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Deletion of the p107/p130-binding domain of Mip130/LIN-9 bypasses the requirement for CDK4 activity for the dissociation of Mip130/LIN-9 from p107/p130-E2F4 complex

Raudel Sandoval[1], **Mark Pilkinton**[1], and **Oscar R Colamonici**[2]
From Department of Pharmacology, University of Illinois, Chicago, IL 60612

Abstract

Mip130/LIN-9 is part of a large complex that includes homologs of the *Drosophila* dREAM (*drosophila* RB-like, E2F, and Myb) and and *C. elegans* DRM complexes. This complex also includes proteins such as Mip40/LIN-37, Mip120/LIN-54, and LIN-52. In mammalian cells, Mip130/LIN-9 specifically associates with the p107/p130-E2F4 repressor complex in G0/G1 and with B-Myb in S-phase. However, little is known about how the transition occurs and whether Mip130/LIN-9 contributes to the repressor effect of p107/p130. In this report, we demonstrate that Mip130/LIN-9, Mip40/LIN-37, Mip120/LIN-54, and Sin3b form a core complex, the Mip Core Complex or LIN Complex (MCC/LINC), which is detectable in all phases of the cell cycle. This complex specifically recruits transcriptional repressors such as p107, p130, E2F4 and HDAC1 in G0/G1, and B-Myb in S-phase. Importantly, we provide strong evidence that the transition between repressors and activators of transcription is mediated by CDK4, through the phosphorylation of the pocket proteins, p107 and p130. The requirement for CDK4 activity is bypassed by the deletion of the first 84 amino acids (Mip130/LIN-9$^{\Delta 84}$), since this mutant is unable to interact with p107/p130 in G0/G1, while maintaining its association with B-Myb. Importantly, the Mip130/LIN-9$^{\Delta 84}$ allele rescues the low expression of G1/S genes observed in CDK4$^{-/-}$ MEFs demonstrating that Mip130/LIN-9 contributes to the repression of these E2F-regulated genes in G0/G1.

INTRODUCTION

The cell cycle progression is driven by the sequential expression of cyclins, which serve as the activating subunit of cyclin dependent kinases (CDK)[3] (for recent reviews see refs. [1–3]). In the G0 and G1 phases, negative regulation is achieved by the pocket proteins family members, pRB, p107, and p130 [1,2,4]. The pocket proteins block transcription of E2F-regulated cell cycle genes in a complementary manner. pRB mainly interacts with E2F1-3 to inhibit transcriptional activation of E2F-regulated genes. On the other hand, p130 and p107 (hereafter p107/p130) primarily bind to E2F4 and E2F5 to repress specific promoters (recently reviewed in [4–11]). Following mitogenic stimulation, cyclin D expression is induced and pRB is phosphorylated by CDK4/6-cyclin D and subsequently CDK2-cyclin E. Unlike pRB, p107 and p130 are phosphorylated by CDK4/6-cyclin D, but not by CDK2/cyclin E [4,12–16].

2Address correspondence to: Oscar R. Colamonici, M.D.,University of Illinois, Department of Pharmacology, 835 S. Wolcott, Room E403 (M/C 868), Chicago, IL 60612. Phone: 312-413-4113, Fax: 312-413-4140 or 312-996-1225 ocolamon@uic.edu.
[1]This authors contributed equally to this manuscript.

Phosphorylation of p107 and p130 dismantles the complex that these pocket proteins form with repressor E2Fs and induces the relocalization of E2F4 to the cytoplasm [12,13,17–19].

The passage through the restriction point allows activating E2Fs (E2F1-3) to induce the transcriptional activation of G1/S genes such as B-Myb and those required for DNA replication (for recent reviews see refs. [4,6–9,11]). Activating E2Fs, in collaboration with B-Myb and Mip130/LIN-9, are responsible for the transcription of a second wave of cell cycle-regulated genes that occurs in S-phase [20–22]. These genes are necessary for the maintenance of S-phase, as well as the G2/M transition and include cyclins A and B, CDK1 CDC25, etc. Interestingly, Mip130/LIN-9 forms mutually exclusive complexes with p107 or p130 and E2F4 in G0/G1, and B-Myb in S-phase [23] suggesting that it may also have a role in the regulation of G1/S genes. This concept is also supported by the finding that a mutation of Mip130/LIN-9 that eliminates the first 84 amino acids of the protein (Mip130/LIN-$9^{\Delta 84}$) corrects the CDK4 null phenotype, which includes fertility and cell cycle defects [24].

The *Drosophila* homologues of Mip130/LIN-9 and B-Myb, Mip130 and dm-Myb, are part of a large multisubunit complex termed the dREAM complex (*drosophila* RB-like, E2F and Myb) [25–27]. A complex containing mammalian orthologs of dREAM that includes pocket proteins, Mip130/LIN-9, Mip40/LIN-37, Mip120/LIN-54, RbAp48 and LIN-52 has also been purified from human cells [28,29]. Although, it is unambiguous that one of the members of this complex, Mip130/LIN-9, is required for the induction of G2/M genes during S-phase [22,30], the role of this protein, as well as Mip120/LIN-54 and Mip40/LIN-37, in the regulation of G1/S genes is not settled. For example, Litovchick et al [28] reported that these proteins bind and repress the promoter of E2F-regulated genes during quiescence and delay cell cycle re-entry, while Schmitt et al [29] indicated that Mip120/LIN-54, Mip130/LIN-9 and Mip-40/LIN-37 specifically function on the activation of G2/M genes in association with B-Myb. Furthermore, two critical questions related to the function of these proteins have not been addressed. First, what is the mechanism that drives their separation from the p107/p130-E2F4 repressor complex and consequently promotes their interaction with B-Myb? Second, do members of the complex such as Mip130/LIN-9 collaborate to the repressor effect that p107/p130 and E2F4 exert on G1/S genes?

In this paper, we demonstrate that the previously reported complex formed by Mip130/LIN-9, Mip120/LIN-54 and Mip40/LIN-37 [28,29] is a core complex that also includes proteins associated with chromatin modification such as Sin3b. This complex termed Mip Core Complex or LIN Complex [29] (MCC/LINC), following the *Drosophila* and *C. elegans* denominations, recruits repressors such as p107/p130, E2F4 and HDAC1 in G0/G1, and B-Myb in S-phase. Our data strongly suggest that CDK4 phosphorylation drives the separation of Mip130/LIN-9 from p107/p130 and E2F4 and promotes the consequent interaction with B-Myb that stabilizes this transcriptional activator of G2/M genes. Importantly, Mip130/LIN-9 also contributes to the repression of G1/S genes mediated by p107 and p130, since the elimination of the first 84 amino acids of Mip130/LIN-9, which encode the p107/p130 binding-domain, releases the repression of G1/S genes observed in $CDK4^{-/-}$ MEFs. Thus, the Mip130/LIN-$9^{\Delta 84}$ mouse model provides a tool to separate the function of Mip130/LIN-9 within the context of the p107/p130-E2F4 repressor complex and its role in the transcription of G2/M genes during S-phase.

MATERIAL AND METHODS

Tissue Culture, Constructs and Transfections

Wild-type, $CDK4^{-/-}$ and $CDK4^{-/-}$Mip130/LIN-$9^{\Delta 84/\Delta 84}$ mouse embryonic fibroblasts (MEF) cells were generated from 12.5 day old embryos and cultured via a 3T3 protocol to establish cell lines. Both NIH/3T3 and the afore mentioned MEF 3T3 cell lines were grown in DMEM

supplemented with 10% FBS, glutamine and non-essential amino acids as previously described [21]. The plasmids used in this study were pEYFP-C1-p107-HA (obtained from Dr. J Wade Harper), pCMV-pRB (obtained from Dr. Pradip Raychaudhuri), pCMV-CDK4D158N, pCMV-CDK2D145N-HA and pBabe-puro-p16 (obtained from Dr. Philipp Kaldis), pCMV-p21 (obtained from Dr. David Ucker), and pcDNA3-B-Myb-FLAG (obtained from Dr. Rob Lewis). The plasmid for HA-tagged Mip40/LIN-37 was purchased from Genecopoeia (Germantown, MD). For transfections, cells were seeded at a density of 2×10^6 in 100 mm dishes and were transfected with 10 μg of plasmid DNA using TurboFect Reagent from Fermentas, Inc. (Glen Bumie, MD) according to the manufacturer's specifications.

Immunoprecipitations and Immunoblotting

Immunoblot analysis was performed as described previously [24]. Antibodies against Mip130/LIN-9, mouse monoclonal (mAb) and rabbit polyclonal (pAb), were previously described [24]. Rabbit anti-peptide polyclonal antibodies were raised against amino acids 32–50 and 70–90 of Mip40/LIN-37, and residues 634–652 and 700–718 of Mip120/LIN-54. The following antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): anti-B-Myb (N-19, sc-724); -p107 (C-18, sc-318), -p130 (C-20, sc-317), -Sin3b (AK-12, sc-768), and -HDAC1 (H-11, sc-8410). The anti-tubulin (ab7291) antibody was obtained from Abcam, Inc. (Cambridge, MA).

Cell cycle synchronization

NIH/3T3 cells were plated at 1×10^6 per 100mm dish and 24 hr later washed with PBS and cultured in serum-free DMEM for 48 hours. Cells were released by changing medium to DMEM supplemented with 10% FBS and then harvested at the indicated post-release time points. Synchronization was assessed by FACS analysis of propidium iodide stained cells.

RT-PCR

Total RNA was isolated using the NucleoSpin RNA II Kit from Clontech Laboratories, Inc. (Mountain View, CA). First Strand synthesis was performed with 3 μg of total RNA using the First Strand cDNA Synthesis Kit from Fermentas, Inc., according to the manufacturer's instructions. Quantitative PCR of the indicated genes was performed using a cDNA equivalent of 50 ng of total RNA. The reactions were performed in a final volume of 20 μl using SYBR ADVANTAGE® qPCR Premix from Clontech Laboratories, Inc. and processed with the ABI 7500FAST Sequence Detection System from Applied Biosystems (Foster City, CA). Expression differences were calculated as previously described [31]. PCR reactions were run in triplicates and experiments were repeated at least twice.

RESULTS AND DISCUSSION

The Mip Core Complex or LIN Complex (MCC/LINC) has specific interactions with p107/p130 in G0/G1, and B-Myb in S-phase

In order to further characterize the interactions of homologs of the *Drosophila* dREAM complex previously shown in mammalian cells [28,29], we performed coupled immunoprecipitations and immunoblots in lysates obtained from mouse NIH/3T3 cells. Supplemental Figure 1 confirms what was reported by others in human cells: Mip130/LIN-9, Mip40/LIN-37 and Mip120/LIN-54 associate with each other, and with p107 and B-Myb [28,29]. More importantly, Mip120/LIN-54 and Mip40/LIN-37 interact with Sin3b, suggesting that they participate in the regulation of gene transcription by recruiting proteins involved in regulating the chromatin structure.

To assess the interaction of these proteins along the cell cycle, NIH/3T3 cells were synchronized by serum starvation followed by release in complete growth medium. Figure 1 shows that Mip130/LIN-9 co-immunoprecipitates with Mip120/LIN-54 and Mip40/LIN-37 throughout the different phases of the cell cycle (Fig. 1, upper and middle panels). A slight decrease in the amount of Mip130/LIN-9 pulled down at 8 and 12 hours after release parallels a decrease in the amount of Mip120/LIN-54 that was immunoprecipitated. In the reciprocal experiment, immunoprecipitations with the anti-Mip130/LIN-9 mAb, confirmed that, unlike B-Myb, the association between Mip120/LIN-54 and Mip130/LIN-9 has little variation along the cell cycle (Fig. 1, IP: Mip130/LIN-9). It is worth mentioning that immunoblotting with the anti-Mip40/LIN-37 antibody after immunoprecipitation is not possible because of the close migration of this Mip/LIN protein within the heavy and light chains of the IgG.

Interestingly, Sin3b also co-immunoprecipitated with Mip120/LIN-54 and Mip40/LIN-37 in all phases of the cell cycle, yet the association has a tendency to decrease at the end of S phase and during mitosis (Fig. 1, upper and middle panels, IP: Mip120/LIN-54 and IP: Mip40/LIN-37, respectively). It is worth noting that an association between Mip130/LIN-9 and Sin3b can only be detected in Sin3b immunoprecipitates (data not show). These results strongly suggest that Mip130/LIN-9, Mip40/LIN-37 and Mip120/LIN-54 are likely to form a core complex that also includes Sin3b. Hereafter, we will refer to this complex as Mip Core Complex or LIN Complex (MCC/LINC).

Importantly, Figure 1 also shows that there is a prominent association of Mip120/LIN-54 with the histone deacetylase HDAC1 during G0 and G1 that rapidly decreases when the cells enter S-phase indicating that at least some elements of this core complex are capable of recruiting other chromatin modifiers such as HDAC1. Moreover, this occurs at stages of the cell cycle when the MCC/LINC has been proposed to function as a repressor of E2F-regulated genes [28].

We previously demonstrated that Mip130/LIN-9 had mutually exclusive interactions with p107/p130-E2F4 or B-Myb [23]. In order to determine if other components of the MCC/LINC also had cell cycle-specific interactions with the pocket protein or B-Myb, we performed immunoprecipitations using the cell lysates described in Figure 1 with antibodies against p107 or B-Myb. Figure 2A shows that p107 associates with Mip120/LIN-54 in G0 and early G1, and the interaction decreases as soon as the cells re-enter G1 and is reestablished in mid S-phase. The association of Mip40/LIN-37 with p107 also decreased after the re-entry in G1; however, the re-association is slightly delayed until cells reach late S-phase. Although a similar pattern is observed for the association between Mip130/LIN-9 and p107, the re-association between these proteins does not occur until the start of the next cell cycle (20 hrs). Interestingly, while association of p107 with HDAC1 decrease as cells re-enter the cell cycle from quiescence, the interaction with Sin3b shows an initial decrease and then remains stable.

Importantly, unlike p107, the association of B-Myb with the three components of the MCC/LINC, Mip130/LIN-9, Mip40/LIN-37 and Mip120/LIN-54 occurs in S-phase (Fig. 2B). These data strongly suggest that the MCC/LINC, as previously described for Mip130/LIN-9 [23], forms distinct complexes: in quiescent cells, it associates with p107, and in S-phase with B-Myb. However, since the dynamics of re-association of Mip120/LIN-54 and Mip40/LIN-37 with p107 are slightly faster than for Mip130/LIN-9, we cannot rule out complexes that include p107, Mip120/LIN-54, and Mip40/LIN-37, but not Mip130/LIN-9. Alternatively, Mip130/LIN-9 may join the complex later.

Altogether, these data show for the first time that the MCC/LINC includes chromatin modifiers such as Sin3b. Additionally, this core complex not only interacts with p107/p130, and E2F4, but also it is likely to recruit HDAC1 in G0/G1, while specifically associating with B-Myb in

S-phase. The interactions of the MCC/LINC with proteins involved in chromatin modification further supports the concept that this complex actively participates in transcriptional regulation in G0/G1.

The release of Mip130/LIN-9 from p107/p130-E2F4 and the stabilization of B-Myb is dependent on CDK4 activity

Although it is evident that the interaction of the components of the MCC/LINC transition from an association with cell cycle repressors in G0 to an association with B-Myb in S-phase, the exact driver of this transition has not been elucidated. The finding that a mutation of Mip130/LIN-9 that lacks the first 84 amino acids corrects the CDK4 and cyclin D2, but not the CDK2 null phenotypes [24] suggests that the type D cyclins-CDK4 complex may be involved in the process. If phosphorylation of p107 and p130 facilitates the dissociation of Mip130/LIN-9 from these pocket proteins and E2F4, it would be expected that the release of Mip130/LIN-9 from this complex would be delayed in CDK4$^{-/-}$ cells and restored to normal in double mutant cells [24]. To test these possibilities, we studied the association of Mip130/LIN-9 with the pocket proteins in CDK4$^{-/-}$ and the double mutant CDK4$^{-/-}$Mip130/LIN-9$^{\Delta 84/\Delta 84}$ cells. In order to estimate the magnitude of the associations, direct lysates corresponding 10% of the input used in the immunoprecipitations, were processed in parallel to assess the total expression of the different proteins. As previously described above for NIH/3T3 cells [23], in spite of the low level of expression, Mip130/LIN-9 associates with p130 in G0 and early G1 (Fig. 3A, 0 and 6 hr time points) in CDK4$^{+/+}$Mip130/LIN-9$^{+/+}$ cells and with B-Myb in S-phase. A significant interaction with p107 occurs only when the cells reenter the next cell cycle. Interestingly, in CDK4-null cells, Mip130/LIN-9 remains associated with p130 until later stages of the cell cycle (Fig. 3B, 12 hours after release). Similarly, the decrease in p130, normally observed in cells that re-enter G1, is delayed in CDK4$^{-/-}$ cells, reflecting the slower cell cycle progression due to the lack of this G1 kinase. Moreover, comparison of the signal detected in immunoprecipitates and direct analysis at 0, 6 and 12 hours shows that larger amounts of p130, and even p107, are co-immunoprecipitated with Mip130/LIN-9 in cells lacking CDK4 than in wild type cells. Similarly, the interaction between B-Myb and Mip130/LIN-9 is delayed 6 hrs (Fig. 3B).Surprisingly, the deletion of the first 84 amino acids of Mip130/LIN-9 in CDK4$^{-/-}$ cells almost eliminated its interaction with p107 and p130 without affecting the association with B-Myb (Fig. 3C), which occurs at similar time points as in wild type cells. It is worth mentioning that in Figure 3C, the expression of Mip130/LIN-9$^{\Delta 84}$ in immunoprecipitates is masked by the heavy chain of the IgG but can be detected in direct immunoblots.

We next tested whether Mip130/LIN-9$^{\Delta 84}$ was able to interact with Mip120/LIN-54 and Mip40/LIN-37. Figure 3D shows that Mip130/LIN-9 was co-immunoprecipitated with the anti-Mip120/LIN-54 antibody. Since Mip130/LIN-9$^{\Delta 84}$ and Mip40/LIN-37 migrate close to the heavy and light chains of the IgG, respectively, we studied the interaction between these proteins by transfecting tagged versions of Mip130/LIN-9 (GFP) and Mip40/LIN-37 (HA). Figure 3D demonstrates that Mip130/LIN-9$^{\Delta 84}$ co-immunoprecipitates with Mip120/LIN-54. Similarly, cotransfection of GFP-Mip130/LIN-9$^{\Delta 84}$ and HA-Mip40/LIN-37 demonstrated that the interaction between these proteins is preserved. Therefore, the deletion of the first 84 amino acids specifically removes the p107/p130 binding-domain without affecting the interaction between Mip130/LIN-9 and Mip120/LIN-54 or Mip40/LIN-37.

These results demonstrate that the requirement for CDK4 activity for the separation of Mip130/LIN-9 from p107/p130-E2F4 complex is bypassed by the specific deletion of the pocket protein binding-domain. Moreover, since the single putative CDK phosphorylation site present within the first 84 amino acids of Mip130/LIN-9 is not phosphorylated by CDK4 or CDK2 [30], it is

likely that Mip130/LIN-9 is released as part of the same mechanism that separates E2F4 from the complex: the phosphorylation of p107 and p130 [12,13,17–19].

To further explore the role of CDK4 in the release of Mip130/LIN-9 from the repressor complex, we studied the stability of ectopically expressed B-Myb in the presence of CDK4 inhibitors, since B-Myb is degraded in the absence of Mip130/LIN-9 in *Drosophila* and mammalian cells [21,26]. Figure 4 shows that the expression of dominant-negative-CDK4 (DN-CDK4) or p16 in T98G cells produced a dramatic reduction in the expression of transfected FLAG-tagged B-Myb (Fig. 4). However, no alteration in FLAG-tagged B-Myb expression was observed when HA-tagged DN-CDK2 or p21 were transfected, indicating that the effect on FLAG-tagged B-Myb degradation was specific for the inhibition of CDK4 activity. Moreover, although p107 and pRB are targets of CDK4, only the overexpression of HA-tagged p107, which specifically associates with Mip130/LIN-9, resulted in a dramatic decrease in FLAG-tagged BMyb expression. The findings that Mip130/LIN-9 is not phosphorylated by CDK4 *in vitro* [30] and that the overexpression of HA-tagged p107 triggered the degradation of FLAG-tagged B-Myb in a similar manner as the inhibition of CDK4 activity, further supports the concept that phosphorylation of p107 or p130 could be responsible for the dissociation of Mip130/LIN-9 from the repressor complex and the stabilization of B-Myb.

Recruitment of Mip130/LIN-9 to the p107/p130-E24 complex contributes to the repression of G1/S genes

Previous reports differ with regard to the role of Mip130/LIN-9 in the regulation of G1/S genes. For example, Litovchick et al [28] reported that overexpression of Mip130/LIN-9 delayed the re-entry of serum starved cells into G1, while Schmitt et al indicated that Mip130/LIN-9 had a specific role in the transcriptional activation of G2/M genes [29]. Moreover, although Mip130/LIN-9 and the pocket proteins are part of the so-called dREAM complex, neither report addressed whether Mip/LIN-9 contributed to the repressor activity of the p107/p130-E2F4 complex. Addressing this issue would require a form of the protein such as Mip130/LIN-9$^{\Delta 84}$, which fails to interact with the p107/p130, but retains the ability to associate with B-Myb. Figure 5 shows that the delay in the transcriptional activation of G1/S genes such as p107, PCNA, CDC6, and E2F3 observed in CDK4-null cells is partially or completely overcome by the deletion of the first 84 amino acids of Mip130/LIN-9. Surprisingly, the rescue is not observed in all E2F-regulated genes since the transcriptional activation of B-Myb was consistently low in cells harboring the mutation Δ84 of Mip130/LIN-9. These results strongly suggest that Mip130/LIN-9 contributes to the repression of E2F-regulated genes. However, this repressor effect does not appear to be exerted on all E2F-regulated genes raising the possibility that Mip130/LIN-9 plays a specific role on the expression of a subset of genes that regulate the G1/S transition.

In this report, we demonstrate that Mip130/LIN-9, Mip120/LIN-54 and Mip40/LIN-37 form a complex that can be detected, although with some variations, throughout the entire cell cycle. This complex previously termed LINC [29] is also referred as to Mip Core Complex (MCC) to reflect the *Drosophila* denomination, where the dREAM complex was first described. Several lines of evidence indicate that the MCC/LINC plays an active role in the repression of G1/S genes. a) The MCC/LINC includes chromatin modifiers such as Sin3b and recruits p107 or p130, E2F4, and possibly HDAC1 in G0/G1. b) The interactions between the MCC/LINC and the p107/p130-E2F4 complex or B-Myb appear to be mutually exclusive as previously described for Mip130/LIN-9 [23]. c) The abrogation of the interaction of Mip130/LIN-9 with the pocket proteins restores the transcription of a set of G1/S genes.

Additionally, our data strongly support the concept that CDK4 is responsible for the separation of Mip130/LIN-9 from the p107/p130-E2F4 complex. First, the absence of CDK4 prolongs the interaction of Mip130/LIN-9 with p107/p130-E2F4. Second, the CDK4 and cyclin D2, but

not CDK2-null phenotypes are rescued by a deletion of the pocket protein-binding domain of Mip130/LIN-9, demonstrating that CDK4 activity is responsible for breaking the complex between these proteins. Third, the expression of p107 or CDK4 inhibitors, which would delay the release of Mip130/LIN-9 form p107/p130-E2F4 similar to the absence of CDK4, triggers the degradation of ectopically expressed FLAG-tagged B-Myb. However, neither CDK2 inhibitors nor pRB has an effect on B-Myb strongly suggesting that CDK4 specifically regulates the release of Mip130/LIN-9 from the complex. Thus, CDK4 activity is responsible for the transition of Mip130/LIN-9, and likely the other components of the MCC/LINC, from transcriptional repressor within the context of the p107/p130-E2F4 complex in G0/G1, to transcriptional activator in association with B-Myb in S-phase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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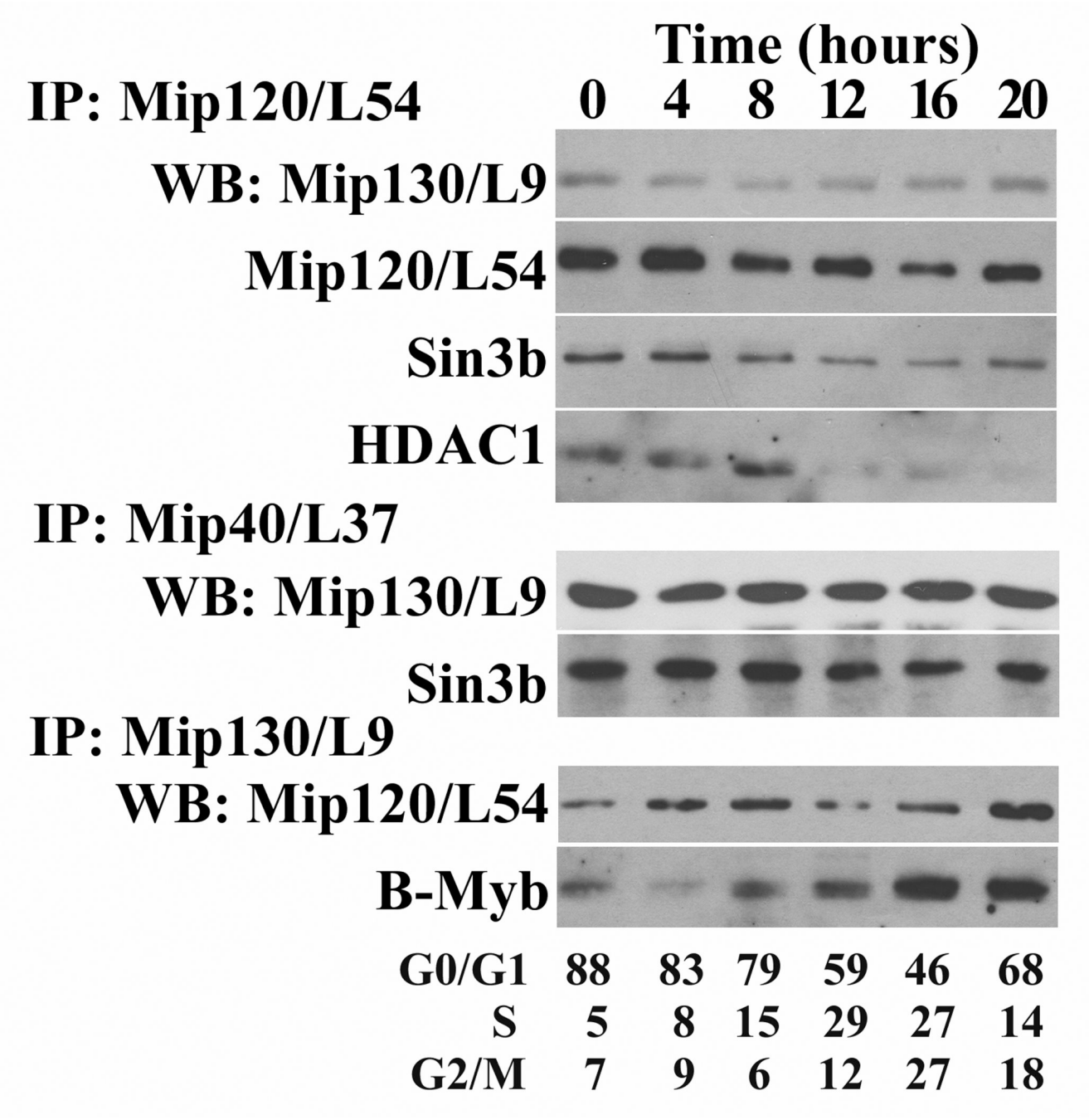

Figure 1. The mammalian orthologs of the dREAM complex form a core complex that includes Sin3b

Serum-starved NIH/3T3 cells were released by the addition of serum, and samples for FACS analysis and protein studies were collected every 4 hours. The cell cycle distribution is shown at the bottom. Nuclear extracts were used for immunoprecipitations (IP) with the indicated antibodies followed by immunoblotting (WB) with antibodies against the indicated mammalian orthologs of the dREAM complex proteins.

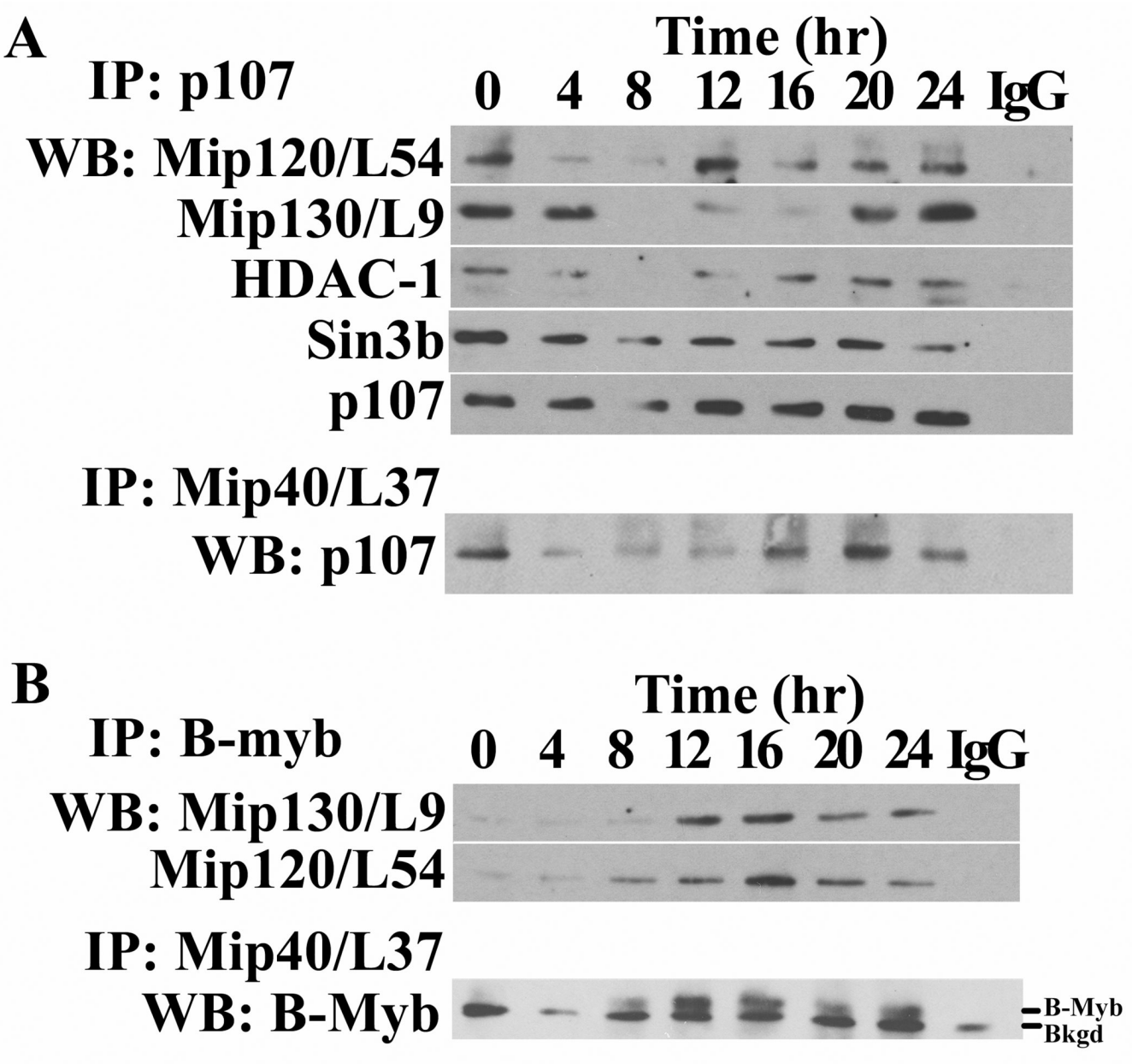

Figure 2. The MCC/LINC interacts with p107 in G0/G1 and B-Myb in S-phase

Protein lysates from the experiment described in Figure 1 were used for immunoprecipitations (IP) with anti-p107 (A) or -B-Myb (B) followed by immunoblotting (WB) with the indicated antibodies. The interactions of B-Myb and p107 with Mip40/LIN-37 were studied by performing immunoprecipitations with the anti-Mip40/LIN-37 serum since the reciprocal experiments cannot be performed due to the close migration of Mip40/LIN-37 to the IgG. The presence of a background band occasionally observed in B-Myb immunoblots is indicated.

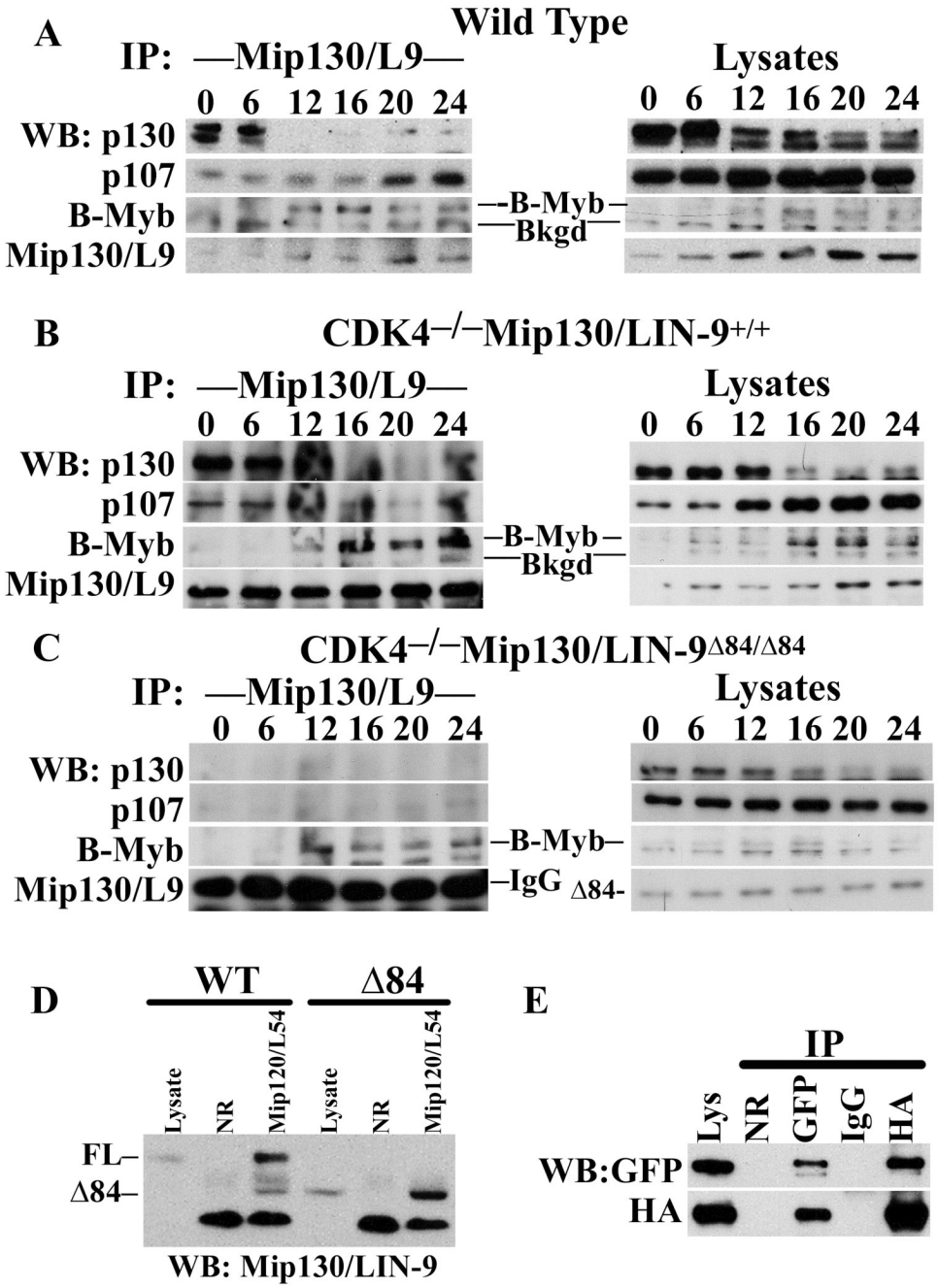

Figure 3. CDK4 drives the separation of Mip/LIN-9 from p107/p130

Synchronized MEFs derived from wild type (A), CDK4$^{-/-}$ (B), or CDK4$^{-/-}$Mip130/LIN-9$^{\Delta 84/\Delta 84}$ (C) animals were used for immunoprecipitations with an anti-Mip130/LIN-9 mAb followed by immunoblotting (WB) with the indicated antibodies (A–C, left panels). In the right panels (A–C), 30 μg of nuclear lysate (10% of the input used in the immunoprecipitations) were processed in parallel to determine the total amount of the different proteins expressed. D. On the left, protein lysates obtained from wild type (WT) or Mip130/LIN-9$^{\Delta 84}$ (Δ84) cells were used for 1immunoprecipitations with the anti-Mip120/LIN-54 serum followed by immunoblotting with the anti-Mip130/LIN-9 mAb. The full-length Mip130/LIN-9 and Mip130/LIN-9 $^{\Delta 84}$ are noted as FL and Δ84, respectively. E. 293FT cells were

transfected with GFP-Mip130/LIN-9$^{\Delta 84}$ and HAMip40/ LIN-37 and immunoprecipitations were performed with anti-GFP or -HA antibodies followed by immunoblotting with the indicated antibodies. Immunoprecipitations with normal rabbit serum (NR) and monoclonal mouse IgG (IgG) were used as controls.

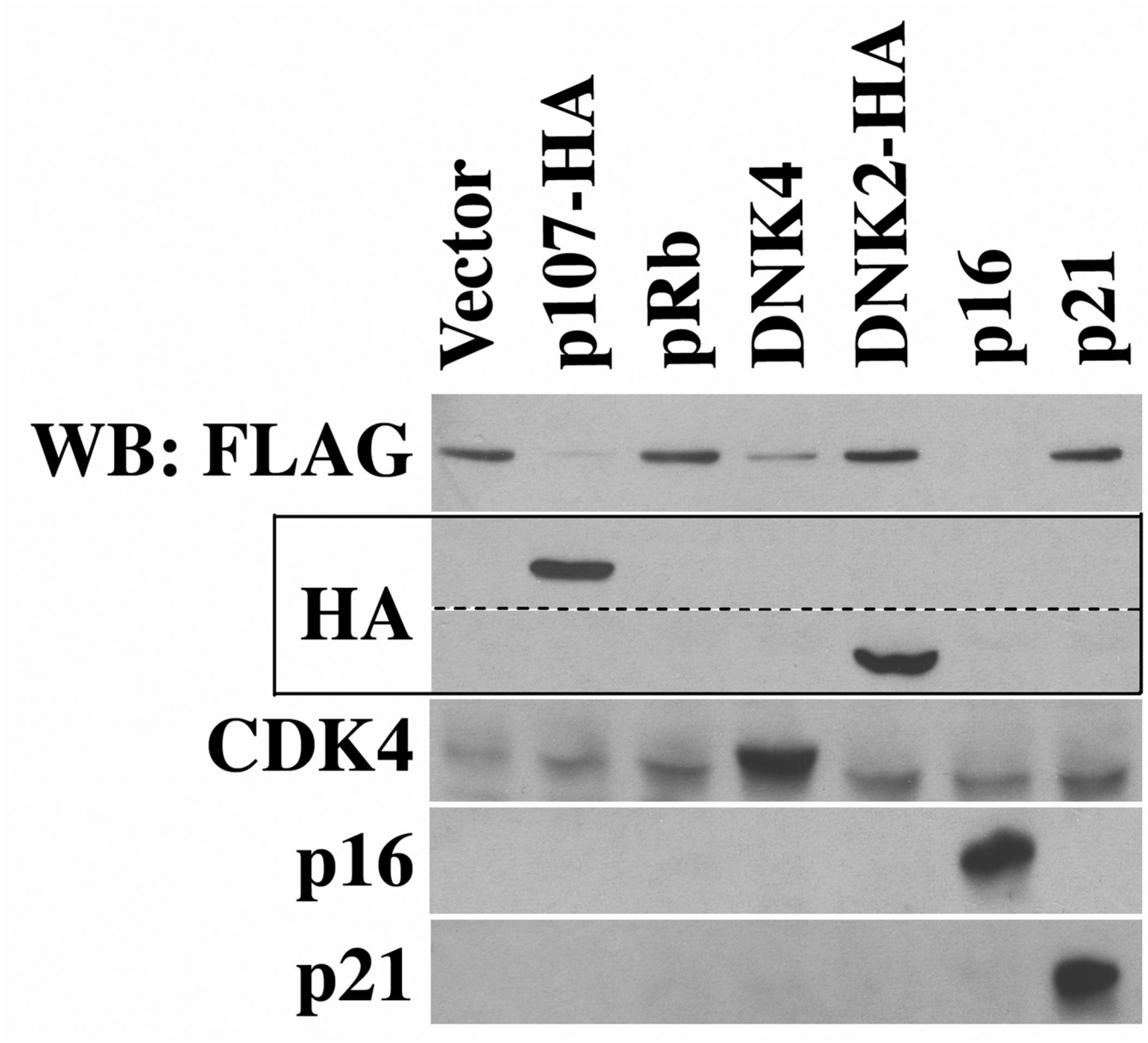

Figure 4. The inhibition of CDK4 or the overexpression of p107 leads to the degradation of B-Myb

T98G cells were transfected with equal amounts of the indicated recombinant constructs and FLAG- tagged B-Myb (10 μg of total plasmid DNA) for 24 hours. Cell lysates (50 μg) were directly analyzed by SDS-PAGE and immunoblotted (WB) first with the FLAG (M2) mAb to assess the expression of FLAG-tagged B-Myb. Subsequent immunoblotting was then performed with the indicated antibodies to verify the transfection of the appropriate recombinant constructs.

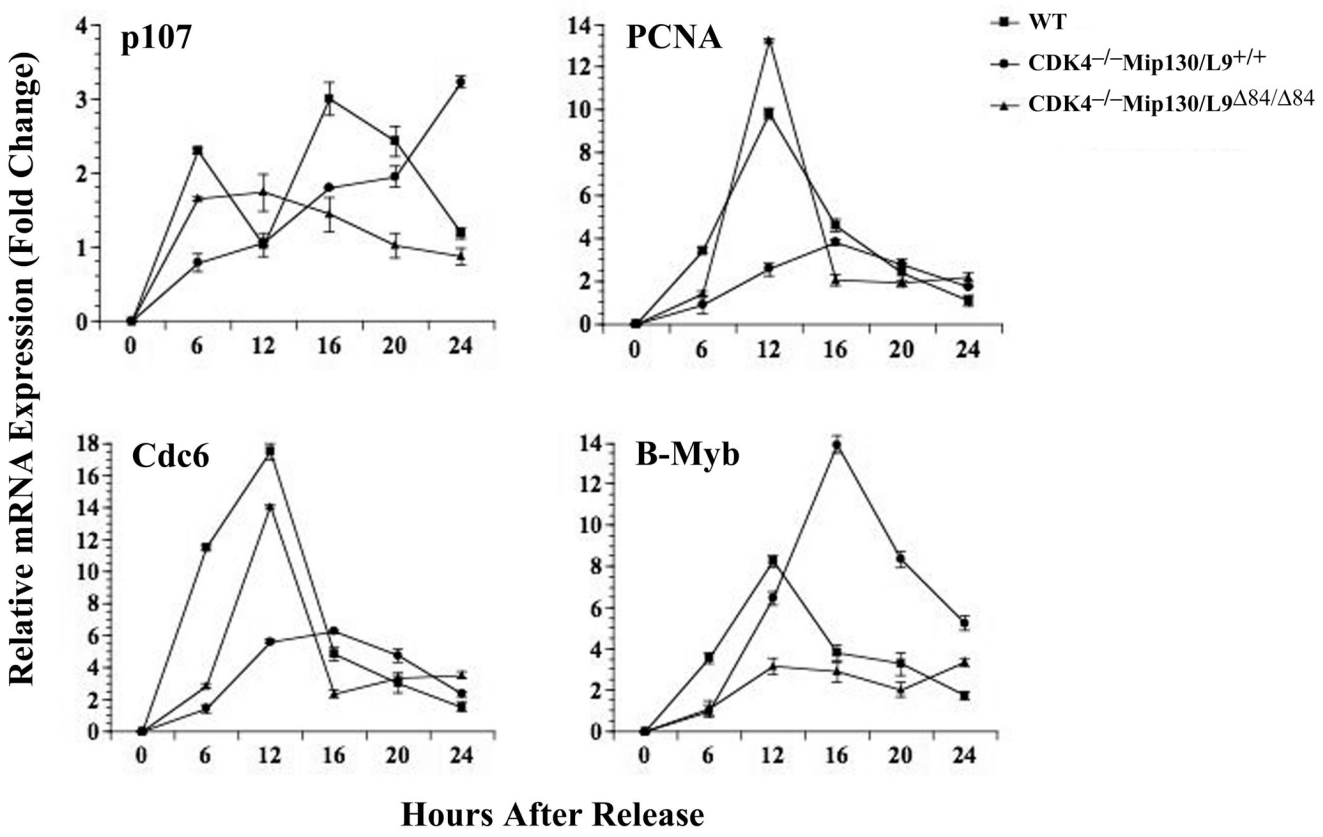

Figure 5. The Mip130/LIN-9$^{\Delta 84}$ allele rescues the defect in the transcription of G1/S genes present in CDK4-null cells

Wild-type, CDK4$^{-/-}$, and CDK4$^{-/-}$Mip130/LIN-9$^{\Delta 84/\Delta 84}$ cells were synchronized by serum starvation and released in growth medium for the indicated times. Total RNA was extracted and RT-PCR was performed as described in Materials and Methods. Expression differences were calculated as previously described [31]. PCR reactions were run in triplicates and experiments were repeated 2–3 times. Of note, no rescue was observed in B-Myb transcription.