DCLK1 autoinhibition and activation in tumorigenesis
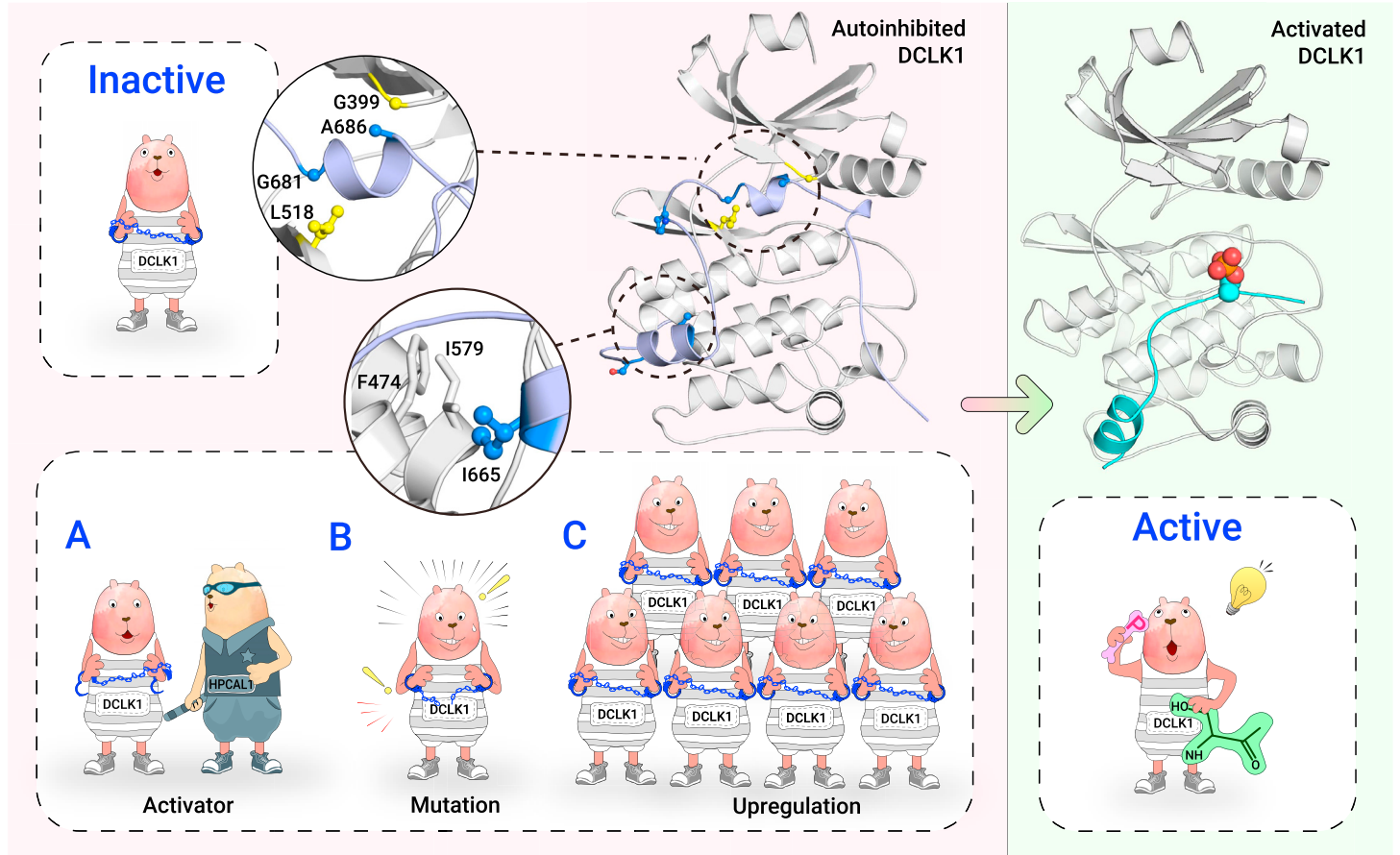
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Graphical abstract

Public summary

- The first reported DCLK1 kinase structure in the autoinhibited state

- The C-terminal autoinhibitory domain functions to block the ATP-binding site

- Cancer-associated mutations significantly upregulate DCLK1 kinase activity

- HPCAL1 activates DCLK1 kinase activity in a Ca^{2+}-dependent manner

DCLK1 autoinhibition and activation in tumorigenesis

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Doublecortin-like kinase 1 (DCLK1) is upregulated in many tumors and is a marker for tumor stem cells. Accumulating evidence suggests DCLK1 constitutes a promising drug target for cancer therapy. However, the regulation of DCLK1 kinase activity is poorly understood, particularly the function of its autoinhibitory domain (AID), and, moreover, no physiological activators of DCLK1 have presently been reported. Here we determined the first DCLK1 kinase structure in the autoinhibited state and identified the neuronal calcium sensor HPCAL1 as an activator of DCLK1. The C-terminal AID functions to block the ATP-binding site and is competitive with ATP. HPCAL1 binds directly to the AID in a Ca^{2+}-dependent manner, which releases the autoinhibition. We also analyzed cancer-associated mutations occurring in the AID and elucidate how these mutations disrupt DCLK1 autoinhibition to elicit kinase activity upregulation. Our results present a molecular mechanism for autoinhibition and activation of DCLK1 kinase activity and provide insights into DCLK1-associated tumorigenesis.

INTRODUCTION

Doublecortin-like kinase 1 (DCLK1) was first described in 1998 as a putative kinase, with homology to doublecortin.[1] DCLK1 upregulation is observed in various cancers, including colorectal,[2,3] pancreatic,[4] hepatocellular,[5,6] renal,[7] breast,[8] and bladder cancers,[9] along with esophageal adenocarcinoma,[10] and its expression has been shown to promote tumorigenesis.[9,11-14] DCLK1 is a marker for tumor stem cells (TSCs) in colon and pancreatic cancers, and is likely a TSC marker in other cancers.[15-17] A recent study also identified DCLK1 as a significantly mutated driver gene in gastric cancer.[18] Specific ablation of Dclk1-positive TSCs resulted in tumor regression without substantial damage to normal tissues.[15] Notably, inhibition of DCLK1 results in suppression of tumor growth in different cancer contexts.[3,19,20] Knockout of DCLK1 inhibits the in vitro migration and invasion of breast cancer cells.[13] Knockdown of DCLK1 significantly reduces NSCLC cell migration/invasion in vitro and inhibits tumor growth in vivo.[21] Moreover, the recent development of a highly selective DCLK1 kinase inhibitor now provides the possibility of rapidly developing DCLK1 as a therapeutic target.[22] Together, these studies rationalize DCLK1 as a promising target for anticancer treatment. However, the relationship between DCLK1 dysfunction and tumorigenesis remains elusive.

Two long (isoforms 1 and 2) and two short (isoforms 3 and 4) variants of DCLK1 are associated with alternate promoter usage, with the shorter variants being selectively expressed by some cancer cells.[1,23] Notably, the 5′(α) promoter, which gives rise to the long DCLK1 isoforms, is hypermethylated in colorectal, lung, pancreatic, and gastric cancer and cholangiocarcinoma, leading to epigenetic silencing.[23-25] In contrast, the alternate (β) promoter is utilized for the expression of the short DCLK1 isoforms in malignancies such as colon cancer.[26] The N terminus of the DCLK1 protein possesses tandem doublecortin domains with a Ser/Thr kinase domain located in the C terminus. The doublecortin domains bind microtubules and regulate microtubule polymerization[27-29] but, notably, the short isoforms lack the doublecortin domains. The C-terminal kinase domain has substantial homology with Ca^{2+}/calmodulin-dependent kinase (CaMK), but lacks calmodulin-binding motifs, and is functionally independent of calmodulin.[30] It is clear that DCLK1 undergoes autophosphorylation,[30,31] and a C-terminal autoinhibitory domain (AID) has been identified in the longer DCLK1 isoforms 2 and 4.[31] The AID consists of an arginine-rich region, which was proposed to function as a pseudosubstrate for DCLK1.[31] The crystal structure of DCLK1 kinase domain lacking the C-terminal AID has recently been reported,[32] but the mechanistic nature of the autoinhibition remains to be elucidated.

The cellular pathways engaged by DCLK1 are poorly understood and the substrates and regulators of DCLK1 remain undefined. Of interest to this report is our discovery that HPCAL1 functions as an activator of DCLK1. HPCAL1, a member of visinin-like-proteins (VILIPs), belongs to the neuronal calcium sensor (NCS) family, which are calcium-binding proteins involved in the regulation of neuronal Ca^{2+} signaling.[33] Besides functioning in the calcium-dependent regulation of signal transduction cascades in the nervous system, other reports have highlighted the signaling role of HPCAL1 in hepatocellular carcinoma,[34] pancreatic cancer,[35] and glioblastoma.[36]

In this report we disclose two related phenomena that advance the understanding of DCLK1 signaling. First, we show the crystal structure of the kinase domain of the human DCLK1 in the autoinhibited state. This structure reveals that the C-terminal AID binds to the cleft between the two lobes of the kinase domain, blocking the ATP and peptide substrate-binding sites and inhibiting DCLK1 kinase activity in an ATP-competitive manner. Second, we establish that HPCAL1 directly binds the AID in a Ca^{2+}-dependent manner to activate DCLK1 kinase activity. Notably, somatic cancer mutations in the AID disrupted the interactions between the AID and kinase domain, and therefore upregulated DCLK1 activity. This proposes that such mutations contribute to tumorigenesis by releasing autoinhibition of DCLK1.

RESULTS

Structure of the autoinhibited kinase domain of DCLK1

We first investigated the autoinhibition mechanism of DCLK1. Different naming schemes have been used to annotate DCLK1 isoforms but, for consistency, we adopted the nomenclature used in the UniProt database (Figure 1A). Based on this information, we prepared the KD-AID construct of isoform 2, consisting of the human DCLK1 kinase domain and the C-terminal AID, and solved the crystal structure of DCLK1 KD-AID to 2.15 Å resolution in space group $P2_12_12_1$.

The catalytic core displayed a similar fold to other kinases. The C-terminal inhibiting tail wraps around the C lobe and the active site of the catalytic domain, blocking the ATP-binding site and the potential substrate-binding site (Figure 1B). There were two molecules per asymmetric unit, associated in a head-to-head, back-to-back arrangement with root-mean-square deviation (RMSD) of 0.202 Å over 259 Cα atoms (Figure 1C). The inhibition segment extended through the active site, wedged between the small and large lobes of the kinase domain and making extensive contacts with the catalytic core, which accounts for autoinhibition (Figure 1D). The C-terminal AID contained two α helixes (R1 and R2) and a 3_{10} helix (R3) (Figure 1E), and each helix formed a hydrophobic core with the catalytic domain (Figure 1D). The AID also made hydrophilic interactions with the kinase domain (Figure 1D). K692, which is adjacent to R3, formed salt bridges with two key residues of the catalytic domain, D511 and D533. Here D511 represents the catalytic aspartate, which is invariant in all kinases. D533 is the first residue of the DFG motif, which is required to coordinate the magnesium ion and position of the ATP phosphate groups for catalysis. R698 and R701, which are located near the C terminus of AID, interact with E595 and D599 via a network
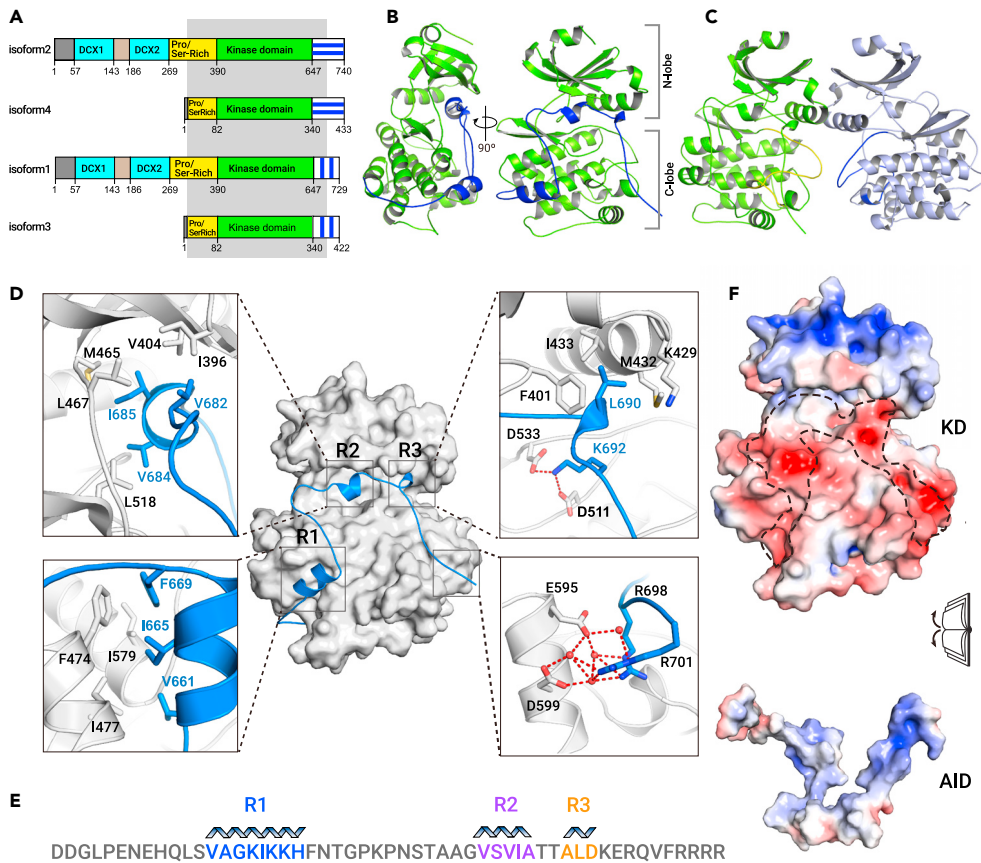
Figure 1. Structure of autoinhibited DCLK1 (A) Domain structure of four DCLK1 isoforms referenced in UniProt. The identical regions within all isoforms are shadowed but notably there are distinct C-terminal regions in different isoforms labeled as blue vertical or horizontal lines. (B) Overall structure of autoinhibited DCLK1 (isoform 2) kinase domain with C-terminal AID. The kinase domain is colored green, and the AID is colored blue. (C) Two molecules of DCLK1 within the asymmetric unit in a head-to-head, back-to-back arrangement. DCLK1 molecules are colored green and light blue, respectively. Their activation loops are highlighted by yellow and deep blue, respectively. (D) Structure of autoinhibited DCLK1 kinase domain. DCLK1 kinase domain is shown as surface (gray), with the AID shown as a ribbon (blue). (E) Residue sequence of the AID observed in the crystal structure. (F) Open-book view of the electrostatic surface potential of DCLK1 kinase domain and the AID. The AID-binding region is marked by dashes. Blue and red (±5 kT/e) indicate the positively and negatively charged areas of the protein, respectively.

AID inhibits the kinase activity of DCLK1

We next used in vitro kinase assays and Phostag SDS-PAGE to quantitatively analyze the autophosphorylation activity of DCLK1. In Phos-tag SDS-PAGE, the phosphorylated and nonphosphorylated forms of a protein can be distinguished by the reduced mobility caused by the presence of phosphorylated motifs.

We prepared the KD construct consisting of the DCLK1 kinase domain lacking the inhibitory C-terminal tail. Instructively, the purified KD-AID remained unphosphorylated, while almost all purified KD was phosphorylated (Figure 3A), indicating that KD-AID was catalytically inactive and KD was active. Treatment of the samples with lambda protein phosphatase eliminated the reduced-mobility bands, confirming that the KD was phosphorylated (Figure 3A). To verify phosphorylation resulted from an auto-phosphorylation event, we mutated the catalytic residue D511 (D511N) of the KD to abolish its catalytic activity.[32] Indeed, no phosphorylation signals were detected for the enzymatically dead D511N mutant (Figure 3A).

To further evaluate the relation between AID binding and DCLK1 activity, we introduced mutations that disrupted the hydrophobic (I685F) or hydrophilic (K692A, R698A, and R701A) interactions between AID and KD. Importantly, analysis of the purified KD-AID mutant proteins showed they were phosphorylated (Figure 3B), indicating mutations that affect AID-KD interactions served to activate DCLK1 kinase activity. Furthermore, kinase activity assays using a modified CaMKI peptide derived from synapsin LRRRLSLANF[31] as an exogenous substrate further confirmed that these mutants enhanced the kinase activity of DCLK1 (Figure 3C). Since the readout of the kinase activity assay was the combination of DCLK1 autophosphorylation and exogenous-substrate phosphorylation, autophosphorylation activity in the absence of the exogenous substrate was treated as the background.

Finally, as seen in the crystal structure, the second regulatory helix of the AID (R2) occupies the ATP-binding site (Figure 1D). We measured ATP binding using isothermal titration calorimetric (ITC), and found that the kinase domain, but not kinase domain-AID, bound to ATP (Figure 3D). Free AID peptide also inhibited the kinase activity of DCLK1 (Figure 3E), with a calculated half maximal inhibitory concentration (IC50) value of 3.22 μM (Figure 3F). Moreover, we found that excess ATP activated KD-AID, suggesting that AID competes with ATP for binding (Figure 3G).

Identification of HPCAL1 as an activator of DCLK1

The DCLK1 kinase domain most closely resembles CaMKs in terms of sequence homology, although DCLK1 has been reported to be calmodulin independent.[30] In the autoinhibited DCLK1 structure, the first helix of the regulatory sequence (R1) occupied a position close to the calmodulin-binding motif of CaMKs (Figure S3A). Notably, the R1 helix contains a cluster of basic and hydrophobic residues (Figure 1E) that is characteristic of Ca2+/calmodulin-binding

of water-mediated hydrogen bonds. However, R701 is only visible in one molecule in the asymmetric unit.

The accessible surface area buried in the interface between the C-terminal inhibiting tail and the kinase domain amounts to 2,402 Å^2 (2,596 Å^2 on the kinase domain and 2,208 Å^2 on the inhibiting segment, calculated using a solvent probe of radius 1.4 Å). The electrostatic potential surfaces of the AID and its binding region in the catalytic core show a complementary pattern (Figure 1F), which ensures the correct binding mode of the autoinhibitory domain. Further confirming interactions between the AID and kinase domains, affinity measurements using surface plasmon resonance (SPR) showed the K_D value was 154.9 μM for the AID-kinase domain interaction (Figure S1).

Comparison between the active and autoinhibited states of DCLK1

The crystal structure of the active DCLK1 kinase domain complexed with the ATP analog, AMP-PNP, has recently been reported.[32] Compared with the active state, the autoinhibited DCLK1 kinase domain underwent remarkable conformational changes, mainly involving the N lobe and αD helix (Figure 2). In the autoinhibited structure, the N lobe rotates about 20° with respect to the C lobe to accommodate the path of the AID (the two molecules were superposed using the C lobe, and then the change in angle upon superimposing the N lobe was calculated). This rotation can be achieved by a hinge motion at the adjacent region between the two lobes. Within the N lobe, the αC helix and connecting loop also undergo a marked conformational change (Figure 2). The αD helix is pushed backward by the first helix of the regulatory sequence (R1) and rotates about 40°.

The activation loop runs from the conserved DFG motif to the conserved APE motif and corresponds to residues 533–557 in DCLK1 isoform 2.[37] It has been observed in many kinases that the activation loop has different conformations in the active and inactive kinase structures.[38] However, in the autoinhibited DCLK1 structure, the activation loop adopts the same configuration as that in the active state (Figure 2). Two nonconsecutive hydrophobic structures termed R spine and C spine reveal the kinase internal architecture. R spine is fully assembled in the active conformation of kinases, but broken in the inactive kinases.[39] However, in autoinhibited DCLK1, R spine is fully assembled as for active DCLK1 (Figure S2).[32]
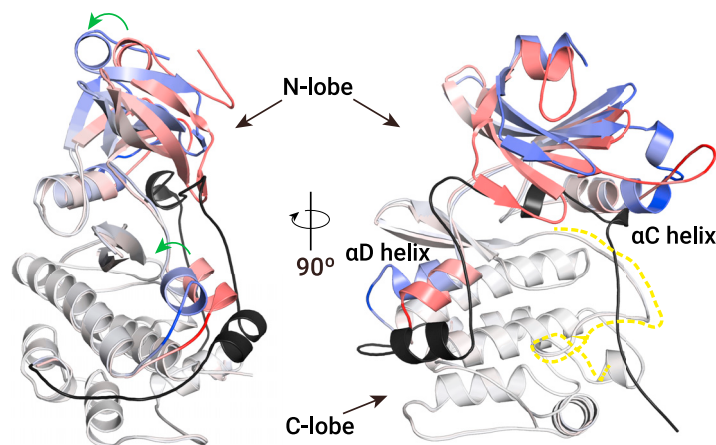
Figure 2. Comparison of the active and autoinhibited kinase domain of DCLK1 The crystal structures of the active DCLK1 (PDB: 5JZJ) and the autoinhibited DCLK1 are aligned by C-alpha atoms of the C lobe. The aligned structures are colored by a color spectrum according to the RMSD, with gray specifying the minimum pairwise RMSD and red (active) and blue (autoinhibited) indicating the maximum for the two structures, respectively. The rotations of the N lobe and αD helix are indicated by green arrows. The AID is colored black. The activation loop is indicated by the yellow dashed line.

sequences.[40] Therefore, we evaluated whether calmodulin could bind to KD-AID and activate DCLK1 through Ca^{2+}/calmodulin akin to the CaMKs. However, neither recombinant human calmodulin or a commercially sourced bovine calmodulin activated DCLK1. Co-expression of human calmodulin with DCLK1 did not enhance DCLK1 autophosphorylation and no interaction was detected between DCLK1 and Ca^{2+}/calmodulin using ITC (Figure S4).

Considering the autoinhibition mode, the observations made with DCLK1 were reminiscent of the inhibition of giant protein kinases twitchin and titin (Figure S3B). Twitchin kinase inhibits muscle activity,[41] while titin is suggested to perform a signaling function as a pseudokinase.[42] Twitchin is known to be activated by Ca^{2+}/S100[43] and we therefore considered if Ca^{2+}/S100 could also activate DCLK1. However, after testing 20 S100 proteins (S100 A1-A16, B, G, P, and Z), we found no significant enhancement of DCLK1 autophosphorylation (Figure S5). Notwithstanding these results, calmodulin and S100 proteins are calcium-binding proteins, which inspired us to evaluate other calcium-binding proteins as DCLK1 activators.

Since DCLK1 is highly expressed in brain and most abundant during the neuronal migration period,[1,44] we expressed and purified NCS family proteins (NCS1, VSNL1, HPCAL1, HPCAL4, HPCA, NCALD, RCVRN, GUCA1A, and KCNIP 1–4). Among these, we found that HPCAL1 activated DCLK1 in a calcium-dependent manner (Figures 4A and S6). SPR assays further suggested that HPCAL1 binds directly to DCLK1 in a calcium-dependent manner (Figure 4B), with their interaction mediated by the AID of DCLK1 (Figure 4C). The interaction between these two proteins was further confirmed by co-immunoprecipitation between endogenous HPCAL1 and DCLK1 (Figure S7). Surprisingly, while displaying high sequence homology to HPCAL1, other members of the VILIP subfamily neither activated nor bound DCLK1 (Figures 4A and S8). These results indicate that Ca^{2+}/HPCAL1 binds directly to the AID and releases the autoinhibition of DCLK1.

Somatic DCLK1 cancer mutants display elevated kinase activity

To explore the impact of DCLK1 somatic mutations in human cancers, we interrogated the COSMIC database and found five mutations (S660L, I665M, P675L, G681E, and A686T) within the DCLK1 C-terminal AID and two mutations (G399E and L518M) in the AID-binding region of the catalytic core (Figure 5A). Details of these mutations are summarized in Table S1. We subsequently expressed and purified these mutants and found that most were significantly phosphorylated (Figure 5B), indicating these mutations cause elevated DCLK1 kinase activity. The findings were further verified by kinase activity assays (Figure 5C).

Based on our structural modeling, mutations from small to large side chains were likely to interfere with AID binding. Interestingly, we found spatial proximity between two pairs of individual mutations involving L518M/G681E and G399E/A686T, respectively (Figure 5A). This indicated that mutation of either residue

to a larger one would disrupt AID binding. Other mutations involving S660L and P675L located at loop regions may generate some rigidity preventing the loops from adopting their proper conformation. We also obtained the crystal structure of the P675L mutant and found that the neighboring part of the mutation site became disordered (Figure S9). Further evidence was derived from proteolysis experiments. Among the mutants tested in the Phos-tag system, G399E, G681E, I685F, and K692A displayed the highest autophosphorylation activity (Figures 3B and 5B) and were more readily digested by trypsin than the wild type (Figure 5D). This indicates that these mutations cause instability in AID binding.

Together these structural and functional results suggest that cancer-associated mutations disrupt AID binding and release the autoinhibition of DCLK1, resulting in increased DCLK1 kinase activity. This contrasts the effects of cancer-associated kinase domain mutants, which display reduced stability and would be expected to have reduced activity.[32]

DISCUSSION

The prevailing hypothesis regarding the mechanism of DCLK1 autoinhibition was that the inhibitory segment acts as a pseudosubstrate blocking the substrate-binding site.[31] However, the autoinhibited DCLK1 structure we report here shows the previously identified pseudosubstrate-like autoinhibitory sequence (VFRRRRNQD) occupies the potential peptide substrate-binding site, while the AID takes up the ATP-binding site (Figure 1D). The buried area between the AID and the catalytic core of DCLK1 (2,402 Å²) is much larger than that in typical protein-protein recognition sites (1,200–2,000 Å²),[45] suggesting a strong interaction. We propose that the complementary electrostatic potential surface pattern on the catalytic core and AID of DCLK1 ensures the correct binding mode of this autoinhibitory domain, and the three hydrophobic cores provide enough binding force to guarantee autoinhibition (Figures 1D–1F). We suspect the binding affinity measured by SPR between AID and DCLK1 (Figure S1) may be an underestimate. First, the covalently tethered AID provides a much higher local concentration of AID than the isolated AID peptide. Second, the isolated AID peptide may not adopt the conformation and orientation favoring interaction with DCLK1.

As noted in the section "introduction," there are four main isoforms of DCLK1, but whether isoforms 1 and 3 of human DCLK1, which lack the arginine-rich region, are also autoinhibited remains unclear. All four isoforms share the same amino acid sequence in their C termini until the middle of R2 (Figure S10), so the C-terminal segment of isoforms 1 and 3 may also potentially occupy the ATP-binding site, resulting in autoinhibition (Figure 1). However, further structural analysis with DCLK1 isoforms 1 and 3 will be necessary to confirm this conjecture. Irrespectively, resolving this point could also be relevant to better understanding the role of DCLK1 in malignancies given that certain cancer cells exhibit preferential overexpression of DCLK1 isoforms 3 and 4, which either possess or lack the arginine-rich region, respectively.

The activation of many kinases involves major conformational changes of the activation loop.[37] It was therefore intriguing to find that the activation loop of DCLK1 in both active and inactive states adopts the same conformation (Figure 2). It is worth noting that the crystal structures of active and inactive DCLK1 have been obtained in two crystal forms ($P2_1$ and $P2_12_12_1$, respectively),[32] indicating that the conformation of DCLK1 activation loop is intrinsic and not influenced by the crystallization environment. R-spine in the autoinhibited DCLK1 is fully assembled (Figure S2), further confirming that the autoinhibited DCLK1 adopts an active conformation while the active site is occupied by the autoinhibitory segment.

Reversible phosphorylation regulates protein function via altering enzymatic activity, binding partners, or cellular localization, and thus plays an essential role in signal transduction. For the autoinhibited KD-AID, although we could not see the phosphorylated protein bands in the Phos-tag SDS-PAGE (Figure 3A), basal kinase activity was evident above the levels measured in kinase assays using the D511N kinase-dead mutant (Figure 3C). Moreover, the various mutants examined showed different phosphorylation patterns in the Phos-tag SDS-PAGE assays, possibly indicating multisite autophosphorylation sites exist, although the exact function remains unknown. One possibility is that multisite autophosphorylation serves as a sensor of kinase activity: as more sites become phosphorylated, kinase activity is increased. Such sensor functions have been observed in the transcription activator Ets-1, which shows a rheostat effect to
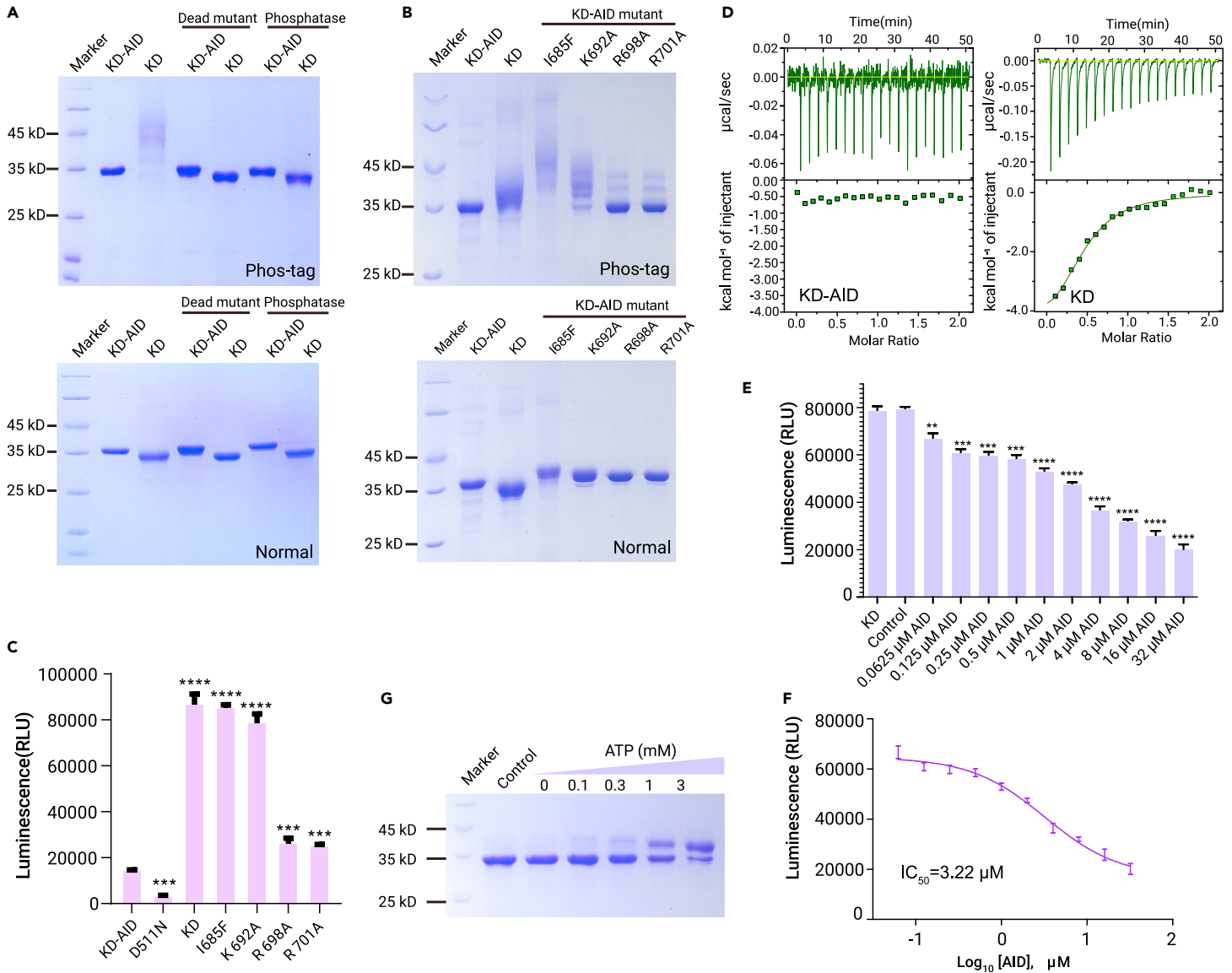
Figure 3. The AID inhibits the autophosphorylation of DCLK1 (A and B) Phosphorylation is detected in (A) KD-AID, KD, D511N kinase-dead mutant, and phosphatase-treated samples, and (B) KD-AID mutants. (Upper) Phos-tag SDS-PAGE. (Lower) Normal SDS-PAGE. (C) Quantification of DCLK1 and mutant kinase activity toward a CaMK1 derivative substrate. Kinase activity is indicated by the consumption of ATP, which is measured using the ADP-Glo kinase assay kit. DCLK1 autophosphorylation activity in the absence of the exogenous substrate is also measured as the background, which is deducted to calculate the kinase activity toward the CaMK1 derivative substrate. Values are means ± SD (n = 3). ***p < 0.001, ****p < 0.0001. (D) The binding of ATP to KD-AID or KD measured by isothermal titration calorimetry. (Upper) The baseline corrected instrumental response. (Lower) The integrated data (solid squares) and the best fit to a 1:1 binding model (solid line). Data are representative of n = 3 experiments. (E) DCLK1 kinase activity is inhibited by synthesized AID peptide. The N-terminal peptide of human importin subunit alpha-1 was used as control, which also has clusters of basic residues but is function unrelated. Different concentrations of AID peptide are pre-incubated with 20 μM KD for 15 min at room temperature and kinase activity is measured using ADP-Glo kinase assays. Values are means ± SD (n = 3). **p < 0.01, ***p < 0.001, ****p < 0.0001. (F) IC_{50} calculation for ATP-competitive AID peptide. The data from (E) were used for the calculation. Curve fitting was performed using GraphPad Prism sigmoidal dose-response software. (G) The AID is competitive with ATP. KD-AID (20 μM) is incubated with increasing concentrations of ATP for 1 h, and then analyzed by Phos-tag SDS-PAGE.

fine-tune cell signaling,[46,47] and also in the cyclin-dependent kinase (CDK) inhibitor SIC1, which elicits a switch-like response when the number of phosphorylated sites reaches a threshold.[48,49]

Autoinhibition has been characterized in many kinases.[50] An AID maintains a kinase in the inactive state, and activation occurs when this domain is sequestered by a specific activator protein. For example, activators represented by Ca^{2+}/calmodulin in myosin light chain kinases (MLCKs) and CaMKs[51] and Ca^{2+}/SI00 in twitchin[43] have been proposed to interact with the inhibitory segment, release autoinhibition, and allow catalytic function. Although the first helix (R1) in the DCLK1 AID is characteristic of a Ca^{2+}/calmodulin-binding motif and the AID-binding mode is structurally similar to twitchin, we failed to detect DCLK1 activation by Ca^{2+}/calmodulin or Ca^{2+}/S100. Broadening our search to other calcium-binding proteins, we turned to evaluating whether NCS family proteins functioned as DCLK1 activators. The NCS family, including the subfamily of VILIPs, are calcium-binding proteins involved in the regulation of neuronal Ca^{2+} signaling.[33]

Among the five VILIP subfamily members in humans, we identified HPCAL1 as an activating partner for DCLK1 (Figure 4). HPCAL1 binds to the AID with a much higher affinity compared with the DCLK1 kinase domain (Figures 4C and S1). The direct interaction between HPCAL1 and DCLK1 appeared specific since none of the other four VILIP subfamily members bound DCLK1 (Figure S8), even though HPCA and NCALD share over 90% protein sequence identity with HPCAL1. Of the five members of the VILIP subfamily, HPCA is most closely related to HPCAL1 (94% amino acid identity), and has been reported to specifically interact with mitogen-activated protein kinase (MAPK).[52] HPCAL1 is more ubiquitously expressed than DCLK1 but their co-expression is detected in many different tissues, and both are enriched in the brain and most abundant in the cerebellum (http://www.proteinatlas.org/). Interestingly, DCLK1 and HPCAL1 are both related to degenerative brain diseases[53,54] and both have been reported in the context of hepatocellular carcinoma[5,6,34] and pancreatic cancer.[4,35] Their interaction, as identified here, may therefore shed new light on their functions in both the neural system and cancer. Furthermore, with the advent of increasingly specific
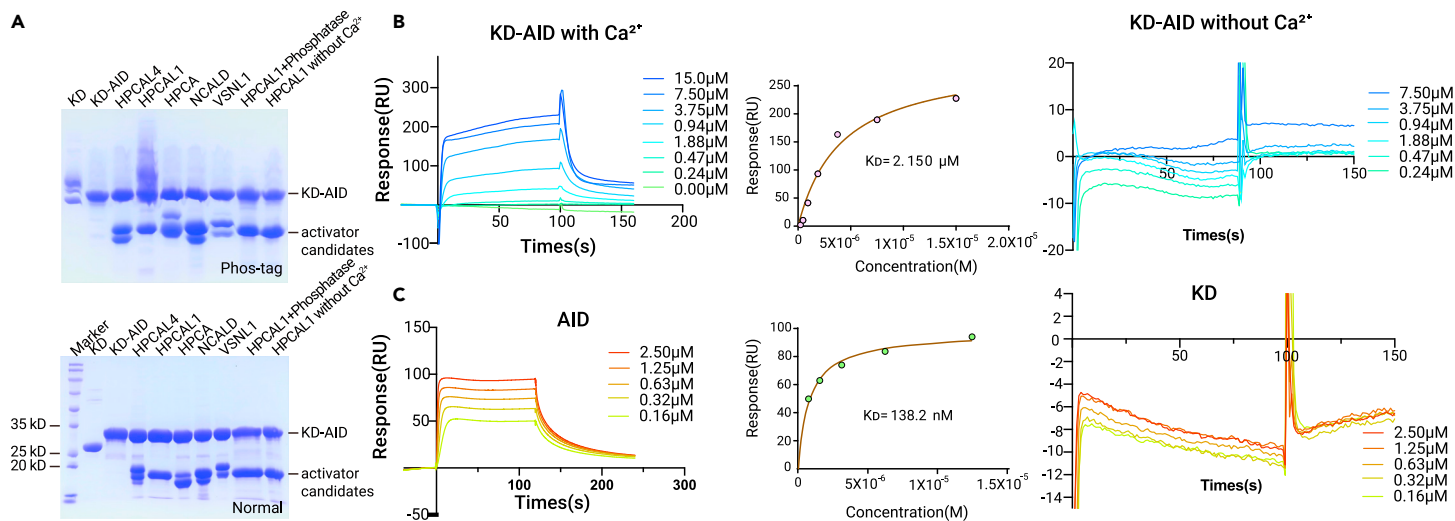
Figure 4. Identification of HPCAL1 as an activator of DCLK1 (A) HPCAL1 activates DCLK1 in a Ca^{2+}-dependent manner. Individual VILIP subfamily members were incubated with KD-AID for 1 h before analysis by Phos-tag SDS-PAGE. HPCAL1 samples were also analyzed after treatment with phosphatase or under calcium-deficient conditions (Phos-tag and normal SDS-PAGE gels are shown in the top and bottom, respectively). (B) SPR analysis showing HPCAL1 binds directly to DCLK1 in a Ca^{2+}-dependent manner. (Left) SPR binding curves under defined Ca^{2+}-containing conditions; (middle) equilibrium response plotted against the DCLK1 concentrations to calculate the K_D value; (right) SPR binding curves under Ca^{2+}-free conditions. (C) Direct interactions between HPCAL1 and DCLK AID or KD measured by SPR. (Left) Binding curves of SPR measurement between HPCAL1 and AID; (middle) Equilibrium response plotted against the AID concentrations to calculate the K_D value. (Right) The binding curves of SPR measurements between HPCAL1 and DCLK1 KD.

inhibitors against DCLK1,[55] it is pertinent to consider how the efficacy of small molecule DCLK1 inhibitors would be affected by the presence of HPCAL1 or other endogenous activators.

It is worth noting that DCLK1 AID was ATP competitive, and that DCLK1 kinase activity was significantly activated *in vitro* by 1 mM ATP (Figure 3G). While further studies are needed to verify whether ATP could activate DCLK1 *in vivo*, it is tempting to speculate that DCLK1 activity is regulated by intracellular local ATP concentrations. Tumor cells predominantly produce energy by aerobic glycolysis rather than oxidative phosphorylation as in most normal cells, known as the Warburg effect. Tumor cells are inclined to have higher ATP levels and more mitochondrial mass per cell relative to normal cells.[56] Although glycolysis has a much lower yield than that of respiration, it significantly increases the rate of ATP production. For example, glycolysis is up to 100 times faster than respiration in striated muscle cells.[57] The Warburg effect might result in an instant increase of local ATP concentration in cancer cells, thereby activating DCLK1.

Finally, we must consider the overall relevance of our findings in the context of malignancy. Kinase activity is tightly regulated both spatially and temporally, with our study proposing that DCLK1 activity is self-restrained through autoinhibition with activation by endogenous regulators such as HPCAL1. However, the inherent genomic instability of cancer cells frequently drives the acquisition of mutations that can either increase expression levels, constitutively activate kinase activity, or release autoinhibition. We demonstrated that cancer-associated mutations found within the AID could render DCLK1 constitutively active, which highlights the likely importance of DCLK1 autoinhibition in tumorigenesis. The basal DCLK1 kinase activity recorded in the autoinhibited state (Figure 3C) also suggests that the general upregulation of DCLK1 expression observed in many tumors would result in DCLK1 activity above normal levels. Our working model illustrating this thesis is shown in Figure 6, where irregularly increased DCLK1 activity acts to promote tumorigenesis. Nevertheless, this projection must be considered in the context of the range of somatic mutations occurring throughout DCLK1 whose functional impact is yet to be determined.
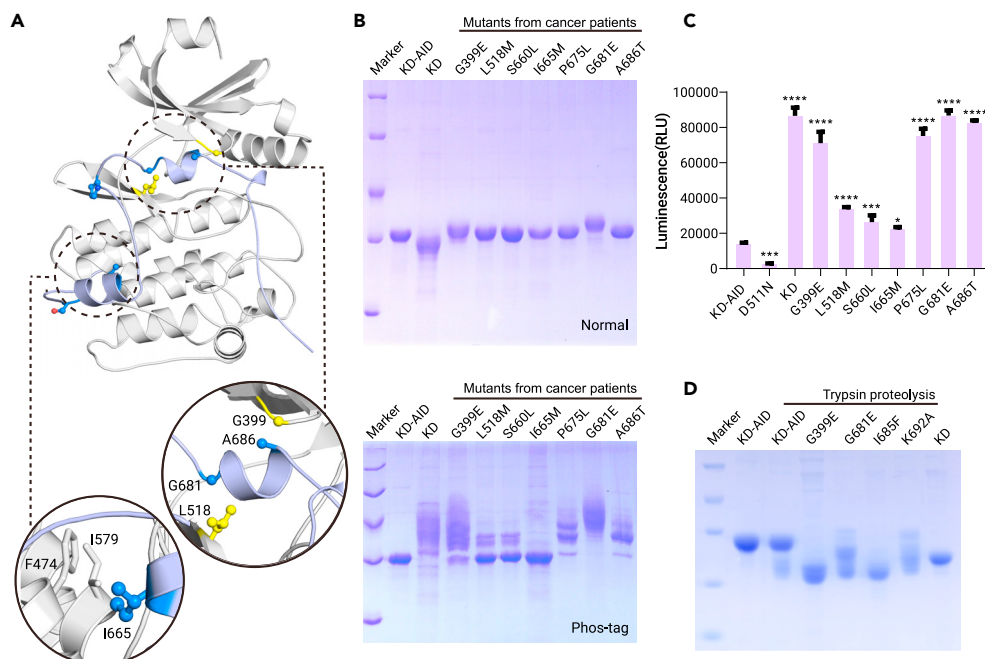

Figure 5. Somatic cancer mutations in the AID activate DCLK1 (A) Visualization of DCLK1 AID mutations identified in cancer tissues from the COSMIC database. The mutation sites are represented in stick form, with the residues located in the AID and the kinase domain are colored blue and yellow, respectively. (B) DCLK1 mutants show increased autophosphorylation activity. (Upper) Normal SDS-PAGE. (Lower) Phos-tag SDS-PAGE. (C) Quantification of the kinase activity of DCLK1 and mutants toward a CaMK1 derivative substrate. The kinase activity was measured by ADP-Glo kinase assay as per Figure 4C. Values are means ± SD (n = 3). **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (D) Proteolytic analysis of wild-type DCLK1 and mutants using trypsin at room temperature for 1 h with samples resolved by SDS-PAGE.
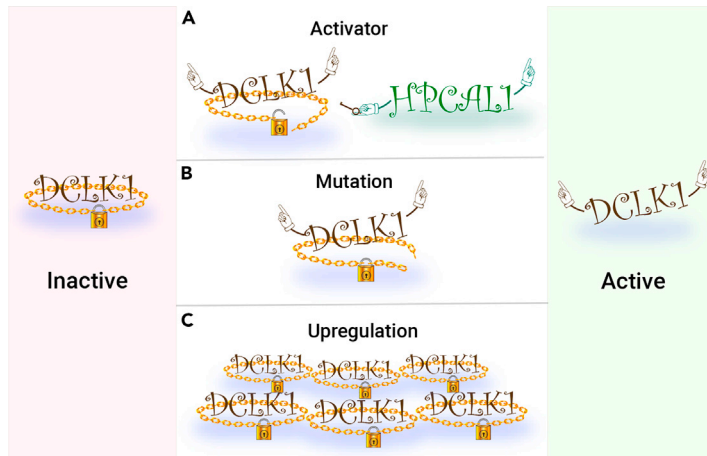
Figure 6. Molecular mechanisms of DCLK1 activation The autoinhibition of DCLK1 could be released via three ways. (A) Activation of DCLK1 by a physiological activator protein such as HPCAL1. (B) Mutational dysregulation interferes with AID binding, releasing autoinhibition to deliver DCLK1 in a constitutively active state. (C) Upregulation of DCLK1 expression levels increases overall kinase activity.

In conclusion, we present the crystal structure of the autoinhibited kinase domain of human DCLK1 and, together with functional studies, reveal that the C-terminal AID occupies the ATP-binding site, resulting in the autoinhibition of DCLK1. We show that HPCAL1 activates DCLK1 *in vitro*, making it a promising candidate as a key physiological activator of DCLK1. Irregular activation of DCLK1, such as expression upregulation or mutations disrupting the interactions between the AID and the kinase domain, would therefore promote tumorigenesis. Our study paves the way for a more comprehensive understanding of the function of DCLK1.

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AUTHOR CONTRIBUTIONS

Q.C. conceived and designed the experiments and wrote the manuscript. L.C. and C.W. performed protein preparation and crystallization. Y.Y. and Q.C. conducted structure determination and analysis. L.C., W.G., S.L., A.T., Z.C., and S.-Y.Y. measured kinase activity and protein binding. Z.Y. performed the activator identification. R.F.T. revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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