concert with activator and repressor members of the E2F family to regulate tissue specific gene expression (17,23).

Mouse models indicate overlapping roles for pRB and p107 in bone and cartilage formation (24), and potential roles for p107 distinct from pRB (4, 25–26). However, beyond these biological phenotypes, very little is known about potential direct tissue-specific gene targets of p107, or the consequences of targeting by p107. ChIP assays indicate *Alpl* is targeted by p107 with the same dynamics as pRB (i.e. selectively during activation) (22), raising the possibility that p107 helps promote osteoblast differentiation by contributing to *Alpl* activation.

The role of p107 at the *Alpl* promoter was probed here in comparison with that of pRB using shRNA-mediated knockdown genetics in an osteoblast progenitor model. The results show that normal induction of *Alpl* is severely impaired in p107-depleted cells. Induction of other recognized targets of pRB-mediated activation, the osteocalcin and osteopontin encoding genes, is independent of p107. Thus, the requirement for cooperation between pRB and p107 for *Alpl* activation is a comparatively specific strategy. At the *Alpl* promoter, p107 is not required for recruitment of transcription factors recognized as co-activation partners with pRB, but p107 is required for efficient recruitment of an activating form of the SWI/SNF chromatin-remodeling complex, an essential event in *Alpl* activation.

Results

p107 is required for efficient activation of the Alpl promoter

The mouse calvaria-derived MC3T3-E1 cell line has a pre-osteoblast phenotype suitable for studying induction of osteoblast-specific gene expression. When stimulated by bone anabolic agents, these non-transformed precursor cells undergo terminal differentiation. The

full differentiation program unfolds over more than two weeks in a highly coordinated successive pattern of gene expression (27, 28). Induction of alkaline phosphatase (*Alpl*) is an early stage event, coordinated closely with differentiation-associated cell cycle arrest. The presence of p107 on the active *Alpl* promoter with the same dynamics as pRB was noted earlier (22) (Fig. 1A) in a pattern suggesting p107 may contribute to *Alpl* activation. Selective pRB knockdown has already shown p107 to be insufficient for *Alpl* activation in pRB-deficient cells (22) but those experiments do not indicate whether p107 is necessary. This issue was addressed here using a parallel shRNA approach to create stable knockdown of p107 in the osteoblast precursors.

Three independent lines were isolated, designated p107_K/D.AA1, p107_K/D.AA3, and p107_K/D.BB1. Western blots show that expression of pRB is relatively unaffected in the p107-depleted lines (Fig 1B). An *In situ* staining assay shows severely impaired induction of alkaline phosphatase activity in each depleted line (Fig. 1C), to an extent similar to that reported previously with pRB depletion (Fig. 1C and Flowers et al., 2010). A representative line (p107_K/D.BB1) was selected for further study here. Quantitative analysis by qRT-PCR shows sharply decreased expression of p107 with no significant effect on pRB expression (Fig. 1D). Conversely, the pRB-depleted line shows normal expression of p107. Side-by-side comparison of *Alpl* expression in induced pRB-depleted and p107-depleted cells shows comparably severe impairment (Fig. 1E). Thus p107 plays a largely non-redundant role with pRB in activation of *Alpl*.

Osteopontin and osteocalcin are induced independently of p107

The osteocalcin and osteopontin encoding genes (*Bglap* and *Spp1*, respectively) are other key osteoblast genes that have been identified as direct pRB targets in differentiating osteoblasts (13). They were screened here to determine whether they are p107 targets as well. Osteopontin expression is already active in committed pre-osteoblasts, and a chromatin immunoprecipitation (ChIP) assay (Fig. 2A) shows pRB on the promoter in non-induced (Day 0) cells, while p107 association was not detected even after induction of differentiation (Day 7). Osteopontin expression increases several-fold more as the committed cells progress through differentiation; this increase is pRB-dependent, but in contrast to induction of alkaline phosphatase, there is no impairment in p107-depleted cells (Fig. 2B).

Targeting by p107 was also considered with respect to *Bglap*. The gene product, osteocalcin, is the best-characterized marker of osteoblast differentiation. It is very tightly regulated, and is an established activation target of pRB (13, 19–21). Repression of osteocalcin prior to differentiation is linked with the presence of p130 on the promoter (17, 23). Osteocalcin is a major component of the mineralized matrix, and gene expression is highly induced during differentiation with delayed kinetics relative to induction of alkaline phosphatase. ChIP assay shows dissociation of p130 and association of pRB on the promoter but again, p107 is not detected (Fig. 2C). Expression monitored by qRT-PCR shows no impairment to induction in p107-deficient cells (Fig. 2D). Thus, not every target of pRB is co-targeted by, or co-dependent on, p107. With regard to p107, regulation of alkaline phosphatase induction is distinct from regulation of osteopontin and osteocalcin. The pRB family members have many overlapping functions but the evolutionary conservation of three distinct proteins

argues that they have unique functions as well. Regulation of the *Alpl* gene is an opportunity to compare the distinct functions of pRB and p107 at a single promoter.

Hypermethylation of H3K4 and hypomethylation of H3K9 at Alpl are pRB-dependent, and independent of p107

Trimethylation at lysine-4 of histone H3 (H3K4) is found at active promoters (29). The repressor histone demethylase, KDM5A (RBP2), which reduces trimethylation of H3K4, is targeted by pRB (21), and a shift to higher order methylation of H3K4 at *Alpl* following the differentiation signal is dependent on pRB (22). *In vitro* studies indicate KDM5A is also targeted by p107 (30), and we considered whether the higher order methylation of H3K4 requires p107 in addition to pRB. However, ChIP assays indicate no required role for p107 in the H3K4 methylation shift on *Alpl* (Fig. 3A, lanes 3 and 4). A distinct histone mark, methylation at lysine-9 of histone H3 (H3K9) is also important for transcriptional activation, and is typically regulated coordinately and inversely with H3K4, such that the mono-methylated form of H3K9 is found on the active promoter where it acts as a buffer between repression and activation by permitting rapid methylation and demethylation, and consequently, a dynamic equilibrium with acetylation (29). H3K9 methylation monitored by ChIP analysis here shows this shift is strikingly dependent on pRB, but again independent of p107 (Fig 3A, lanes 7–8). The success of pRB-dependent events like these confirms that pRB is still active in the p107-depleted cells.

Dissociation of p130 from Alpl at the onset of activation requires either p107 or pRB

The repressed *Alpl* promoter is occupied by p130, along with repressor members of the E2F family (E2F4 and E2F5) and a module of the development-regulating LIN complex (LINC) (23), together forming an entity similar to the DREAM complex that targets cell cycle promoters during arrest (31). Depletion of p130 results in constitutive dissociation of repressor E2Fs and the LINC proteins, and to precocious transcriptional activation of *Alpl* (23). Differentiation-associated dissociation of p130, repressor E2Fs, LINC proteins and histone deacetylases (HDACs), is independent of pRB (17, 23). A potential role for p107 at this point was assessed here in the p107-depleted cells, but the ChIP assays show p107 is not necessary for dissociation of these various repressor modules: p130, E2F4, E2F5, the signature LINC components LIN9 and RBBP4, and HDAC1 and HDAC2, all dissociate normally (Fig. 3B).

Although neither pRB nor p107 is required for dissociation of p130, a model in which at least one of them is required is not excluded. The possibility that only one or the other is required, such that either is sufficient for this step, was examined using a viral vector to knock down pRB in the p107-depleted cells, which were then analyzed at day 7 post-differentiation. qRT-PCR shows about 50% reduction in pRB expression at this point (Fig 3C). (Knockdown of p107 in these cells was shown in Fig. 1D). In cells infected with a control virus carrying a non-targeting scrambled shRNA sequence (scrambled virus), ChIP analysis (Fig 3D) shows successful dissociation of the repressor p130 and concomitant dissociation of E2F4, E2F5 and BRM (lanes 5–8). In contrast, in p107-depleted cells infected with virus carrying the authentic pRB-targeting shRNA sequence, pRB is no longer detected at the promoter (lane 3), and dissociation of p130, E2F4, E2F5 and BRM is

impaired. Uncovering this redundancy reveals an important mechanistic step for the pRB family members in displacing p130 from the repressed promoter. While this finding reveals a significant activity of p107, it does not explain the impaired activation of *Alpl* in p107-depleted, pRB-competent, cells. The genetics indicate a required function unique to p107.

p107 is required for recruitment of BRG1-SWI/SNF

While pRB is regarded as a repressor of E2F functions in the cell cycle, recent results show that pRB and E2F1 cooperate to co-occupy and activate certain genes, including tissue specific promoters (17, 32). E2F1 and pRB are required jointly for efficient *Alpl* occupation by other specific transactivators, notably RUNX2 and Sp1. We considered whether this function likewise requires p107. Probing the *Alpl* promoter in parental or p107-depleted cells shows pRB, E2F1, RUNX2, and SP1 all occupy the promoter successfully with the onset of differentiation in sharp contrast to the promoter occupation profile seen in pRB-deficient cells (Fig. 4A).

The success of pRB-dependent changes in histone methylation status and transcription factor occupation of the promoter, argues that the p107-dependent step is downstream of de-repression and initial activation events. The final steps in *Alpl* activation involve the SWI/SNF chromatin-remodeling complex. SWI/SNF has both repressor and activator functions on osteogenic promoters, and in osteoblasts these opposing functions are largely linked with the difference between the two possible ATPases in the complex, BRM or BRG1. BRM-containing SWI/SNF targets osteogenic promoters to repress their premature activation in progenitor cells (33). Upon induction, the *Alpl* promoter converts from the repressed state characterized by BRM-SWI/SNF occupation to an activated state occupied by BRG1-containing SWI/SNF (33; seen here in Fig. 1A). BRG1-containing SWI/SNF is required for recruitment of RNA polymerase-II to *Alpl* (22) and for normal induction of alkaline phosphatase expression (33). Results in Fig. 3D show that dissociation of the BRM complex does not require p107 as long as pRB is sufficient. Subsequent recruitment of BRG1 and RNA polymerase-II requires pRB (22), but a requirement for p107 is also possible. Probing this here reveals that recruitment of BRG1 and RNA polymerase-II to *Alpl* is strikingly impaired in the p107-depleted cells (Fig. 4B). Thus, normal activation of *Alpl* in differentiating osteoblasts requires both p107 and pRB acting in a largely non-overlapping manner to recruit BRG1-SWI/SNF to the promoter.

Role of p107 prior to osteoblast commitment

A prior study looked for p107 effects on induction of osteogenic proteins in p107 knockout mouse-derived mouse embryo fibroblasts (MEFs) induced with bone morphogenic protein 2 (BMP2), and found a different effect of p107 deficiency. In the pluripotent cell model, p107 deficiency correlated with enhanced induction of alkaline phosphatase, paralleling the effects of p130 depletion rather than pRB depletion (13). To address the possibility that p107 acts as a repressor in tandem with p130 at the earlier multipotent stage, the dynamics of p107 association with the *Alpl* promoter were evaluated here in an established multipotent mesenchymal stem cell model, C3H10T1/2 cells. The cells were treated with retinoic acid to induce expression of the osteogenic transcription factor RUNX2 (Fig. 5A) and initiate commitment to the osteoblast lineage without full osteoblast differentiation (34). ChIP assay

(Fig. 5B) shows the promoter dynamics for the pRB family are the same as observed in the MC3T3-E1 pre-osteoblasts: the p107 protein is not detected with p130 on the repressed promoter before induction (lane 3 *versus* lane 4), and occupies the promoter in tandem with pRB after induction. Even sensitive qRT-PCR analysis (lower panels) does not detect appreciable promoter sequences associated with p107 prior to induction (sample 3). These dynamics do not support a direct repressor role for p107 on *Alpl* prior to commitment; the accelerated induction of *Alpl* observed in p107-deficient multipotent cells (13) may be an indirect effect. On the other hand, the selective presence of p107 on the induced promoter in the precursor cells leaves open the question of why deficiency of p107 impedes induction of *Alpl* in committed pre-osteoblasts but not in the multipotent precursors. To ascertain whether p107 is required for *Alpl* association of BRG1-SWI/SNF in the precursor cells, we constructed a C3H10T1/2 cell line stably depleted of p107 (p107_K/D.TB7) (Fig. 6A). The p107-depleted line was induced with retinoic acid as above, and the promoter occupation pattern was determined by visualization of gel-isolated PCR fragments and qRT-PCR analysis (Fig. 6B). The results confirm depletion of p107 at the protein level (lane 3), yet with respect to BRG1, the results differ from the pattern seen in pre-osteoblasts. Here, BRG1 is readily detected on the induced promoter (lane 8, day 3) in the absence of detectable p107. This curious finding indicates that BRG1-SWI/SNF recruitment to *Alpl* is independent of p107 at the commitment stage in multipotent precursor cells, while it is p107-dependent in the pre-osteoblast cells.

Osteoblasts pass through discrete steps of alkaline phosphatase induction on the path from commitment to maturation. MC3T3-E1 pre-osteoblasts are derived from newborn mouse calvaria, whose borders are rich with pre-osteoblasts. These cells *in situ* are restrained from differentiation until the proper time for fusion of the cranial bones. Direct qRT-PCR comparison of the relative level of *Alpl* induction in the precursor and pre-osteoblast models (Fig 6C) shows that *Alpl* expression in the retinoic acid-induced precursor cells, while many-fold induced with respect to the uncommitted precursors, is still at a level of induction below that of fully differentiating osteoblasts. If pre-osteoblasts are destined to be temporally restricted from further differentiation, the *Alpl* promoter apparently is reconfigured into another repressor conformation with p130 and BRM-SWI/SNF re-occupying the promoter (the pattern seen in non-induced MC3T3-E1 cells), while expression remains active at the commitment level. To permit differentiation to the actual osteoblast phenotype, the configuration must be changed again to a higher activation state, again dependent on BRG1-SWI/SNF recruitment, at this stage requiring a contribution from p107.

Discussion

The p107 protein is the least studied member of the pRB family, particularly with regard to tissue-specific gene expression. Mouse models indicate a role for p107 in cartilage and bone formation (4, 24, 26), but in whole animal models it is difficult to know whether bone effects are an indirect consequence of cartilage defects, and to what extent tissue effects involve regulation of tissue specific genes rather than proliferative functions. A direct gene target of p107 occupation and regulation has not previously been characterized in osteoblasts. The role of p107 in *Alpl* activation is notable for its specificity with respect to timing and to other osteogenic genes recognized as pRB targets. Induction of alkaline phosphatase can be

at least a two-step process, with cells held at the post-commitment, pre-osteoblast level for an indeterminate period of time. In cells paused at the pre-osteoblast level represented by MC3T3-E1 cells, p107 has a unique role in the induction of *Alpl* to the full expression level seen in differentiating osteoblasts. The activation step requiring p107 occurs relatively late in the *Alpl* induction program, as p107 is not required for multiple pRB-dependent events prior to recruitment of BRG1-SWI/SNF, a finding that demonstrates unimpeded activity of pRB in the p107-depleted cells. Transcription factors linked with BRG1 recruitment occupy the *Alpl* promoter successfully in p107-depleted cells, but the genetics reveal an additional prerequisite for BRG1 association that is p107-dependent in the pre-osteoblast context of the promoter. Direct association of p107 with BRG1 is a possibility, given that the SWI/SNF ATPases contain an LXCXE amino acid motif, a signature motif for association with the pRB family proteins (35). This motif is bound by pRB as well, but context-specific interactions might be a factor at specific times at individual promoters. Another possibility is a potential role for p107 in introduction or removal of an unrecognized transcription factor or chromatin mark at the promoter specifically in the pre-osteoblast configuration.

The mild phenotype of mice null only for the p107 member of the family (4, 36,37) is typical of the greater level of compensation seen in long-term animal development in comparison to cell culture models. In the whole organism, alkaline phosphatase activity is sufficient in the absence of p107 to support bone development, but the value of the cell culture models lies in their ability to cast light on individual roles via acute short-term effects. Cell culture models can reveal discreet actions otherwise obscured in the full developmental program.

In addition to the unique contribution of p107 to recruitment of BRG1-SWI/SNF in pre-osteoblasts, the present study reveals important dynamics in the switch from *Alpl* promoter occupation by p130 to the fully activated configuration. The overlapping roles of pRB and p107 in this function imply direct displacement of p130. This most likely occurs through temporary binding with E2F4 and/or E2F5 at the promoter, given that an intermediate stage with pRB and p107 on the promoter together with E2F4 and E2F5 can be seen when further activation is stalled by sequestration of the p300 coactivator (17, 23).

Recent transcriptional profiling in mesenchymal stem cells showed unique as well as overlapping effects for p107 with respect to pRB and p130 (38). The profile showed more genes down-regulated than up-regulated when p107 was depleted, suggesting a greater role for p107 in activation, with the caveat that transcriptional profiling does not distinguish direct from indirect targets. The few other studies identifying direct targets of p107 have found both activation and repression targets (37, 39–41). In a roughly similar tissue-specific dynamic, pRB and p107 are both present on the promoter of the pro-opiomelanocortin encoding *POMC* gene in pituitary cells, and shRNA-mediated inhibition of expression of either pRB or p107 decreases *POMC* mRNA levels (39). An integral role in SWI/SNF recruitment can support either activation or repression. An important emerging point is that generalizations on pRB family function from one tissue type or differentiation stage may not apply to all. For example, levels of p107 tend to decline during differentiation (37). This can be seen in the adipocyte lineage, where pRB and p107 both initially favor selection of white adipogenesis over brown adipogenesis (16; 42), but post-commitment, expression of p107 is

down-regulated during maturation of white adipocytes, while pRB is required (10; 43). In contrast, p107 expression does not decline when pre-osteoblasts progress from proliferation to differentiation (23), supporting a positive role for p107 in osteoblast differentiation.

The strong conservation of p107 in evolution argues for the selective advantage of its presence, even though it does not play a critical role in development or tumor suppression. Distinguishing a specific transcriptional activity of p107 enhances our understanding of the functions of this important protein family.

Materials and Methods

Materials

Ascorbic acid, β-glycerol phosphate, sodium phosphate mono and dibasic, Alizarin red S, puromycin, and protease inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). Penicillin and streptomycin (P/S) was purchased from Mediatech (Herndon, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, α-modified essential medium (α-MEM) was purchased from Irvine Scientific (Santa Ana, CA). Retinoic acid was purchased from R&D Systems (Minneapolis, MN).

Expression assays and cells

MC3T3-E1 cell passaging, authentication, differentiation by exposure to ascorbic acid and β-glycerol phosphate, immunoblotting, and *in situ* staining for alkaline phosphatase activity were described previously (22, 28, 44,45), as was generation of the pRB.Seq-1.AA1 depleted line (22). C3H10T1/2 cells (clone 8) were obtained from the ATCC (catalog# CCL-226). Cells were maintained in BME plus 10% fetal bovine serum supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin. Differentiation of C3H10T1/2 to osteoblast was induced by the addition of 1 μM Retinoic acid. The p107-depleted lines for both MC3T3-E1 and C3H10T1/2 were generated using the Origene HuSH system and puromycin selection. The p107 knockdown sequence is 5′-TGG TAT CGC CAA TGA TGC TGG AGA AAT CA-3′.

Chromatin immunoprecipitation(ChIP) assay

ChIP assays and primer sequences for *Alpl* (alias *Akp2*), and *Bglap* (osteocalcin), have been described (22, 33). The primers for the *Spp1* (osteopontin) gene are: Forward 5′-TTC CGC GAT TCT AAA TGC AGT CTA- 3′ and Reverse 5′-CTC CCA GAA TTT AAA TGC TGG TCC- 3′. Antibodies were obtained either from Santa Cruz: pRB (sc-50x), p107 (sc-318), E2F1 (sc-193), SP1 (sc-59), RNA polymerase-II (sc-9001), p300 (sc-584), E2F4 (sc- 1082), E2F5 (sc-968), Lin9 (sc-130571), RBBP4 (sc-33170), BRM (sc-6450), BRG1 (sc-10768), HDAC2 (sc-7899), HSC70 (sc-7298), and RUNX2 (sc12488); from Invitrogen monomethyl-histone H3 lysine 9 (49-1006) trimethyl-histone H3 lysine 9 (49-1008); from BD Transduction: p130 (610261); from Millipore: monomethyl-histone H3 lysine 4 (07-436), trimethyl-histone H3 lysine 4 (04-745); or from Cell Signaling: HDAC1 (2062); normal mouse IgG is a component of the EZ ChIP system (Upstate Cell Signaling Solutions). Individual protein associations were typically analyzed at least twice in independent experiments, and at two discrete time points in differentiating cells. Pre and post induction

samples were always assayed in parallel. The knockdown lines provide additional controls for confirming and/or contrasting results consistent with expression patterns. Differentiation was monitored with complementing biological assays. Quantification was performed in triplicate by SYBR green based quantitative PCRs carried out using an ABI prism 7500 real time PCR system (Applied Biosystems, Foster City, CA), and normalized using the percent input method.

qRT-PCR

Quantitative reverse transcription PCR (qRT-PCR) was conducted as described previously with the *Alpl*, *Bglap*, *Rb1* and *Gapdh* expression primers (17, 23, 46). Other primers used were: p107 (Forward 5′ AGG AAG TTC GCA CTG ACA GTG G- 3′; Reverse 5′-CAG TCT CCT CTT AGC and OPN (Forward 5′-GCT TGG CTT ATG GAC TGA GGT C- 3′ and Reverse 5′-CCT TAG ACT CAC CGC TCT TCA TG). Results were performed in triplicate and normalized to *Gapdh* expression. Fold change data are presented as means ± SEM.

Construction of Adenovirus vector targeting pRB

Adenovirus constructs for the pRB knockdown and the scrambled control were made using the BLOCK-iT Adenoviral RNAi Expression system (Invitrogen) according to manufacturer's instructions. The knockdown sequence inserted for the pAd/Block-iT-DEST-pRB vector is: 5′-CCT GTG CTC TTG AAG TTG TAA TGG CTA CG- 3′and for the pAd/Block-iT-DEST-scrambled vector is 5′-CTC CCA ATC TTC GCG CGT CT-3′. The pRB knockdown virus or the scrambled control virus were added to p107-depleted MC3T3-E1 cells at a multiplicity of infection of 100 plaque-forming units per cell, five days prior to the addition of induction medium. Virus was re-added with fresh medium every two days. Cells were harvested for assays at seven days post-infection.

Acknowledgments

Research reported in this publication was supported by the National Institute of General Medical Science and the National Cancer Institute of the National Institutes of Health under award numbers R01GM073257 (EM), T32-CA134268 (SF), and R25-CA-019536 (PJP and KA).

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Highlights

- The pRB-related protein p107 is implicated in bone development, but very little is known about its role.
- Results here show the alkaline phosphatase gene *(Alpl)* is a direct promoter activation target of p107 in differentiating osteoblasts.
- pRB and p107 are each sufficient to displace the p130 repressor complex from *Alpl* during activation.
- Certain *Alpl* activation steps require pRB, but p107 serves specifically to recruit an activating form of the SWI/SNF chromatin-remodeling complex.
- *Alpl* expression can reach a commitment level without p107, but p107 is required for full activation during terminal osteoblast differentiation.

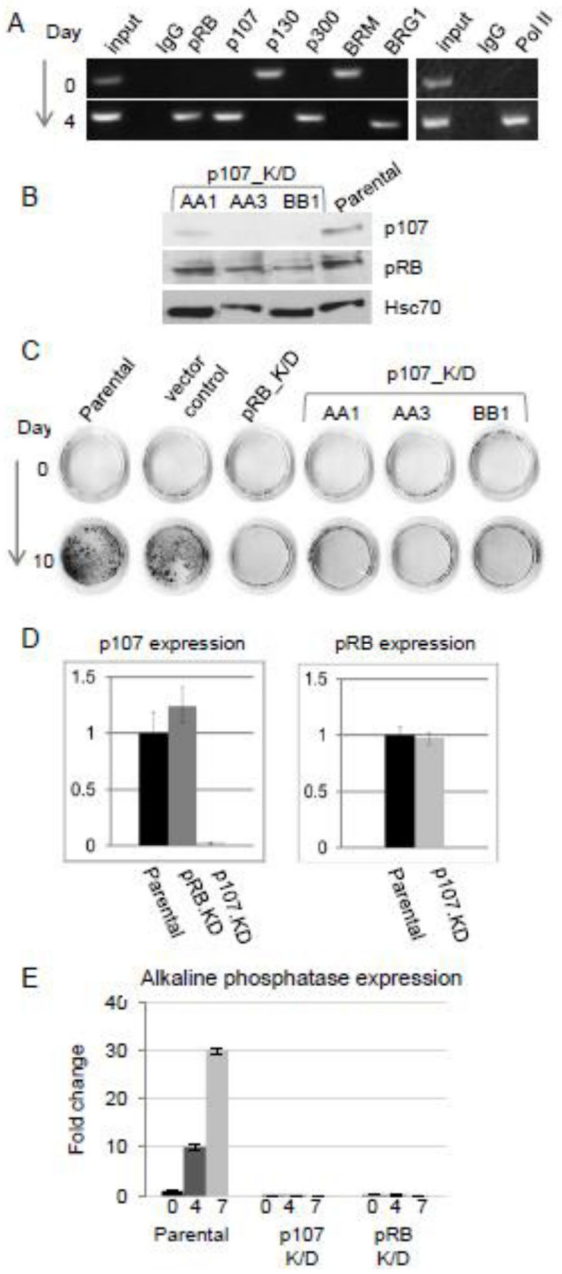

Figure 1. Depletion of p107 in osteoblast progenitors impairs induction of the alkaline phosphatase gene Alpl

A. ChIP analysis conducted on the *Alpl* promoter in normal parental preosteoblasts before and after induction of differentiation (4 and 7 days postinduction) shows recruitment of the pRB and p107 proteins specifically on the activated promoter, indicated by the presence of the p300 co-activator and RNA polymerase II (pol II). During activation the promoter changes from a repressed form characterized by the presence of p130 and BRM-SWI/SNF to an activated form characterized by recruitment of BRG1-SWI/SNF. The dynamics, assayed in parallel provide strong negative controls for the antibodies.

B. Western blot analysis shows reduced levels of p107 in each of three cell lines isolated independently after stable transfection with a plasmid encoding a p107-targeting shRNA sequence. Constitutively expressed heat shock protein hsc70 was used as a loading control.
C. Alkaline phosphatase activity, monitored colorimetrically *in situ*, shows sharply increased activity by day 10 postinduction in parental cells, or cells stably transfected with a vector control. The three independently isolated p107-depleted (p107_K/D cell lines all show impaired induction similar to that seen with pRB deficiency (pRB_K/D).
D. p107 and pRB gene expression determined by qRT-PCR in a representative p107 knockdown line is shown relative to normal parental cells and the pRB knockdown line.
E. Alkaline phosphatase gene expression determined by qRT-PCR before (day 0) and at 4 and 7 days post-induction in differentiating osteoblast progenitors. The induction pattern in a p107 knockdown line is shown relative to the patterns in normal parental cells and the pRB knockdown line.

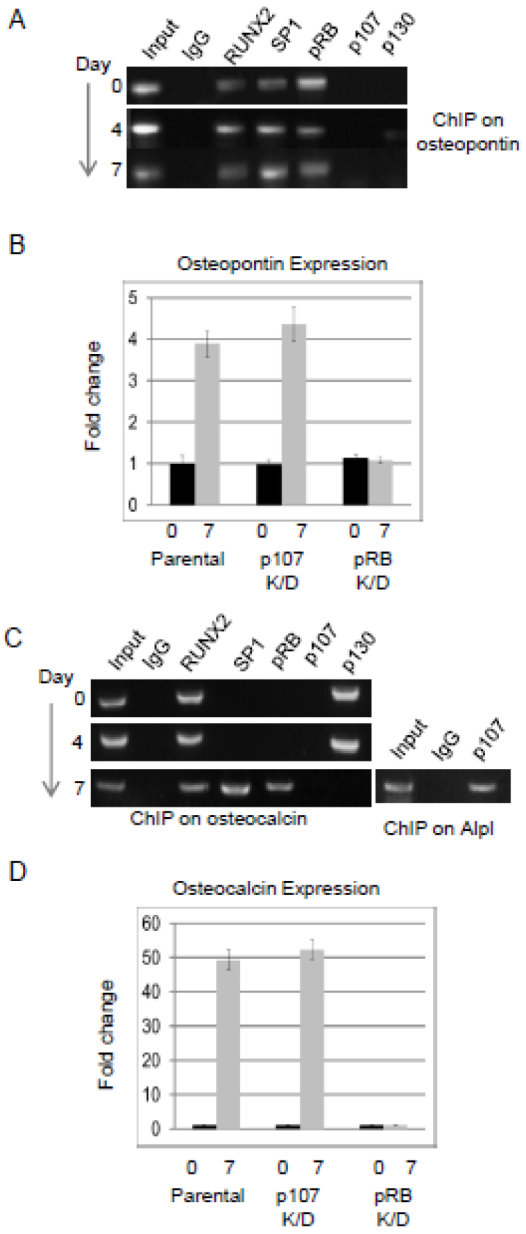

Figure 2. Depletion of p107 in osteoblast progenitors does not affect expression of the osteopontin or osteocalcin genes

A. ChIP analysis conducted on the osteopontin *(Ssp1)* promoter in normal parental preosteoblasts before (day 0) and during differentiation (4 and 7 days post-induction) shows the presence pRB but not p107. The osteopontin gene is already active in pre-osteoblasts, but is induced several-fold more during differentiation. The samples in panels A and C were assayed in parallel, providing cross-controls.

B. Osteopontin gene expression analysis by qRT-PCR before (day 0) and during (day 7) differentiation shows an approximately 4-fold increase in parental cells, dependent on pRB, but unaffected by depletion of p107.

C. ChIP analysis conducted on the osteocalcin *(Bglap)* promoter in normal parental preosteoblasts before (day 0) and during differentiation (4 and 7 days post-induction) shows promoter association by pRB specifically on the active promoter, but no detectable accompanying association of p107. Recruitment of the transcription factor Sp1 correlates with the dynamics of pRB association; this and the constitutive presence of RUNX2 were noted previously (17). The samples in panels A and C were assayed in parallel, providing cross-controls. A signal for the p107 immune complex was confirmed with the *Alpl* primers at day 7 as a positive control.

D. Osteocalcin gene expression analysis by qRT-PCR before (day 0) and during (day 7) differentiation shows a sharp increase in parental cells dependent on pRB, but unaffected by depletion of p107.

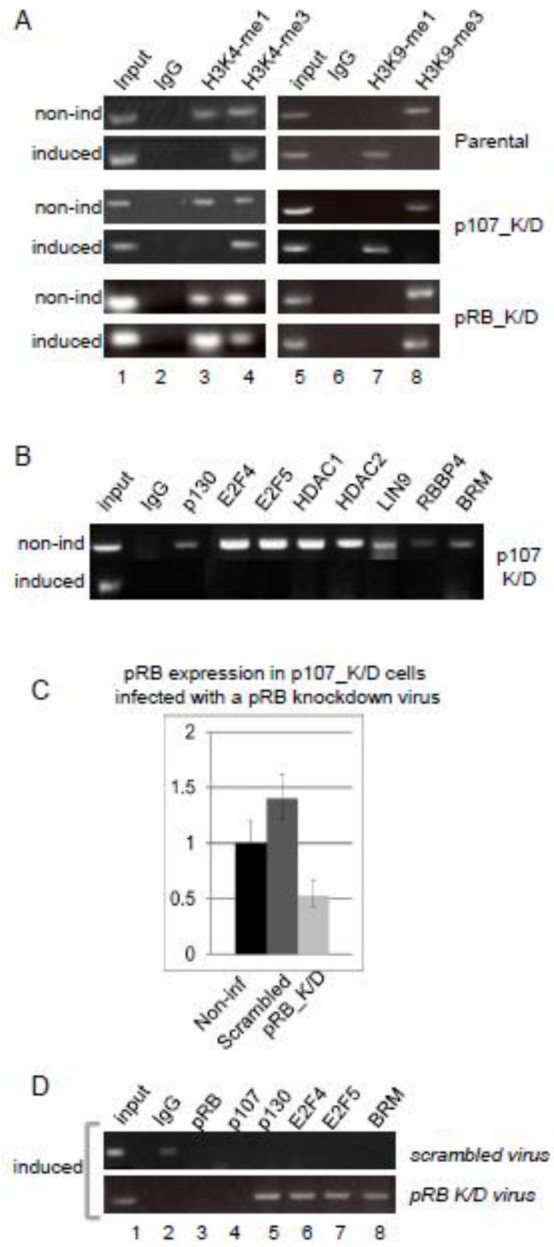

Figure 3. Distinct roles of pRB and p107 in Alpl activation

A. The dynamics of H3K4 and H3K9 methylation were analyzed by ChIP on the *Alpl* promoter before (day 0) and during (day 4) induction of differentiation. Normal parental osteoblast progenitors show a decline in mono-methylated H3K4 and a rise in mono-methylated H3K9 at the *Alpl* promoter during activation. These changes are pRB-dependent, and p107-independent.

B. Prior to activation the *Alpl* promoter is occupied by repressor complex components including p130, repressor E2Fs (E2F4 and E2F5), HDACs, LINC complex members Lin9 and RBBP4, and BRM-SWI-SNF. Dissociation of these repressors is independent of p107; dissociation independent of pRB was reported earlier (17).

C. An Adenovirus vector was used to transiently deplete pRB from the stable p107 knockdown line. qRT-PCR analysis shows about 50% reduction in expression of pRB is relative to the level in non-infected cells.

D. ChIP analysis of induced cells shows impaired dissociation of p130 from *Alpl* in the double-knockdown cells, compared with panel B.

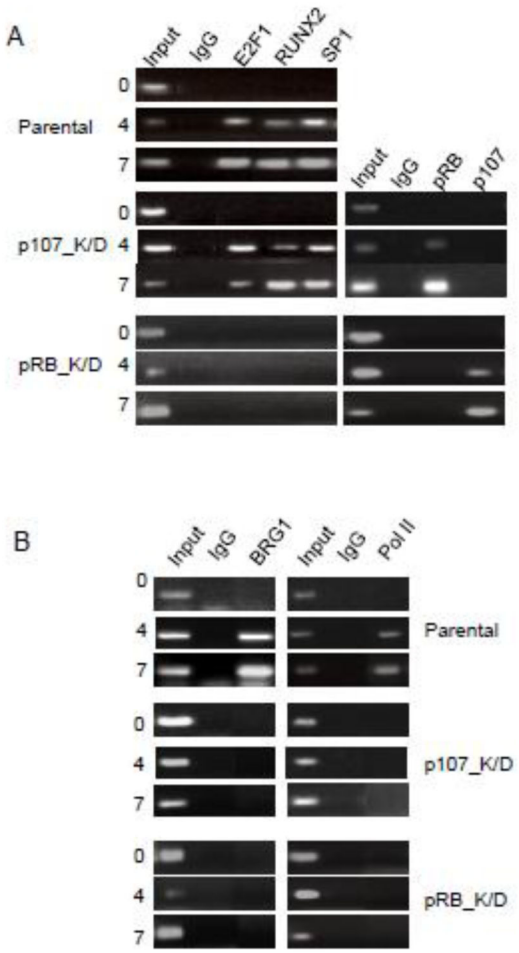

Figure 4. Requirement for p107 in recruitment of BRG1-SWI/SNF during Alpl activation

A. ChIP analysis shows recruitment of activating transcription factors E2F1, RUNX2, and Sp1 to *Alpl* is independent of p107. Recruitment of these factors is dependent on pRB (Flowers et al., 2013), shown here in parallel analysis for control.

B. ChIP analysis shows recruitment of the activating BRG1-SWI/SNF chromatin remodeling complex and RNA polymerase II is sharply dependent on p107. Recruitment of these factors is similarly dependent on pRB (22), shown here in parallel analysis for control.

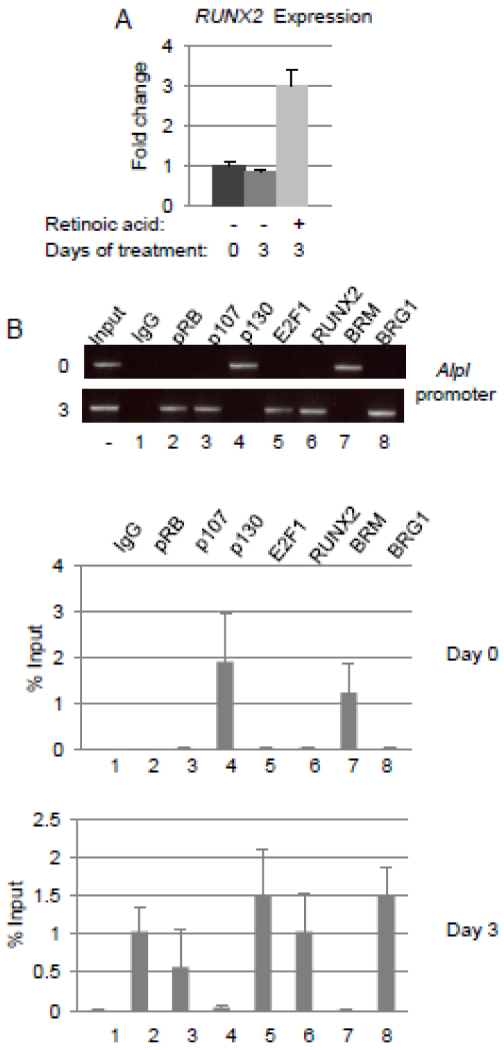

Figure 5. Occupation pattern on the Alpl promoter in multipotent precursor cells

A. Cells of the multipotent precursor cell line C3H10T1/2 were treated with retinoic acid to induce commitment to the osteoblast phenotype, indicated by induction of the osteogenic transcription factor RUNX2.

B. ChIP analysis by gel isolation of the sequenced promoter fragment and by qRT-PCR shows the promoter dynamics for the pRB family are the same as observed in the MC3T3-E1 pre-osteoblasts: the p107 protein is not detected with p130 on the repressed promoter before induction (day 0), and occupies the promoter in tandem with pRB after induction (day 3). Likewise, BRM and BRG1 show the same reciprocal relationship seen in MC3T3-E1 cells, with BRM occupying the promoter pre-induction and BRG1 occupying the promoter post induction.

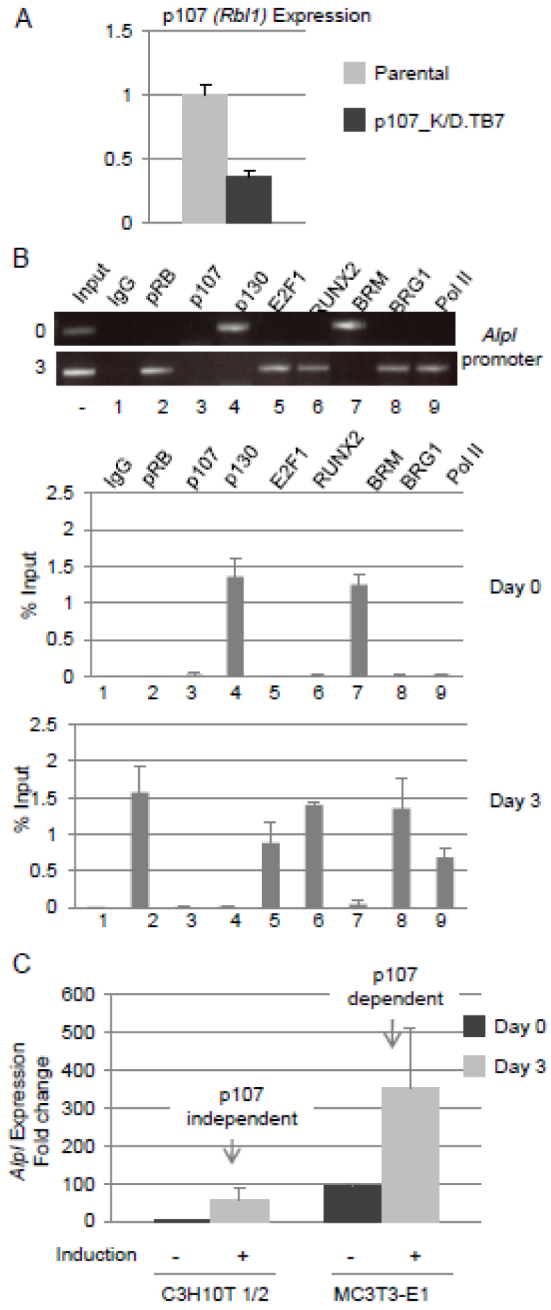

Figure 6. BRG1 recruitment to Alpl in p107-depleted multipotent precursor cells

A. Expression analysis by qRT-PCR of the p107 gene (*Rbl1*) in parental C3H10T1/2 cells and in a line (P107_K/D.TB7) stably incorporating the same p107-targeting shRNA sequence as the MC3T3-derived line. Results are expressed as a fraction of the parental value.

B. ChIP analysis by gel isolation of the promoter fragment and by qRT-PCR confirms depletion of p107 at the protein level (lane 3, day 3), and shows successful recruitment of BRG1 to the induced promoter (lane 8, day 3).

C. Direct comparison of *Alpl* expression levels in multipotent precursor cells induced with retinoic acid, and in pre-osteoblasts induced as in panel 1E, shows that non-induced MC3T3-E1 cells are held at a level of *Alpl* expression similar to that reached in C3H10T1/2 cells induced to commitment. The promoter configuration maintaining the interim expression level in the MC3T3-E1 pre-osteoblasts involves reassembly of repressor factors including p130 and BRM-SWI/SNF. The figures above show efficient recruitment of BRG1 for induction past this level requires p107.