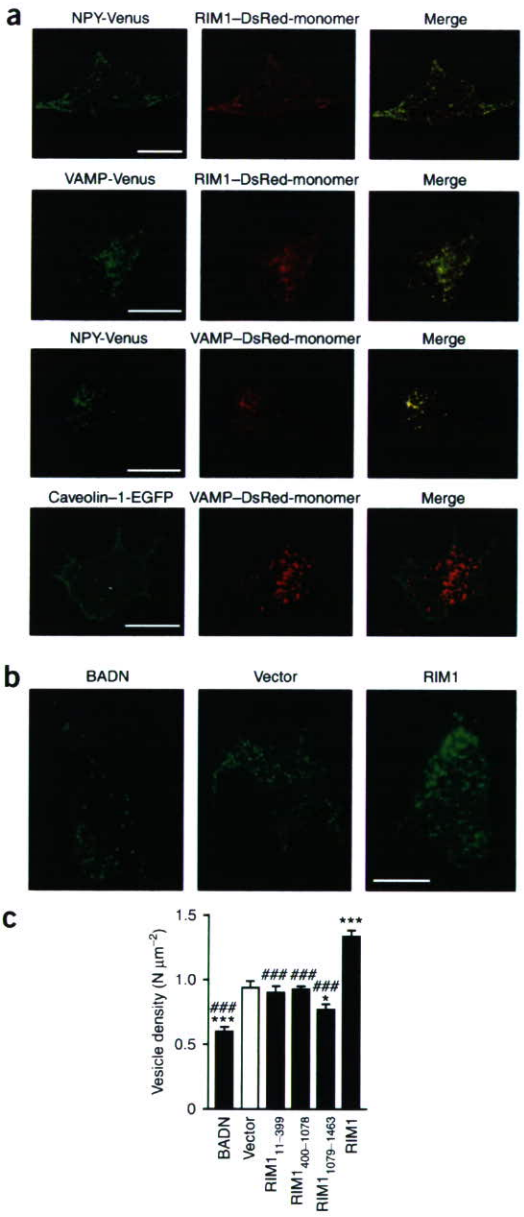

Figure 7 RIM1 and β subunits associate to anchor neurotransmitter vesicles to VDCCs at the plasma membrane. (**a**) NPY-containing secretory vesicles were colocalized with RIM1 and VAMP that was not colocalized with caveolin-1 in PC12 cells. NPY-Venus and RIM1–DsRed-monomer, VAMP-Venus and RIM1–DsRed-monomer, NPY-Venus and VAMP–DsRed-monomer, or caveolin-1–EGFP and VAMP–DsRed-monomer were coexpressed in PC12 cells and live images of the cells were obtained by confocal microscopy. Scale bar, 10 µm. (**b**,**c**) Effects of RIM1 constructs and BADN on the density of docked vesicles. Typical TIRF images of plasma membrane–docked vesicles containing NPY-Venus are shown in **b**. Left, BADN-cotransfected PC12 cell. Middle, vector-cotransfected PC12 cell. Right, RIM1-cotransfected PC12 cell. Scale bar, 10 µm. In **c**, the vesicle density was determined by counting the vesicles in each image ($n = 20$ cells in each). $^{*}P < 0.05$, $^{***}P < 0.001$ versus vector. $^{###}P < 0.001$ versus RIM1.

neurons are indistinguishable between wild-type and lethargic mice[32]. This has been attributed to the rescue of β_4 deficiency by the remaining subunits, β_1, β_2 and β_3. We expect a similar compensatory mechanism may also occur in the RIM1-β interaction in lethargic mice. Therefore, the β_4 isoform is unlikely to exclusively mediate the RIM1-VDCC association in the brain.

The RIM1-β association enables RIM1 to have a dual physiological function in neurotransmitter release: sustaining Ca^{2+} influx through the functional regulation of VDCCs and anchoring vesicles to VDCCs (**Supplementary Fig. 8** online). Among the functional parameters of VDCCs, those that are related to voltage-dependent inactivation are most prominently modified by RIM1 through the β interaction (**Fig. 4**). The inactivation kinetics are markedly decelerated, resulting in the predominance of high-voltage inactivation and an inactivation-resistant current component in the 2-s prepulse protocol. The similarly modulated inactivation properties of P/Q-type VDCCs by RIM2α, RIM3γ or RIM4γ (unpublished data), which all carry the C_2B domain, suggest that this function is a common feature for the RIM family[5]. Suppression by BADN of the RIM1-mediated inactivation in both the recombinant (**Supplementary Fig. 5**) and native VDCCs (**Fig. 5c**) provides evidence that RIM1 exerts observed effects through the RIM1-β association. This is supported by our finding that β_4-GK, which binds to α_1 (ref. 37) but not to RIM1, did not mediate RIM1 effects on N-type channels (**Supplementary Fig. 5**). Although the detailed molecular mechanisms underlying inactivation have yet to be determined, previous mapping of the molecular determinants for voltage-dependent inactivation kinetics[43] suggest that RIM1-β complexes bound to the I-II linker AID further act on adjacent segment S6 of repeat I to hinder its conformational transition to the inactivated state. Alternatively, RIM1 may immobilize the β subunit and the process of inactivation by slowing the movement of the I-II loop[44,45]. When voltage-dependent inactivation is thus suppressed, the responses of Ca^{2+} sensors such as synaptotagmins to Ca^{2+} influx may be potentiated at depolarizing membrane potentials that induce voltage-dependent inactivation when RIM1 is absent. Because RIM1 virtually abolished VDCC inactivation that was elicited by a train of action potential waveforms (**Fig. 4f**), certain forms of synaptic depression via closed-state inactivation[36] may be minimized by RIM1 at presynaptic active zones. The impact of the RIM1-β association on delaying VDCC inactivation may explain recent findings with the RIM1α knockout mouse showing that RIM1 is important for the late-stage asynchronous neurotransmitter release, whereas synaptotagmin I is involved in the earlier synchronous release[12]. With P/Q- and N-type channels, the RIM1-β association significantly affected channel activity as well; current densities were nearly doubled by RIM (**Fig. 6c**). Thus, RIM1 can maintain and enhance depolarization-induced Ca^{2+} influx to support neurotransmitter release at presynaptic active zones.

Regarding the role of RIM1 in vesicle anchoring to VDCC at the plasma membrane, full-length RIM1 is required to mediate vesicle anchoring, in contrast to the RIM1 suppression of VDCC inactivation, which requires only the RIM1 C terminus. Taking into consideration the direct RIM1-Rab3 association and the regulation of tethering and/or priming of synaptic vesicles by Rab3 (ref. 40), it is likely that simultaneous interactions of RIM1 with vesicle-associated Rab3 and Munc13 via the N-terminal Zn^{2+}-finger domain and with the VDCC β subunit via the C-terminal C_2B domain underlie, at least in part, the maintenance of the close proximity of VDCCs to vesicles, thereby regulating the dynamic properties of synaptic transmission[3]. Supporting this idea, BADN and $RIM1_{1079–1463}$ significantly suppressed vesicle docking in PC12 cells (**Fig. 7c**). In our experiments, however, RIM1 and β subunit targeting to the presynaptic site was observed only after early synapse formation (**Fig. 3**). It is possible that the interaction of Mint and CASK with VDCC α_1 subunits via their C termini[22,23] may

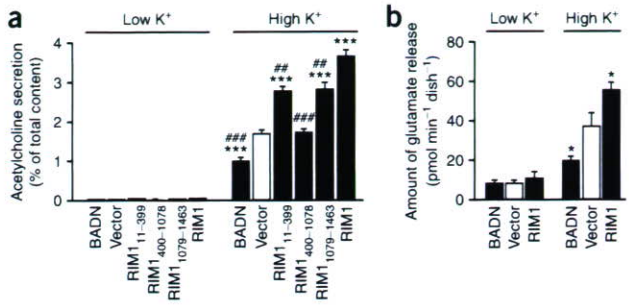

Figure 8 The RIM1-β association enhances neurotransmitter release in PC12 cells and cultured cerebellar neurons. (**a**) Effects of RIM1 constructs and BADN on depolarization-dependent release of ACh from ChAT-cotransfected PC12 cells. Three days after transfection, PC12 cells were incubated for 30 s with low-K^+ solution (5.9 mM K^+) at 37 °C. The release of ACh during this period was considered to be basal release. To measure ACh release, the cells were then incubated for 30 s with a high-K^+ solution (51.1 mM K^+). The amount of secreted ACh was determined as a percentage of the cellular content for each dish. $^{***}P < 0.001$ versus vector. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus RIM1. (**b**) Effects of RIM1 and BADN on depolarization-dependent release of glutamate from cultured cerebellar neurons. Twenty-four hours after the introduction of cDNAs, cerebellar neurons (9–11 DIV) were incubated for 1 min with the low-K^+ solution (5.9 mM K^+) at 37 °C. The release of glutamate during this period was considered to be basal release. To measure glutamate release, the cells were then incubated for 1 min with a high-K^+ solution (51.1 mM K^+). $^*P < 0.05$.

also direct channel targeting in parallel of, or before, RIM1–β subunit complex formation. Notably, our observation is consistent with a previous report[46] stating that the loss of the RIM homolog UNC10 caused a reduction in membrane-contacting synaptic vesicles within 30 nm of the dense projection at *Caenorhabditis elegans* neuromuscular junctions. Furthermore, in RIM1α-deficient mice, the decay of excitatory postsynaptic currents (EPSCs) during 14-Hz trains of presynaptic stimulation is abolished, whereas the rate at which the readily releasable vesicle pool is refilled is indistinguishable between the wild-type and RIM1α mutant mice[12]. These data suggest that RIM1-mediated vesicles anchoring to VDCCs may enable a rapid depletion of vesicle pools such that the available vesicles are exhausted, leading to EPSC decay. In this scenario, RIM1 knockout would minimize rapid vesicle release, enabling the readily releasable pool to be maintained and thus prevent EPSC decay. More recently, it has been reported that mice deficient in both RIM1α and RIM2α show lethality due to defects in Ca^{2+}-triggered release despite normal presynaptic active zone length and normal spontaneous neurotransmitter release[47]. In combination, these studies support our model (**Supplementary Fig. 8**), predicting a dual function for the RIM1-β interaction in neurotransmitter release by coordinating the molecular constituents and Ca^{2+} signaling in presynaptic active zones.

Previous reports have demonstrated the functional impact of syntaxin, synaptosome-associated protein (SNAP-25) and synaptotagmin on VDCCs through physical association with the 'synprint' region in the II-III linker of α_1-proteins[19–21]. It has been reported[48] that RIM associates with the synprint[10] directly via the C_2A domain, and with the α_1 C-terminal tail indirectly via the RIM-binding protein (RIM-BP) (**Fig. 1a**). However, RIM1 regulation of VDCCs may be independent of the synprint- or RIM-BP–mediated association, because $RIM1_{1079–1463}$, which lacks both the C_2A domain necessary for synprint binding[10] and the PXXP motif for the RIM-BP binding, is still sufficient to inhibit VDCC inactivation (**Fig. 4a,c**). This is supported by our observation that BADN, or replacement of β with β_4-GK, was sufficient to disrupt the RIM1 effects on inactivation (**Supplementary Fig. 5**). Syntaxin and SNAP-25 have been proposed to inhibit VDCC-mediated Ca^{2+} influx via a hyperpolarizing shift of the inactivation curve in the absence of vesicle docking at VDCC sites[19,21], whereas our finding implies enhancement or maintenance of the Ca^{2+} influx via interaction with RIM1 during the docking of vesicles. Notably, previous reports suggest that RIM1 is involved in the modification of the release apparatus at a late stage in the vesicle cycle[9], particularly in the postdocking step[49]. In early phases of vesicle docking, VDCC α_1 interactions with syntaxin and SNAP25 (refs. 19–21) and Mint and CASK[22,23] may be important, eliciting partial resistance to BADN suppression of vesicle docking (**Fig. 7c**). Thus, the α_1 protein associations and the RIM1-β association may be distinct interactions that contribute at different stages of vesicle cycling to controlling the Ca^{2+} supply from the source, namely the VDCC, in addition to regulating the proximity between the Ca^{2+} source VDCC and the target Ca^{2+} sensors at the presynaptic active zone.

METHODS

cDNA expression, cell culture, molecular modeling, recombinant proteins and infection with Sindbis viruses. Methods for cDNA cloning and expression, cell culture, molecular modeling of BADN, preparation of GST fusion proteins and purified β_{4b}-subunit recombinants, and the preparation and infection of Sindbis viruses can be found in the **Supplementary Methods** online.

Yeast two-hybrid screening and β-galactosidase assay. We subcloned rat β_{4b} subunit (GenBank accession number XM_215742) into pGBK-T7 and used it as a bait to screen a mouse brain pACT2 library in the yeast strain AH109 (Clontech). We plated transformants (1.5×10^6) on synthetic medium lacking adenine, histidine, leucine and tryptophan and assayed His^+ colonies for β-galactosidase activity with a filter assay. Of the transformants, 103 were His^+, and 21 of these were also $LacZ^+$. We isolated prey clone no. 2–5, encoding $RIM1_{1079–1463}$ (NM_053270).

***In vitro* binding of the purified proteins, GST-pulldown and coimmunoprecipitation experiments.** RIM1-GST fusion proteins at various concentrations were incubated with 50 pM purified recombinant β_4 subunits for 3 h at 4 °C. Proteins were subjected to western blotting with an antibody for β_4 raised against the peptide ENYHNERARKSRNRLS. The densities of protein signals, obtained using NIH Image (National Institute of Mental Health) under the linear relationship with the applied amount of proteins (**Supplementary Fig. 2**), were normalized to the densities from the maximal binding. For the pulldown assays, the cell lysate was incubated with glutathione-Sepharose beads bound to purified fusion proteins. The proteins were characterized by western blotting with antibody for Myc (Invitrogen). For coimmunoprecipitation, the cell lysate was incubated with anti-FLAG M2 monoclonal antibody (Sigma), and the immunocomplexes were characterized by western blotting with antibody for Myc. For details, see **Supplementary Methods**.

Biochemistry of native neuronal VDCC complexes. VDCC complexes were partially purified from the brains of C57BL/6 or lethargic mice (B6EiC3Sn-*a/A-Cacnb4*^{lh}/J, Jackson Laboratory) as previously reported[25]. KCl washed microsomes (50 mg) were solubilized in buffer I (for buffers, see **Supplementary Table 8** online) and centrifuged at 142,000*g* for 37 min. The supernatant was incubated with heparin-agarose (Sigma); agarose was washed with buffer II and III before elution with buffer IV. After elution, samples were diluted to 150 mM NaCl by addition of buffer V and concentrated using centrifugal filter devices (Millipore). The samples were applied to 5–40% sucrose density gradients (buffer VI) and were centrifuged at 215,000*g* for 90 min. Western blots were performed using antibodies for RIM (BD Biosciences), $Ca_v2.1$ (Alamone), syntaxin (Sigma) and β_4 (described above). Western blot band densities (NIH image) were normalized from four to five independent experiments.

For immunoprecipitation, partially purified neuronal VDCC complexes were incubated with protein A–agarose coupled to antibodies for β_4 or RIM1[4]. Immunoprecipitated proteins were subject to western blotting with

antibodies for RIM or $Ca_v2.1$. To disrupt the physiological association of native RIM1 with VDCC β_4, partially purified VDCC complexes were incubated with 200 nM GST-BADN and GST-$RIM1_{1079–1463}$ for 8 h at 4 °C before immunoprecipitation.

Confocal imaging. At 32 h after transfection, HEK293 cells or PC12 cells were plated on poly-L-lysine–coated glass coverslips. Hoechst 33342 (1 μg ml^{-1}, Dojindo) was added to stain nuclei 56 h after transfection. The imaging was performed in modified Ringer's buffer that contained 130 mM NaCl, 3 mM KCl, 5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.4). Fluorescence images were acquired with a confocal laser-scanning microscope (FV500, Olympus). For details, see the **Supplementary Methods**.

TIRF imaging. PC12 cells cotransfected with 1 μg pVenus-N1-NPY and RIM1 expression plasmids at the equal molar quantity (5.0 μg $RIM1_{11–399}$, 5.7 μg $RIM1_{400–1078}$, 5.0 μg $RIM1_{1079–1463}$ or 7.5 μg RIM1) and BADN (10 μg) using OptiFect (Invitrogen) were plated onto poly-L-lysine–coated coverslips. PCR analysis showed that the RIM plasmids were transfected at the equal level (**Supplementary Fig. 7**). The imaging was performed in modified Ringer's buffer. Fluorescence images of NPY-Venus were taken at the single vesicle level using an inverted microscope (IX71, Olympus). Incident light for total internal reflection illumination was introduced from the high numerical objective lens through a single mode optical fiber. Images were captured by a cooled CCD camera (EM-CCD, Hamamatsu Photonics). Area calculations and fluorescent spot counting were performed using Metamorph software (Molecular Devices). The cells showing distribution of vesicles with <10 μm^2 dark circle area that can be placed in between vesicles were selected as cells with uniformly distributed vesicles. The maximal dark circle area in the images from BADN-transfected cells with uniform vesicle distribution was 10 μm^2. For details, see the **Supplementary Methods**.

Immunostaining of cultured hippocampal neurons. Culture and transfection of mouse hippocampal neurons were carried out as described[50]. EGFP-tagged $Ca_v2.1$, Myc-tagged RIM1 and FLAG-tagged β_{4b} were detected with a Zeiss LSM510META confocal microscope using a combination of antibodies: FLAG M2 monoclonal, Myc polyclonal (Cell Signaling) and Alexa488- and Alexa594-conjugated secondary antibodies (Invitrogen). See the **Supplementary Methods** for further quantification procedures.

Current recordings. Whole-cell mode of the patch-clamp technique was carried out at 22–25 °C. An external solution contained 3 mM $BaCl_2$, 155 mM tetraethylammonium chloride (TEA-Cl), 10 mM HEPES and 10 mM glucose for BHK cells, and 10 mM $BaCl_2$, 153 mM TEA-Cl, 10 mM HEPES and 10 mM glucose for PC12 cells (pH 7.4). The pipette solution contained 95 mM CsOH, 95 mM aspartate, 40 mM CsCl, 4 mM $MgCl_2$, 5 mM EGTA, 2 mM disodium ATP, 5 mM HEPES and 8 mM creatine phosphate (pH 7.2). To characterize Ca^{2+}-dependent inactivation, external solutions contained 5 mM $CaCl_2$ or $BaCl_2$, 153 mM TEA-Cl, 10 mM HEPES and 10 mM glucose (pH 7.4). The pipette solution contained 135 mM Cs-$MeSO_3$, 5 mM CsCl, 0.5 mM EGTA, 5 mM $MgCl_2$, 4 mM disodium ATP and 10 mM HEPES (pH 7.2). Single-channel currents were recorded using cell-attached patch mode. The bath solution contained 150 mM KCl, 5 mM HEPES, 0.2 mM EGTA and 10 mM glucose (pH 7.4). The pipette solution contained 110 mM $BaCl_2$ and 10 mM HEPES (pH 7.4). 750-ms voltage steps were given every 5 s from a V_h of –100 mV. Details of current recordings and analyses, including voltage dependence of inactivation and activation and action potential train, are described in the **Supplementary Methods**.

Suppression of the action of endogenous RIMs using siRNAs and BADN in PC12 cells. The sense siRNA sequences 5′-AAGAATGGACCACAAATGCTT-3′ and 5′-AAGGTGATTGGATGGTATAAA-3′ for rat RIM1, and 5′-AAGGC CCAGATACTCTTAGAT-3′ and 5′-AAGAACTATCCAACATGGTAA-3′ for rat RIM2, were used. Suppression of RNA expression was confirmed by RT-PCR analyses (**Supplementary Fig. 7**). The cells transfected with 8.0 μg pCI-neo-BADN or 8.0 μg pCI-neo-RIM1 were subjected to current recordings 72–96 h after transfection. For details, see the **Supplementary Methods**.

Release assay and RNA analysis in PC12 cells. RNA expression of the α_1 subunits, β subunits, RIM1 or RIM2 in PC12 cells was determined by RT-PCR (**Supplementary Fig. 7**; see **Supplementary Table 9** online for primers). ACh secretion experiments were carried out as previously reported with slight modifications[42]. PC12 cells were plated in poly-D-lysine–coated 35-mm dishes (BD Bioscience) with 5×10^5 cells per dish. Cells were cotransfected with 1 μg of pEFmChAT encoding mouse *Chat* cDNA and RIM1 plasmids at equal molar quantity (3.4 μg of $RIM1_{11–399}$, 3.8 μg of $RIM1_{400–1078}$, 3.4 μg of $RIM1_{1079–1463}$ or 5.0 μg of RIM1) and BADN (10 μg) using Lipofectamine 2000 (Invitrogen). ACh secretion experiments were carried out 3 d after transfection. For details, see the **Supplementary Methods**.

Glutamate release assay using cerebellar neuron primary cultures. Cerebellar granular cells were plated on polyethylenimine-coated 35-mm diameter culture dishes (BD Falcon) at a density of 4.5×10^6–5.0×10^6 cells per dish. BADN or RIM1 cDNAs were introduced with Sindbis viruses in cerebellar neurons. At 24 h after infection, high K^+–evoked glutamate release was carried out. For details, see the **Supplementary Methods**.

Statistical analysis. All data accumulated under each condition from at least three independent experiments are expressed as means ± s.e.m. Student's *t*-test, Kolmogorov-Smirnov test or ANOVA followed by Fisher's test were employed.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

S.K., M.W., T.M. and Y.U. contributed to the acquisition, analysis and interpretation of data, and drafting of the manuscript. H.B., A.M.B., M.T. and K.P.C. contributed to the analysis and interpretation of data, and drafting of the manuscript. E.M., Y.H., M.N., M.D.W., M.K. and M.I. contributed to the acquisition, analysis and interpretation of data. Y.M. contributed to the analysis and interpretation of data, and drafting and critical review of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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Protein A003011

Protein Overview

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Published 15 Nov 2007

Abstract
Full Text
Network Map
States
Transitions
Functions
Protein Classes

Automated Data
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Protein Records
Interaction DBs
Domains & Motifs
Protein Structure
Gene Info
Orthologs

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DCLK1

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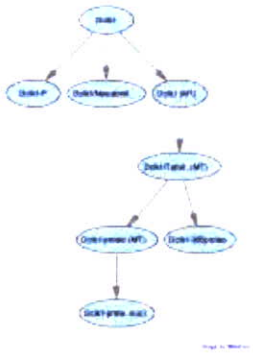
Doublecortin-like kinase-1 (DCLK1, or previously known under the symbol DCAMKL1) is a serine/threonine kinase which contains two major functional domains: an N-terminal doublecortin-like domain, and a C-terminal kinase domain with striking homology to CaMKI/CaMKIV and CaMKII. DCLK1 expression is largely specific to the central nervous system. Its level of expression is high in the embryo but it is also present in adult brain. DCLK1 function is likely to be mediated by the regulated expression of multiple splice variants, including a full-length isoform (DCLK1/DCAMKL1/KIAA0369), a DCX-domain-only isoform (DCL/DCLK DCX-like), a kinase-domain only isoform (CPG16/CLICK-I) and a CaMK-related peptide (CARP/Ania-4). DCLK1 acts as a microtubule-associated protein, through the DCX-like domain, and gene deletion studies confirm involvement of DCLK1 in microtubule-mediated neuronal migration, morphogenesis and layer formation.

Alternative names for this molecule: CLICK-I; CLICK-I beta; Cpg16; Dcamkl1; DCDC3A; Dcl; Dclk; Dclk1; DCLK1; Double cortin and calcium/calmodulin-dependent protein kinase-like 1; Doublecortin and calcium/calmodulin-dependent protein kinase-like 1; Doublecortin and CaM kinase-like 1; Doublecortin-like and CaM kinase-like 1; Doublecortin-like kinase; Doublecortin-like kinase 1; KIAA0369; Serine/threonine-protein kinase DCAMKL1



This molecule exists in 8 states, has 7 transitions between these states and has 1 enzyme function.

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Protein Function

The nomenclature of the doublecortin-like kinase/doublecortin and Ca^{2+}/calmodulin-dependent protein kinase-like (*Dclk/Dcamkl*) genes has recently been unified to *Dclk*. This family comprises three serine/threonine protein kinases (DCLK1-3/DCAMKL1-3), which are characterized by the presence of a single or double doublecortin (DCX) domain and a C-terminal kinase domain bearing similarity to the Ca^{2+}/calmodulin-dependent protein kinases CaMKI/CaMKIV and CaMKII. The *Dclk1* gene, which encodes a tandem repeat of two DCX domains and a kinase domain, generates a large number of splice variants (including those completely lacking either the repeat or the kinase domain). *Dclk1* splice variants were discovered by several groups, which is reflected in the use of numerous names for specific isoforms in various species (see Abstract and Splice Variants section). The roles of the DCX and kinase domains of DCLK1 have now been extensively examined.

Microtubule Stabilization Activity

The N-terminal DCX tandem in DCLK1 is most homologous to the doublecortin protein, with 78% amino acid identity. Like DCX, DCLK1 can also function as a microtubule-associated protein (MAP). The DCX tandem of DCLK1 binds to microtubules, stabilizes polymerized microtubules *in vitro* and stimulates microtubule bundling in intact cells. Kinase activity of DCLK1 is dispensable for its ability to bind and bundle microtubules, and indeed, a kinase-absent doublecortin-only isoform has been reported. As a MAP, DCLK1 appears to share some roles with DCX in the formation of axonal projections and in neuronal cell migration, in a partially redundant manner. Zyg-8, the DCLK orthologue in *C. elegans*, has a key role in spindle positioning during asymmetric division of one-cell stage embryos by promoting microtubule assembly during anaphase. In mammalian cells, including cortical progenitors, DCLK1 regulates the formation of bipolar mitotic spindles and the proper transition from prometaphase to metaphase during mitosis.

Kinase Activity

The C-terminal kinase domain of DCLK1 resembles members of the family of calcium/calmodulin-dependent protein kinases, such as CaMKI, CaMKII and CaMKIV, but lacks a canonical calmodulin-binding site. Consistently, DCLK1 has been shown to phosphorylate intact myelin basic protein and to autophosphorylate in the absence of calcium. Interestingly, despite a large structural similarity in the kinase domain, the functional consequence of DCLK1 kinase activity appears to be distinct from CaMKI/CaMKIV and CaMKII. Unlike CaMKI/CaMKIV, which are known Ca^{2+}/cAMP-response element (CRE) binding protein (CREB) activating kinases, a DCX-lacking form of DCLK1 rather inhibited CRE-dependent gene expression by a dominant mechanism that bypassed CREB and was instead mediated by phosphorylated CRTC (CREB regulated transcription coactivator). It is also possible that CREB repression might be additionally mediated via DCX in a microtubule-dependent manner.

As described above, Zyg-8 controls the stabilization of microtubules as several mutations in the kinase domain of Zyg-8 caused an anaphase spindle positioning defect and embryonic death in nematodes. The exact mechanism by which kinase inactivation leads to this phenotype remains to be determined. Mutations in non-kinase domains also led to similar phenotypes.

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Regulation of Activity

Although the C-terminal kinase domain of DCLK1 shows significant homology with CaMKI and CaMKIV, it lacks a canonical CaM-binding motif and significant CaM binding affinity. Although DCLK1 can phosphorylate myelin basic protein (MBP) and other synthetic peptides *in vitro*, MBP is not expected to be a target for DCLK1 kinase activity *in vivo*. A *bona fide* target for DCLK1 still remains to be identified. However, MBP and synthetic peptides can be used as *in vitro* substrates for enzymatic assays. The autophosphorylation and phosphorylation activity for MBP and synthetic peptide substrates was found in the absence of calcium. A DCLK1β splice variant was reported to possess a reduced autophosphorylation activity, whereas its kinase activity toward MBP remained intact. A truncation of the C terminus in a candidate plasticity-related gene-16 (CPG16) splice variant enhances its kinase activity, and furthermore, a point mutation T239E in CPG16 also augments its kinase activity, indicating the existence of phosphorylation-mediated CPG16 regulation. The autophosphorylation activity of CPG16 can be stimulated by forskolin and PKA in an indirect manner.

The endoproteases caspase-3, caspase-8 and calpain can catalyze DCLK1 cleavage. Calpain-triggered cleavage of DCLK1 generates a fragment equivalent to CPG16 that dissociates from microtubules and translocates to the nucleus.

Overexpression of a kinase-dead DCLK1 mutant causes an arrest in M phase progression in HEK293 cells that is comparable to overexpression of wild-type DCLK1. Thus, it is unlikely that the doublecortin

domain is regulated by DCLK1 kinase activity itself. However, it remains to be elucidated if, and to what extent, the function of the doublecortin domain in DCLK1 is regulated by heterologous phosphorylation and dephosphorylation, in a manner closely resembling DCX.

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11473121	Burgess HA, Reiner O	Cleavage of doublecortin-like kinase by calpain releases an active kinase fragment from a microtubule anchorage domain.	J Biol Chem, 276, 39	28 Sep 2001
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Interactions with Ligands and Other Proteins

Similar to the *Dcx* gene product, the *Dclk1* gene product functions as a microtubule-associated protein (MAP) with the N-terminal domain binding to polymerized microtubules (MTs) and stabilizing them. In addition to MTs, DCX domains can interact with neurabinII/spinophilin, an F-actin binding protein. The C-terminal kinase domain of DCLK1 resembles members of the family of calcium/calmodulin-dependent protein kinases, but lacks a canonical calmodulin-binding site and does not bind CaM. The physiological substrates of DCLK1 kinase activity are unknown.

From structural studies of the DCX domains and from functional analyses of the strikingly homologous DCX protein, it has been suggested that the N-terminal DCX domain binds to assembled MTs, whereas the C-terminal domain binds to both MTs and unpolymerized tubulin.

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Regulation of Concentration

The expression of the *Dclk1* gene appears to be regulated developmentally, although the mechanisms of its spatiotemporal regulation are unknown. Interestingly, strong stimulation of neurotransmitter receptors triggered the expression of a truncated *Dclk1* isoform, CaMK-related peptide (*Carp*). Furthermore, a rat splice variant of *Dclk1* lacking the doublecortin domain, but sharing an identical N terminus with *Carp*, was isolated as a differentially upregulated gene from kainate-treated rat hippocampus and named CPG16. Transcription of both *Carp* and *CPG16* starts from an alternative promoter following the DCX domains of the *Dclk1* gene.

PM ID	Authors	Title	Journal	Pub Date
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Subcellular Localization

A DCLK1 isoform that contains an intact doublecortin domain associates with microtubules and leads to microtubule polymerization and bundling. In primary neuronal cultures, the localization of such a DCLK1 isoform is fibrillar and shows some overlap with anti-tubulin antibody immunoreactivity. By contrast, a splice variant lacking the doublecortin domain, CPG16 for example, shows diffuse cytoplasmic localization when overexpressed in NIH 3T3 cells. Some enrichment in the nucleus was reported for the kinase domain after forskolin stimulation or after calpain cleavage.

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Major Sites of Expression

Unlike *Dcx*, *Dclk1* has varying levels of expression throughout embryonic and adult life, through regulated expression of multiple splice variants, including a full-length isoform (DCLK1/DCAMKL1/KIAA0369), a DCX-domain-only isoform (DCLK DCX-like/DCL), a kinase domain only isoform (CPG16/CLICK-I), and a CaMK-related peptide (Carp/Ania-4). Expression of *Dcl* appears in mouse embryonic brain as early as day 8. Expression of *Dclk1* starts later, it is widely expressed by embryonic day 16, and continues throughout life whereas *Dcl* disappears in adult brain. Adult expression of *Dclk1* is high in the cortex, in the hippocampus and in the cerebellum, but lower in the basal ganglia, and very low in the thalamus. *Carp* is detected only in adult brain. *CPG16* is expressed throughout the

adult central nervous system, in a neuron-specific fashion. In addition, *Dclk1* and *CPG16* variants are strongly expressed in testis in adult mouse.

In chick, *Dclk1* is widely expressed in pallial and subpallial structures, including the telencephalon, optic tectum and cerebellum. In zebrafish, expression of *Dclk1* starts as early as 24 hours post-fertilization in brain and eye, and persists until adulthood. In nematode, *Zyg-8* acts as a maternal protein in one-cell stage embryos.

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Phenotypes

The *Dclk1* gene functions in a partially redundant pathway with DCX during brain development. The *Dcx* gene, which encodes a microtubule-associated protein, is essential for normal human brain development as various mutations in *Dcx* cause X-linked lissencephaly. Brain cortical surfaces of affected males are abnormally smooth. Although females that are heterozygous for *Dcx* have milder clinical features than males, most still suffer from mental retardation and seizures. Affected females have a 'double cortex' syndrome showing a relatively normal cerebral cortex with a subcortical band heterotopia, that is, a second layer of grey matter within the subcortical white matter. *Dcx*-null mice showed decreased viability, but some (-/y or -/-) can survive to adulthood. They have a seemingly normal neocortical lamina. However, layer formation was abnormal in the hippocampus. Thus, at least in the mouse, loss of the *Dcx* gene may be compensated for by the function of other genes.

Two knockout mouse lines have been reported for the *Dclk1* gene. One type of *Dclk1*-null mutant lacks

exon 3; splice variants containing the DCX domain are disrupted but expression of the DCX domain-lacking kinase-like variants remain intact. In the other line, exons 9-11 are deleted; splice variants containing the kinase domain are selectively disrupted. Expression of the DCL variants was preserved in mice of the latter line. Both mutant lines are viable and fertile, and have preserved neocortical lamination. In contrast, irrespective of the type of mutation involved, *Dclk1/Dcx* double-null mice show profound disorganization in cortical layer formation and widespread axonal defects in the corpus callosum, anterior commissure, subcortical fiber tracts and internal capsules. An absence of the corpus callosum was observed in the mouse model lacking the DCX domain of *Dclk1*, but not in mice in which the kinase domain was deleted. Perturbation of zebrafish *Dclk1* using antisense morpholinos during embryogenesis resulted in increased apoptosis during early development of the central nervous system.

A defect in neuronal migration is responsible for abnormal cortical lamination. For this phenotype, a clear gene dosage-dependency exists between *Dcx* and *Dclk1*, indicating a strong genetic interaction between the two alleles. Neuronal migration is also affected in mice subjected to *in utero* RNAi-mediated gene knockdown, however, specific reduction in the kinase-only isoforms has little effect. In neuronal culture systems, disruption of both *Dclk1* and *Dcx* function leads to abnormal dendritic development, shorter axons and disrupted axonal transport of synaptic vesicle proteins during axon growth.

A role for DCLK1 during mouse neurogenesis is also suggested by *in utero Dclk1* gain-of-function and loss-of-function analyses, as the former promotes differentiation of cortical neuronal progenitors whereas the latter altered the fate choice of neuronal progenitors.

In vitro analyses in HEK293 cells demonstrated that DCLK1 may regulate the formation of bipolar mitotic spindles and the proper transition from prometaphase to metaphase during mitosis via a dynein-dependent mechanism. Similarly, mutations in *Zyg-8*, a nematode orthologue of *Dclk1*, resulted in disorganized and smaller spindles in nematode eggs. Thus, Zyg-8 might be required for the positioning and assembly of mitotic spindles during asymmetrical division at one-cell stage in lower eukaryotic systems.

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16387639	Koizumi H, Tanaka T, Gleeson JG	Doublecortin-like kinase functions with doublecortin to mediate fiber tract decussation and neuronal migration.	Neuron, 49, 1	5 Jan 2006
17498644	Shimomura S, Nagamine T, Nimura T, Sueyoshi N, Shigeri Y, Kameshita I	Expression, characterization, and gene knockdown of zebrafish doublecortin-like protein kinase.	Arch Biochem Biophys, 463, 2	15 Jul 2007
16387637	Shu T, Tseng HC, Sapir T, Stern P, Zhou Y, Sanada K, Fischer A, Coquelle FM, Reiner O, Tsai LH	Doublecortin-like kinase controls neurogenesis by regulating mitotic spindles and M phase progression.	Neuron, 49, 1	5 Jan 2006

Splice Variants

The *Dclk1* gene consists of 20 exons and is extensively alternatively spliced, resulting in the production of a variety of isoforms. At least nine alternative splice variants have been reported, which can be categorized in four classes: functional full-length form (containing both doublecortin and kinase domains), kinase-like form (lacking doublecortin domain), doublecortin-like form (lacking kinase domain) and CaMK-related peptide form (truncated peptide). Full-length and doublecortin-like forms use the start codon in exon 2, whereas the kinase-like and CaMK-related peptide forms share the second start codon

in exon 6. In the kinase domain, alternative splicing of exon 9 causes deletion of an arginine-rich region (R) and that of exon 19 causes a modification in the C-terminal sequence. The whole spectrum of the tissue specificity and developmental changes, as well as the functional characterization of each splice variant, still remains to be studied.

Full-length forms:

DCLKα/DCLK-long-A/DCK-α2/KIAA0369-AL/DCAMKL1/Zyg-8

$DCLK^{R+}$α/DCLK-long-C/DCK-α2-R

DCLKβ/DCLK-long-B/DCK-α1/KIAA0369-AS

Kinase-like forms:

Click-1α/KIAA0369-BS/CaMK-VI/DCLK-short-B/DCK-β1

Click-1β/CPG16/KIAA0369-BL/DCLK-short-A/DCK-β2

$CPG16^{R+}$/DCLK-short-C/DCK-β2-R

Doublecortin-like form:

DCLK DCX-like/DCL

Truncated CaMK-related peptide form:

Carp/Ania-4

PM ID	Authors	Title	Journal	Pub Date
10533048	Burgess HA, Martinez S, Reiner O	KIAA0369, doublecortin-like kinase, is expressed during brain development.	J Neurosci Res, 58, 4	15 Nov 1999
11083916	Burgess HA, Reiner O	Doublecortin-like kinase is associated with microtubules in neuronal growth cones.	Mol Cell Neurosci, 16, 5	Nov 2000
14741399	Engels BM, Schouten TG, van Dullemen J, Gosens I, Vreugdenhil E	Functional differences between two DCLK splice variants.	Brain Res Mol Brain Res, 120, 2	5 Jan 2004
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11124993	Lin PT, Gleeson JG, Corbo JC, Flanagan L, Walsh CA	DCAMKL1 encodes a protein kinase with homology to doublecortin that regulates microtubule polymerization.	J Neurosci, 20, 24	15 Dec 2000
10550327	Mizuguchi M, Qin J, Yamada M, Ikeda K, Takashima S	High expression of doublecortin and KIAA0369 protein in fetal brain suggests their specific role in neuronal migration.	Am J Pathol, 155, 5	Nov 1999
9205841	Nagase T, Ishikawa K, Nakajima D, Ohira M, Seki N, Miyajima N, Tanaka A, Kotani H, Nomura N, Ohara O	Prediction of the coding sequences of unidentified human genes. VII. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro.	DNA Res, 4, 2	28 Apr 1997
9747029	Omori Y, Suzuki M, Ozaki K, Harada Y, Nakamura Y, Takahashi E, Fujiwara T	Expression and chromosomal localization of KIAA0369, a putative kinase structurally related to Doublecortin.	J Hum Genet, 43, 3	1998
16869982	Reiner O, Coquelle FM, Peter B, Levy T, Kaplan A, Sapir T, Orr I, Barkai N, Eichele G, Bergmann S	The evolving doublecortin (DCX) superfamily.	BMC Genomics, 7	2006
12590608	Shang L, Kwon YG, Nandy S, Lawrence DS, Edelman AM	Catalytic and regulatory domains of doublecortin kinase-1.	Biochemistry, 42, 7	25 Feb 2003
9915791	Silverman MA, Benard O, Jaaro H, Rattner A, Citri Y, Seger R	CPG16, a novel protein serine/threonine kinase downstream of cAMP-dependent protein kinase.	J Biol Chem, 274, 5	29 Jan 1999
10036192	Sossey-Alaoui K, Srivastava AK	DCAMKL1, a brain-specific transmembrane protein on 13q12.3 that is similar to doublecortin (DCX).	Genomics, 56, 1	15 Feb 1999

10213452	Vreugdenhil E, Datson N, Engels B, de Jong J, van Koningsbruggen S, Schaaf M, de Kloet ER	Kainate-elicited seizures induce mRNA encoding a CaMK-related peptide: a putative modulator of kinase activity in rat hippocampus.	J Neurobiol, 39, 1	Apr 1999
11597766	Vreugdenhil E, Engels B, Middelburg R, van Koningsbruggen S, Knol J, Veldhuisen B, de Kloet ER	Multiple transcripts generated by the DCAMKL gene are expressed in the rat hippocampus.	Brain Res Mol Brain Res, 94, 1-2	19 Oct 2001

Antibodies

DCLK1/DCAMKL1 antibodies are available from several commercial providers. Depending on whether the epitopes on which the antibodies were raised are located in the N-terminal or C-terminal part of the molecule, the antibodies can differentiate between splice variants that possess N-terminal or C-terminal domains.

1. DCAMKL1 antibody (N terminus), Abgent (San Diego, CA, USA).
2. DCAMKL1 antibody (C terminus), Abgent (San Diego, CA, USA).
3. Anti-CaM Kinase Like Kinase (1-55 of rat Carp), Millipore (Billerica, MA, USA).
4. Monoclonal DCAMKL1 antibody, Novus Biologicals (Littleton, CO, USA).

Protein A003011

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Abstract
Full Text
Network Map
States
Transitions
Functions
Protein Classes

Automated Data
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As At Publication

Protein Records
Interaction DBs
Domains & Motifs
Protein Structure
Gene Info
Orthologs

Automated Data
Not Reviewed
Latest from 1 Apr 2008

Protein Records
Interaction DBs
Domains & Motifs
Protein Structure
Gene Info
Orthologs
Blast Data

UCSD-Nature Molecule Pages
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DCLK1

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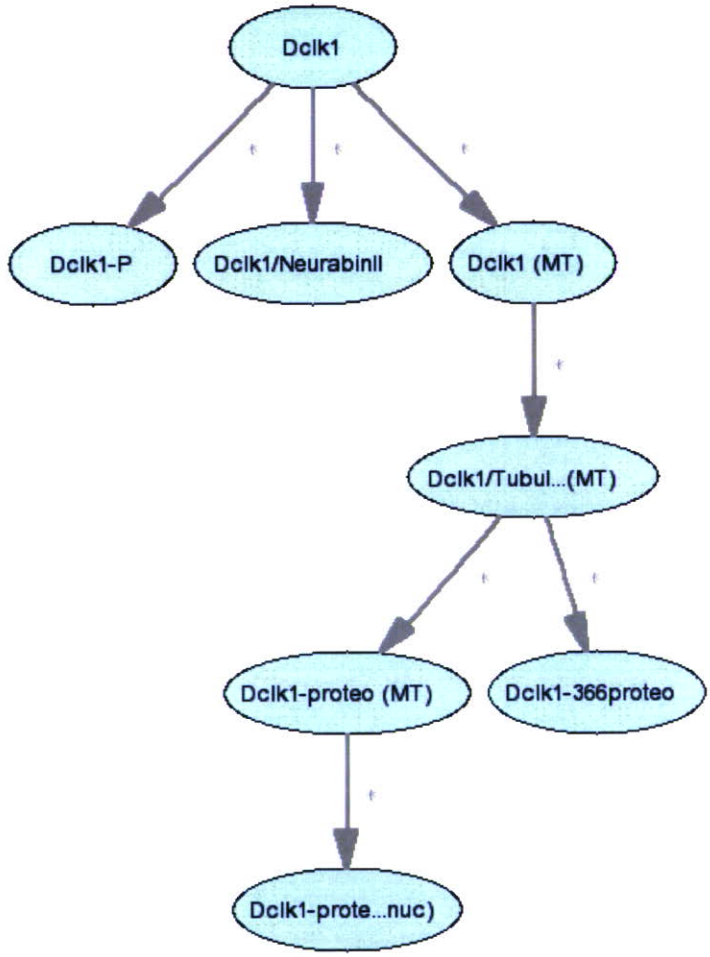
Network Map

Click on ovals to see state details and on asterisks to see transition details.

See SVG version



Graph by WebDot

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Domains & Motifs
Protein Structure
Gene Info
Orthologs

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Functional states

State description	State name	Location	Transition graph
Dclk1	(Dclk1)[2]	cytoplasm	
Dclk1-P	(Dclk1 P)	Unknown	
Dclk1/NeurabinII	(Ppp1r9b) (Dclk1)	Unknown	
Dclk1 (MT)	(Dclk1)[3]	microtubule cytoskeleton	
Dclk1/Tubulin (MT)	(Tuba1a) (Dclk1)	microtubule cytoskeleton	
Dclk1-proteo (MT)	(Dclk1 PR)[2]	microtubule cytoskeleton	
Dclk1-proteo (nuc)	(Dclk1 PR)[3]	nucleus	
Dclk1-366proteo	(Dclk1 PR)[1]	cytoplasm	

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Transitions
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Interaction DBs
Domains & Motifs
Protein Structure
Gene Info
Orthologs

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view transition network
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Transitions

(Dclk1)[2] --> (Dclk1 P)

(Dclk1)[2] --> (Ppp1r9b) (Dclk1)

(Dclk1)[2] --> (Dclk1)[3]

(Dclk1)[3] --> (Tuba1a) (Dclk1)

(Tuba1a) (Dclk1) --> (Dclk1 PR)[2]

(Tuba1a) (Dclk1) --> (Dclk1 PR)[1]

(Dclk1 PR)[2] --> (Dclk1 PR)[3]

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Transitions
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Protein Classes

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Domains & Motifs
Protein Structure
Gene Info
Orthologs

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Not Reviewed
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Enzyme

Reaction catalyzed	States that catalyze this reaction	Function detail
(Dclk1)[2] + ATP -> (Dclk1 P) + ADP	(Dclk1)[2]	F

Note: Many proteins also function as binding proteins, adaptors, or scaffolding proteins. This functional information is captured in the States and Transitions sections associated with this molecule.

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Interaction DBs
Domains & Motifs
Protein Structure
Gene Info
Orthologs

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Latest from 1 Apr 2008

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Author-defined classes

Class name	Class description
Dclk[1-3]	Dclk[1-3]_A003011