Adaptive cyclin D1 upregulation in response to CDK4/6 inhibitors in cancer

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ABSTRACT

Cyclin-dependent kinases 4 and 6 (CDK4/6) are key serine-threonine kinases activated by D-type cyclins to drive G1 to S phase cell-cycle progression. Their activity is often dysregulated in cancers resulting in aberrant cell proliferation. Therefore, CDK4/6 are attractive drug targets for cancer therapy. Palbociclib, ribociclib, and abemaciclib are CDK4/6 inhibitors approved by the Food and Drug Administration (FDA) to treat estrogen receptor–positive (ER^+) breast cancers. In addition, abemaciclib has shown promising antitumor activities for treating both *KRAS*-mutant non–small cell lung cancer (NSCLC) patients. Furthermore, we recently demonstrated that CDK4/6 inhibitors are also effective in suppressing cancer growth in preclinical models of SMARCA4-mutant cancers including small cell carcinoma of the ovary, hypercalcemic type (SCCOHT; ~100%) and a subset of NSCLC (~10%). Despite these encouraging potential clinical utilities, adaptive response to CDK4/6 inhibitors has been observed and likely contributes to the development of drug resistance. For example, treatment of CDK4/6 inhibitors is known to result in upregulation of cyclin D1 expression in cancer cells, but the mechanism is not understood. Furthermore, we demonstrated that cyclin D1 overexpression confers resistance to CDK4/6 inhibition in cancer cells. Here, we aim to dissect the molecular mechanism underlying this adaptive cyclin D1 upregulation in ER^+ breast cancer and *KRAS*-mutant NSCLC. We demonstrate that treatment with CDK4/6 inhibitors in these cancer cells results in elevated cyclin D1 expression in part through downregulation of *MIR17HG*, a microRNA cluster encoding for several miRNAs known to target cyclin D1. Furthermore, we show that CDK6 directly regulates *MIR17HG* expression by binding to its gene locus; this likely occurs through the NF-κB RELA subunit known to complex with CDK6. Finally, our data indicate that CDK6 may promote phosphorylation of MYC, which is also known to stimulate the transcriptional activity of MYC in inducing *MIR17HG* expression. Collectively, these results suggest a model where CDK6 is recruited to the *MIR17HG* locus through RELA and also stimulates MYC to promote *MIR17HG* transcription. Together, our work elucidates a novel mechanism underlying the adaptive upregulation of cyclin D1 in ER^+ breast cancer and *KRAS*-mutant NSCLC in response to CDK4/6 inhibitors.

RÉSUMÉ

Les kinases 4 et 6 dépendantes des cyclines (CDK4/6) sont des sérine-thréonine kinases activées par les cyclines de type D qui jouent un rôle majeur dans la régulation du cycle cellulaire lors de sa progression de la phase G1 à la phase S. Leur activité est dérégulée dans de nombreux cancers, ce qui entraîne une prolifération cellulaire aberrante. Par conséquent, les inhibiteurs de CDK4/6 sont des cibles thérapeutiques attrayantes pour le traitement du cancer. Trois inhibiteurs de CDK4/6 : le palbociclib, le ribociclib et l'abémaciclib, ont déjà été approuvés par la Food and Drug Administration américaine (FDA) afin de traiter les cancers du sein positifs aux récepteurs d'œstrogènes (ER^+). L'abémaciclib a également montré des activités anti tumorales prometteuses pour le traitement de patients atteints d'un cancer du poumon non à petites cellules (CPNPC) qui présentent une mutation du gène *KRAS*. De plus, nous avons récemment démontré que les inhibiteurs de CDK4/6 sont également efficace pour bloquer la croissance de cancers déficients en SMARCA4, comprenant le carcinome à petites cellules de l'ovaire de type hypercalcémique (SCCOHT ; ~100%) et un sous-ensemble de CPNPC (~10%). Malgré l'utilité clinique encourageante de ces inhibiteurs, une réponse adaptative a été observée et contribue vraisemblablement au développement de résistance aux médicaments. Par exemple, le traitement avec des inhibiteurs de CDK4/6 induit une régulation positive de l'expression de la cycline D1 dans les cellules cancéreuses. Toutefois, ce mécanisme n'est pas encore compris. De plus, nous avons démontré que la surexpression de cycline D1 confère une résistance aux inhibiteurs de CDK4/6 dans les cellules cancéreuses. Ici, nous visons à investiguer le mécanisme moléculaire sous-jacent à cette régulation positive de la cycline D1 dans les cancers du sein ER^+ ainsi que les CPNPC mutants en *KRAS*. Ici, nous démontrons que le traitement avec des inhibiteurs de CDK4/6 dans ces cellules cancéreuses induit une expression élevée de cycline D1, en partie causée par une régulation négative du polycistron *MIR17HG*. Celui-ci encode plusieurs microARN connus pour inhiber la cycline D1. De plus, nous montrons que CDK6 régule directement l'expression de *MIR17HG* en se liant à son promoteur de gène : cela se produit vraisemblablement par le biais de la sous-unité RELA du NF-kB connue pour former un complexe avec CDK6. Enfin, nos données indiquent que CDK6 peut favoriser la phosphorylation de MYC, qui est également connu pour stimuler l'activité transcriptionnelle de MYC en induisant l'expression de *MIR17HG*. Collectivement, nos résultats suggèrent un modèle où CDK6 est recruté au promoteur du

polycistron *MIR17HG* par RELA et stimule également MYC afin de promouvoir la transcription de *MIR17HG*. Ensemble, nos travaux élucident un nouveau mécanisme sous-jacent de la régulation positive de la cycline D1 observé lorsque des cellules du cancer ER^+ ainsi que les CPNPC mutant en *KRAS* sont traitées avec des inhibiteurs de CDK4/6.

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CONTRIBUTION OF AUTHORS

L.P. analyzed the data and wrote the thesis. S.H. provided substantial feedback and editorial help on the thesis. L.P., T.K. and S.H. designed the experiments. A.N and J.D. provided substantial direction on the project. L.P. performed all the experiments unless otherwise stated. T.K. performed palbociclib, abemaciclib and ribociclib treatment assays and analyzed publicly available data sets, where indicated.

LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
ATRT	Atypical teratoid rhabdoid tumor
CAPTUR	Canadian profiling and targeted agent utilization trial
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
CDK4/6	Cyclin dependent kinases 4 and 6
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation sequencing
CTD	Carboxy terminal domain
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GSEA	Gene Set Enrichment Analysis
$HER2^-$	Human epidermal growth factor 2 negative
HR^+	Hormone receptor positive
IC_{50}	Half-maximal inhibitory concentration
IL-1	Interleukin-1
Mcl-1	Myeloid cell leukemia 1
miRNA	microRNA
miRNA-seq	micro-RNA sequencing
NF-κB	Nuclear factor kappa b
NPC	Nasopharyngeal carcinoma
NSCLC	Non-small cell lung cancer
PLA	Proximity ligation assay
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma protein

RELA	Rel-associated protein
RISC	RNA-induced silencing complex
RNA Pol II	RNA polymerase II
SCC	Squamous cell carcinoma
SCCOHT	Small cell carcinoma of the ovary, hypercalcemic type
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor alpha
VEGFA	Vascular endothelial growth factor A

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INTRODUCTION

Cyclin-dependent kinases and cell cycle regulation

All living organisms are the product of repeated rounds of cell growth and division. During this process known as the cell cycle, the faithful transmission of genetic material to daughter cells is highly regulated and consists of a series of sequential events. In eukaryotes, the cell cycle consists of four main phases (**Figure 1A**): during the synthesis (S) phase, the genetic material is duplicated, and subsequently divided into two identical daughter cells during mitosis (M); during the so-called gap phases (G_1 and G_2), the cell grows in size and cellular organelle contents are doubled. These four phases are progressed in a consecutive order of G_1, S, G_2, and M. The G1 and G2 phases provide essential time for the cell to ensure that internal and external conditions are suitable before the cell commits itself to DNA replication and mitosis. If extracellular conditions are unfavorable during the G_1 phase, the cell can delay progression and may even enter a specialized resting state known as G zero (G_0). Once conditions are favorable and signal the cell to proceed to S phase, it progresses through a commitment step known as the G_1/S phase checkpoint or restriction point. Following this, the cell is committed to DNA replication, even if the extracellular signals stimulating cell proliferation are removed [1].

Progression through the cell cycle relies on the activity of key serine-threonine kinases known as cyclin-dependent kinases (CDKs). They drive the transitions between phases, including regulation of cell-cycle checkpoints and transcriptional events in response to intracellular and extracellular signals, by phosphorylating various substrates. The activity of CDKs is subject to positive or negative regulation by their association with cyclins or CDK inhibitors, respectively (**Figure 1A**) [1]. At the start of G_1, the tumor suppressor retinoblastoma protein (RB) is hypo-phosphorylated and binds to E2F transcription factors, blocking their transactivation domain and effectively sequestering them to prevent transcription of S phase genes [2, 3]. In response to regulatory signals, levels of D-type cyclins (D1, D2, and D3) rise and activate CDK4 and CDK6 [4, 5]. Activated CDK4/6-cyclin D complexes partially phosphorylate RB at various phosphorylation sites, weakening its interaction with E2F transcription factors. As G_1 progresses, cyclin E levels rise and activate CDK2. Once CDK2 is activated by cyclin E, the complex reinforces activity of CDK4/6

to complete phosphorylation of RB on additional sites [6]. This results in RB dissociation from E2F leading to subsequent transcription of genes such as cyclin E and cyclin A, which marks progress through the G_1/S checkpoint (**Figure 1B**) [7, 8]. More simply, transcription of such genes changes the program of the cell to reduce its dependency on regulatory signals allowing it to complete cell cycle, and, in this sense, irreversibly commits the cell to enter S phase. During S phase, RB phosphorylation is maintained by CDK2-cyclin A complexes, while CDK1-cyclin A/B maintain hyper-phosphorylation of RB during the G_2 phase. Once the cell enters mitosis, cyclin A and B are degraded resulting in the loss of sustained RB phosphorylation, which returns the system to ground state marked by hypo-phosphorylation of RB (**Figure 1A**) [9, 10]. The cell enters G_1 phase, which re-establishes a period where it is dependent on regulatory signals to progress through the next cell cycle, including the requirement for cyclin D.

In conjunction with CDK activation by cyclins, the cell cycle is also tightly regulated through the action of two classes of CDK inhibitors (CDKIs). The first class consists of four INK4 proteins ($p16^{INK4\alpha}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$), which bind exclusively to CDK4 and CDK6 to repress complex formation with cognate cyclins. The second class of inhibitors consists of more broadly acting proteins known as the Cip/Kip family, including $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$. These inhibitors bind both cyclin and CDK subunits to inhibit the activities of cyclin E- and cyclin A-dependent kinases. For example, $p21^{Cip1}$ and $p27^{Kip1}$ inhibit cell cycle by binding to CDK2-cyclin E complexes. As the cell progresses through G_1 phase, CDK4/6-cyclin D complexes also function to sequester both $p21^{Cip1}$ and $p27^{Kip1}$, thus promoting CDK2 activity (**Figure 1B**). The emergence of CDK2-cyclin E complexes requires partial phosphorylation of RB and inactivation of both $p21^{Cip1}$ and $p27^{Kip1}$ and is therefore dependent on the activation of the cyclin D pathway [5]. Through their interaction with CDK4/6-cyclin D complexes, CDKIs of the Cip/Kip family have been proposed to act as positive regulators of these heterodimers and enhance their stability. Thus, this complex and tight regulation of progression through the cell cycle relies on the two functions of CDK4/6-cyclin D complexes: RB phosphorylation and CDKI sequestration.

In addition to their essential role in regulating cell cycle, CDKs have also been shown to have functions in regulating transcription. For example, CDK6 was found to act as a transcriptional regulator at the promoter of both *CDKN2A,* encoding the CDK4/6 inhibitor $p16^{INK4\alpha}$, and the

vascular endothelial growth factor A (*VEGFA*), encoding a known angiogenic factor that stimulates the formation of new blood vessels [11]. CDK6 was also shown to physically and functionally interacts with the nuclear factor kappa B (NF-κB) subunit RELA, also known as p65, to regulate the expression of a wide array of inflammatory genes [12].

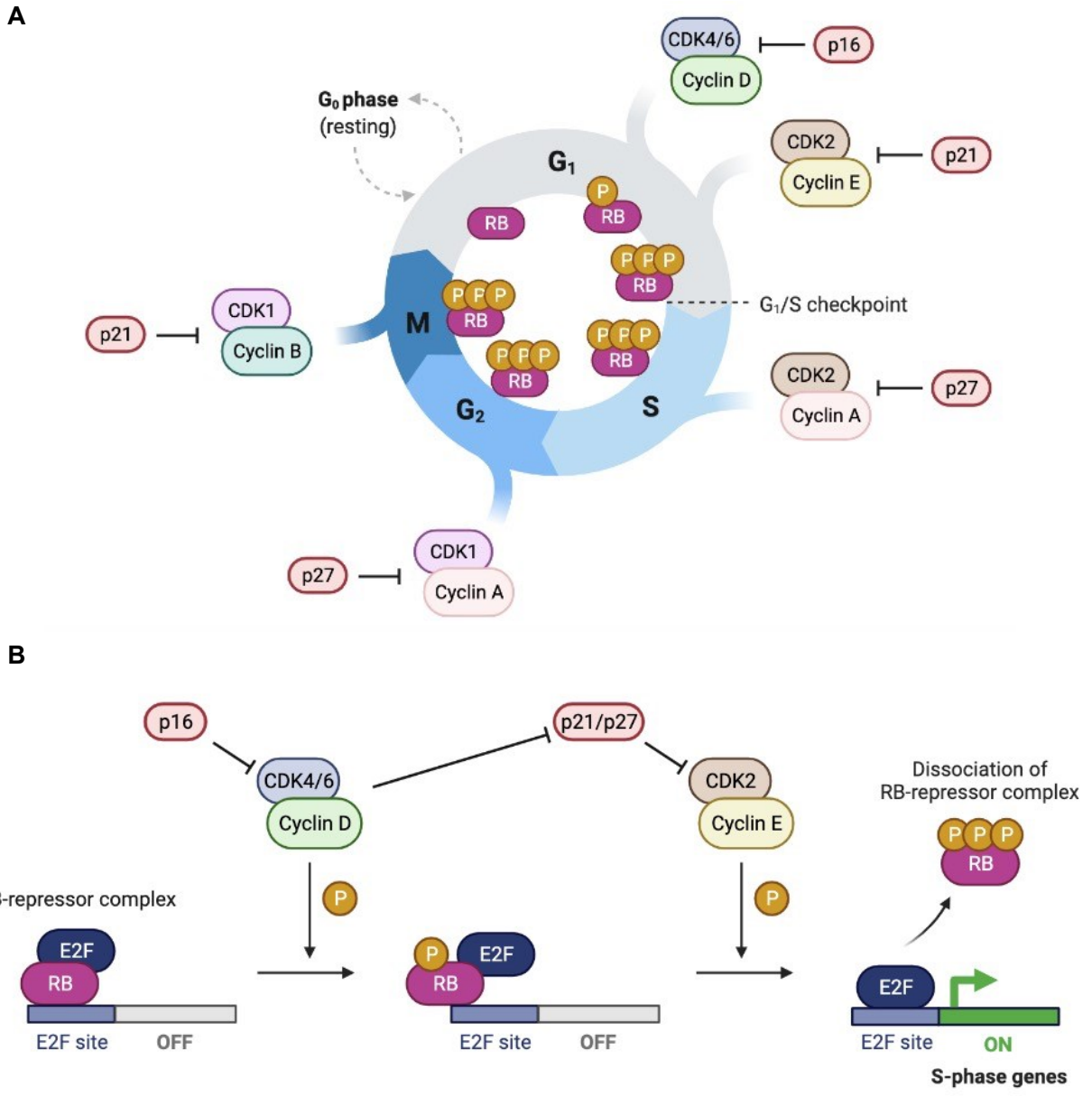


Figure 1. Cyclin-dependent kinases (CDKs) regulate cell cycle progression. (A) Schematic overview of the cell cycle and its regulation by specific CDK-cyclin complexes and phosphorylation status of RB throughout each phase. **(B)** Overview of the G_1/S checkpoint. Created with Biorender (https://biorender.com).

Cell cycle alterations in cancer

Given the crucial role of the RB/E2F pathway in controlling cell cycle progression, it is not surprising that alterations in this pathway resulting in unrestrained cell proliferation have been implicated in a multitude of human cancers. It has been estimated that approximately 90% of human cancers display abnormalities in some component of the RB signaling pathway [13, 14].

For example, *CDK6* gene amplification and overexpression have been described in lymphomas, leukemias, squamous cell carcinoma, gliomas and medulloblastoma. *CDK4* amplification and overexpression have been reported in sporadic melanoma, sporadic breast carcinoma, refractory rhabdomyosarcoma, osteosarcoma, liposarcoma, glioblastoma and neuroblastoma [15]. Additionally, the CDK4 R24C point mutation results in constitutive activation of CDK4 by causing loss of CDK4 binding to $p16^{INK4a}$ in familial melanoma and in a subset of lung cancers and lymphomas [15]. The *CDKN2A* gene which encodes for the naturally occurring CDK4/6 inhibitor $p16^{INK4\alpha}$, is inactivated in a high percentage of human tumors by a variety of mechanisms including deletion, point mutations and hypermethylation [16-18]. Additionally, due to its central role as a mitogen sensor to govern G_1 phase progression, amplification and translocation of the *CCND1* gene encoding for cyclin D1 has been reported in many human cancers [19]. Aberrations in the *CCNE1* gene encoding for cyclin E have also been found [20]. Lastly, the most important CDK substrate, RB, frequently sustains deleterious mutations or epigenetic silencing in human cancers, resulting in constitutively active E2F transcription factors and unrestrained cell proliferation [14, 21].

Given the central role of CDK4/6-cyclin D complexes in governing G_1 phase progression, proof that inactivation of *CCND1*, CDK4 and CDK6 genes can prevent tumor development in mouse models reinforced the view that CDKs are promising cancer-specific therapeutic targets [22, 23].

The development of CDK4/6 inhibitors

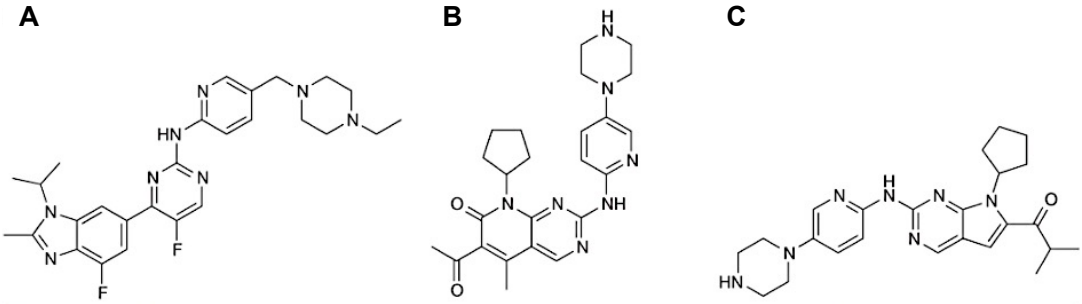
In the early 1990s, broad inhibitors of CDKs such as roscovitine, or flavopiridol, the first CDK inhibitor to enter clinical trials in humans, provided encouraging results in experimental systems

[24, 25]. Although flavopiridol was later approved by the Food and Drug Administration (FDA) for the treatment of acute myeloid leukemia (AML), serious toxicity associated with roscovitine and other non-selective CDK inhibitors limited their clinical applications. In 1994, the discovery of the CDK4 homolog, CDK6, prompted the development of more selective, second generation inhibitors of CDK4 and CDK6 [26].

By 1995, scientists at Parke-Davis began working on the development of a selective CDK4/6 inhibitor, the product of which, palbociclib, formerly known as PD-0332991, was first synthesized in 2001 [27]. Preclinical results showed that of a panel of breast cancer cell lines, estrogen receptor positive (ER^+) cell lines were most sensitive to palbociclib treatment [28]. In 2009, encouraging results of combinatorial use of palbociclib with the standard antiestrogen inhibitor, letrozole, led to a phase II clinical trial, PALOMA-1 [29]. As a result, in early 2015, the FDA approved palbociclib in combination with letrozole, as a first-line treatment for ER^+/human epidermal growth factor 2 negative ($HER2^-$) breast cancer in postmenopausal women [30]. Soon thereafter, two additional CDK4/6 inhibitors were approved by the FDA: ribociclib (LEE011) was approved in March 2017, for the treatment of hormone receptor (HR^+)/$HER2^-$ advanced or metastatic breast cancer in combination with letrozole in women after menopause [31]; this was followed by FDA approval of abemaciclib (LY2835219) in September 2017 for use in combination with fulvestrant, an estrogen receptor degrader, as a second-line treatment in HR^+/$HER2^-$ advanced breast cancer patients displaying disease progression following endocrine therapy. Abemaciclib is also the first CDK4/6 inhibitor to be approved as a single therapy agent for HR^+/$HER2^-$ metastatic breast cancer patients, who have previously received chemotherapy and endocrine therapy [32].

In preclinical models, palbociclib, ribociclib and abemaciclib inhibit CDK4/6 at nanomolar concentrations and effectively arrest cells in the G_1 phase by decreasing phosphorylation of RB. Although these three compounds are all orally available and highly selective ATP-competitive inhibitors of CDK4/6, they have distinct chemical structures and characteristics (**Figure 2**). Structurally, palbociclib is a pyridopyrimidine derivative and among 36 kinases profiled, the CDK4 and CDK6 kinases were the only kinases that palbociclib inhibited in the nanomolar range [33]. Ribociclib is a pyrrolo-pyrimidine derivative and exhibits the highest CDK4/6 selectivity among the three inhibitors. Finally, abemaciclib is a structurally more distinct inhibitor that, with

palbociclib, can penetrate the blood-brain barrier, thus widening their potential application to brain cancers and secondary brain metastases [34, 35]. Abemaciclib inhibits CDK4 and CDK6 at the lowest nanomolar concentrations and is also active against CDK2, CDK7 and CDK9 at nanomolar concentrations. Interestingly, these off-target activities of abemaciclib have been shown to be clinically relevant: CDK7 and CDK9 respectively promote the initiation and elongation of transcription by phosphorylating the carboxy terminal domain (CTD) of RNA polymerase II (RNA Pol II) at various sites [36, 37]. Inhibitors that target CDK7/9, such as flavopiridol, lead to transcriptional suppression of myeloid cell leukemia 1 (Mcl-1), a pro-survival member of the Bcl-2 family that was identified as a key driver of cell survival in diverse cancers, including AML [38].



	Abemaciclib (LY-2835219)	Palbociclib (PD-0332991)	Ribociclib (LEE011)
IC_{50}	CDK1: >1 μM	CDK1: >10 μM	CDK1: >100 μM
	CDK2: >500 nM	CDK2: >10 μM	CDK2: >50 μM
	CDK4: 2 nM	CDK4: 9–11 nM	CDK4: 10 nM
	CDK5: ND	CDK5: >10 μM	CDK5: ND
	CDK6: 5 nM	CDK6: 15 nM	CDK6: 39 nM
	CDK7: 300 nM	CDK7: ND	CDK7: ND
	CDK9: 57 nM	CDK9: ND	CDK9: ND

Figure 2. Highly selective CDK4/6 inhibitors. The chemical structure of CDK4 and CDK6 inhibitors, including **(A)** abemaciclib, **(B)** palbociclib, and **(C)** ribociclib. The half-maximal inhibitory concentration (IC_{50}) of these three inhibitors for a number of cyclin-dependent kinases (CDKs) are also shown. Adapted from O' Leary *et al.* [39].

In addition to breast cancer, CDK4/6 inhibitors have shown promising antitumor activities for the treatment of various types of cancers. Clinical trials are currently evaluating the combination of palbociclib and a MEK inhibitor, PD-0325901 (NCT02022982) or binimetinib (NCT03170206, NCT03981614) for the treatment of *KRAS*-mutant non-small cell lung cancer (NSCLC) and

metastatic unresectable colorectal cancer. The combination of palbociclib, binimetinib and the BRAF-inhibitor encorafenib is also being evaluated in patients with *BRAF*-mutant metastatic melanoma (NCT04720768). Lastly, our group recently showed that preclinical models of SMARCA4-mutant cancers including small cell carcinoma of the ovary, hypercalcemic type (SCCOHT; ~100%) and a NSCLC subset (~10%) are also highly sensitive to CDK4/6 inhibitors [40, 41]. The efficacy of palbociclib in treating SMARCA4-mutant cancers is currently being tested as part of the Canadian Profiling and Targeted Agent Utilization Trial (CAPTUR) (NCT03297606).

Drug resistance to CDK4/6 inhibitors

Despite the recent approval of CDK4/6 inhibitors for the treatment of an advanced breast cancer subtype in combination with antiestrogen therapy and its encouraging anti-tumour activities in various types of cancers, increasing evidence reports the inevitable emergence of resistance to CDK4/6 inhibitors [42, 43]. Cancer therapies are particularly susceptible to the development of resistance and subsequent patient relapse; the same mechanisms that drive cancer initiation and progression also enable tumor cells to adapt rapidly and escape pressures applied by the drug. To date, mechanisms and strategies to combat these resistant cancers have not been extensively investigated and represent the next major clinical challenge with CDK4/6 inhibitors. A better understanding of molecular mechanisms underlying drug resistance is required to help identify novel clinical therapeutic targets and provide possible strategies to overcome this clinical challenge.

Various factors govern the emergence of drug resistance. These include the pre-existence of cancer cell populations intrinsically resistant to treatment (intrinsic resistance), and/or the induction of adaptive responses in cancer cells to counter drug treatment, resulting in the survival of a subset of cells and decreased overall sensitivity to therapy over time (acquired resistance). These adaptations and resistance to treatment are the result of genetic, epigenetic, and/or other molecular events unmasked or directly induced by the pressure of therapies. In the context of CDK4/6 inhibitors, several studies have observed that in response to initial treatment, cells first undergo adaptive changes that may affect durability of therapeutic response, and this is later followed by

acquisition of resistance mechanisms after prolonged drug pressure [44-46]. Although mutations in the *RB1* gene have been observed and result in an irreversible mechanism of resistance, few genetic changes are associated with CDK4/6 inhibitors resistance in patients. Interestingly, no reports of mutations in either *CDK4* or *CDK6* that reduce the binding affinity of CDK4/6 inhibitors have been identified in patients so far. Adaptive response to initial treatment is postulated to play a role in the acquisition of resistance, notably through kinase re-wiring [45]. For example, in ER^+ breast cancer cell lines acutely exposed to palbociclib, cell cycle inhibition was temporary and continued RB phosphorylation was associated with increased cyclin D1 expression; cyclin D1 was found to form non-canonical complexes with CDK2, which was proposed to be the cause of sustained RB phosphorylation [44]. Consistent with this key role of cyclin D1 in modulating drug responses, our group demonstrated that cyclin D1 overexpression confers resistance to CDK4/6 inhibitors in ER^+ breast cancer cells and SMARCA4-deficient ovarian and NSCLC cancer cells, whereases cyclin D1 knockdown can sensitize cancer cells to treatment with these inhibitors [41, 47]. In addition to cyclin D1, decreased $p27^{Kip1}$ expression was shown to contribute to elevated CDK2 activity in acute myeloid leukemia cell lines [48]. Overexpression of *CCNE1* [44], CDK6 [49], and CDK4 [50], are also mechanisms by which cancer cells can bypass CDK4/6 inhibitor mediated G_1 arrest in both experimental models of cancer and patients. Interestingly, a recent study revealed a microRNA (miRNA)-mediated resistance mechanism resulting in increased CDK6 expression in response to CDK4/6 inhibitors in ER^+ breast cancer cells, which was reversible by miRNA inhibition [51].

The role of microRNAs in cancer drug resistance

miRNAs are short, generally 19 to 27 nucleotides, single-stranded non-coding RNAs that regulate gene expression at a post-transcriptional level. Most miRNAs are transcribed from DNA sequences into a primary miRNA transcript (pri-miRNA) which is then processed into a stem-loop structure called precursor miRNA (pre-miRNA). Following export from the nucleus to the cytoplasm, the pre-miRNA is cleaved to yield the mature miRNA, which is then incorporated into the RNA-induced silencing complex (RISC) and functions as a guide by base-pairing with target mRNA to negatively regulate its expression [52]. A wide body of studies have demonstrated that miRNA expression is dysregulated in human cancer and depending on the context, miRNAs may function

as either oncogenes or tumor suppressors. Various mechanisms including amplification or deletion of miRNA genes, abnormal transcriptional regulation of miRNAs and defects in the miRNA biogenesis machinery have been implicated in dysregulated miRNA expression in cancer [53]. Additionally, cumulating evidence now demonstrates that miRNAs can be a major determinant of cancer drug responses, and as a result can also serve as biomarkers for prognosis and survival in response to treatment.

MIR17HG, the gene for the polycistronic *miR-17-92* cluster encoding for six miRNAs (*miR-17*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b-1,* and *miR-92a-1*), is overexpressed in human lymphomas and has also been implicated in contributing to drug resistance [54-57]. Feng *et al*. found that overexpression of *miR-17-5p* induces resistance to paclitaxel, an anti-microtubule agent, and promotes migration and invasion in ovarian cancer cells [58]. Increased *miR-17-5p* expression enhanced epithelial to mesenchymal transition (EMT) by targeting phosphatase and tensin homolog (PTEN) signaling in ovarian cancer cells. Consistent with this, another study found that overexpression of *miR-17-5p* enhances resistance of nasopharyngeal carcinoma (NPC) to radiotherapy through the PTEN/AKT pathway [59]. We recently showed that three miRNAs of the *MIR17HG* cluster (*miR-17*, *miR-19a*, and *miR-20a*) known to target cyclin D1 [60-62], contribute to cyclin D1 deficiency induced by SMARCB1 loss in atypical teratoid rhabdoid tumor (ATRT) [63].

In addition, Cornell *et al.* found that resistance to CDK4/6 inhibitors is centered on increased CDK6 expression in ER^+ breast cancer cells, which is achieved via the suppression of the transforming growth factor β (TGF-β) pathway mediated by exosomal *miR-432-5p* expression [51]. Exosomes are membrane-bound extracellular vesicles secreted by cells and originating from the endosomal pathway; they can protect miRNAs from degradation, enabling them to be stably expressed in the extracellular space. A wide body of studies suggest that exosomes play an important role in establishing a fertile environment to support tumor proliferation, angiogenesis, invasion and premetastatic niche formation [64, 65]. Supporting these *in vitro* findings, expression of *miR-432-5p* was found to be significantly higher in biopsies from ER^+ breast cancer patients with intrinsic or acquired CDK4/6 inhibitor resistance compared to those who were sensitive to treatment. Additionally, analysis of pre-treatment and post-progression biopsies from a patient

with parotid cancer harboring *CDKN2A/B* loss and who had achieved a partial response to ribociclib, displayed an 88-fold increase in *miR-432-5p* expression in the post-progression biopsy relative to the pre-treatment biopsy. These findings support a mechanism of CDK4/6 inhibitor resistance that is independent of inherent genetic mutations but is dependent on secretion of miRNAs by exosomes; this was reversible in resistant-ER^+ breast cancer cell lines and xenografts following a prolonged drug holiday [51]. Consistent with previous observations, the authors also noted that CDK4/6 inhibitor-resistant cells also display increased cyclin D1 expression; but the underlying mechanism is unknown and remains to be investigated.

AIMS

Previous studies have shown that treatment with CDK4/6 inhibitors in cancer cells induces upregulation of cyclin D1 expression, but the mechanism of this adaptive response is not understood. Furthermore, we recently demonstrated that cyclin D1 overexpression conferred drug resistance in experimental models of multiple cancer types and that *MIR17HG* contributes to cyclin D1 deficiency induced by SMARCB1 loss in atypical teratoid rhabdoid tumor. Here, I sought to investigate the role of *MIR17HG* in regulating adaptive cyclin D1 upregulation in response to CDK4/6 inhibition and uncover the underlying mechanism.

METHODS

Cell culture and Viral Transduction

A549, H358, and MCF-7 cancer cell lines were obtained from the ATCC. T47D was obtained from Dr. Morag Park (Goodman Cancer Research Center, Montreal). A549, and H358 were cultured in RPMI-1640 Medium (Gibco) supplemented with 6% fetal bovine serum (FBS), 1% penicillin-streptomycin antibiotics, and 2mM L-glutamine. MCF-7, and T47D were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 6% fetal bovine serum (FBS), 1% penicillin-streptomycin antibiotics, and 2mM L-glutamine. All cell lines were maintained at 37°C in 5% CO_2, were validated by STR profiling and regularly tested for Mycoplasma using a Mycoalert Detection Kit from Lonza (Basel, Switzerland).

Lentiviral transduction was performed following the protocol as described at http://www.broadinstitute.org/rnai/public/resources/protocols. Infected cells (30 hours post-infection) were selected with 2 μg/ml puromycin or 20 μg/ml blasticidin for 2-4 days and plated immediately after selection for further assays.

Compounds and antibodies

Abemaciclib (S7158), palbociclib (S1116) and ribociclib (S7440) were purchased from Selleck Chemicals (Houston, Texas, USA). Antibody against RB (554136) was from BD Pharmigen (San José, CA, USA). Antibodies against CDK6 (13331), Cyclin D1 (2978), MYC (5605), p-MYC S62 (13748), p-RB S795 (9301), and RELA (8242) were from Cell Signalling Technology (Danvers, MA, USA). Antibody against V5 tag (37-7500) was from Thermo Fisher Scientific (Waltham, MA, USA). Antibody against HSP90 (13119) was from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against HSP90 and V5 tag were used at a 1:10000 dilution while all others at a 1:1000 dilution.

Plasmids

Individual shRNA vectors were cloned into pLKO.1-blast by Y. Xue using the following sequence:
shMIR17HG#1: $^{5'}$CCGGCACTTGAGACTTCAGATTATTCTCGAGAATAATCTGAAGTCTC AAGTGTTTTTG$^{3'}$;
shMIR17HG#2: $^{5'}$CCGGGCCACGTGGATGTGAAGATTTCTCGAGAAATCTTCACATCCA CGTGGCTTTTTG$^{3'}$.

The human CRISPR knockout sgRNA targeting RELA (TEDH-1066733) was from TransOMIC provided by the McGill Platform for Cellular Perturbation (MPCP) service of the Goodman Cancer Research Centre and Biochemistry at McGill University (Montreal, QC, Canada).

pLX304-*GFP* and pLX304-*CDK6* were from the TRC3 ORF collections from TransOMIC and Sigma provided by the MPCP. pLX304-*CDK6*^{D163N} kinase-inactive mutant construct was generated by site-directed mutagenesis by Y. Xue. pLX304-*CDK6* was used as a template and PCR amplified using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) using the following primers designed with NEBase Changer (NEB):

pLX304-*CDK6*^{D163N}_Forward, $^{5'}$AAAACTCGCTAACTTCGGCCTTG$^{3'}$;
pLX304-*CDK6*^{D163N}_Reverse, $^{5'}$ATTTGTCCGCTGCTGGTC$^{3'}$.

PCR products were treated with KLD Enzyme Mix from NEB and transformed into Stbl3 competent cells. Clones were verified by sequencing prior to experiments.

Protein lysate preparation and immunoblots

Cells were first seeded in 6-well plates. After 24 hours, cells were washed with cold PBS, lysed with protein sample buffer and collected. For drug assays, the medium was replaced with media containing inhibitors 24 hours post-seeding and collected 24 hours post-treatment, unless otherwise indicated. HSP90 serves as a loading control.

RNA isolation and RT-qPCR

Cells were first seeded in 6-well plates. After 24 hours, cells were washed with cold PBS and harvested for RNA isolation using TRIzol (Invitrogen). According to manufacturer protocols, synthesis of cDNA was performed using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), qRT-PCR assays were carried out using the SYBR® Green master mix (Roche), and Taqman assays (Thermo Fisher Scientific) were used to quantify mature *miR-19a* (Catalog #4427975). Relative mRNA and miRNA levels of each gene were normalized to the expression of the housekeeping gene *ACTB,* and *U6*, respectively. The sequences of the primers for qRT-PCR assays are as follows:

*ACTB*_Forward, $^{5'}$GTTGTCGACGACGAGCG$^{3'}$;
*ACTB*_Reverse, $^{5'}$GCACAGAGCCTCGCCTT $^{3'}$;
*CCND1*_Forward, $^{5'}$GGCGGATTGGAAATGAACTT$^{3'}$;
*CCND1*_Reverse, $^{5'}$TCCTCTCCAAAATGCCAGAG$^{3'}$;
*MIR17HG*_Forward, $^{5'}$GGCCTCCGGTCGTAGTAAAG$^{3'}$;
*MIR17HG*_Reverse, $^{5'}$GCTACTGGTGCAGTTAGGTC$^{3'}$;
*U6*_RT: $^{5'}$AAAATATGGAACGCTTCACGAATTTG$^{3'}$;
*U6*_Forward: $^{5'}$CTCGCTTCGGCAGCACATATACT$^{3'}$;
*U6*_Reverse: $^{5'}$ACGCTTCACGAATTTGCGTGTC$^{3'}$.

Chromatin immunoprecipitations (ChIP)

T47D cells were transduced with pLX304-*CDK6* that carries a V5 epitope tag. Forty million cells were fixed in complete media containing 0.3% formaldehyde for 30 min at 4°C rocking and quenched for 5 min at room temperature by adding 1/10 volume of 2 M glycine. Cells were then pelleted, washed twice with PBS, snap-frozen on dry ice and stored at -80°C. ChIP was performed as previously described with modifications [66]. Frozen pellets were thawed on ice for 30 min, resuspended in 1 mL swelling buffer (25 mM HEPES-NaOH pH 7.5, 10 mM KCl, 0.1% NP-40, 1.5 mM $MgCl_2$ containing 1X protease inhibitor cocktail (P.I.C) from Roche) and incubated for 10 min on ice. Cells were dounced using a B type homogenizer for 20 strokes followed by addition

of 9 mL of MNase digestion buffer (15 mM HEPES-NaOH pH 7.9, 15 mM NaCl, 60 mM KCl, 0.32 M sucrose containing 1X P.I.C) and subsequent centrifugation at 2000 rpm for 7 min at 4°C. The pellet (nuclei) was resuspended in 1 mL of MNase digestion buffer per 4 x 10^7 cells + 3.3 µl $CaCl_2$ per mL of MNase digestion buffer containing 1X P.I.C and then incubated for 15 min at 37°C. MNase (2,000 U/µl) was added at 0.5 µl per 1 x 10^7 cells and incubated for 15 min at 37°C. MNase was then chelated using 10 µM EGTA and placed on ice for 5 min. This was followed by the addition of a 1X volume of a 2X immunoprecipitation (IP) buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 2 mM EDTA, 1 mM EGTA, 0.2% Na-Deoxycholate, 1% N-lauroylsarcosine supplemented with 1X P.I.C) and subsequent addition of Triton X-100 to a 1% final concentration. The lysate was passed through a 21G needle 5 times and centrifuged at 13,000 rpm for 15 min at 4°C. Antibody against V5 was added (5 µg, 37-7500, Thermo Fisher Scientific) to the supernatant (digested chromatin) and incubated overnight at 4°C on an end-over-end rotator. Protein G magnetic beads (Thermo Fisher Scientific) were washed three times with 1X IP buffer prior to capture antibody/chromatin complexes for 2 hours at 4°C on an end-over-end rotator. Beads were washed 4 times for 5 min each time with RIPA buffer (50 mM HEPES-KOH pH 7.5, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% N-lauroylsarcosine) and once with 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl. DNA was eluted at 65°C for 17 min with fresh 100 µl of 100 mM $NaHCO_3$, 1% SDS. The supernatant was recovered, 5 µl of 5 M NaCl was added and crosslinks were reversed overnight at 65°C. DNA was treated with 3 µl RNAseA for 30 min at 37°C followed by 5 µl proteinase K (20 mg/mL) for 1 hour at 56°C. Finally, DNA was purified using the DNA Clean & Concentrator-5 kit (D4014) from Zymo Research and used for subsequent RT-qPCR assays. The sequence of the primers used are as follows:

*MIR17HG*_Upstream_Forward, $^{5'}$TGGACTATGTTGTGCCTTCCCTG$^{3'}$;
*MIR17HG*_Upstream_Reverse, $^{5'}$GTCTCGGGCGCATCTTACAAT$^{3'}$;
*MIR17HG*_Promoter_Forward, $^{5'}$GGAGTGGGGCTTGTCCGTA$^{3'}$;
*MIR17HG*_Promoter_Reverse, $^{5'}$CGAAGGACCATGTGGGTGAATG$^{3'}$;
*EGR1*_Promoter_Forward, $^{5'}$TGTACGTCACGACGGAGGC$^{3'}$;
*EGR1*_Promoter_Reverse, $^{5'}$GATCTCTCGCGACTCCCCG$^{3'}$;
*VEGFA*_Promoter_Forward, $^{5'}$GAACCTTGGTGGGGGTCGAG$^{3'}$;
*VEGFA*_Promoter_Reverse, $^{5'}$CTGCGGACGCTCAGTGAAG$^{3'}$.

Statistical analysis

Statistical significance was calculated by one-way ANOVA, Dunnett's multiple comparison test. Prism 8 software was used to generate graphs and statistical analyses. Error bars represent mean ± standard deviation (SD). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

RESULTS

Treatment of CDK4/6 inhibitors results in adaptive upregulation of cyclin D1 by suppressing *MIR17HG* in cancer cells

We and others have shown that treatment with CDK4/6 inhibitors induces upregulation of cyclin D1 expression in breast and pancreatic cancer cells [44, 47, 67]. However, the mechanism underlying this adaptive cyclin D1 feedback upregulation remains to be understood. We recently showed that *MIR17HG,* a microRNA cluster that encodes for six miRNAs, three of which have been shown to target cyclin D1, contributes to cyclin D1 deficiency induced by SMARCB1 loss in atypical teratoid rhabdoid tumor (ATRT) [63]. To test whether CDK4/6 inhibition upregulates cyclin D1 by suppressing *MIR17HG*, a former lab member, Tim Kong (T.K.), treated ER^+ breast cancer cell lines (T47D, MCF-7) and *KRAS*-mutant NSCLC cell lines (H358, A549) with three different FDA-approved CDK4/6 inhibitors: palbociclib (300 nM), abemaciclib (150 nM), or ribociclib (1 μM) for 24 hours and examined mRNA expression levels of *CCND1* and *MIR17HG.* Supporting our hypothesis, RT-qPCR analysis indeed showed that *CCND1* mRNA was inversely correlated with *MIR17HG* expression upon CDK4/6 inhibition (Figure 3A, B). To further investigate this, I treated T47D and H358 cells with increasing doses of palbociclib, ranging from 50 nM to 300 nM, for 24 hours. Western blot analysis showed that expression of phosphorylated RB at serine 795 (S795) was inversely proportional to palbociclib dose used for treating these cells (Figure 3C), which reflects dosage-dependent inhibition of CDK4/6 kinase activity; however, maximum upregulation of cyclin D1 protein expression was already observed when treated with 50 nM of palbociclib (Figure 3C). Consistently, this 50nM palbociclib treatment was sufficient to result in full upregulation of *CCND1* mRNA expression and associated downregulation of *MIR17HG* expression (Figure 3D). This adaptive regulation of cyclin D1/*MIR17HG* preceding full suppression of phospho-RB S795 suggests a potential E2F-independent mechanism induced by CDK4/6 inhibitors.

This suppression of *MIR17HG* upon CDK4/6 inhibition is predicted to lead to reduction of its coding miRNAs, which in turn would result in elevation of their target genes. To assess this, I examined the expression of predicted target genes of miRNAs coded by *MIR17HG* in a publicly available RNA-seq dataset generated in T47D cells treated with 1 μM palbociclib for 48 hours [68]. Specifically, I investigated the predicted target genes of *miR-17*, *miR-19a* and *miR-20a*, three mature miRNAs generated from *MIR17HG* known to target cyclin D1 in several other contexts [60-62]. Gene Set Enrichment Analysis (GSEA) shows that palbociclib treatment indeed upregulated mRNA expression of genes known to be targeted by these miRNAs (Figure 3E), which is consistent with downregulation of *MIR17HG* upon CDK4/6 inhibition. In keeping with this, in this same dataset, palbociclib treatment specifically reduced expression of *MIR17HG* by almost 8 folds, while cyclin D1 expression was upregulated by 2 folds compared to the untreated condition, which is similar to our observations (Figure 3A, D).

To confirm the role of *MIR17HG* in regulating cyclin D1 in ER^+ breast cancer and *KRAS*-mutant NSCLC, I knocked down *MIR17HG* using two independent shRNAs in T47D and H358 cells (Figure 3G). Indeed, cyclin D1 protein and mRNA expression was upregulated in both cell lines when *MIR17HG* was suppressed (Figure 3F, G), which is consistent with the well-established role of miRNAs in inducing mRNA degradation and inhibiting translation [69]. Next, I performed rescue experiments to evaluate the contribution of *MIR17HG* downregulation to the adaptive upregulation of cyclin D1 upon CDK4/6 inhibition. I ectopically expressed *MIR17HG* in T47D and H358 cells in combination with palbociclib treatment. To account for effective processing of exogenous *MIR17HG*, I measured expression of mature *miR-19a*. Consistent with Figure 1A-D, CDK4/6 inhibition suppressed *miR-19a* in both T47D and H358 cells (Figure 3I). Ectopic expression of *MIR17HG* in palbociclib-treated cells led to an expected increase in *miR-19a* expression and blunted cyclin D1 upregulation at both mRNA and protein levels (Figure 3H, I). Further repeats of this experiment are required for statistical analysis of results (Figure 3I). Together, these data demonstrate that treatment of CDK4/6 inhibitors results in upregulation of cyclin D1 at least in part through suppressing *MIR17HG* expression in ER^+ breast cancer and *KRAS*-mutant NSCLC.

If treatment with CDK4/6 inhibitors results in suppression of *MIR17HG* leading to upregulation of cyclin D1, this adaptive response may contribute to acquired resistance to CDK4/6 inhibition since we have shown that increased cyclin D1 expression confers resistance to CDK4/6 inhibitors in ER^+ breast cancer and SMARCA4-deficient SCCOHT cells [40, 47]. To address this, T.K. analysed a publicly available data set where micro-RNA sequencing (miRNA-seq) was performed on 44 tumors biopsies from patients who received CDK4/6 inhibitor treatment [51]. As shown in Figure 3J, we found that tumor biopsies from breast cancer patients who developed resistance to CDK4/6 inhibitor treatment showed a marked decrease of miRNAs encoded by *MIR17HG* compared to sensitive tumors. These findings in patient tumor samples are in line with the model that this adaptive response of feedback regulation of cyclin D1/*MIR17HG* may contribute to the development of acquired resistance to CDK4/6 inhibitors.

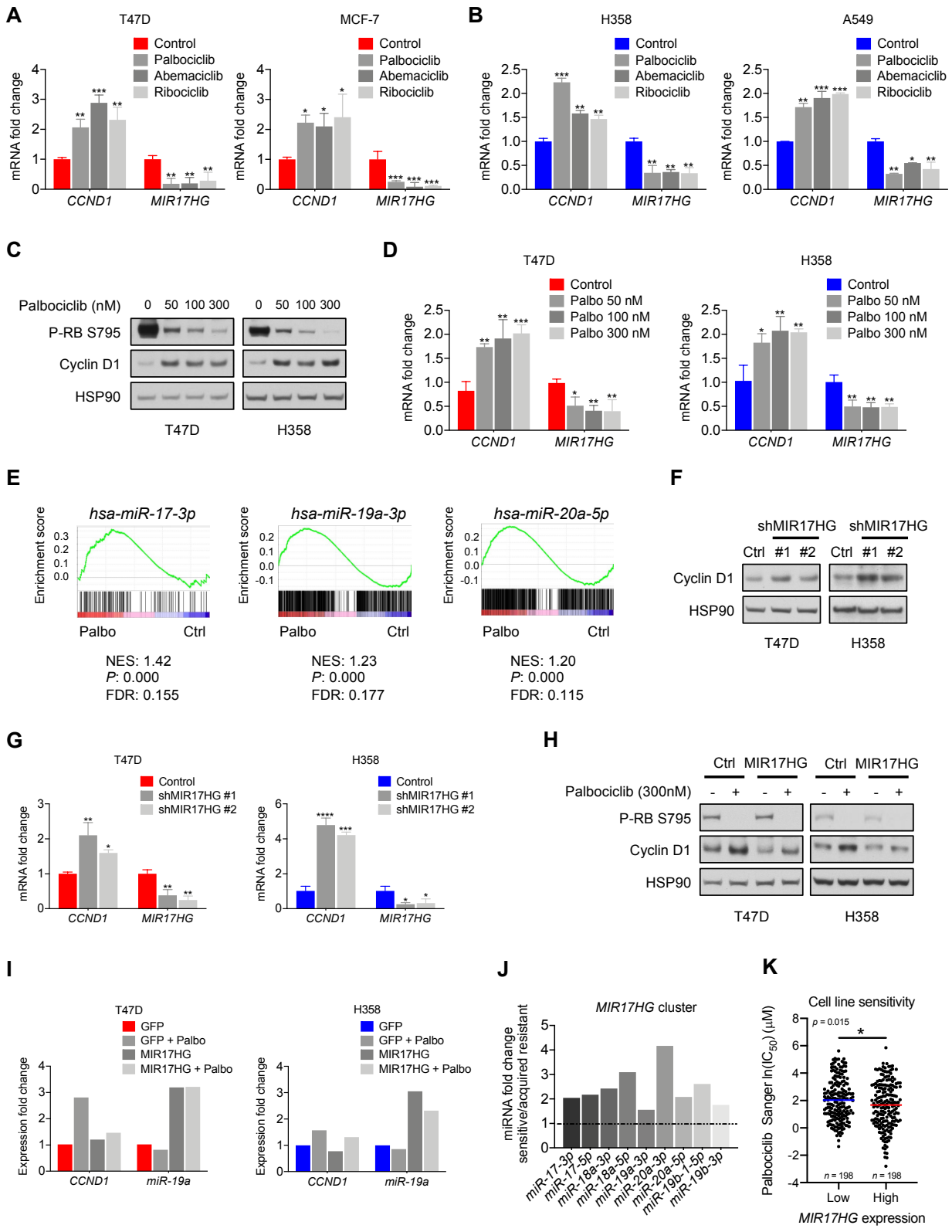


Figure 3. Treatment with CDK4/6 inhibitors results in adaptive upregulation of cyclin D1 by suppressing *MIR17HG* in cancer cells. (A) RT-qPCR analysis of *CCND1* and *MIR17HG* mRNA

expression after 24-hour treatment with 300 nM palbociclib, 150 nM abemaciclib, or 1 μM ribociclib in ER^+ breast cancer (T47D, MCF-7), **(B)** and *KRAS*-mutant NSCLC (H358, A549). *CCND1* and *MIR17HG* mRNA expression was normalized to *ACTB*. **(C)** Cyclin D1 protein expression in T47D, and H358 upon treatment with 0, 50, 100, and 300 nM palbociclib for 24 hours, **(D)** and RT-qPCR analysis of *CCND1* and *MIR17HG* mRNA expression normalized to *ACTB*. **(E)** Analysis of RNA-seq data in T47D cells treated with 1 μM palbociclib for 48 hours [68], using Gene Set Enrichment Analysis (GSEA). GSEA plots for the target genes of 3 mature miRNAs generated from *MIR17HG* known to target cyclin D1 with their respective normalized enrichment score (NES) and false discovery rate (FDR). **(F)** RT-qPCR analysis of *CCND1* and *MIR17HG* mRNA expression normalized to *ACTB*, **(G)** and cyclin D1 protein expression after knockdown of *MIR17HG* using two independent shRNAs in T47D and H358. **(H)** RT-qPCR analysis of *CCND1* and *miR-19a* mRNA expression. *miR-19a* is a mature miRNA generated from *MIR17HG*. *CCND1* mRNA was normalized to *ACTB*, and *miR-19a* expression was normalized to *U6*, (**I**) and cyclin D1 protein expression in T47D and H358 cells expressing *MIR17HG* and/or treated with 300 nM palbociclib for 24 hours. **(J)** Analysis of miRNAseq data in tumor biopsies taken from patients who received CDK4/6 inhibitor treatment and were grouped based on their response into sensitive or acquired resistance [51]. Fold change in the expression of miRNAs encoded by the *MIR17HG* cluster of sensitive over acquired CDK4/6 inhibitor resistant patient tumor biopsies. **(K)** The half-maximal inhibitory concentration (IC_{50}) to palbociclib of two groups of cancer cell lines, categorized based on their expression levels of *MIR17HG* [70].

CDK6 regulates *MIR17HG* expression

CDK6 has been shown to have alternative functions beyond its key role in regulating cell cycle, including direct functions in transcription [11, 71]. To investigate the potential role of CDK6 in regulating transcription of *MIR17HG*, T.K. examined the correlation between *CDK6* mRNA expression and *MIR17HG* expression. Pearson correlation plots from publicly available datasets in BRCA (n=994) and METABRIC (n=1904) cohorts [72, 73] showed a mild but significant positive correlation between *CDK6* mRNA and *MIR17HG* expression (Figure 4A), which is consistent with the notion that CDK6 may play a role in regulating *MIR17HG* expression. Additionally, published chromatin immunoprecipitation sequencing (ChIP-seq) data also showed a clear enrichment of CDK6 occupancy in the promoter region of *MIR17HG* in squamous cell carcinoma (SCC) KB cells (Figure 4B) [12]. This suggests that CDK6 may directly regulate

transcription of *MIR17HG.* To confirm this CDK6 occupancy in our cell line models, I expressed a CDK6 wild-type construct fused to a V5 epitope tag in T47D cells and performed ChIP experiments using an anti-V5 antibody. The use of a tagged CDK6 construct was necessary due to the fact that currently available antibody against endogenous CDK6 was not suitable for ChIP. As shown in Figure 4C, ChIP-qPCR detected significant CDK6 occupancy at the *MIR17HG* promoter but not at an upstream region of the locus. This enrichment of CDK6 occupancy at the *MIR17HG* promoter was comparable to its occupancies detected at *EGR1* and *VEGFA* promoter regions, which served as positive controls for CDK6 binding [11, 74]. Together, these data indicate that CDK6 promotes *MIR17HG* expression by directly interacting with its gene locus. As a result, CDK4/6 inhibition downregulates *MIR17HG,* which contributes to increased cyclin D1 expression in ER^+ breast cancer and *KRAS*-mutant NSCLC cells.

To further demonstrate that the kinase activity of CDK6 is required for the regulation of *MIR17HG*, I ectopically expressed a CDK6 wild-type and catalytically inactive $CDK6^{D163N}$ mutant construct in T47D and H358 cells. As expected, ectopic expression of the wild-type construct led to increased *MIR17HG* expression and associated suppression of cyclin D1 at both mRNA and protein levels (Figure 4D, E). Interestingly, expression of the catalytically inactive mutant construct resulted in the reverse observation where *MIR17HG* expression was suppressed and cyclin D1 mRNA and protein was upregulated. These results suggest that the catalytically inactive mutant may act in a dominant negative fashion, likely by competing with endogenous CDK6 for cyclin D and possibly also the occupancy at the *MIR17HG* promoter; this remains to be validated. Nevertheless, these findings show that the kinase activity of CDK6 is required to promote transcription of *MIR17HG*.

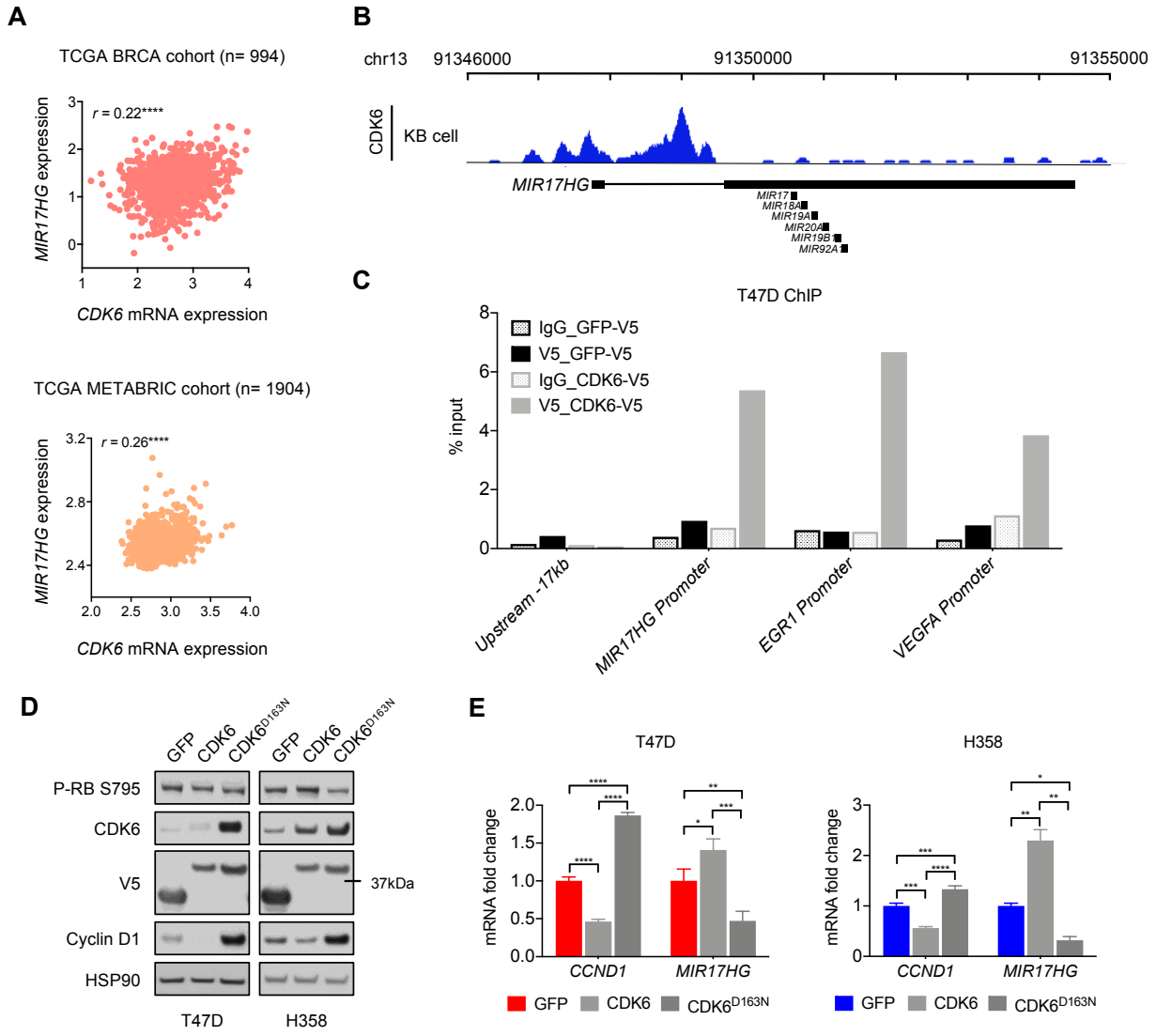


Figure 4. CDK6 regulates *MIR17HG* expression. (A) Pearson correlation of *CDK6* mRNA and *MIR17HG* expression in BRCA (n=994) and METABRIC (n=1904) cohorts. **(B)** ChIP-seq data in vicinities of the *MIR17HG* locus indicate enhanced CDK6 binding at promoter regions in KB cells. **(C)** V5 ChIP-qPCR for regions at *MIR17HG* promoter and upstream control site in T47D cells overexpressing a GFP or CDK6 construct fused to a V5 epitope tag. IgG served as an antibody control. *EGR1* and *VEGFA* promoter regions are positive controls for CDK6 binding. **(D)** Cyclin D1 protein expression in T47D and H358 cells overexpressing a CDK6 wild-type construct or a catalytically inactive mutant CDK6^{D163N} construct, **(E)** and RT-qPCR analysis of *CCND1* mRNA and *MIR17HG* expression normalized to *ACTB*.

CDK6 and the NF-κB RELA subunit may function together to activate *MIR17HG* expression

Next, I sought to further study the mechanism by which CDK6 regulates transcription of *MIR17HG*. CDK6 lacks nuclear localization sequences and DNA-binding domains, and thus needs to contact its specific sites indirectly through DNA-binding proteins. Here, I aimed to extend my findings by investigating which cofactor(s) CDK6 interacts with to regulate *MIR17HG* expression (Figure 5).

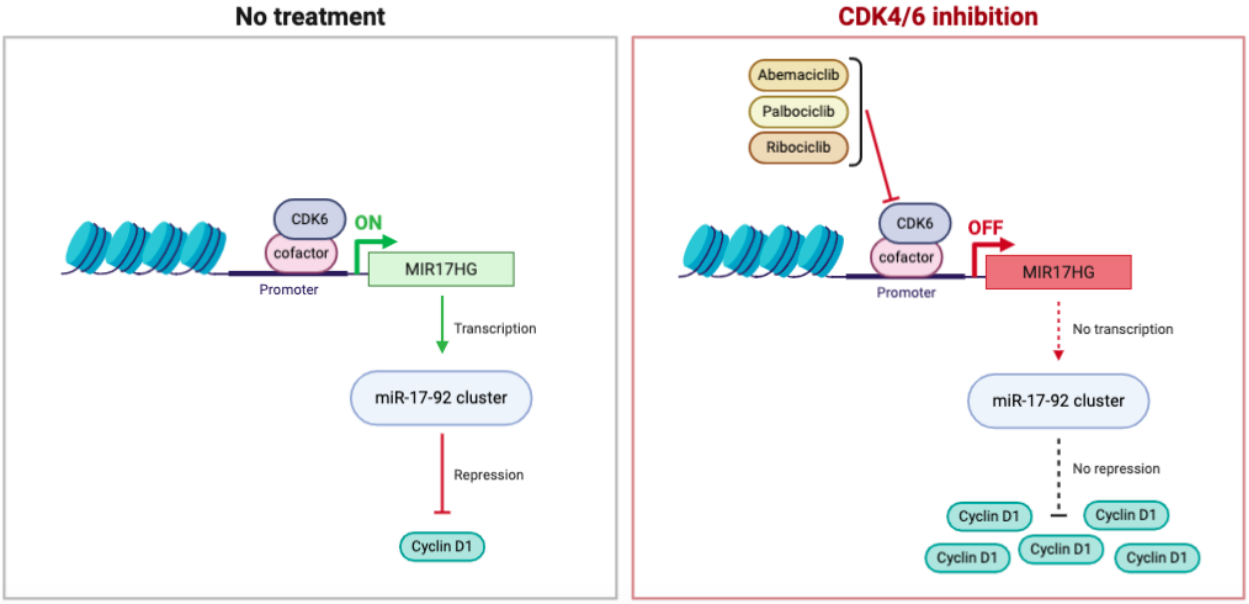


Figure 5. A schematic diagram representing the adaptive regulation observed upon CDK4/6 inhibition in cancer. CDK6 interacts directly at the *MIR17HG* locus and promotes its transcription. *MIR17HG* encodes a cluster of six miRNAs (*miR-17-92*), three of which (*miR-17, miR-19a,* and *miR-20a*) have been shown to induce mRNA degradation and inhibit translation of cyclin D1. Upon CDK4/6 inhibition with abemaciclib, palbociclib or ribociclib, the catalytic activity of CDK6 is compromised, resulting in suppression of *MIR17HG* expression and associated cyclin D1 upregulation. Created with Biorender (https://biorender.com).

The coactivator repertoire underlying the transcriptional functions of nuclear CDK6 is still incompletely understood. Handschick *et al.* showed that CDK6 physically and functionally interacts with the nuclear factor kappa B (NF-κB) subunit RELA, also known as p65 [12]. NF-κB consists of a family of inducible transcription factors that regulate a large array of genes involved

in different processes of the immune and inflammatory responses [75]. It is activated by phosphorylation-dependent proteolytic degradation of inhibitors of NF-κB (IκBs) in response to inducers such as pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) [76]. ChIP-seq experiments in KB cells revealed a striking overlap of CDK6 and RELA at defined chromatin regions, thus providing evidence for a specific role of nuclear CDK6 in mediating expression of numerous NF-κB genes [12]. Exploring these existing data sets, I examined the *MIR17HG* locus and found an enrichment of RELA occupancy in KB and A549 cells, which directly overlapped with CDK6 occupancy in KB cells (Figure 6A). These observations suggest a potential interaction between CDK6 and RELA at the *MIR17HG* promoter region [12, 77].

To evaluate the role of the CDK6/RELA interaction in regulating *MIR17HG* expression, I utilized the CRISPR-Cas9 system to knockout *RELA* in T47D cells (in a polyclonal fashion). I found that *RELA* knockout led to decreased *MIR17HG* expression (Figure 6C), similar to CDK4/6 inhibition in control cells. This suggests that RELA is required for *MIR17HG* expression. However, in *RELA*-knockout but not control cells, this marked decrease in *MIR17HG* expression was not associated with cyclin D1 protein and mRNA upregulation even upon palbociclib treatment in T47D cells (Figure 6B, C). This can be explained by the fact that NF-κB has been shown to directly stimulate cyclin D1 transcription in T47D cells [78], which may be dominant over *MIR17HG* in regulating cyclin D1. Nonetheless, these findings indicate that RELA plays a role in regulating *MIR17HG* expression upon CDK4/6 inhibition.

Next, I examined the promoter region of *MIR17HG* for RELA consensus binding sequence, $^{5'}$NGGRNYYYCC$^{3'}$, where N denotes any nucleotide, R a purine (A or G), and Y a pyrimidine (C or T) [79]. However, there is no RELA consensus binding sequence in the vicinity of the *MIR17HG* locus, suggesting that CDK6 and RELA may be recruited to the *MIR17HG* locus through other factor that directly binds to this region. Supporting this, Handschick *et al.* found that 78% of top CDK6 ChIP-seq peaks have no consistent *cis*-element requirements and upon analysis of motifs for top 1000 peaks representing RELA or CDK6 binding, only 51% were enriched for NF-κB binding sites, including RELA [12].

To investigate how CDK6 and RELA are recruited to the *MIR17HG* locus, I first examined transcription factors known to have binding sites in the *MIR17HG* locus. *MYC*, a member of the MYC family proto-oncogenes, encodes a transcription factor, MYC, that regulates cell proliferation and apoptosis [80] and has been shown to bind to a conserved $^{5'}$CATGTC$^{3'}$ motif in the first intron of *MIR17HG* and stimulate its transcription [81, 82]. Interestingly, I found that ChIP-seq peaks for MYC occupancy in A549 and MCF-7 cells directly overlap with enriched RELA and CDK6 occupancy at the *MIR17HG* locus (Figure 6A) [12, 77, 83, 84]. This raises the possibility that CDK6/RELA may be recruited to the *MIR17HG* promoter by MYC.

A recent study showed that phosphorylation of MYC at S62 by CDK2 promotes its association with the miR-571 promoter region, but the MYC^{S62A}-mutant is unable to associate at these regions [85]. Additionally, CDK4/6 was shown to directly phosphorylate MYC at serine 62 (S62) [86]. Consistently, I found that CDK4/6 inhibition downregulated phosphorylation of MYC at S62 in T47D and H358 cells (Figure 6D). Interestingly, knockout of RELA in T47D cells also downregulated MYC phosphorylation, which was further supressed in control and RELA-knockout cells treated with palbociclib (Figure 6B). Furthermore, phospho-MYC levels also correlated with *MIR17HG* expression (Figure 6B, C), suggesting that phosphorylation status of MYC might modulate its capacity to act as a transcriptional factor at the *MIR17HG* locus. Taken together, these data suggest that CDK6, likely through RELA, modulates phosphorylation of MYC at S62, which may be required for its association to the *MIR17HG* locus and subsequent transcription.

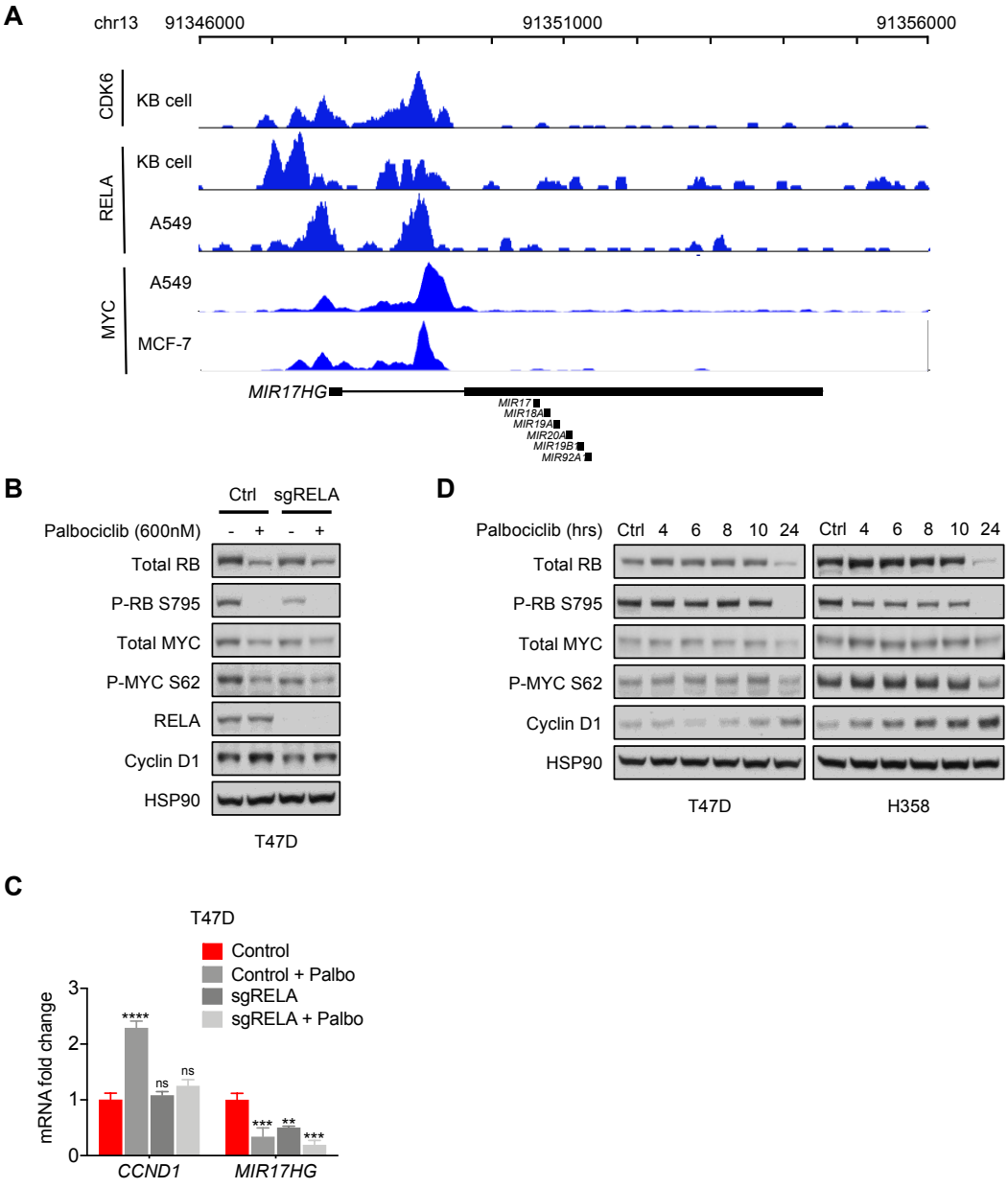


Figure 6. CDK6 and the NF-κB RELA subunit modulate phosphorylation of MYC at S62, regulating its capacity to promote *MIR17HG* transcription. (A) ChIP-seq data in vicinities of the *MIR17HG* locus indicate enhanced CDK6 binding at promoter regions, which directly overlaps with RELA and MYC binding in KB, A549 and MCF-7 cells [12, 77, 83, 84]. **(B)** Cyclin D1, total MYC and phospho-MYC S62 expression in RELA knockout T47D cells using the CRISPR-Cas9 system, **(C)** and RT-qPCR analysis of *CCND1* and *MIR17HG* mRNA expression normalized to *ACTB*. **(D)** Cyclin D1, total MYC, phospho-MYC S62 expression after 4 to 24 hour-treatment with 300 nM palbociclib in T47D and H358 cells.

Since CDK6 has been shown to phosphorylate MYC at S62, I first wanted to check if CDK6/MYC interactions could be directly detected. I performed coimmunoprecipitations experiments where I expressed a wild-type CDK6-V5 construct and used an anti-V5 antibody as well as used an anti-MYC antibody to pull-down CDK6. However, both approaches failed to show an interaction between CDK6 and MYC (data not shown). This suggests that their interaction may be too transient to be stabilized or alternatively, CDK6 may be brought in close proximity to MYC via the NF-κB RELA subunit, which may act as a bridging factor between CDK6 and MYC at the *MIR17HG* locus (Figure 7), but this remains to be confirmed.

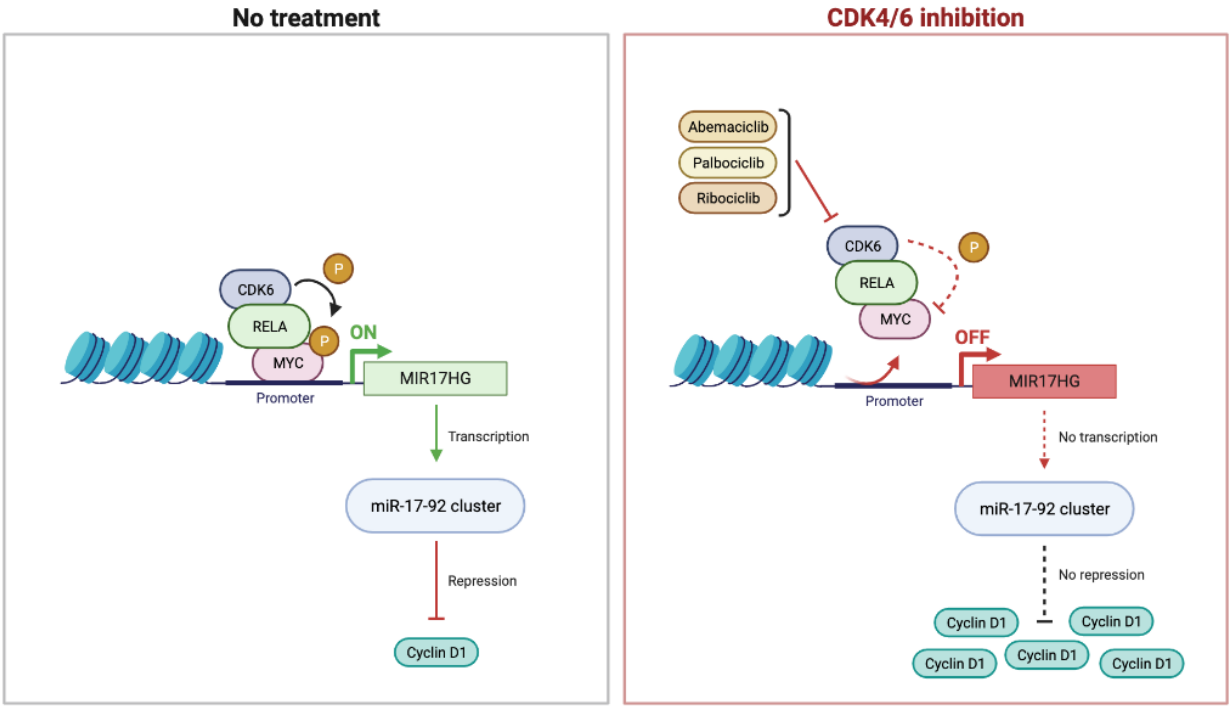


Figure 7. Proposed model for the adaptive cyclin D1 upregulation observed upon CDK4/6 inhibition in cancer. CDK6 is recruited to the *MIR17HG* locus likely via the NF-κB RELA subunit and also stimulates MYC in a phosphorylation dependent manner to promote *MIR17HG* transcription. *MIR17HG* encodes a cluster of six miRNAs (*miR-17-92*), three of which have been shown to induce mRNA degradation and inhibit translation of cyclin D1. Upon CDK4/6 inhibitor treatment, the catalytic activity of CDK6 is compromised, resulting in its inability to phosphorylate MYC at the S62 residue, which may be crucial for the association of MYC to the *MIR17HG* promoter and subsequent transcription of the cluster. As a result, CDK4/6 inhibition leads to decreased *MIR17HG* expression and associated cyclin D1 upregulation. Created with Biorender (https://biorender.com).

DISCUSSION

CDK4/6 inhibitors in combination with endocrine therapy is the new standard of care for patients with ER^+ advanced breast cancer [23]. These inhibitors have also shown promising antitumor activities for treating *KRAS*-mutant NSCLC patients and preclinical models of SMARCA4-mutant cancers including SCCOHT (100%) and a NSCLC subset (~ 10%) [40, 41, 87]. However, adaptive response to CDK4/6 inhibitors has been observed and likely contributes to the development of drug resistance seen in patients [42, 43]. Much effort is now under way to enable the development of treatment options to overcome this clinical challenge, which necessitates a thorough understanding of the mechanisms of resistance to CDK4/6 inhibitors. Relevant to this thesis, previous studies have shown that treatment with CDK4/6 inhibitors in breast and pancreatic cancer cells induces upregulation of cyclin D1 expression [44, 47, 67]. Furthermore, we recently demonstrated that cyclin D1 overexpression conferred drug resistance, while cyclin D1 knockdown sensitized cells to CDK4/6 inhibition in experimental models of multiple cancer types [40, 47]. In this thesis, we aimed to uncover the underlying mechanism of adaptive cyclin D1 upregulation in response to CDK4/6 inhibitors.

Previously, we showed that upregulation of the microRNA cluster *MIR17HG* contributes to cyclin D1 deficiency induced by SMARCB1 loss in ATRT [63]. *MIR17HG* encodes for six miRNAs, three of which have been shown to target cyclin D1 [60-62]. Therefore, we hypothesized that *MIR17HG* may be involved in the adaptive upregulation of cyclin D1 in response to CDK4/6 inhibition. Indeed, we showed that cyclin D1 upregulation is associated with decreased *MIR17HG* expression upon CDK4/6 inhibition in ER^+ breast cancer and *KRAS*-mutant NSCLC cells. Further supporting the potential role of *MIR17HG* in modulating drug responses to CDK4/6 inhibitors in patients, we found that breast tumor biopsies that developed resistance to a CDK4/6 inhibitor showed significant decrease of miRNAs encoded by *MIR17HG* compared to sensitive tumors, in an existing miRNA-seq data set of patient tumors [51]. Interestingly, we also noted that sensitive patients compared to patients who were intrinsically resistant to abemaciclib did not display an increase in the expression of *MIR17HG*- encoded miRNAs, suggesting that adaptive response such as decreased *MIR17HG* expression/upregulation of cyclin D1 may contribute to the development of acquired drug resistance. However, RNA-seq was not performed in the same tumor samples in

this study, which prevents the validation of the inverse expression correlation between *MIR17HG* and cyclin D1 in these tumor biopsies. Nevertheless, these observations are consistent with the potential role of feedback regulation along the *MIR17HG*-cyclin D1 axis in acquired resistance to CDK4/6 inhibitors. Moreover, analysis of the palbociclib IC_{50} of a panel of cancer cell lines revealed that cell lines expressing higher levels of *MIR17HG* are more sensitive to palbociclib treatment (Figure 3K) [70]. To further support this, we are generating palbociclib-resistant clones in cell line models of ER^+ breast cancer (T74D) and *KRAS*-mutant NSCLC (H358) through chronic exposure as we have done previously [47]. It would be important to assess if some of the resistant clones exhibit decreased *MIR17HG* expression/upregulation of cyclin D1, and also evaluate if overexpression of *MIR17HG* expression can restore their sensitivity to CDK4/6 inhibitors. This would help to better establish the causal role of *MIR17HG*-mediated cyclin D1 contribution to CDK4/6 inhibitors resistance in ER^+ breast cancer and *KRAS*-mutant NSCLC.

The major focus of this thesis was to dissect the molecular mechanism underlying this adaptive regulation of the *MIR17HG*-cyclin D1 axis in response to CDK4/6 inhibition. To this end, we found that CDK6 regulates *MIR17HG* expression at least in part by directly interacting with its gene locus as shown by the ChIP experiments in T47D cells. Additionally, our experiments using wild-type and kinase inactive mutant if CDK6 confirms that CDK6 kinase activity is required to promote transcription of *MIR17HG*, which is in line with our observations that CDK4/6 inhibitors induce downregulation of *MIR17HG* expression. Despite the high level of homology between CDK4 and CDK6, increasing evidence have demonstrated that CDK6 have alternative functions beyond its key role in regulating cell cycle, notably in transcription regulation. CDK6 was found to act as a transcriptional regulator at the promoter of both *CDKN2A* and *VEGFA* [11]. Handschick *et al.* also found that, while CDK4/CDK6 co-regulate expression of a set of inflammatory genes [12], CDK6, but not CDK4, occupies active promoters across the genome, including the *IL-8* gene, a major inflammatory mediator. This suggests that CDK4 may indirectly regulate gene expression of these targets. To address whether *MIR17HG* regulation is specific to CDK6, the role of CDK4 in regulating *MIR17HG* needs to be evaluated. First, RNAi-mediated knockdown of CDK6 or CDK4 needs to be performed in T47D and H358 cells to analyze their individual contribution to the regulation of the *MIR17HG*-cyclin D1 axis. Furthermore, overexpression experiments using CDK4 wild-type and catalytically inactive $CDK4^{D158N}$ mutant construct can also be conducted to

further collaborate with the finding of RNAi. Additionally, CDK4 occupancy at the *MIR17HG* locus can be examined using the same configuration as for CDK6 to allow direct comparison, by expressing CDK4 constructs fused to the same V5 epitope tag followed by ChIP-qPCR experiments using an anti-V5 antibody.

While our results show that CDK6 regulates *MIR17HG* expression in part by binding to its gene locus, it does not rule out the contribution of other cell cycle related transcription factors in the regulating *MIR17HG* expression upon CDK4/6 inhibition. In fact, chromatin immunoprecipitation experiments in HeLa cells revealed that endogenous E2F1, E2F2, and E2F3 bind to the promoter of the *MIR17HG* gene and regulate its transcription [88]. Given that CDK4/6 inhibitors function by preventing the induction of E2F-target genes, *MIR17HG* may also be downregulated in our cell models through E2F repression upon CDK4/6 inhibition. However, our findings suggest an alternative mechanism through which CDK6 regulates *MIR17HG* expression by directly interacting with its gene locus. Supporting this, low dose of palbociclib treatment (50 nM) in T47D and H358 cells was sufficient to result in full downregulation of *MIR17HG*/upregulation of *CCND1*, which preceded full suppression of phospho-RB S795 achieved in higher dose of palbociclib (300 nM). Additionally, ectopic expression of CDK6 upregulated *MIR17HG* expression but was not associated with increased phospho-RB S795 expression. This suggests that, in our model systems tested, CDK6-mediated regulation of *MIR17HG* may also occur through an E2F-indpendent mechanism. Furthermore, we have previously generated MCF-7 palbociclib-resistant clones, some of which show loss of RB and phospho-RB S795 expression but display increased cyclin D1 expression [47], which may be mediated by *MIR17HG,* but remains to be confirmed. To further support this, it would be important to use the CRISPR-Cas9 system to generate RB1-knockout in T47D and H358 cells, which would display constitutively active E2Fs given the inability of these cells to form RB/E2F-repressor complexes and examine whether palbociclib treatment in these clones can still results in adaptive feedback regulation of the *MIR17HG*-cyclin D1 axis. These experiments are important to further establish the contribution of CDK6 in directly regulating *MIR17HG* expression.

Since CDK6 lacks DNA-binding domains, we also investigated which co-factors are involved in CDK6-mediated *MIR17HG* regulation. The NF-κB RELA subunit has been shown to physically

and functionally interact with CDK6 to regulate expression of inflammatory genes [12]. Analysis of ChIP-seq experiments performed in squamous cell carcinoma KB cells provides evidence that the CDK6/RELA interaction is also found at the *MIR17HG* locus. To evaluate the contribution of RELA in regulating *MIR17HG* expression, we used the CRISPR-Cas9 system to knockout RELA in ER^+ breast cancer cells and found that loss of RELA results in decreased *MIR17HG* expression, similar to treatment with CDK4/6 inhibitors. This indicates that RELA is indeed required for *MIR17HG* expression in these breast cancer cells, which remains to be confirmed in *KRAS*-mutant NSCLC cells. In addition, ChIP experiments need to be performed to confirm RELA binding to the *MIR17HG* locus in our cell models.

Interestingly, it was found that translocation of RELA to the nucleosolic and chromatin fractions was enhanced in G1-released cells [12]. Given that CDK4/6 inhibitors function by preventing G1 phase progression, decreased translocation of RELA to the nucleosol and chromatin upon CDK4/6 inhibition may prevent recruitment of CDK6 to the *MIR17HG* locus. To confirm that CDK6 recruitment to the *MIR17HG* locus is mediated by RELA, ChIP experiments need to be conducted in these RELA knockout cells. If CDK6 indeed is recruited to this region predominantly through RELA, CDK6 occupancy at the *MIR17HG* locus will be reduced if not abolished when RELA is knockout.

While our data and existing ChIP-seq data set indicate that CDK6/RELA binds to the promoter and the first intron region of the *MIR17HG* locus, a consensus RELA binding sequence is absent where CDK6/RELA are found. Our current data suggest that CDK6/RELA may be recruited to the *MIR17HG* locus by MYC, which is known to bind to a conserved $^{5'}$CATGTC$^{3'}$ motif in the first intron of *MIR17HG* and stimulate its transcription [81, 82]. This is supported by superimposed binding peaks of CDK6, RELA and MYC on the same region of the *MIR17HG* locus from multiple existing ChIP-seq data sets in A549, MCF-7 and KB cells. However, these remain to be confirmed in our cell systems.

Our preliminary data also suggest that CDK6 may promote the activity MYC by phosphorylation of MYC at S62. In response to mitogen signaling, the stability of MYC is regulated by phosphorylation at S62 and T58 residues. Extracellular signal-regulated kinase (ERK) or CDK2

increase the stability of MYC by phosphorylating it at S62 while GSK3β-mediated T58 phosphorylation signals for dephosphorylation of S62 by protein phosphatase 2A (PP2A), ubiquitination by the SCF-Fbw7 E3 ligase and subsequent proteasomal degradation [89-91]. Indeed, we found that palbociclib treatment in both T74D and H358 cells suppressed phosphorylation of MYC at S62 and associated with reduced total MYC expression. Since palbociclib targets CDK4/CDK6, both of which have been shown to be able to modulate phosphorylation of MYC *in vitro*, RNAi-mediated silencing of CDK4 or CDK6 will need to be performed to dissect their relative contribution to phosphorylation of MYC at S62 in our model systems.

Interestingly, it has been shown that phosphorylation of MYC at S62 by CDK2 promotes its association with the miR-571 promoter region, but expression of a mutant MYC^{S62A} construct is unable to associate at these regions [85]. Given that CDK6/RELA binds to the same regions of *MIR17HG* locus where MYC also occupies, we speculated that a mechanism similar to CDK2-MYC regulation may also be in place for CDK6 in stimulating MYC activity at the *MIR17HG* locus. Indeed, we show that RELA depletion led to partial reduction of phosphorylation of MYC at S62. To further support this model, ChIP experiments are needed to examine the MYC occupancy at the *MIR17HG* locus before and after RELA depletion. In addition, phosphorylation site mutant of MYC (MYC^{S62A}) and wildtype control may also be used to evaluate the effect of this phosphorylation on the binding of MYC to the *MIR17HG* locus. It is also worth pointing out that decreased *MIR17HG* expression in response to CDK4/6 inhibitors may be mediated by decreased phosphorylation of MYC at S62, which is known to increase stability of MYC, and thus results in total downregulation of MYC expression. Finally, while coimmunoprecipitation experiments failed to detect CDK6/MYC interactions, proximity ligation assays (PLA) need to be performed to assess endogenous CDK6/MYC, CDK6/RELA, and RELA/MYC interactions. It is expected that treatment with CDK4/6 inhibitors decreases abundance of these interactions in the nucleus. Ultimately, ChIP-re-ChIP studies are required to provide conclusive results to show the co-bindings of CDK6/RELA/MYC to the regulatory regions of the *MIR17HG* locus.

CONCLUSIONS

Our study has provided mechanistic insight into the adaptive upregulation of cyclin D1 in response to CDK4/6 inhibition in ER^+ breast cancer and *KRAS*-mutant NSCLC cells. We reveal that this feedback is mediated in part through downregulation of *MIR17HG* due to inhibition of CDK6 activity upon treatment with CDK4/6 inhibitors. Furthermore, we show that CDK6 regulates *MIR17HG* expression by binding to its gene locus, which likely occurs through the NF-κB RELA subunit known to complex with CDK6. Moreover, our preliminary data suggest that CDK6 may modulate phosphorylation of MYC which is also known to directly induce *MIR17HG* expression. Collectively, we propose a model where CDK6 is recruited to the *MIR17HG* locus through RELA and also stimulates MYC to promote *MIR17HG* transcription through phosphorylation at S62. Together, our work elucidates a novel mechanism underlying the upregulation of cyclin D1 in ER^+ breast cancer and *KRAS*-mutant NSCLC, which is of clinical relevance since cyclin D1 expression has been shown to contribute to the development of acquired resistance to CDK4/6 inhibitors.

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