# Biochemical Studies of CDK16


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**TABLE OF CONTENTS**

**ABSTRACT**

Cyclin-dependent kinases (CDKs) are protein kinases regulated by cyclin subunits and are a popular target for cancer therapeutics due to the important role they play in the cell cycle. CDK16 is activated by cyclin Y and is a vital regulator of neuronal cell proliferation, brain development, neuronal migration, dendrite development, neurite outgrowth, and spermatogenesis. Garwain et al. (2018) identified Phospholipase Cβ1 (PLCβ1), a G-protein regulated enzyme important for cell differentiation, as an inhibitor of CDK16 activity. This interaction prevents uncontrolled cell growth. Our study sought to understand the mechanism through which PLCβ1 inhibits CDK16. Our approach is to express the proteins in cells and isolate the complex through immunoprecipitation. We were able to make complexes at high enough concentrations for structure resolution by cryo-EM where purity was confirmed by mass spectroscopy. We are currently assessing CDK16 kinase activity using a FRET-based commercial assay.

**ACKNOWLEDGEMENTS**

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## BACKGROUND

Cyclin-dependent kinases (CDKs) are proline-directed serine/threonine protein kinases activated by cyclin regulatory subunits with an important role in cell cycle regulation (Morgan, 1995). The human genome contains 26 different CDK family members that can also control various cellular activities including mRNA processing, DNA damage and repair, and neuronal function (Malumbres et al., 2009; Wang et al., 2023). The PCTAIRE subgroup of the CDK family have a highly conserved catalytic domain and are characterized by N- and C-terminal extensions, not found in other CDKs, that mediate protein-protein interactions and regulate kinase activity (Mikolcevic et al., 2012; Shehata et al., 2015; Dixon-Clarke et al., 2017).

PCTAIRE1, also known as CDK16, is activated by the binding of phosphorylated membrane-bound cyclin Y complexed with 14-3-3 protein (Mikolcevic et al., 2012; Shehata et al., 2015). CDK16 is present in cells throughout the body, however, expression is highest in the brain and testes. Therefore, the protein plays a key role in neurite outgrowth, exocytosis, neuronal proliferation, brain development, neuronal migration, and nerve system regeneration (Graeser et al., 2002; Pedersen et al., 2021; Palmer et al., 2005; Wang et al., 2023; Mokalled et al., 2015). Prior studies have identified CDK16 as a key player in various cell signaling pathways and protein interactions that influence the cell cycle (Figure 1).

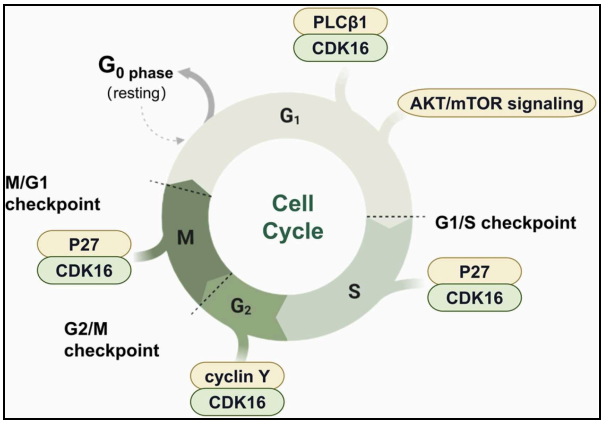



**Figure 1. Regulatory mechanisms and checkpoints of CDK16 throughout the cell cycle (Wang et al., 2023)** CDK16/PLCβ1 interaction and AKT/mTOR signaling play a role in cell growth which can both be affected by changes in CDK16 expression. P27 is a CDK inhibitor that has shown to induce positive and negative effects on the cell cycle (Chu et al., 2008). CDK16 phosphorylates P27 during DNA replication (S phase) and cell division (M phase) (Yanagi et al., 2014).

Additionally there is a growing interest in the role of CDK16 in cancer progression as tumors result from uncontrolled cell growth. CDK16 has been assessed in a variety of cancers including lung cancer, breast cancer, melanoma, liver cancer, cervical cancer, and prostate cancer (Yanagi & Matsuzawa, 2015; Li et al., 2022; Wang et al., 2023).

Phospholipase Cβ1 (PLCβ1) is a $G_{\alpha q}$-regulated enzyme that localizes on the plasma membrane and has an important role in intracellular calcium signaling. Garwain et al. (2018) established PLCβ1 binds to and inhibits CDK16 preventing unchecked proliferation by keeping cells in the G1 phase (Figure 2).

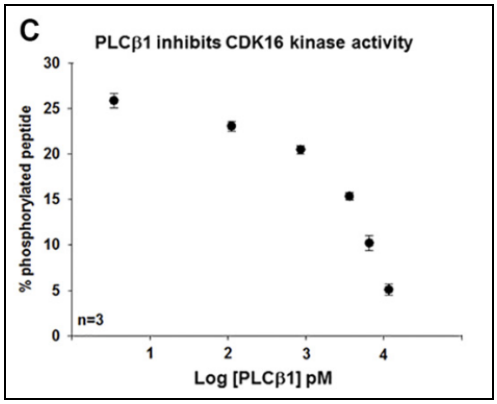



**Figure 2. PLCβ1 inhibits CDK16 (Garwain et al., 2018).** FRET-based commercial assay shows relative phosphorylation of 40 nM of CDK16 decreases with an increase in PLCβ1.

To further understand the mechanism of PLCβ1 inhibition of CDK16, we first assessed the known structures of CDK16 and PLCβ3, another member of the phospholipase C family since the independent structure of PLCβ1 has not yet been determined. Then, we isolated the PLCβ1/CDK16 complex for cryo-EM analysis and conducted an additional kinase assay to ensure CDK16 functionality.

## METHODS

*Cell Culture*

HEK-293 cells were bioengineered with a tetracycline promoter for increased PLCβ1 expression. These cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin streptomycin, and antifungal. PC-12, a neuronal cell line, was cultured in high-glucose DMEM with 10% inactivated horse serum (HI), 5% FBS, 1% penicillin streptomycin, and antifungal. Both cell lines were incubated at 37°C at 5% $CO_2$ and washed with phosphate buffered saline (PBS). Trypsin (0.005%) was used to detach PC12 cells from 150 mm x 21 mm Nunc™ EasYDish™ Dishes. Cells were cultured to ~80% confluence before transfection.

*Transfection by Electroporation*

CDK16 plasmid tagged with human influenza hemagglutinin (HA) was purified by Guanyu Lin. For both HEK-293 and PC12 cell lines, ~30,000 ng of HA-tagged CDK16 plasmid was added per large plate. Electroporator was set to 110 V, 25.0 msec, one pulse, zero intervals, and 2 mm cuvette. Cells were returned to the incubator to restore growth to ~80% confluence.

*Immunoprecipitation*

Both cell lines were lysed in 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 5% glycerol, and Pierce™ Protease Inhibitor Mini Tablets and centrifuged at 2,500 xg for 5 minutes. PC12 cells with overexpressed CDK16 remained as a complete lysate, whereas HEK-293 cells continued the purification process to obtain CDK16/PLCβ1 complex. To prepare the magnetic beads for protein isolation,

anti-CDK16 antibody was incubated with the magnetic beads which were then used to separate the supernatant. Cells were washed with a lysis buffer and then washed using an elution buffer. A Bicinchoninic Acid (BCA) protein assay was used to determine the protein concentration of both the total lysate (PC12 and HEK-293) and the isolated sample (HEK-293). Protein electrophoresis was performed on the isolated sample from HEK-293 to separate remaining proteins in the sample.

*Kinase Assay*

CDK16 activity from PC12 cells with CDK16 overexpression was measured using commercially available Z'-LYTE™ Kinase Assay Kit with Ser/Thr 1 Peptide (Thermo Fisher Scientific). The assay utilizes a synthetic peptide substrate labeled with Förster resonance energy transfer (FRET) donor (coumarin) and a FRET acceptor (fluorescein). CDK16 phosphorylates the Tyr, Ser, or Thr on the synthetic peptide, then an introduced protease has a stronger affinity for the non phosphorylated peptides (Figure 3). Only uncleaved peptides maintain FRET which was imaged on a plate reader using 445 nm for coumarin and 520 nm for fluorescein. Reaction progression was quantitated by calculating emission ratio and relating it to phosphorylation percentage.

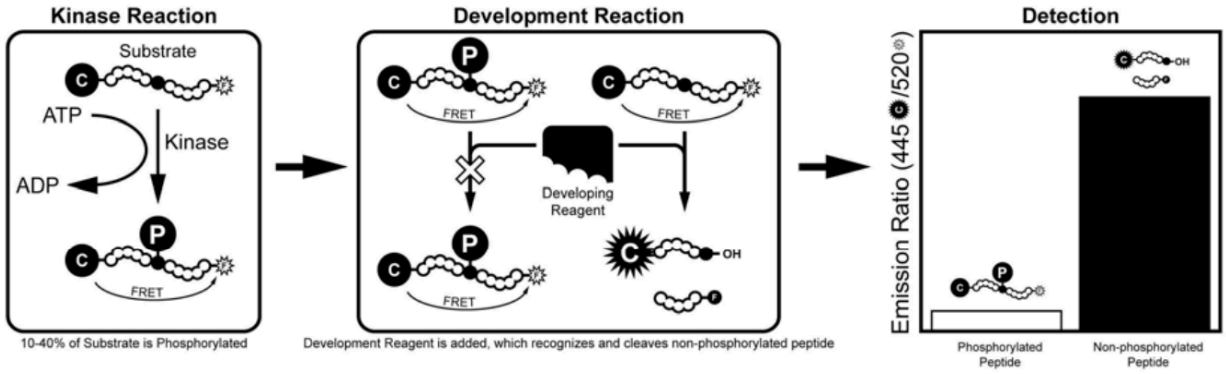
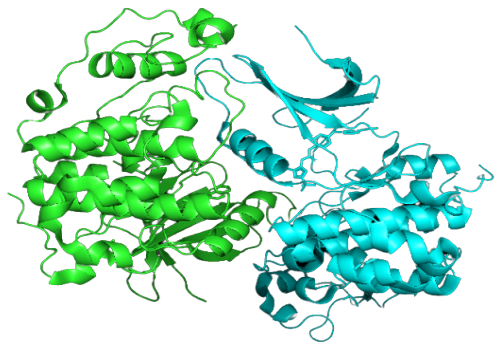



**Figure 3. Kinase assay yields an emission ratio that can express phosphorylation as a percentage.** Increased phosphorylation indicates increased kinase activity and vice versa.

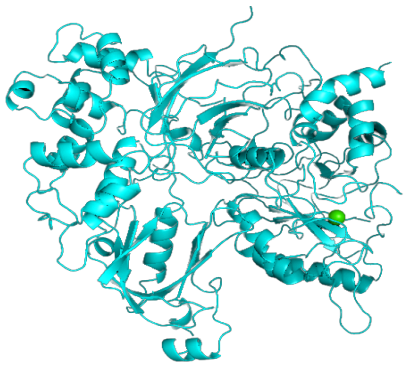
## RESULTS AND DISCUSSION

Known structures of CDK16 and PLCβ3 (since PLCβ1 is unknown) were assessed to further understand a potential mechanism for CDK16 inhibition by PLCβ1. Falzone and MacKinnon (2023) determined the structure of PLCβ3 in a study to assess activation of the enzyme by the $G_{\alpha q}$ protein (Figure 3). However, it is unknown if PLCβ1 is activated by $G_{\alpha q}$ when it interacts with CDK16. The ATP binding pocket of CDK16 can accommodate both type I inhibitors, bind when kinase is active, and type II inhibitors, bind when kinase is inactive (Figure 3; Dixon-Clarke et al., 2017). This finding indicates PLCβ1 may interact with active CDK16 or inactive CDK16. Successful extraction of the PLCβ1/CDK16 complex then assessment by cryo-EM may provide further information regarding the conformational state of both proteins in the complex.



**CDK16 Structure**

(Dixon-Clarke et al., 2017)



**PLCB3 Structure**

(Falzone and MacKinnon, 2023)

**Figure 3. Three dimensional protein structures of CDK16 and PLCB3**

Initially, we considered protein modeling software, a beneficial tool to assess protein interactions with small molecules/ligands, to determine the possible interactions between CDK16 and

PLCβ1. However, it is difficult to obtain accurate assessments of protein-protein interactions due to the large size of both molecules.

Following isolation of CDK16/PLCβ1 from HEK-293 cells, protein gel electrophoresis was performed to assess sample purity and determine complex size. PLCβ1 independently has a size of ~140 kDa (UniProt Q9NQ66). CDK16 independently has a size of ~55 kDa (UniProt Q00536). Therefore, the complex is shown by band I with a size ~160 kDa (Figure 4).

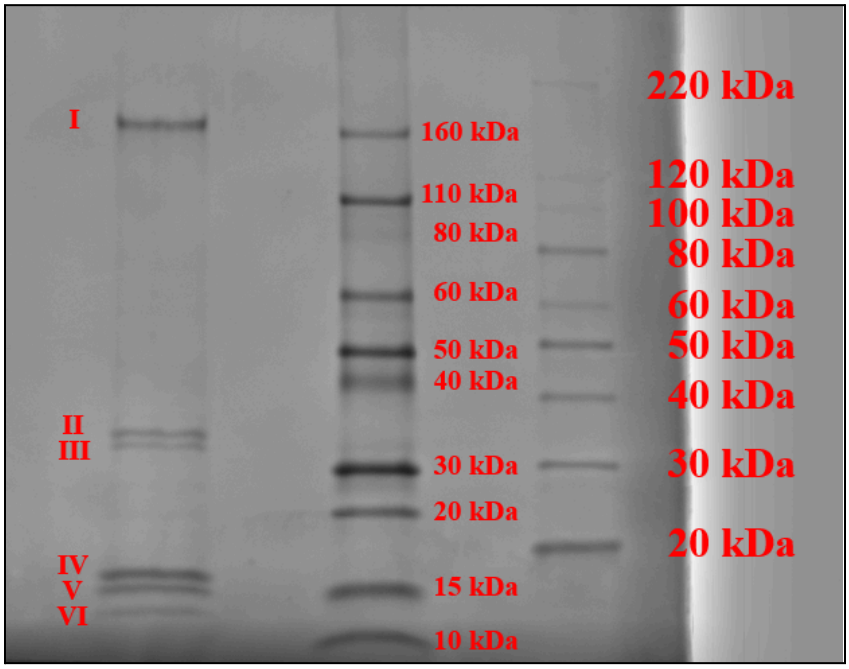



**Figure 4. PLCβ1/CDK16 complex has a size of 160 kDa.** Protein electrophoresis was used to assess the eluate obtained from HEK-293 cells with CDK16 overexpression. The complex is shown by the 160 kDa band in the left column and the middle and right columns show standard protein ladders.

To assess CDK16 kinase activity in PC12 cells with CDK16 overexpression, a Z'-LYTE™ kinase assay was used. PC12 cells without CDK16 overexpression were used as a control to

ensure the protein is still functional following electroporation. A 12-point serial dilution of each lysate was used to establish the phosphorylation curve of PC12 cells with CDK16 overexpression and control PC12 cells. The left side of a 384-plate was used for the experimental group, PC12 with CDK16 overexpression and the right side was used for the PC12 control (Figure 5).

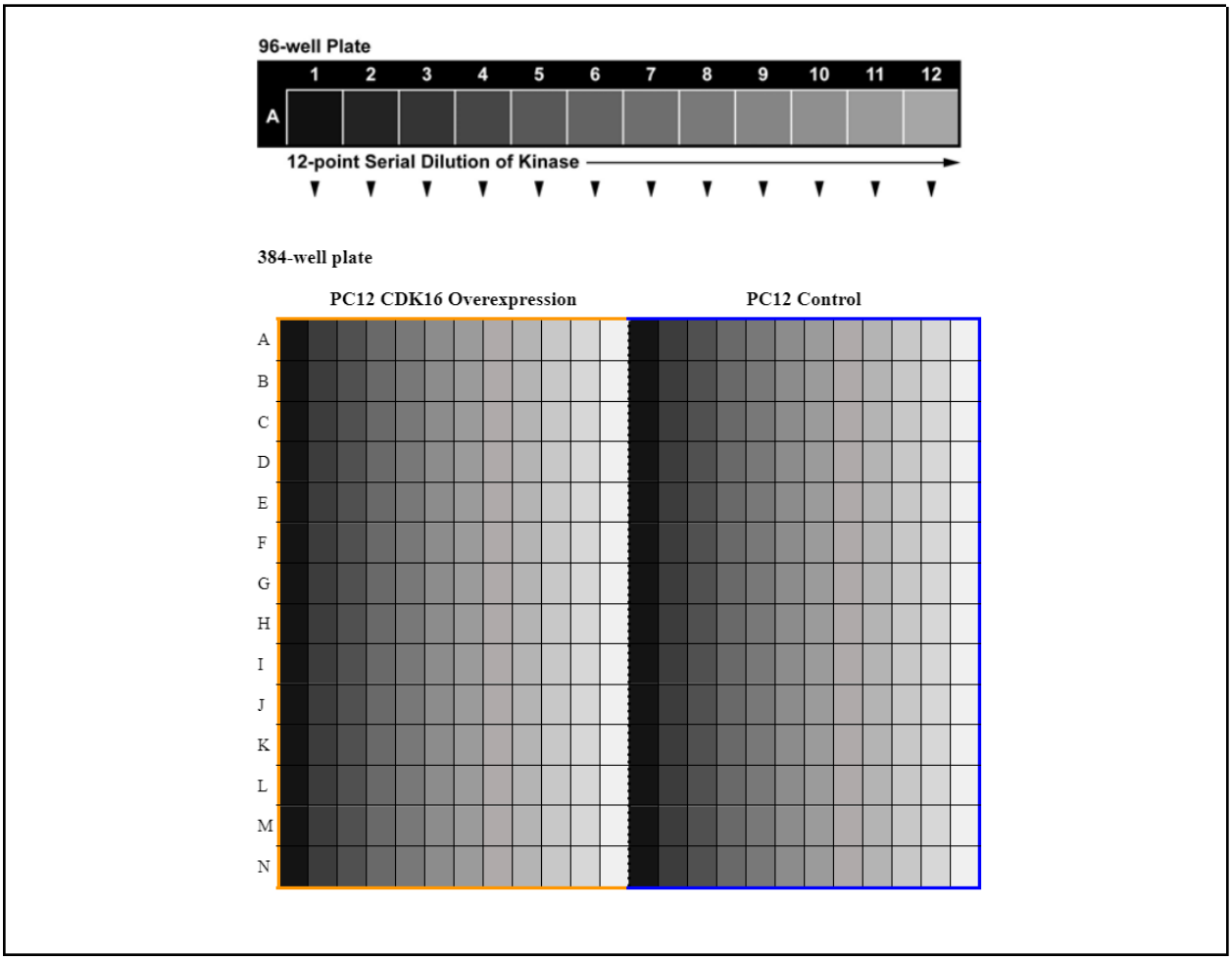



**Figure 5. Kinase assay plate set-up.** This arrangement for the kinase assay allows for a comparison of PC12 with CDK overexpression and PC12 control cells on the same plate to minimize variation.

Garwain et al. (2018) established that the PLCβ1/CDK16 interaction occurs in the cytosol close to but not on the plasma membrane. Cyclin Y, the activator protein of CDK16, is located in the nucleus and $G_{\alpha q}$, the activator protein of PLCβ1, is present in the nuclear membrane. Based on the subcellular locations of each protein, it is possible that PLCβ1 competes with cyclin Y as the nuclear membrane begins to break down since the PLCβ1/CDK16 interaction occurs close to the plasma membrane. In addition to the cryo-EM data, a follow up study to image cyclin Y and $G_{\alpha q}$ could be beneficial to understand more about the PLCβ1/CDK16 complex and the conditions regarding their interaction.

**REFERENCES**

Chu, I.M., Hengst, L., and Slingerland, J.M. (2008) The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy, *Nature Reviews Cancer, 8*, 253–267. https://doi.org/10.1038/nrc2347

Dixon-Clarke, S.E., Shehata, S.N., Krojer, T., Sharpe, T.D., von Delft, F., Sakamoto, K., and Bullock, A.N. (2017) Structure and inhibitor specificity of the PCTAIRE-family kinase CDK16, *Biochem J. 474*(5), 699-713. https://doi.org/10.1042/bcj20160941

Falzone, M.E., and MacKinnon, R. (2023) The mechanism of $G_{\alpha q}$ regulation of PLCβ3-catalyzed PIP2 hydrolysis, *PNAS*, *120*(48), e2315011120. https://doi.org/10.1073/pnas.2315011120

Garwain, O., Valla, K., and Scarlata, S. (2018) Phospholipase Cb1 regulates proliferation of neuronal cells, *The FASEB Journal*, *32*, 2891-2898. https://doi.org/10.1096/fj.201701284R

Graeser, R., Gannon, J., Poon, R.Y.C., Dubois, T., Aitken, A., and Hunt, T. (2002) Regulation of the CDK-related protein kinase PCTAIRE-1 and its possible role in neurite outgrowth in Neuro-2A cells, *J Cell Sci*. *115*(17) 3479–3490. https://doi.org/10.1242/jcs.115.17.3479

Li, X., Li., J., Xu, L., Wei, W., Cheng, A., Zhang, L., Zhang, M., Wu, G., and Cai, C. (2022) CDK16 promotes the progression and metastasis of triple-negative breast cancer by phosphorylating PRC1, *J Exp Clin Cancer Res. 41*(1), 149. https://doi.org/10.1186/s13046-022-02362-w

Malumbres, M., Harlow, E., Hunt, T., Hunter, T., Lahti, J.M., Manning, G., Morgan, D.O., Tsai, L., and Wolgemuth, D.J. (2009) Cyclin-dependent kinases: a family portrait, *Nat Cell Biol*. 11, 1275–1276. https://doi.org/10.1038/ncb1109-1275

Mikolcevic, P., Sigl, R., Rauch, V., Hess, M.W., Pfaller, K., Barisic, M., Pelliniemi, L.J., Boesl, M., and Geley, S. (2012) Cyclin-dependent kinase 16/PCTAIRE kinase 1 is activated by cyclin Y and is essential for spermatogenesis, *Mol Cell Biol. 32*(4), 868-79. https://doi.org/10.1128%2FMCB.06261-11

Mokalled, M.H., Johnson, A., Kim, Y., Oh, J., and Olson, E.N. (2010) Myocardin-related transcription factors regulate the Cdk5/Pctaire1 kinase cascade to control neurite outgrowth, neuronal migration and brain development, *Development, 137*(14), 2365–2374. https://doi.org/10.1242/dev.047605

Morgan, D. (1995) Principles of CDK regulation, *Nature*, 374, 131–134. https://doi.org/10.1038/374131a0

Palmer, K.J., Konkel, J.E., and Stephens, D.J. PCTAIRE protein kinases interact directly with the COPII complex and modulate secretory cargo transport, *J Cell Sci*. *118*(17), 3839–3847. https://doi.org/10.1242/jcs.02496

Pedersen, A., Pfeiffer, A., Karemore, G., Akimov, V., Bekker-Jensen, D.B., Blagoev, B., Francavilla, C., and Olsen, J.V. (2021) Proteomic investigation of Cbl and Cbl-b in neuroblastoma cell differentiation highlights roles for SHP-2 and CDK16, *iScience*, *24*(4), 102321. https://doi.org/10.1016/j.isci.2021.102321

Scarlata, S., Singla, A., and Garwain, O. (2018) Phospholipase Cβ interacts with cytosolic partners to regulate cell proliferation, *Advances in Biological Regulation*, *67*, 7-12. http://dx.doi.org/10.1016/j.jbior.2017.09.004

Shehata, S.N., Deak, M., Morrice, N.A., Ohta, E., Hunter, R.W., Kalscheuer, V.M., and Sakamoto, K. (2015) Cyclin Y phosphorylation- and 14-3-3-binding-dependent activation of PCTAIRE-1/CDK16, *Biochem J. 469*(3) 409-20. https://doi.org/10.1042%2FBJ20150486

UniProt. Q9NQ66 · PLCB1_HUMAN. https://www.uniprot.org/uniprotkb/Q9NQ66/entry

UniProt. Q00536 · CDK16_HUMAN. https://www.uniprot.org/uniprotkb/Q00536/entry

Wang, X., Liu, R., Li, S., Xia, W., Guo, H., Yao, W., Liang, X., Lu, Y., and Zhang, H. (2023) The roles, molecular interactions, and therapeutic value of CDK16 in human cancers, *Biomedicine & Pharmacotherapy*, *164*, 114929. https://doi.org/10.1016/j.biopha.2023.114929

Yanagi, T., Krajewska, M., Matsuzawa, S., and Reed, J.C. (2014) PCTAIRE1 phosphorylates p27 and regulates mitosis in cancer cells, *Cancer Res. 74*(20) 5795-807. https://doi.org/10.1158%2F0008-5472.CAN-14-0872

Yanagi, T., and Matsuzawa, S. (2015) PCTAIRE1/PCTK1/CDK16: a new oncotarget?, *Cell Cycle*, 14:4, 463-464. https://doi.org/10.1080/15384101.2015.1006539