CDK2 Inhibition Causes Anaphase Catastrophe in Lung Cancer through the Centrosomal Protein CP110

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Running Title: CDK2 causes anaphase catastrophe through CP110

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Abstract

Aneuploidy is frequently detected in human cancers and is implicated in carcinogenesis. Pharmacological targeting of aneuploidy is an attractive therapeutic strategy as this would preferentially eliminate malignant over normal cells. We previously discovered that CDK2 inhibition causes lung cancer cells with more than two centrosomes to undergo multipolar cell division leading to apoptosis, defined as anaphase catastrophe. Cells with activating KRAS mutations were especially sensitive to CDK2 inhibition. Mechanisms of CDK2-mediated anaphase catastrophe and how activated KRAS enhances this effect were investigated. Live-cell imaging provided direct evidence that following CDK2 inhibition, lung cancer cells develop multipolar anaphase and undergo multipolar cell division with the resulting progeny apoptotic. Small interfering RNA (siRNA)-mediated repression of the CDK2 target and centrosome protein CP110 induced anaphase catastrophe of lung cancer cells. In contrast, CP110 overexpression antagonized CDK2 inhibitor-mediated anaphase catastrophe. Furthermore, activated KRAS mutations sensitized lung cancer cells to CDK2 inhibition by deregulating CP110 expression. Thus, CP110 is a critical mediator of CDK2-inhibition-driven anaphase catastrophe. Independent examination of murine and human paired normal-malignant lung tissues revealed marked upregulation of CP110 in malignant versus normal lung. Human lung cancers with KRAS mutations had significantly lower CP110 expression as compared to KRAS wild-type cancers. Thus, a direct link was found between CP110 and CDK2 inhibitor antineoplastic response. CP110 plays a mechanistic role in response of lung cancer cells to CDK2 inhibition, especially in the
presence of activated KRAS mutations.

Introduction

Cyclin-dependent kinases (CDK) regulate cell cycle progression (1). CDK2 is activated by the temporal upregulation of cyclin E promoting DNA duplication, entry and progression through the cell cycle (2). Cyclin E-CDK2 deregulation is frequent in epithelial carcinogenesis, including in lung cancer where it is associated with a poor prognosis (3). Transgenic mouse models were engineered with surfactant C–targeted cyclin E expression in the lung (4). This conferred chromosomal instability and lung cancers in mice with tumors recapitulating key features of human lung carcinogenesis (4).

Our prior work reported CDK2 inhibition caused anaphase catastrophe and apoptosis in lung cancer cells (5). Results from a high-throughput screen system testing the effect of seliciclib in 270 cancer cell lines revealed that in non-small cell lung cancer (NSCLC) cell lines, the most sensitive lines frequently had activated KRAS, while the 15 least sensitive cell lines all had wild-type KRAS, indicating that KRAS mutant lung cancer cell lines are most sensitive to CDK2 inhibition (5). Notably, lung cancer cases with activated KRAS are chemoresistant and have a poor prognosis (6). Therapeutic strategies for lung cancers with KRAS mutations are needed. This study sought to elucidate mechanistic pathways through which CDK2 inhibition confers anaphase catastrophe, and how KRAS mutation enhances this effect.

Anaphase catastrophe is observed in cancers with extra centrosomes that segregate
chromosomes with multipolar spindles into non-viable cells (7,8). Centrosome amplification occurs in diverse cancers and is associated with chromosome instability, anaphase catastrophe, aneuploidy and tumorigenesis (9-13). Agents that cause anaphase catastrophe, including CDK2 inhibitors, exploit the fact that cancer cells with supernumerary centrosomes can undergo multipolar cell division, leading to aneuploidy and cell death (14).

To identify potential mediators of anaphase catastrophe engaged by CDK2 inhibition, several CDK2 targets were examined. Among them, the centrosomal protein CP110 was highlighted since CP110 knockdown increased anaphase catastrophe in lung cancer cells. CP110 is a direct target of cyclin E-CDK2, cyclin A-CDK2, and cyclin B-CDC2 (15). CP110 has differing roles dependent on cell cycle phase (15-20). During the G1/S phase, CP110 regulates centrosome duplication and maturation (15,16) and during the M phase it is involved in cytokinesis (17). In non-cycling cells and cells in G0 phase, CP110 inhibits primary cilia formation (18,19). CP110 knockdown prevents centrosome reduplication in S-phase-arrested cells and induces premature centrosome separation (15), resulting in tetraploidy and binucleate cells indicating cytokinesis failure (17).

This study demonstrates in lung cancer cells that CDK2 inhibition causes multipolar anaphase that temporally precedes apoptosis and cell death. We found that CP110 is a mediator of CDK2-inhibitor-mediated anaphase catastrophe. Intriguingly, KRAS mutations sensitized lung cancers to CDK2-inhibitor-mediated anaphase catastrophe by deregulating CP110 expression. Translational relevance of these CP110 findings was established by comprehensively examining human malignant lung tissue arrays with an associated clinical
database and by investigating lung cancers from engineered mouse models. Findings presented here reveal a direct role for CP110 in lung cancer response to CDK2 inhibition, especially when KRAS mutations were detected.

Materials and Methods

Chemicals and antibodies

Seliciclib (CYC202, R-roscovitine) was provided by Cyclacel (stock solution 10mM in dimethyl sulfoxide, DMSO). Dosages of seliciclib used in the study (5μM, 10μM and 15μM) are clinically achievable (21) and biological effects of seliciclib at those dosages were due to CDK2 inhibition rather than to CDK7/9 blockade (5). Antibodies used were: cytochrome C (556432, BD Pharmingen. 1:1000), α-tubulin (T6199, Sigma Aldrich. 1:10000), CP110 (sc-136629 Santa Cruz Biotechnology, Inc. 1:1000), actin (sc-1615, Santa Cruz Biotechnology, Inc.1:3000), KRAS (sc-30, Santa Cruz Biotechnology, Inc. 1:1000), Texas red anti-mouse IgG (H+L) (TI-2000, Vector Laboratories, Inc.), ECL anti-rabbit IgG (NA934V, GE Healthcare), ECL anti-mouse IgG (NA931V, GE Healthcare) and horseradish peroxidase–conjugated donkey anti-goat IgG (sc-2020, Santa Cruz Biotechnology, Inc.). Pro-Long Gold anti-fade reagent with 4’,6-diamidino-2-phenylindole (DAPI) (P36935, Invitrogen) preserved immunofluorescence.

Cell culture

The murine lung cancer cell line ED-1 was derived and cultured, as described (22). LKR13, 344p and 393p murine lung cancer cell lines were provided by others (23). Human
lung cancer cell lines Hop62, A549, H460 and H522 were purchased from ATCC and cultured as described (22).

**Live cell imaging**

Cells plated on coverslips were treated with seliciclib (15μM) or vehicle for 24 hours before live cell imaging. Multipolar metaphase cells were individually selected for time-lapse live cell imaging, as described (8). DIC images were acquired with a Nikon Eclipse Ti microscope and an Andor cooled CCD camera using a 60x1.4NA oil immersion objective. For Hop62 cell imaging, 21 z-axis optical sections of 0.5 micron were acquired at 10 min intervals for 25 hours.

Following time-lapse imaging, cells were fixed with 3.5% paraformaldehyde and stained with DAPI and a cytochrome C-specific antibody. Fluorescent images were acquired using 11 z-axis optical sections of 1.0 micron. Image stacks and full volume renderings were performed using Nikon Elements and contrast enhancement was aided by Adobe Photoshop software. Cytochrome C immunofluorescence images were quantified by calculating average (mean) of the mean pixel intensity of at least 20 regions of interest (area= 0.8micron²) within each cell to avoid any mitochondrial staining and quantify only cytoplasmic cytochrome C. Background levels for each image were subtracted.

**Expression plasmids and transient transfection**

HA-tagged wild-type pcDEF3-CP110 (CP110-WT) vector and a CP110 vector with 8 phosphorylation sites mutated pcDEF3-CP110 (CP110-MUT) were generous gifts from others (15). Logarithmically growing ED-1, LKR13, Hop62, H522, A549 and H460 cell lines
were each transiently transfected using TransIT-LT1 reagent (Mirus), following the manufacturer's instructions. Each experiment was independently replicated at least three times.

Indicated lung cancer cells were transfected with small interfering RNAs (siRNAs) using Lipofectamine 2000 (Invitrogen). The siRNAs targeting murine CP110 (Dharmacon), human or murine CDK2 (IDT) and human or murine KRAS (Thermo Scientific) species and RISC control siRNA (Dharmacon, IDT and Thermo Scientific) were purchased and validated for effects of each knock-down by immunoblot and qPCR assays. SiRNA sequences appear in Supplementary File. S1. Each experiment was independently replicated at least three times.

**Multipolar anaphase assay**

Indicated lung cancer cells were fixed in cold methanol and stained with DAPI and an anti-α-tubulin–specific antibody and examined using an Eclipse TE 2000-E microscope (Nikon). Anaphase cells that contained three or more spindle poles were scored as multipolar. Data were expressed as percent multipolar versus total anaphase cells.

**Generation of stable KRAS transfectants**

Logarithmically growing ED-1 cells (3×10^6) were plated in each 10 cm tissue culture dish, 24 hours before transfection. Twelve μg each of the pCGN K-RasG12V, 188L plasmid (Addgene) with the pPUR expression plasmid (Clontech) or an empty vector with the pPUR plasmid were individually transfected into ED-1 cells using Lipofectamine 2000 (Invitrogen). Puromycin selection began 24 hours after transfection. Engineered KRAS overexpression was confirmed by immunoblot analysis.
Proliferation and apoptosis assays

Logarithmically growing cells were plated onto individual 12-well tissue culture plates (5×10^3 cells/well). Twenty-four hours later, cells were treated with seliciclib over a range of concentrations versus vehicle controls. Three independent wells were seeded in each experiment with triplicate independent replicates. Proliferation was measured using the CellTiter-Glo Assay Kit (Promega), as described (24). Trypan blue viability assays were performed (22). Cellular apoptosis was measured by Annexin V:PE positivity detected using the Annexin V assay kit (Southern Biotech).

Immunohistochemistry

Thirty lung cancers (17 adenocarcinoma, 8 squamous cell, 5 other histologies) from the New Hampshire State Cancer Registry and Dartmouth Tumor Registry in addition to lung cancer specimens obtained from Dartmouth’s Department of Pathology archives were used in paired normal and malignant lung tissue microarrays. This study was reviewed and approved by the Dartmouth Committee for Protection of Human Subjects.

A larger set of tissue microarrays was from a lung cancer tissue bank at M. D. Anderson Cancer Center. Institutional Review Board approved these studies of 558 surgically resected NSCLC (369 adenocarcinomas, 176 squamous cell carcinomas, 13 histology information not available).

Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections using a Leica BOND-MAX™ automated stainer (Leica Microsystems Inc.) and Leica Bond Polymer Refine Detection reagents to detect CP110 protein. Antibody specificity was
confirmed using a blocking peptide (sc-136629, Santa Cruz Biotechnology Inc.). The immunohistochemistry scoring system was similar to prior work (22, 25). CP110 immunohistochemical expression was scored by a reference pathologist who was unaware of clinical findings. Both average staining intensity and percentages of immunoreactive cancer cells were recorded.

CP110 immunohistochemical analyses were independently performed in paired normal-malignant lung tissues from cyclin E as well as KRAS-driven lung cancers in engineered mouse models (4, 26).

**Immunoblot analyses**

Cells were lysed with ice-cold radioimmunoprecipitation (RIPA) buffer with protease inhibitors (Biosciences), and immunoblot analyses performed as previously described (27). Lysates were size-fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to nitrocellulose membranes (Schleicher and Schuell Bioscience) and probing with indicated antibodies.

**Real-time reverse transcription-PCR assays**

Total RNA was isolated from cells using the RNA easy kit (Invitrogen). Reverse transcription (RT) was done using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with a Peltier Thermal Cycler (MJ Research). Quantitative real-time polymerase chain reaction (PCR) assays were done using SYBR Green PCR Mastermix (Applied Biosystems) and the 7500 Fast Real time PCR System (Applied Biosystems) for quantitation. Reverse transcription-PCR assays were conducted following the
manufacturer’s protocol (Applied Biosystems). Three replicate experiments were done. Primers sequences appear in Supplementary File S1.

Statistical analyses

Results of independent experiments were pooled to assess statistical significance. Two-tailed t-tests were used. Statistical significance was noted with *, P < 0.05 and **, P < 0.01.

Results

Live cell imaging after CDK2 inhibition

Prior work established CDK2 inhibition caused anaphase catastrophe in lung cancer cells (5,7). While prior work revealed most progeny of multipolar cell divisions died or arrested regardless of cell origin (8), direct evidence linking anaphase catastrophe with induced cell death after CDK2 inhibition in lung cancer cells remained to be shown. To determine the outcomes of cells undergoing multipolar anaphase, we used live-cell imaging to follow their fates following CDK2 inhibitor treatment. A representative Hop62 human lung cancer cell displaying a multipolar metaphase was selected by DIC imaging and followed by time lapse microscopy. After 25 hours of imaging (Fig.1A, whole video is provided in Supplementary File S2), Hop62 cells were fixed and stained for cytochrome C and DAPI. Cytoplasmic cytochrome C was quantified in progeny. In Fig.1B, representative negative and positive control cells appear in the examined field. Compared to these cells, two of the daughter cells had significant cytoplasmic cytochrome C release, as quantified in Fig.1C, indicating apoptosis was initiated. The third daughter cell did not show a significant
cytoplasmic cytochrome C signal. However, the third daughter cell displayed marked DNA fragmentation and micronuclei, features seen before cell death or senescence (28). These results provide direct evidence that progeny of lung cancer cells undergoing multipolar anaphases can undergo apoptosis.

**CP110 regulates anaphase catastrophe**

Since CP110 regulates centrosome function and is a direct CDK2 phosphorylation target (15), it was hypothesized CDK2 inhibition induced anaphase catastrophe via CP110. To investigate whether CP110 knockdown caused anaphase catastrophe, two different siRNAs targeting murine CP110 and a scrambled control siRNA were each used. Real-time quantitative RT-PCR assays validated knockdown of targeted mRNAs. Marked knockdown of CP110 mRNA was achieved at both 24 and 48 hours following transfection (Fig.2A, left panel). CP110 knockdown significantly increased percentages of cells undergoing multipolar anaphase in murine ED-1 lung cancer cells; overexpression of wild-type CP110 abrogated anaphase catastrophe conferred by CP110 knockdown (Fig.2A, middle panel).

To investigate whether CP110 knockdown augmented anaphase catastrophe by seliciclib-mediated CDK2 inhibition, CP110-depleted ED-1 murine lung cancer cells were treated with seliciclib for 24 hours and scored for multipolar anaphase (Fig.2A, right panel). CP110 knockdown increased multipolar anaphases induced by seliciclib treatment in ED-1 cells.

To examine whether increased CP110 levels affected anaphase catastrophe via CDK2 inhibition, CDK2 activity was repressed, genetically or pharmacologically, and CP110 was
simultaneously overexpressed in ED-1 cells (Fig.2B). CP110 overexpression was confirmed by immunoblot analyses (Supplementary Fig.S1A) and CDK2 knockdown was validated by real-time quantitative RT-PCR assays and immunoblot analyses (Fig.2B, left panel). Overexpression of CP110 significantly antagonized multipolar anaphases induced by either Cdk2 knockdown (Fig.2B, middle panel) or seliciclib treatment (Fig.2B, right panel). CP110 overexpression also significantly reduced apoptosis caused by seliciclib treatment (Supplementary Fig.S1B).

To examine whether seliciclib treatment affected CP110 expression level, we examined basal levels of CP110 at 24 and 48 hours after seliciclib treatment. Treatment of seliciclib did not appreciably affect CP110 protein levels in human and murine lung cancer cells (Supplementary Fig.S1E).

To investigate whether CP110 phosphorylation was critical for protecting cells from undergoing anaphase catastrophe induced by CDK2 inhibition, a mutant CP110 species with all potential CDK2 phosphorylation sites transversed to alanines (15) was transfected into murine lung cancer cells. Mutant CP110 over-expression did not antagonize induction of multipolar anaphase (Supplementary Fig.S1C) or apoptosis (Supplementary Fig.S1D) in ED-1 cells treated with the CKD2 inhibitor seliciclib. Similar results were observed in LKR13 murine lung cancer cells (Supplementary Fig.S2) and A549 human lung cancer cells (data not shown). Together, these studies indicated that increasing wild-type CP110 protein could override CDK2 inhibition and protect cells from anaphase catastrophe. Cells with lower CP110 levels are particularly sensitive to CDK2 inhibition.
The working model hypothesized that inhibition of CDK2 decreases CP110 phosphorylation levels, which leads to anaphase catastrophe. Moreover, activated KRAS can downregulate CP110 basal levels, increase lung cancer cellular response to CDK2 inhibitors (Schematic diagram in Supplementary Fig.S3A).

**Engineered CP110 overexpression in human lung cancer cells**

To investigate whether CP110 overexpression can rescue anaphase catastrophe caused by CDK2 inhibition in human lung cancer cells, A549, Hop62, H460 and H522 cells were each engineered with CP110 overexpression (Supplementary Fig.S3B) before seliciclib treatment (15μM) for 4, 8 and 24 hours. Induction of multipolar anaphases by CDK2 inhibition was observed as early as 4 hours after drug treatment of all four cell lines (Fig.3A), while apoptosis induction did not occur until 24 hours of this treatment (Fig.3B). CDK2 knockdown was achieved by independent siRNAs following CP110 overexpression in A549 and Hop62 cell lines. Real-time quantitative RT-PCR assays validated CDK2 knockdown (Fig.4A and 4B, left panel) and multipolar anaphases were scored 24 hours after transfection (Fig.4A and B right panel). Consistent with results from murine cells, engineered CP110 overexpression substantially reduced multipolar anaphases and apoptosis in all examined human lung cancer cell lines, despite seliciclib treatment or CDK2 knockdown. Engineered gain of wild-type CP110 expression protected both human and murine lung cancer cell lines from undergoing anaphase catastrophe and apoptosis induced by pharmacologic inhibition or genetic knockdown of CDK2.

**KRAS sensitizes cancer cells to anaphase catastrophe by decreasing CP110 levels**
To investigate the role of KRAS in seliciclib-mediated cytotoxicity, a KRAS\textsuperscript{G12V} expression vector or an empty vector was independently stably transfected into ED-1 cells. Expression of oncogenic KRAS was confirmed by immunoblot analyses (Fig. 5A). KRAS\textsuperscript{G12V} transfected ED-1 cells (KRAS-ED-1) exhibited marked growth inhibition (Supplementary Fig. S4A), increased multipolar anaphases (Fig. 5A) and apoptosis (Fig. 5B) after seliciclib treatment as compared to control transfectants. Thus, KRAS activation sensitized lung cancer cells to pharmacological CDK2 inhibition, as is consistent with previous work (5,7).

CP110 protein levels were down-regulated in KRAS-ED-1 cells, but CP110 mRNA levels were not appreciably affected as compared to control cells (Fig. 5C). CP110 protein levels were also lower in 344p, 393p and LKR13 murine lung cancer cells that harbor activating KRAS mutations as compared to ED-1 cells that express wild-type KRAS (Supplementary Fig. S4C). To further explore the role of KRAS in regulating CP110 expression, transient KRAS knockdown was achieved in 344p and Hop62 cells using siRNAs. Decreased KRAS expression was detected at 48 and 72 hours after transfection. Increased CP110 expression was evident 72 hours after transfection for 344p cells and 96 hours after transfection for Hop62 cells (Supplementary Fig. S4D and E). This delay in a change in CP110 expression implied that CP110 expression was regulated by targets downstream of KRAS.

To learn whether CP110 overexpression could reverse KRAS-ED-1 cells sensitivity to CDK2 inhibition, wild-type CP110 was overexpressed in them. This reduced both multipolar anaphases and apoptosis (Fig. 5D and E). Therefore, KRAS activation repressed CP110
expression, which enhanced lung cancer cell response to CDK2 inhibition.

**CP110 expression in lung cancers**

To investigate whether CP110 was differentially expressed in human lung cancers with different KRAS mutation status, tumor histology, size, age or stage CP110 immunohistochemistry assays were performed (Fig.6A).

In murine lung cancer cell lines driven by KRAS expression (LKR13) or not (ED-1), CP110 levels were 2.6 fold higher in ED-1 than in LKR13 cells (Supplementary Fig.S4C). Immunohistochemical expression profiles were also examined in the normal versus malignant lung tissues from KRAS or cyclin E-driven murine transgenic lung cancers (Supplementary Fig.S5). Notably, the intensity of CP110 staining was much lower in the KRAS-driven lung cancers as compared to lung cancers with wild-type KRAS status.

CP110 expression was higher in human malignant versus adjacent normal lung tissues (Fig.6A and 6B). A logistic regression model using CP110 intensity as a dichotomous outcome (CP110 intensity ≥ 200, median) revealed that a larger proportion of adenocarcinomas with KRAS mutations were low-CP110-intensity as compared with KRAS wild-type adenocarcinomas (Fig.6C). However, average CP110 intensity did not show a difference between KRAS mutant and wild-type lung tumors (data not shown). No significant differences were observed in CP110 expression in lung cancer cases when stratified for survival (Fig.6D), tumor stage or age at diagnosis (data not shown).

**Discussion**

This study revealed that CP110 expression determines the extent of anaphase...
catastrophe when CDK2 levels or activity are inhibited. Reducing CP110 levels promotes anaphase catastrophe and overexpression of CP110 significantly reduces anaphase catastrophe conferred by genetic or pharmacological inhibition of CDK2. Time-lapse live-cell imaging provided direct evidence demonstrating that anaphase catastrophe results in apoptosis in human lung cancer cells.

CP110 interacts with distinct protein complexes that regulate centrosome duplication and separation, chromosome segregation and cilia formation (16-20). It is not known to have enzymatic activity, but is thought to function structurally to regulate microtubule growth and centriole length (29). CP110 is a direct target of cyclin E-CDK2 (15), however, functional consequences of specific CP110 sites of phosphorylation by CDK2 are not yet known. Studies presented in Fig. 2 revealed CP110 repression caused anaphase catastrophe. The impact of CP110 on CDK2 inhibition-mediated anaphase catastrophe was studied further.

Intriguingly, engineered KRAS expression down-regulated CP110 levels in lung cancer cells, which provided an explanation for the observed enhanced sensitivity of lung cancer cells with KRAS mutations to CDK2 inhibition (5). KRAS mutations are linked to centrosome amplification (30,31) and chromosomal instability (32). The deregulation of CP110 linked to activated KRAS found here likely contributes to these processes.

CP110 is expressed ubiquitously in normal tissues (15). Expression of CP110 changes in the cell cycle and is repressed when cells enter G0 phase (15). The upregulation of CP110 expression evident in malignant versus normal lung tissues could reflect an
increased proliferation of lung cancer cells or the presence of inflammation since proinflammatory cytokines enhance CP110 expression (33).

High CP110 expression induces centrosome amplification (20) but then delays centrosome separation and promotes centrosome clustering. High CP110 expression should protect cancer cells with supernumerary centrosomes from undergoing multipolar cell division. High CP110 expression also inhibits primary cilia formation (18). This contributes to cilia defects in cancer cells (32). Primary cilia are crucial for signaling pathways through PDGFα, Hedgehog and Wnt, which are essential for growth and differentiation (34,35). Loss of cilia in cancer cells likely contributes to insensitivity of cancer cells to environmental repressive signals (34). KRAS mutations can play a role in primary cilia formation in pancreatic cancers (36). Of note, loss of primary cilia is important in cytogenesis in polycystic kidney disease and seliciclib treatment is reported to block cystogenesis in cultured cells and in mouse models of polycystic kidney disease, but ability to restore primary cilia formation was not examined (37). The relationships between KRAS mutation, primary cilia formation, CDK2 inhibition, CP110 and lung carcinogenesis warrant further investigation.

The biological relevance of these findings was confirmed in lung cancers from engineered mice where KRAS is the driver mutation. These lung cancers exhibited lower CP110 levels as compared to lung cancers from mice with wild-type KRAS (Supplementary Fig.S5). Clinical relevance was established by determining that a larger proportion of lung tumors with KRAS mutations were low-CP110-expression as compared with KRAS
wild-type tumors when using logistic regression model categorizing tumors into high or low CP110 intensity (Fig.6C). This finding supports the hypothesis that lung cancers with \textit{KRAS} mutations are likely to be sensitive to a CDK2 inhibitor therapeutic strategy. One of the mechanisms underlying that is low CP110 levels increasing anaphase catastrophe. Although the overall average CP110 levels between \textit{KRAS} mutant and wild-type lung cancers did not show a significant difference, it is possible that the immunohistochemical assay used was not sufficiently sensitive to appreciate subtle differences in CP110 expression.

The effect of \textit{KRAS} mutations on CP110 expression is likely complex and other proteins that interact with CP110 could be affected by \textit{KRAS} mutations. For instance, CP110 expression is controlled by two independent ubiquitination pathways, SCF\textit{cyclinF}-mediated pathway (20) and NEURL4-HERC2 complex-mediated pathway (38). Recently, a centriolar deubiquitinating enzyme USP33 was found to regulate CP110 expression by countering cyclin-F-mediated destruction (25).

In summary, this study identified CP110 as a key mediator of anaphase catastrophe induced by CDK2 inhibition that temporally precedes apoptosis. \textit{KRAS} mutations sensitized lung cancer cells to seliciclib-mediated CDK2 inhibition, inducing anaphase catastrophe in part by down-regulating CP110 levels. The translational relevance of this finding is underscored by the fact that CP110 is frequently overexpressed in NSCLCs and a larger percentage of lung cancers with \textit{KRAS} mutation are low-CP110-expressing. Taken together, these findings indicate a mechanistic link between CP110 expression and \textit{KRAS} mutation. It
is proposed that these species are important to prioritize selection of a CDK2 inhibitor for the clinical treatment of KRAS mutant lung cancers.

Authors’ Contributions

Conception and design S. Hu, A.V. Danilov, F. Galimberti, D.A. Compton, E. Dmitrovsky

Development of methodology S. Hu, A.V. Danilov, K. Godek, B. Orr, L.J. Tafe


Writing, review and/or revision of the manuscript S. Hu, A.V. Danilov, K. Godek, B. Orr, A.S. Andrew, S. Freemantle, D.A. Compton, E. Dmitrovsky

Administrative, technical, or material support D. Sekula, I.I. Wistuba, S. Freemantle, D.A. Compton, E. Dmitrovsky

Study supervision I.I. Wistuba, S. Freemantle, D.A. Compton, E. Dmitrovsky

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References


9. Levine DS, Sanchez CA, Rabinovitch PS, Reid BJ. Formation of the tetraploid intermediate is associated with the development of cells with more than four centrioles in the


Figure Legends

Fig.1. Live-cell imaging revealed the fate of cells undergoing multipolar anaphase. (A) A representative Hop62 multipolar metaphase cell was selected and filmed for 25 hours. Hop62 cells were treated with seliciclib (15μM) for 24 hours before filming and was in medium with seliciclib (15μM) during filming. (B) Hop62 cells filmed in panel A were fixed and stained for cytochrome C (green) and DAPI (blue). The signals of cytoplasmic cytochrome C of daughter cells were displayed enlarged. (C) The quantification of cytoplasmic cytochrome C in each progeny is shown.

Fig.2. Gain or loss of CP110 function in murine lung cancer cells implicates it in CDK2-inhibition-mediated anaphase catastrophe. (A) Effect of knock-down CP110 on anaphase catastrophe. Left panel, confirmation of CP110 mRNA knock-down by real-time RT-PCR assays performed 24 and 48 hours after transfection. Immunoblot confirmation of CP110 knockdown is shown in middle panel. Middle panel, ED-1 cells overexpressing wild-type-CP110 or an empty vector were transfected with each of two different CP110 targeting siRNAs and control siRNA. Twenty-four hours later, cells were fixed and scored for multipolar anaphases. Right panel, 24 hours after siRNA transfection, ED-1 cells were treated with indicated seliciclib dosage and scored for multipolar anaphases. (B) Effect of CP110 overexpression on CDK2 inhibitor activity. Left panel, confirmation of CDK2 mRNA knock-down by real-time RT-PCR and immunoblot analyses, respectively. Middle panel, ED-1 cells overexpressing wild-type...
CP110 or an empty vector were transfected with two different CDK2-targeting siRNAs and control siRNA and scored for multipolar anaphases 24 hours after transfection. Right panel, ED-1 cells overexpressing wild-type CP110 or an empty vector were treated with indicated seliciclib dosages. ED-1 cells were scored for multipolar anaphases 24 hours later.

Fig.3. Overexpression of CP110 rescues anaphase catastrophe induced by CDK2 inhibition in human lung cancer cells. A549, Hop62, H460 and H522 human lung cancer cells overexpressing CP110 were each treated with seliciclib (15μM) for indicated hours and (A) scored for multipolar anaphase and (B) analyzed for apoptosis as detected by Annexin V:FITC and 7-Aminoactinomycin D (7-AAD) staining.

Fig.4. Overexpression of CP110 rescues anaphase catastrophe caused by genetic CDK2 inhibition in human lung cancer cells. Left panel, confirmation of CDK2 mRNA knock-down by real-time RT-PCR assays and immunoblots 24 hours after transfection. Right panel, A549 and Hop62 human lung cancer cells overexpressing CP110 were transfected with siRNA-targeting CDK2 or control siRNA and scored for multipolar anaphase 24 hours after transfection.

Fig.5. KRAS mutation affects CDK2 inhibitor activity and deregulates CP110. KRAS^{G12V} stably transfected ED-1 cells (KRAS-ED-1) showed a marked increase of (A) multipolar
anaphase and (B) apoptosis as compared with empty vector-transfected ED-1 cells (EV-ED-1) after 24 hours seliciclib treatment. Increased KRAS protein level was confirmed by immunoblot. (C) CP110 expression in KRAS-ED-1 cells as compared to EV-ED-1 cells on the protein (left panel) and mRNA (right panel) levels. (D and E) KRAS-ED-1 and empty vector (EV)-ED-1 cells overexpressing wild-type CP110 were treated with seliciclib (10\(\mu\)M) for 24 hours and scored for multipolar anaphase (D) and apoptosis (E, NS, Not significant).

Fig.6. CP110 expression in human normal versus malignant lung. (A) Representative CP110 immunostaining of normal adjacent lung versus malignant lung. (B) Left panel, quantification of CP110 expression in malignant (T) as compared with normal lung (N). Right panel, quantification of CP110 expression in malignant lung versus adjacent normal lung in adenocarcinoma (AD), squamous cell carcinoma (SCC) and other histological types. (C) Logistic regression model using CP110 intensity (CP110 intensity \(\geq\) 200, median) as a dichotomous outcome comparing KRAS mutant and wild-type adenocarcinomas. The abbreviations are: Ref, reference; WT, wild-type; OR, odds ratio; CI, confidence interval. This model was adjusted for race, gender, tobacco use, and stage. (D) The overall survival of CP110 high expressing as compared to CP110 low expressing lung cancer cases.
Figure 1

**A**

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<td>3h 10min</td>
<td>7h 40min</td>
</tr>
<tr>
<td>T</td>
<td>16h 40min</td>
<td>20h 50min</td>
<td>25h 30min</td>
</tr>
</tbody>
</table>

**B**

Transmitted light

DAPI

Cytochrome-C

1 5X 3

2

3

4

25μm

**C**

<table>
<thead>
<tr>
<th></th>
<th>Cytoplasmic Cytochrome C units (×100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control (Cell 1)</td>
<td><strong>P &lt; 0.01</strong></td>
</tr>
<tr>
<td>Daughter Cell 1 (Cell 2)</td>
<td><strong>P &lt; 0.01</strong></td>
</tr>
<tr>
<td>Daughter Cell 2 (Cell 3)</td>
<td><strong>P &lt; 0.01</strong></td>
</tr>
<tr>
<td>Positive Control (Cell 4)</td>
<td><strong>P &lt; 0.01</strong></td>
</tr>
</tbody>
</table>

25μm
Figure 2

A

% Multipolar Anaphase (Fold of Control)

% Relative mRNA Level vs Control

CDK2

Tubulin

Control siRNA

CDK2.1 siRNA

CDK2.2 siRNA

24

48

Control siRNA

CP110 siRNA 1

CP110 siRNA 2

**

**

**

**

0

5

10

15

Seliciclib (µM)

Hours after Transfection

B

% Relative mRNA Level vs Control

CDK2

Tubulin

Control siRNA

CDK2.1 siRNA

CDK2.2 siRNA

0

5

10

15

Seliciclib (µM)

Multipolar Anaphase (Fold of Control)

Vector

WT-CP110

* P < 0.05

** P < 0.01

Research.
Figure 3

A

Multipolar Anaphase (Fold of Control)

Hours of Seliciclib Treatment (15 µM)

Vector

WT-CP110

A549

Hop62

H460

H522

B

Apoptosis (Fold of Control)

Hours of Seliciclib Treatment (15 µM)

Vector

WT-CP110

A549

Hop62

H460

H522

* P < 0.05  ** P < 0.01
Figure 5

A

Multipolar Anaphase (Fold of Control)

<table>
<thead>
<tr>
<th>Seliclib (µM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Vector</td>
<td>*</td>
<td>*</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>KRAS</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

B

Apoptosis (Fold of Control)

<table>
<thead>
<tr>
<th>Seliclib (µM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Vector</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

CP110 mRNA level

<table>
<thead>
<tr>
<th>CP110 mRNA level vs control</th>
<th>EV-ED-1</th>
<th>KRAS-ED-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D

Multipolar Anaphase (Fold of Control)

<table>
<thead>
<tr>
<th>Seliclib (µM)</th>
<th>0</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV+Vector</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS+Vector</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS+WT-CP110</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E

Apoptosis (Fold of Control)

<table>
<thead>
<tr>
<th>Seliclib (µM)</th>
<th>0</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV+Vector</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS+Vector</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS+WT-CP110</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05
** P < 0.01
NS
Figure 6

A  

- Peptide  + Peptide

Normal Lung  Malignant Lung

B  

CP110 Staining

** N = 30

C  

Logistic regression model in lung adenocarcinomas using CP110 intensity as a dichotomous outcome

<table>
<thead>
<tr>
<th></th>
<th>CP110 intensity &lt; median</th>
<th>CP110 intensity ≥ median</th>
<th>OR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS WT</td>
<td>118</td>
<td>126</td>
<td>1.0 (ref)</td>
<td>0.36 - 0.98</td>
<td>0.044</td>
</tr>
<tr>
<td>KRAS Mutant</td>
<td>54</td>
<td>40</td>
<td>0.59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D  

Overall Survival

** P < 0.01
CDK2 Inhibition Causes Anaphase Catastrophe in Lung Cancer through the Centrosomal Protein CP110


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