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## MEN1-tumorigenesis in the pituitary and pancreatic islet requires *Cdk4* but not *Cdk2*

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### Abstract

Recent studies suggest that physiological and tumorigenic proliferation of mammalian cells is controlled by multiple cyclin-dependent kinases (CDKs) largely in tissue-specific manners. We and others previously demonstrated that adult mice deficient for the D-cyclin-dependent kinase CDK4 (*Cdk4*<sup>-/-</sup> mice) exhibit hypoplasia in the pituitary and pancreatic islet due to primary postnatal defects in proliferation. Intriguingly, those neuroendocrine tissues affected in *Cdk4*<sup>-/-</sup> mice are the primary targets of tumorigenesis in the syndrome of multiple endocrine neoplasia type-1 (MEN1). Mice with heterozygous disruption of the tumor suppressor *Men1* gene (*Men1*<sup>+/-</sup>) develop tumors in the pituitary, pancreatic islets and other neuroendocrine tissues, which is analogous to humans with *MEN1* mutations. To explore the genetic interactions between loss of *Men1* and activation of CDKs, we examined the impact of *Cdk4* or *Cdk2* disruption on tumorigenesis in *Men1*<sup>+/-</sup> mice. A majority of *Men1*<sup>+/-</sup> mice with wild-type CDKs developed pituitary and islet tumors by 15 months of age. Strikingly, *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice did not develop any tumors, and their islets and pituitaries remained hypoplastic with decreased proliferation. In contrast, *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> mice showed pituitary and islet tumorigenesis comparable to those in *Men1*<sup>+/-</sup> mice. Pituitaries of *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice showed no signs of loss of heterozygosity

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

(LOH) in the *Men1* locus, while tumors in *Men1*<sup>+/-</sup> mice and *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> mice exhibited LOH. Consistently, CDK4 knockdown in INS-1 insulinoma cells inhibited glucose-stimulated cell cycle progression with a significant decrease in phosphorylation of retinoblastoma protein (RB) at specific sites including Ser780. CDK2 knockdown had minimum effects on RB phosphorylation and cell cycle progression. These data suggest that CDK4 is a critical downstream target of MEN1-dependent tumor suppression and is required for tumorigenic proliferation in the pituitary and pancreatic islet, whereas CDK2 is dispensable for tumorigenesis in these neuroendocrine cell types.

## Keywords

cell cycle; menin; insulinoma; pituitary; knockout mice

## INTRODUCTION

Functional loss of a tumor suppressor gene is a key step of tumor initiation, triggering multi-step oncogenic events. A number of hereditary tumor syndromes result from germline mutations of tumor suppressor loci, providing implications about the mechanisms of tumor development. One of such examples is the cancer syndrome multiple endocrine neoplasia type 1 (MEN1)<sup>1,2</sup>. Individuals with germline mutations of the *MEN1* gene are predisposed to develop hyperplasia and tumors in the endocrine pancreas, anterior pituitary and parathyroid. The *MEN1* gene encodes a ubiquitously expressed transcriptional cofactor menin. Menin regulates gene transcription at least partly by modifying chromatin structure through its physical association with the mixed lineage leukemia (MLL) gene products MLL and MLL2, which are SET domain-containing histone lysine methyltransferases. The Menin-MLL complexes mediate tri-methylation of histone H3 at Lysine-4 (H3K4me3), a histone modification mark observed predominantly at transcriptionally active loci. Consistent with the role of menin as a *bona fide* tumor suppressor in neuroendocrine tissues, somatic *MEN1* mutations are frequently found in sporadic pancreatic tumors and parathyroid tumors, and also in some pituitary tumors<sup>1</sup>. While homozygous mice with targeted disruption of the *Men1* gene are embryonic lethal, heterozygous mice are viable and develop tumors in the endocrine pancreas and parathyroid within 9 months of age, and pituitary tumors within 12 months<sup>3-5</sup>. Tumors developed in *Men1*<sup>+/-</sup> mice display loss of heterozygosity (LOH), which closely resembles MEN1 individuals. These data suggest that the functional loss of menin confers a selective advantage for pre-tumorigenic neuroendocrine cells, whereas the exact mechanism of such neuroendocrine-specific tumor initiation remains unclear.

Cyclin-dependent kinases (CDKs) form central machinery for eukaryotic cell cycle progression<sup>6-8</sup>. While yeast cells express a single CDK catalytic subunit differentially activated by multiple Cyclin regulatory subunits, mammalian cells depend on several CDKs for cell cycle control. During the G<sub>1</sub> phase, D-type Cyclins bind and activate CDK4 or CDK6, followed by Cyclin E-mediated activation of CDK2. From early S-phase through G<sub>2</sub>, Cyclin A becomes a predominant activator of CDK2. CDK4/CDK6 and CDK2 collaborate in phosphorylating retinoblastoma protein (RB) and related pocket binding proteins p107

and p130, which is critical for transactivation of S-phase specific E2F target genes. These G<sub>1</sub>/S-specific CDKs are negatively regulated by several CDK inhibitors. The INK4-family inhibitors, such as p16, p15, p18, and p19, specifically inhibit CDK4 and CDK6. In contrast, the Cip/Kip-family inhibitors such as p21, p27 and p57 bind promiscuously to various Cyclin-CDK complexes. These CDK inhibitors play not only developmental roles but also tissue-specific tumor-suppressive roles, as demonstrated by the phenotypes of mice with targeted disruption of the CDK inhibitor genes. For instance, both p27-null mice and p18-null mice spontaneously develop pituitary adenomas in the intermediate lobe, and p27; p18-double null mice exhibit MEN1-like tumorigenesis in the endocrine pancreas, pituitary and parathyroid<sup>9-13</sup>. Recent studies using mice with targeted disruption of CDK genes have suggested that CDK proteins have substantial redundancies in developmental functions<sup>7, 8</sup>. Mice with disruption of *Cdk2*, *Cdk4* or *Cdk6* are viable with defects in highly specific tissues, despite almost ubiquitous expression of these proteins. Combined CDK deficiencies result in more severe developmental defects and lethality, suggesting functional redundancies. Importantly, *Cdk4*-null mice display progressive hypoplasia in pancreatic islets and the anterior pituitary postnatally after several weeks<sup>14-17</sup>. The tissue spectrum affected by *Cdk4* deficiency is similar to that associated with the MEN1 tumorigenesis. In contrast, *Cdk6*-null mice and *Cdk2*-null mice exhibit no developmental or functional defects in the neuroendocrine tissues, while they show some defects in hematopoietic and gonadal tissues, respectively<sup>18-20</sup>. Thus, we hypothesized that CDK4 plays a unique essential role in driving proliferation of neuroendocrine cells, and menin may negatively regulate the CDK4 action. To evaluate this hypothesis, we examined the impact of *Cdk4*- or *Cdk2*-deficiency upon neuroendocrine tumorigenesis in *Men1* heterozygous mice.

## RESULTS

### Pituitary tumorigenesis in *Men1*<sup>+/-</sup> mice depends on *Cdk4*

Previous studies demonstrated that *Men1*<sup>+/-</sup> mice develop tumors in the anterior pituitary with a latency of 9-13 months<sup>3, 4</sup>. *Cdk4*<sup>-/-</sup> mice exhibit hypoplasia of the anterior pituitary and pancreatic islets during postnatal periods, while embryonic development of these endocrine tissues occurs normally<sup>21-23</sup>. To determine whether CDK4 activity is required for tumorigenesis initiated by the loss of menin, *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice were generated by crossbreeding and characterized in comparison with *Men1*<sup>+/-</sup>; *Cdk4*<sup>+/+</sup> (*Cdk* wild-type) littermates. Mice were euthanized at 15 months of age for pathological examinations. None of 12 wild-type (*Men1*<sup>+/+</sup>) mice generated from the breeding displayed pituitary tumors, while 45% of *Men1*<sup>+/-</sup> mice (n=31) showed pituitary tumors (Fig. 1A, B). It is noteworthy that no *Men1*<sup>+/-</sup> males exhibited macroscopic pituitary tumors at 15 months of age (n=11), whereas 14 (70%) of 20 *Men1*<sup>+/-</sup> female mice had developed pituitary tumors. This gender-specific effect on pituitary tumorigenesis is consistent with a previous study, which showed 79% of *Men1*<sup>+/-</sup> mice that developed pituitary tumors were females<sup>4</sup>. Strikingly, none of 30 *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice (11 males and 19 females) displayed pituitary tumors. These observations indicate that pituitary tumorigenesis induced by menin deficiency depends on CDK4 activity. Furthermore, the pituitaries from the entire cohort of *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice remained hypoplastic, in a manner similar to the single *Cdk4*<sup>-/-</sup> mice throughout their 15-month lifespan (Fig. 1A). Our previous study demonstrated that CDK4 is an essential

driving force for estrogen- or GHRH-induced proliferation in murine pituicytes<sup>23</sup>. Thus, these data indicate that CDK4 is required for both physiological and tumorigenic control of cell cycle progression in this particular endocrine tissue.

To determine whether the requirement for CDK4 in menin-associated tumorigenesis is unique among the G<sub>1</sub>-regulatory CDKs or there is functional overlap, we next examined the impact of *Cdk2*-deficiency on tumorigenesis of *Men1*<sup>+/-</sup> mice, by generating *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> and *Men1*<sup>+/-</sup>; *Cdk2*<sup>+/+</sup> (*Cdk* wild-type) littermates. In sharp contrast to *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice, 51% of examined *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> mice (n=29) showed macroscopic pituitary tumors comparable to those in *Men1*<sup>+/-</sup>; *Cdk* wild-type mice (Fig. 1A, 1B). Interestingly, we observed tumors in 4 (24%) of 17 *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> male mice and 11 (92%) of 12 *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> female mice. This may imply that *Cdk2* deficiency modestly promoted pituitary tumorigenesis under the conditions of the study, although examinations of a larger cohort will be necessary to be conclusive. Regardless, these data indicate that the absence of CDK2 can be compensated, and has no impact on restraining the process of pituitary tumorigenesis induced by menin deficiency.

### Pancreatic islet tumorigenesis in *Men1*<sup>+/-</sup> mice depends on *Cdk4*

Previous studies reported that 30-50% of *Men1*<sup>+/-</sup> mice exhibited tumors in the endocrine pancreas, mostly insulinomas, during 8-18 months of age, and almost 80% of *Men1*<sup>+/-</sup> mice developed islet adenomas or carcinomas by 18-26 months<sup>3-5</sup>. To determine the effects of *Cdk4* or *Cdk2* deficiency on islet tumorigenesis, we examined pancreatic tissues from 15-month-old *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice, *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> mice and control littermates with *Men1*<sup>+/+</sup> and/or *Cdk* wild-type genotypes. Histological analyses showed that 62% of *Men1*<sup>+/-</sup>; *Cdk* wild-type mice (n=29) had islet tumors (Fig. 2A, C). Of the samples examined, islet adenomas were found in 12 (67%) of 18 *Men1*<sup>+/-</sup>; *Cdk* wild-type females and 6 (55%) of 11 *Men1*<sup>+/-</sup>; *Cdk* wild-type males. Essentially all islet tumors displayed insulin immunoreactivity (Fig. 2B), indicating these were insulinomas. No glucagonomas were observed in the examined groups of mice. Spontaneous islet tumors could not be found in *Men1*<sup>+/+</sup> mice regardless of the *Cdk4* or *Cdk2* genotypes. In sharp contrast to *Men1*<sup>+/-</sup>; *Cdk* wild-type mice, none of 30 *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice (19 females and 11 males examined) had islet tumors or showed evidence of dysplasia. Indeed, pancreatic islets of *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice were markedly hypoplastic, essentially identical in appearance to the hypoplasia in the islets of *Men1*<sup>+/+</sup>; *Cdk4*<sup>-/-</sup> mice. On the other hand, 10 (77%) of 13 *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> females and 12 (57%) of 21 *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> males exhibited islet tumors, again demonstrating the lack of restraining effect of *Cdk2* deficiency on both normal growth of the islets and neoplastic transformation..

We then examined proliferation of islet cells using immunohistochemistry with the Ki67 proliferation marker (Fig. 3A, B). In normal islets of wild-type mice, approximately 0.2% of endocrine cells stained positive for Ki67 immunoreactivity (Fig. 3B, Group 1), which is consistent with previous reports<sup>24, 25</sup>. In *Men1*<sup>+/-</sup>; *Cdk* wild-type mice, even islets that appeared normal in size showed higher percentages (0.7%) of Ki67-positive endocrine cells (Group 2). In hyperplastic or dysplastic *Men1*<sup>+/-</sup> islets, about 1.5% of cells showed Ki67 immunoreactivity (Group 3), and 6.5% of cells in islet adenomas were Ki67-positive (Group

4). In *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> islets and *Men1*<sup>+/+</sup>; *Cdk4*<sup>-/-</sup> islets, Ki67-positive endocrine cells were virtually undetectable (Group 5), indicating that the absence of CDK4 severely impaired proliferation of islet cells in adult mice. In *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> mice, 0.5%, 2.2% and 6.0% of cells were Ki67-positive in normal, hyperplastic/dysplastic and adenomatous islets, respectively (Groups 6-8). Thus, increased proliferation is correlated with tumorigenic changes in *Men1*<sup>+/-</sup> islets, regardless of the *Cdk2* genotype. Previous studies showed that adult murine islets express relatively high levels of CDK4 protein<sup>22</sup>, consistent with its role in  $\beta$  cell proliferation. Our immunohistochemical analysis not only confirmed readily detectable expression of CDK4 in wild-type and *Cdk2*<sup>-/-</sup> islets, but also demonstrated robust CDK4 expression in islet tumors of *Men1*<sup>+/-</sup>; *Cdk* wild-type and *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> mice (Fig. 3C). CDK4 expression was not detected in *Cdk4*<sup>-/-</sup> islets, confirming the specificity of the immunoreactivity. These data indicate that *Cdk4* is required for islet tumorigenesis initiated by hemizygous loss of *Men1*, while *Cdk2* function can be compensated in this process.

### LOH of the Men1 locus in pituitary tumors

It is well established that endocrine tumors that arise in *MEN1*<sup>+/-</sup> humans and *Men1*<sup>+/-</sup> mice exhibit LOH<sup>3,4</sup>, which is consistent with the classical “two-hit” model of tumorigenesis operative with inactivation of tumor suppressor genes<sup>26</sup>. In order to determine whether *Cdk2* or *Cdk4* deficiency had a differential impact on *Men1* LOH, we examined whether LOH had occurred at the *Men1* locus in pituitary tissues and tumors from *Men1*<sup>+/-</sup> mice with *Cdk4*<sup>-/-</sup>, *Cdk2*<sup>-/-</sup> and *Cdk*-wild type backgrounds. Genomic PCR analyses unambiguously indicated that pituicytes in *Men1*<sup>+/-</sup>; *Cdk* wild-type mice and *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> mice underwent LOH, i.e., the loss of the wild-type *Men1* allele in genomic DNA, while livers from those mice as non-tumorigenic control tissues retained both wild-type and mutant alleles (Fig. 4, compare lanes 4, 8 to lanes 3, 7). In contrast, pituitary tissues from *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice showed no sign of LOH (Fig. 4, lane 6). These results suggest that *Cdk4* deficiency impedes early tumorigenic changes in *Men1*<sup>+/-</sup> pituicytes that normally occur in prior to the complete loss of menin expression. In contrast, *Cdk2* deficiency does not alter LOH that occurs as part of the multi-step tumorigenic process.

### CDK4 depletion inhibits cell cycle progression of insulinoma cells with suppressed Rb phosphorylation

To obtain insight into the mechanism underlying the requirement for CDK4 in neuroendocrine tumorigenesis, we examined INS-1 insulinoma cells, which express very low levels of menin and have been widely used for functional studies by forced expression of wild-type and mutant menin proteins<sup>27,28</sup>. At 24 hrs after transfection with siRNA against CDK2 or CDK4, cells were treated with medium containing a low level of glucose (0.1 mM) and incubated for 48 more hours to suppress proliferation. Cell cycle progression was then stimulated by switching to medium with a high glucose concentration (11 mM). Immunoblotting (Fig. 5A) demonstrated that both CDK2 and CDK4 siRNAs successfully downregulated the expression of the target proteins. The stimulation of cells with 11 mM glucose resulted in marked upregulation of Cyclin D1 and substantial increases in Cyclin D3 expression, for which the knockdown of *Cdk2* or *Cdk4* had minimal impact. The expression

levels of Cyclin E and CDK6 did not significantly alter during glucose starvation and stimulation. It was noted that CDK2 knockdown resulted in modest increases in CDK4 levels (2<sup>nd</sup> panel, C vs. K2 at 0, 6 and 24h). Immunoblots for total RB showed accumulation of the slower migrating hyperphosphorylated forms of RB after the switch to 11 mM glucose, and CDK4 knockdown clearly inhibited the hyperphosphorylation of RB. In contrast, CDK2 knockdown resulted in slightly accelerated kinetics of RB phosphorylation, compared with control cells. Immunoblots using phospho-specific RB antibodies showed that phosphorylation of RB at Ser780 was delayed in cells with CDK4 knockdown (8<sup>th</sup> panel, K4-6 h vs. C-6 h and K4-24 h vs. C-24 h), whereas phosphorylation at Ser807/811 was minimally affected by CDK2 or CDK4 siRNA. We did not observe major effects of CDK2 or CDK4 knockdown in phosphorylation of p130 at Ser952. Consistently with the suppression of hyperphosphorylated forms of RB, CDK4-depleted cells exhibited a substantial delay in glucose-mediated cell cycle stimulation relative to control or CDK2 depleted cells, as shown as partially inhibited G<sub>1</sub>-S transition in flow cytometric analysis using DNA staining with propidium iodide and immunodetection of incorporated bromodeoxyuridine (BrdU)(Fig. 5B). Using another insulinoma cell line, Min6 cells, we confirmed that siRNA-mediated silencing of CDK4 significantly decreased Ser780 phosphorylation of RB, as well as hyperphosphorylated forms of the protein (Fig. 5C). Thus, these data using insulinoma cells demonstrate that CDK4 depletion inhibits site-specific RB phosphorylation and cell cycle stimulation in this cell type, whereas CDK2 depletion has minimal effect on these molecular processes.

## DISCUSSION

The present study demonstrated that *Cdk4* disruption completely inhibited pituitary and islet tumorigenesis in *Men1*<sup>+/-</sup> mice. This is in contrast with the finding that *Cdk2* disruption did not affect pituitary and islet tumorigenesis. *Cdk4* deficiency markedly inhibited islet proliferation in both wild-type and *Men1*<sup>+/-</sup> mice, while *Cdk2* deficiency showed no appreciable effects. LOH at the *Men1* locus was observed in *Cdk*-wild type or *Cdk2*-null pituitary tumors, whereas no sign of LOH was detected in *Cdk4*-null pituitaries. These observations suggest that CDK4 plays a unique and essential role in islet and pituitary tumorigenesis initiated by the loss of menin.

MEN1 tumors in humans are restricted to endocrine tissues such as the pituitary, pancreatic islet and parathyroid, although the tumor suppressor menin is ubiquitously expressed. The mechanism of the tissue-specific tumor suppression is yet to be elucidated. Our data indicating the unique requirement for CDK4 suggest that CDK4 activation is essential for a pre-LOH stage of the oncogenic process in the neuroendocrine cell types. While parathyroid tumorigenesis was not examined in the present study, complete inhibition of pituitary and islet tumor development in the *Cdk4*-null background indicates tight linkage between menin loss and CDK4 activation. Activating mutations of the human *CDK4* gene in pituitary, islet or other neuroendocrine tumors have not been reported, whereas activating germline mutations of *CDK4* have been described in melanoma-prone families<sup>29-31</sup>. These mutations in melanomas affect the Arg24 residue and render the mutant protein resistant to INK4-mediated inhibition. For MEN1 tumorigenesis, downregulation of CDK inhibitors seems to be involved in CDK4 activation. In particular, p18 and p27 have been proposed to play



important roles. Menin together with its partner MLL has been shown to control histone methylation at the p18 (*CDKN2C*) and p27 (*CDKN1B*) loci for transactivation<sup>32, 33</sup>. The levels of p18 and p27 mRNAs in pancreatic islets of *Men1*<sup>+/-</sup> mice decline within 28 weeks of age<sup>33</sup>. Intriguingly, *Cdkn1b*<sup>-/-</sup> mice develop pituitary tumors spontaneously<sup>9</sup>, and *Cdkn1b*<sup>-/-</sup>; *Cdkn2c*<sup>-/-</sup> mice exhibit tumorigenesis with the tissue spectrum overlapping with that of the MEN1 syndrome, including the endocrine pancreas, parathyroid, thyroid, adrenal and pituitary glands<sup>13</sup>. A gene expression profiling study, which analyzed pituitary and pancreatic tumors from *Men1*<sup>+/-</sup> mice, confirmed p18 downregulation<sup>34</sup>. Thus, it is conceivable that downregulation of the CDK inhibitors accounts substantially for ectopic CDK4 activation in pre-oncogenic menin-deficient neuroendocrine cells.

The requirement for CDK4 in tumorigenesis of *Men1*<sup>+/-</sup> mice expands upon its physiologic role previously demonstrated in the pituitary and pancreatic islets in *Cdk4*<sup>-/-</sup> mice<sup>14, 15</sup>. It is important that this phenotype is restricted to adult mice, while embryonic development of these organs is completely intact<sup>18-20</sup>. Regarding murine pancreatic islets, it is possible that CDK4 activity is essential for proliferation of differentiated  $\beta$  cells, which is thought to be a major mechanism for the adaptive regulation of islet mass in response to homeostatic requirement for insulin secretion<sup>35</sup>. A similar scenario might apply to the regulation of adult pituitary proliferation. A recent study proposed that downregulation of menin promotes  $\beta$  cell proliferation during pregnancy<sup>36</sup>. Thus, menin could be a limiting factor for not only tumorigenesis but also physiological proliferation in adult endocrine tissues, presumably via CDK4 inhibition. Pancreatic islets and pituitaries in *Cdkn2c*<sup>-/-</sup>; *Cdk4*<sup>-/-</sup> double mutant mice are as hypoplastic as *Cdk4*<sup>-/-</sup> mice<sup>37</sup>, indicating a linear functional relationship between p18 and CDK4. While CDK6 is another target of p18 and this kinase has also been implicated in the control of human islet proliferation<sup>38</sup>, genetic evidence suggests that murine islet cells depend solely on CDK4 for proliferation and tumorigenesis. Moreover, CDK4 expression was robust in islet tumors of *Men1*<sup>+/-</sup> mice, suggesting that upregulation of CDK4 protein may cooperate with downregulation of the CDK inhibitors in *Men1*<sup>+/-</sup> neuroendocrine cells. This notion is consistent with a recent report that a number of human pancreatic neuroendocrine tumors expressed high levels of CDK4<sup>39</sup>. We hypothesize that CDK4 activation not only accelerates proliferation but also generates a selective pressure for cells with LOH at the *Men1* locus. To further delineate the role of CDK4 in pituitary tumorigenesis triggered by the loss of menin, we recently generated mice with pituitary-specific disruption of *MEN1*, using Cre transgenic mice under the growth hormone promoter (*rGHP-Cre*<sup>tg+</sup>) crossed with mice with a floxed *Men1* allele (*Men1*<sup>lox/lox</sup>). While *rGHP-Cre*<sup>tg+</sup>; *Men1*<sup>lox/lox</sup>; *Cdk4*<sup>+/+</sup> mice displayed large pituitary tumors by 7 months of age with complete penetrance, no tumor was detected in *rGHP-Cre*<sup>tg+</sup>; *Men1*<sup>lox/lox</sup>; *Cdk4*<sup>-/-</sup> mice autopsied at 9 months (Gillam et al., unpublished observations). Since the GH promoter becomes active during late embryogenesis, homozygous loss of menin in this model occurs earlier than any detectable impact of *Cdk4* deficiency on pituitary proliferation or differentiation. Thus, the abrogation of tumorigenesis observed in *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice results from the requirement for CDK4 in neuroendocrine tumor initiation, and is unlikely to reflect other functional defects in hypoplastic pituitary or islets in *Cdk4*<sup>-/-</sup> mice before LOH occurs in the *Men1* locus.

Unlike *Cdk4* deficiency, *Cdk2* deficiency does not affect the homeostasis and function of the neuroendocrine tissues, and does not inhibit menin-associated tumorigenesis. These results are congruent with our previous observation that *Cdk2* deficiency does not prevent p27-deficient mice from developing pituitary tumors<sup>40</sup>. In sharp contrast, *Cdk2* deficiency and *Cdk4* deficiency are both inhibitory on *HER2/neu*-induced murine mammary tumorigenesis<sup>41,42</sup>. The embryonic lethality of *Cdk4*<sup>-/-</sup>; *Cdk2*<sup>-/-</sup> double mutant mice<sup>43</sup> implies that these two kinases have overlapping function in embryonic tissues as well as in most non-endocrine organs. CDK4 abundantly expressed in islet cells of *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> mice may be sufficient to compensate for the absence of CDK2 during tumorigenesis. It is thought that CDK4 and CDK2 collaborate in phosphorylating the critical substrate RB, sharing different phosphorylation sites on the protein<sup>44</sup>. In the present study using the islet cell lines, CDK4 depletion inhibited glucose-stimulated hyperphosphorylation of RB, including Ser780 phosphorylation, and G<sub>1</sub>-S progression, whereas CDK2 depletion had no effect. Collectively, these data underscore the significance of the CDK4-RB pathway in the control of neuroendocrine cell proliferation. Further investigations are necessary to understand how multiple CDK complexes control phosphorylation of RB at the 16 CDK-consensus sites<sup>44</sup> in neuroendocrine-specific manners, and whether non-RB substrates of CDK4 are involved in MEN1 tumorigenesis.

## MATERIALS AND METHODS

### Animals

All mice were maintained under identical conditions recommended by the Institutional Animal Care and Use Committee of Northwestern University. *Men1*<sup>+/-</sup> (*FVB*; *129S-Men1*<sup>tm1Cre</sup>) mice were obtained from the Jackson Laboratory. These mice were developed on a mixed FVB/N, 129S6 background<sup>3</sup>. *Cdk4*<sup>+/-</sup> mice and *Cdk2*<sup>+/-</sup> mice were generated in the *C57BL/6*; *129sv* mixed background as previously described<sup>14,18</sup>. *Men1*<sup>+/-</sup> mice were bred with *Cdk4*<sup>+/-</sup> mice or *Cdk2*<sup>+/-</sup> mice to generate mice doubly heterozygous for *Men1* and *Cdk4*, or *Men1* and *Cdk2*. Subsequently, *Men*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice and *Men*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> mice were generated by intercross breeding of double heterozygotes in each group. Mice were genotyped by PCR and monitored as described previously<sup>3,14,18</sup>. Cohorts were housed and analyzed in a common setting. All mice were sacrificed and subjected to necropsy at 15 months of age. At least 10 animals were analyzed per genotype. Our previous study demonstrated that a majority of *Cdk4*<sup>-/-</sup> males and some females develop diabetes mellitus within 8 weeks of age and many of them die in several months with hyperglycemia<sup>14</sup>. Thus, blood glucose levels were measured weekly on male *Cdk4*<sup>+/-</sup> mice beginning at 6 weeks of age, and periodically on female *Cdk4*<sup>+/-</sup> mice using a Precision Extra glucometer (Abbott Laboratories). To prevent diabetic *Cdk4*<sup>+/-</sup> mice from premature death, Linbit insulin implants (LinShin Canada, Inc) were delivered subcutaneously to transiently anesthetized mice when random blood glucose measurements exceeded 250 mg/dl. Physical conditions of all mice were carefully monitored on daily basis until the 15-month endpoint.



## Histopathology, Immunofluorescence, and Immunohistochemistry

Tissues of organs were removed and fixed in 10% neutral buffered formalin, or snap frozen in liquid nitrogen. The gross sizes of pituitaries or pituitary masses were measured using an electronic ruler. After macroscopic evaluation during necropsy, tissues were embedded in paraffin, sectioned at 5  $\mu$ m thickness and stained with hematoxylin and eosin (H&E). Pituitary and pancreatic sections were examined and photographed using a Zeiss Axiovert microscope and Axiovision software. Additional sections were taken for immunofluorescence and immunohistochemical analyses, performed as previously described<sup>45</sup>. To measure proliferating and mitotic cells, sections were blocked with normal goat serum in phosphate-buffered saline and incubated with a polyclonal antibody against Ki67 (NCL-Ki67 at a dilution of 1:1,000; Novocastra Laboratories) and with biotin-conjugated secondary antibody (Vector Laboratories). Other primary antibodies used were anti-insulin (Zymed) and anti-Cdk4 (Santa Cruz). Immunocomplexes were detected using the Vectastain ABC alkaline phosphatase kit according to the manufacturer's instructions (Vector Laboratories). For quantification of Ki67-positive islet cells, at least 1,000 cells in total were counted within each category of the following three: nonhyperplastic islets, hyperplastic islets and islet tumors.

## Assay for loss of heterozygosity

Pituitary tumors or non-tumorigenic pituitary tissues were sampled from 15-month-old mice, and genomic DNA was extracted. DNA samples from livers of each mice were also prepared as non-tumorigenic controls. The wild-type and mutant alleles of *Men1* were amplified by PCR (95°C, 1 min; 95°C, 30 s; 60°C, 30 s; 72°C, 1 min, for 35 cycles; 72°C, 10 min; 4°C hold), which yielded 300-bp and 638-bp amplicons, respectively. The three primers used for the reactions were oIMR1484: CCCACATCCAGTCCCTCTTCAGCT; oIMR1485: CCCTCTGGCTATCAATGGCAGGG; oIMR1486: CATAAAATCGCAGCAGGTGGCAA, as suggested by JAX.

## Cell culture, flow cytometry and immunoblotting

Rat insulinoma INS-1 cells were cultured in RPMI-1640 medium (Invitrogen), supplemented with 10% heat inactivated fetal bovine serum (HyClone/Thermo Fisher Scientific), 1 mM sodium pyruvate, 2 mM L-glutamate, 0.05 mM 2-mercaptoethanol, 10 mM HEPES and penicillin/streptomycin. Cells were transfected with Smart Pool Cdk2 siRNA or Cdk4 siRNA or control dsRNA (Dharmacon/Thermo Fisher Scientific), using the RNAiMax lipofection reagent (GiBCO/Invitrogen). For stimulation of cell cycle progression, cells at 48 h posttransfection were incubated in the medium with low (0.1 mM) glucose for 48 h, followed by incubation in the medium with high (11 mM) glucose. At various times after stimulation with high glucose, cells were harvested for immunoblotting and flow cytometry. At 30 min before each harvesting point, bromodeoxyuridine (BrdU, Sigma/Aldrich) was added to the medium. Immunoblotting and flow cytometry were performed as described previously<sup>14</sup>. Anti-BrdU antibody for flow cytometry was obtained from Pharmingen/BD Biosciences. Antibodies against CDK2, CDK4, CDK6, Cyclin D1, Cyclin D3, Cyclin E and Actin were purchased from Santa Cruz Biotechnology. Anti-total

RB antibody was from Pharmingen/BD Biosciences, and the phospho-specific antibodies against RB and p130 were from Cell Signaling Technology.

### Statistical Analyses

The data on tumor incidences in mice with different genotypes were examined statistically by Fisher's exact test.

### ACKNOWLEDGMENT

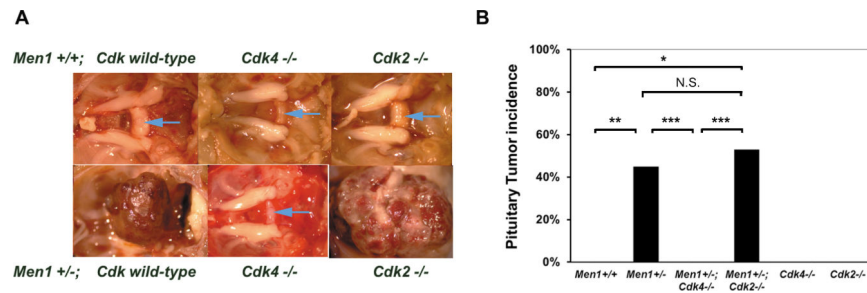
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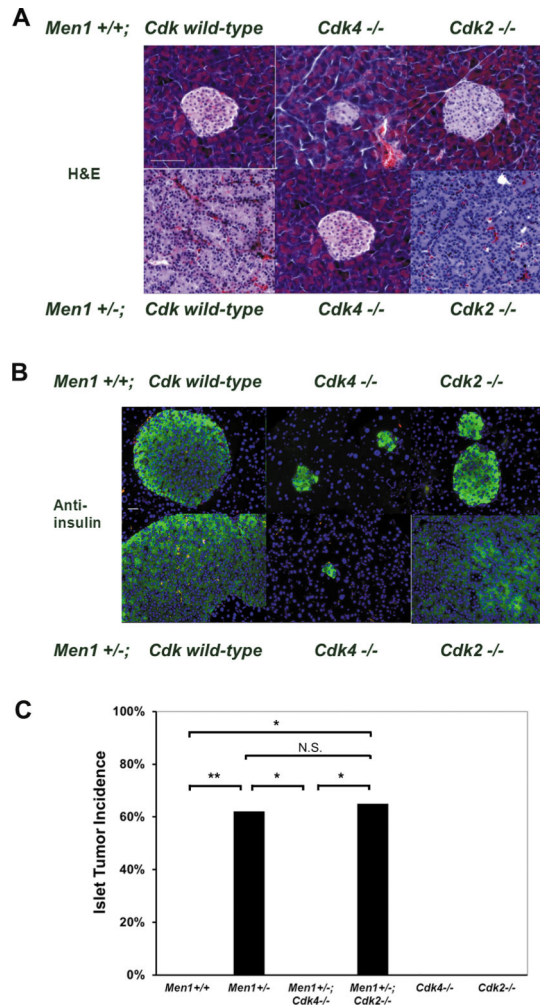
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**Fig. 1.**

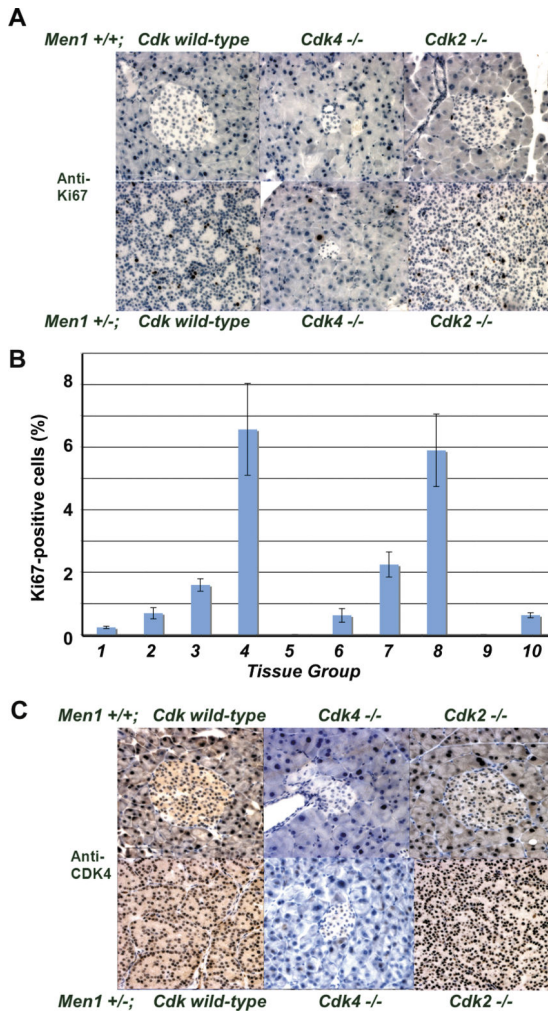
Pituitary tumorigenesis in *Men1*<sup>+/-</sup> mice is abrogated in the *Cdk4*<sup>-/-</sup> background, but not in the *Cdk2*<sup>-/-</sup> background. **(A)** Macroscopic pictures of pituitary organs or tumors from mice with the indicated genotypes. Blue arrows indicate non-tumorigenic pituitaries in *Men1*<sup>+/+</sup> (wild-type) mice in the *Cdk* wild-type (i.e., *Cdk4*<sup>+/+</sup>; *Cdk2*<sup>+/+</sup>), *Cdk4*<sup>-/-</sup> or *Cdk2*<sup>-/-</sup> background and in *Men1*<sup>+/-</sup> mice in the *Cdk4*<sup>-/-</sup> backgrounds. Note that pituitaries of mice in the *Cdk4*<sup>-/-</sup> backgrounds are smaller with hypoplastic changes as we reported previously. Large pituitary tumors in *Men1*<sup>+/-</sup>; *Cdk* wild-type and *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> mice are shown as large masses in dark red color occupying much of the fields. **(B)** Incidences of pituitary tumors determined at 15 months of age. The total numbers of *Men1*<sup>+/+</sup> mice, *Men1*<sup>+/-</sup> mice, *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> mice and *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> mice examined for tumorigenesis were 12, 31, 30 and 29, respectively (see Results for details). \*, p=0.001; \*\*, p=0.004; \*\*\*, p<0.0001; N.S., not significant.



**Fig. 2.**

Pancreatic islet tumorigenesis in *Men1*<sup>+/-</sup> mice is inhibited in the *Cdk4*<sup>-/-</sup> background, but not in the *Cdk2*<sup>-/-</sup> background. (A) Histological examinations of pancreatic islets and tumors after hematoxylin-eosin staining. Pancreas samples were obtained from 15-month-old mice with the indicated genotypes. (B) Immunofluorescence microscopy for insulin. Green, insulin; Blue, DAPI. (C) Incidences of pancreatic islet tumors at 15 months. The total numbers of *Men1*<sup>+/+</sup> mice, *Men1*<sup>+/-</sup> mice, *Men1*<sup>+/-</sup>;*Cdk4*<sup>-/-</sup> mice and *Men1*<sup>+/-</sup>;*Cdk2*<sup>-/-</sup> mice examined for tumorigenesis were 12, 29, 30 and 34, respectively (see Results for details). \*, p=0.0003; \*\*, p<0.0001; N.S., not significant.





**Fig. 3.** Effects of *Cdk4*- or *Cdk2*-deficiency on proliferation of endocrine cells in *Men1*<sup>+/-</sup> and *Men1*<sup>+/+</sup> pancreatic islets. **(A)** Immunohistochemistry for the proliferation marker Ki67 with pancreatic islets and tumors from 15-month-old mice with the indicated genotypes. **(B)** Percentages of Ki67<sup>+</sup> cells in the following tissue groups: group 1, wild-type islets; group 2, *Men1*<sup>+/-</sup> islets with normal morphology; group 3, *Men1*<sup>+/-</sup> dysplastic/hyperplastic islets; group 4, *Men1*<sup>+/-</sup> islet adenomas; group 5, *Men1*<sup>+/-</sup>; *Cdk4*<sup>-/-</sup> islets; group 6, *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> islets with normal morphology; group 7, *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> dysplastic/hyperplastic islets; group 8, *Men1*<sup>+/-</sup>; *Cdk2*<sup>-/-</sup> islet adenomas; group 9, *Cdk4*<sup>-/-</sup> islets; group 10, *Cdk2*<sup>-/-</sup> islets. At least 1,000 cells were counted within each category. **(C)** The expression of CDK4 protein in pancreatic islets and tumors from 15-month-old mice with the indicated genotypes.

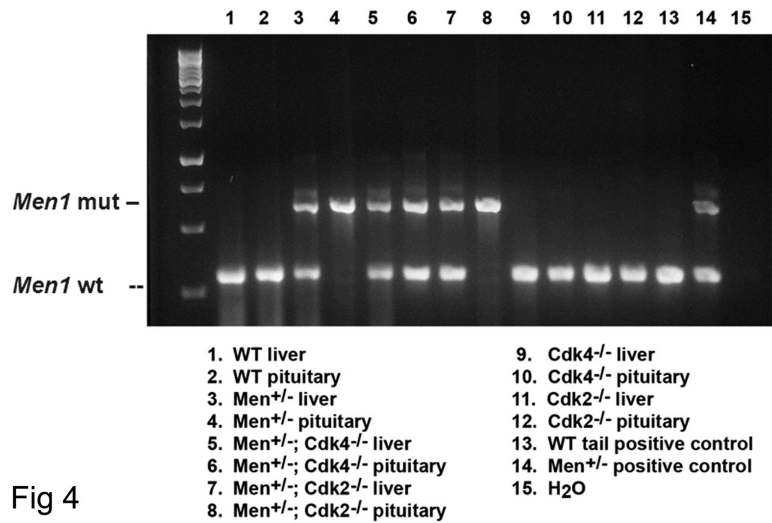
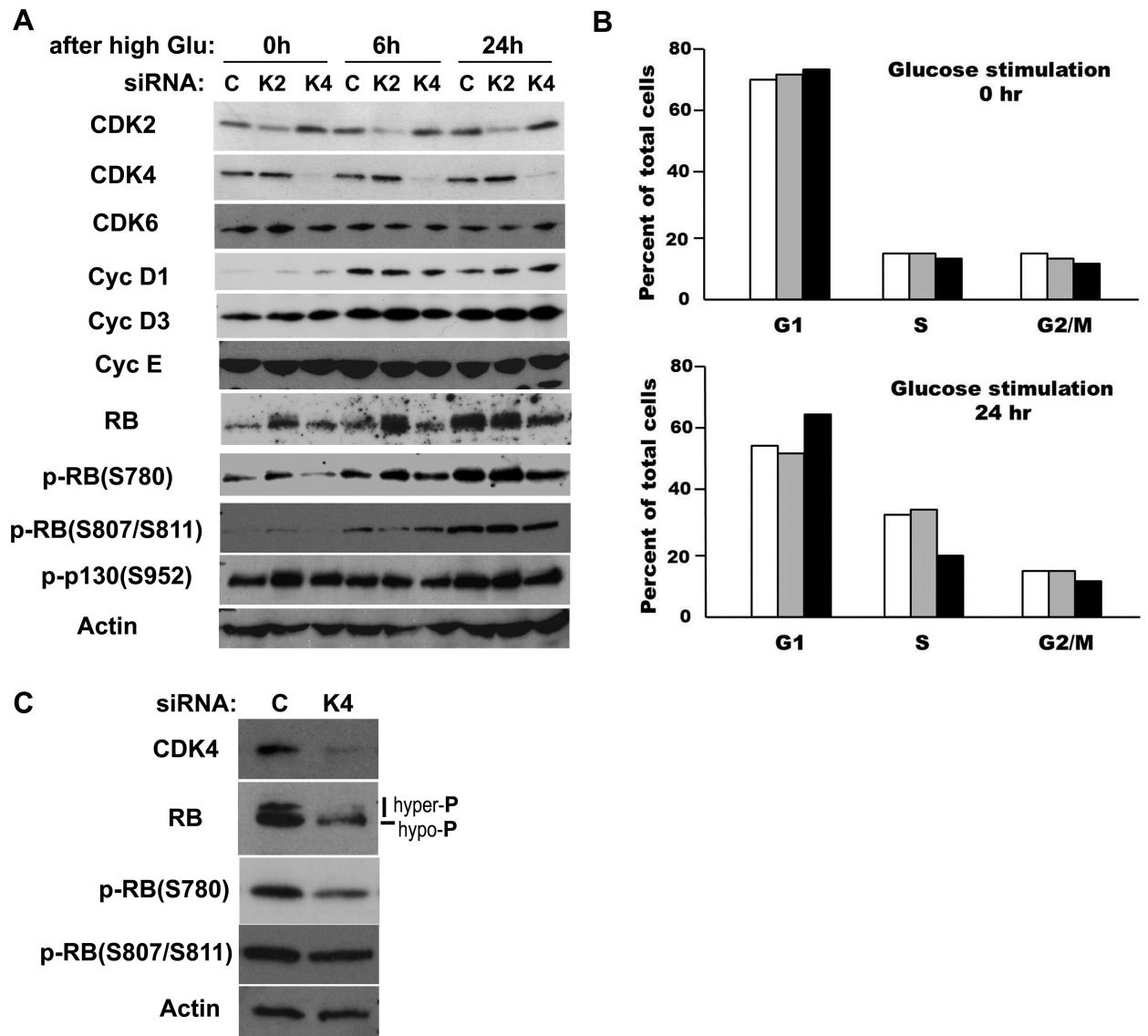


Fig 4

**Fig. 4.** Examination of loss of heterozygosity at the *Men1* locus in pituitary tumors and livers (as a non-tumorigenic control) from mice with the indicated genotypes. Chromosomal DNA samples prepared from tissues were analyzed by genomic PCR as described in Methods. The wild-type (wt) *Men1* allele and the targeted mutant (mut) allele were amplified as the indicated bands on agarose gel electrophoresis.



**Fig. 5.** Cdk4 knockdown in insulinoma cells results in delayed S phase entry with diminished RB phosphorylation, while Cdk2 knockdown shows minimum effects. (A) Expression of cell cycle proteins and phosphorylation of RB and p130 in INS-1 insulinoma cells transfected with Cdk2 or Cdk4 siRNA. Cells at 24 h post transfection (C, control dsRNA; K2, Cdk2 siRNA; K4, Cdk4 siRNA) were cultured in low (0.1 mM) glucose for 48 h, followed by incubation in high (11 mM) glucose for the indicated times. Immunoblotting was performed using the indicated antibodies. (B) Cell cycle profiles of INS-1 cells transfected with control dsRNA (white columns), Cdk2 siRNA (grey columns) and Cdk4 siRNA (black columns) determined by flow cytometry with assessment of BrdU incorporation. Data are representative of three independent experiments. (C) Effects of Cdk4 silencing on RB phosphorylation in Min6 insulinoma cells. Cells were transfected with control dsRNA (C) or Cdk4 siRNA (K4), incubated with low glucose from 24 to 72 h posttransfection, and then

treated with high glucose for 24 more hrs. Immunoblotting was performed using the indicated antibodies.

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