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Deficiency of Cks1 leads to learning and long-term memory defects and p27 dependent formation of neuronal coflin aggregates

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Brief running title: Memory defects in Cks1 null mice

Keywords: cyclin-dependent kinase / hippocampus / long-term potentiation / synaptic plasticity / RhoA
Abstract

In mitotic cells, the cyclin-dependent kinase (CDK) subunit protein CKS1 regulates S phase entry by mediating degradation of the CDK inhibitor p27. Although mature neurons lack mitotic CDKs, we found that CKS1 was actively expressed in post-mitotic neurons of the adult hippocampus. Interestingly, Cks1 knockout (Cks1−/−) mice exhibited poor long-term memory, and diminished maintenance of long-term potentiation in the hippocampal circuits. Furthermore, there was neuronal accumulation of coflin-actin rods or coflin aggregates, which are associated with defective dendritic spine maturation and synaptic loss. We further demonstrated that it was the increased p27 level that activated coflin by suppressing the RhoA kinase-mediated inhibitory phosphorylation of coflin, resulting in the formation of coflin aggregates in the Cks1−/− neuronal cells. Consistent with reports that the peptidyl-prolyl-isomerase PIN1 competes with CKS1 for p27 binding, we found that inhibition of PIN1 diminished the formation of coflin aggregates through decreasing p27 levels, thereby activating RhoA and increasing coflin phosphorylation. Our results revealed that CKS1 is involved in normal glutamatergic synapse development and dendritic spine maturation in adult hippocampus through modulating p27 stability.
The establishment of long-term memory requires structural plasticity of dendritic spines, which contributes to altered synaptic strength between neurons. Recent studies have begun to unravel an increasing number of molecular partners that underscore this process (Rochefort NL and A Konnerth 2012), some of which are also involved in non-neuronal cellular processes. The functions of these molecules range from sculpting the cytoskeleton during mitosis and cellular migration, to control of the cell cycle (Odajima J et al. 2011).

CKS1 was first identified as a cyclin-dependent kinase (CDK) interacting protein that participates in cell cycle control (Pines J 1996). In the mitotic cell cycle, mammalian CKS1 binds CDK1 and CDK2. Expression of *CKS1* is particularly elevated in many aggressive human cancers, where its oncogenic role has been ascribed to a second function for the protein as a cofactor to the ubiquitin ligase complex, SCFSKP2 (Hao B et al. 2005). SKP2 ubiquitylates and therefore mediates proteasomal-dependent degradation of a number of important substrates that control G1 - S phase transition of the cell cycle. This includes the CDK inhibitor, p27. In mice knocked down for *Cks1*, high levels of p27 accumulate (Spruck C et al. 2001). Another level of post-translational regulation of p27 level is mediated by peptidyl-prolyl-isomerase PIN1. PIN1 binds specifically to its substrates at the serine/threonine-proline motifs when the serine or threonine residue is phosphorylated, and catalyzes isomerization of serine/threonine-proline peptide bond, resulting in conformational and functional changes of its substrate (Liou YC et al. 2011; Cheng CW et al. 2013). It has been shown that PIN1 binds p27 and induces conformational change that hinders the binding between p27 and CKS1 (Zhou W et al. 2009). As a result, PIN1 positively regulates p27 level in competition with CKS1.

Apart from inhibiting cell cycle entry, p27 is known to have cell-cycle independent roles in controlling neuronal migration in the developing mouse neocortex. In particular, stabilization of p27 by the neuronal-specific CDK, CDK5, has been shown to be crucial in this context (Kawauchi T et al. 2006). Studies on mitotic as well as neuronal cells have indicated that during migration, p27 mediates actin cytoskeleton reorganization through inhibition of RhoA, a small GTPase involved in the Rho/ROCK kinase signaling cascade (Besson A et al. 2004). An important downstream effector of this pathway is cofilin, which acts as an actin severer. LIM and other kinases have been shown to negatively regulate the activity of cofilin by phosphorylation of the serine residue at amino-acid position 3 (Mizuno K 2013). Precise
control of cofilin activity is important in cellular migration as well as in processes involving actin cytoskeleton remodeling, such as dendritic spine maturation (Pontrello CG and IM Ethell 2009; Tolias KF et al. 2011). Non-phosphorylated or activated cofilin promotes the formation of cofilin-actin rods or aggregates that are typically identified in neurons in neurodegenerative disorders such as Alzheimer’s disease (Huang TY et al. 2008).

In this study, we demonstrated that CKS1 possesses a cell-cycle independent role in controlling dendritic spine maturation. Through mediating p27 degradation, and thus RhoA activation, CKS1 negatively controls cofilin activity. CKS1 is expressed strongly in the adult hippocampus. The sequelae of Cks1 deletion, hence cofilin over-activation and formation of cofilin-actin aggregates, include impaired maturation of hippocampal dendritic spines, an inability to establish late phase long-term potentiation (LTP) and impaired hippocampal-based spatial learning.

Materials and methods

Animals
All experiments were conducted in 3-4 month old male Cks1 knock-out mice (Cks1−/−) (Frontini M et al. 2012), and their wild type littermate controls. All procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986 following institutional ethical approval at the University of Oxford and University of Sussex.

X-Gal histochemistry
Mice were anesthetized with chloroform and fix-perfused transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS). Brains were removed and incubated in cold fix for an additional 15 minutes. After rinsing 3 times in rinse buffer (100 mM sodium phosphate (pH 7.3), 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% Nonidet P40), sections were stained (rinse buffer plus 5 mM potassium ferrocyanide, 5mM potassium ferricyanide and 1mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) overnight at 4 °C. Sections were post-fixed before sectioning and mounting.

Novel Object Recognition (NOR)
The Novel Object Recognition (NOR) task was performed in an open-field apparatus (40 cm
wide x 60 cm long x 40 cm high) placed under a dim light. In the habituation phase, mice were individually allowed to explore the box for 10 min per day for three days. In the training trial, mice were presented with two identical, emotionally neutral plastic objects (such as a red cube), placed in opposite corners of the box and allowed to explore for 5 min. In the test trial performed 24 h later, we replaced one of the familiar object with a new one (e.g. yellow pyramid). Objects were similar in size and emotionally neutral, but varied in shape and color. Mice were allowed to explore for 5 min and exploratory behavior recorded, i.e. head orientation, sniffing, nose to object distance ≤ 1cm. All trials were video-tracked using Ethovision XT 7.1 (Noldus Information Technology). We determined a preference index (PI), which is a difference in the new object exploration time divided by the total time spent exploring the two objects. The open-field box and objects were thoroughly cleaned with 70 % ethanol solution, dried and ventilated between tested mice to eliminate odor cues.

**Barnes Maze Test**

The Barnes maze consisted of a white, polypropylene disk of 100 cm diameter, mounted 50 cm above the floor. The disk has 20 holes of 5 cm diameter and evenly spaced around the periphery. A black acrylic drawer was located beneath one of the hole and served as the escape box. Fixed spatial cues were placed on the walls and a bright light (100 W) provided the aversive stimuli. The method was modified from Patil et al. (Patil SS et al. 2009). For each trial, mice were allowed to freely explore the maze for 2 min or until reaching the escape box, where they would remain for 1 min before being returned to their home cage. If a mouse failed to find the escape box within 2 min, the experimenter would gently direct the animal to the escape box where it would remain for 1 min. During five consecutive days mice had either three trials per day (with a retention time of 15 min) or one trial per day (retention time of 24 h). The time spent before finding the escape box was measured as an indication of spatial learning. All trials were video-tracked using Ethovision XT 7.1 (Noldus Information Technology), which recorded distance travelled and latency to find the escape box. The maze and escape box were cleaned carefully with a 70 % ethanol solution between each trial to dissipate odor cues.

**Electrophysiology**

Mice were anesthetized with pentobarbitone prior to decapitation and preparation of slices. Transversal hippocampal slices (350 µm) were cut with Microm HM650V slicer in ice-cold
artificial cerebrospinal fluid (aCSF) comprising (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl$_2$, 1.3 MgSO$_4$, 1.25 NaH$_2$PO$_4$, 25 NaHCO$_3$, 11 glucose (pH 7.2-7.4, equilibrated with 95% O$_2$/5% CO$_2$), stored at 20-24°C for at least 1 hour, then transferred to a submerged recording chamber perfused with aCSF at 32°C for at least 30 min before recording. An extracellular concentric tungsten stimulation electrode was positioned in the CA1 stratum radiatum. A cut was made between the CA3 and CA1 areas to prevent polysynaptic activity. Single-shock stimuli were applied at 15 s interval, and field potential EPSPs (fEPSPs) were recorded in the CA1 stratum radiatum using glass capillary electrodes (5-10 MΩ) filled with aCSF. Stimulation intensity was set to 50 % of maximal fEPSP amplitude. Theta-burst stimulation (TBS) comprised 10 trains of 5 pulses at 100 Hz separated by 200 ms. 20-80 % fEPSP slope was analyzed and magnitude of LTP was defined as % of baseline mean. Paired t-test was used to test significance. Miniature EPSCs (mEPSCs) were recorded in voltage clamp at -60 mV with Multiclamp 700B amplifier (Axon Instruments) and using filling solution (in mM): 135 CsCl, 10 KOH-HEPES, 10 BAPTA, 8 NaCl, 2 Mg-ATP, 0.3 GTP (pH 7.2, 290 mOsm/L). Neurobiotin (Vector Labs, UK) (0.3% w/v) was present for post hoc anatomical analysis. mEPSCs were analyzed from at least 5 min recording episodes, with stable access resistance (<25 MΩ). Data were low-pass filtered (4–5 kHz) and acquired at 10–20 kHz for off-line analysis. The GABA$_A$R blocker picrotoxin (100 µM) and tetrodotoxin (TTX) (1 µM) were present in all mEPSC experiments. Data were analyzed using pClamp 10 (Axon Instruments).

**Dendritic spine density and structure analysis**

Slices were fixed with 4 % PFA and 0.2 % picric acid solution overnight at 4 °C, and then washed in 0.1 M phosphate buffer. Neurobiotin was visualized by incubating with Alexa Fluor 488-conjugated streptavidin (Invitrogen, UK; diluted 1:1000) in TBS with 0.3 % Triton XC-100. Slices were mounted in Vectashield (Vector Laboratories). Fluorescent images of dendrites were collected using a confocal laser scanning microscope (Leica TCS SP5). At least three randomly selected areas with 50-70 µm length each were imaged from a single pyramidal cell. Three or more cells were analyzed from each animal and having at least 3 animals per genotype. Dendritic spines morphology was analyzed using the NeuronStudio software (Rodriguez A et al. 2008).

**siRNA Transfection**
Specific siRNA targeting Cks1 (siCks1) and control siRNA (siCrtl) were purchased from Dharmacon (SMARTpools) and the cells were transfected with Oligofectamine transfection reagent (Life-Technologies-Invitrogen) following the manufacturer's instructions. Cells were analyzed 48 hr after transfection.

**Real-time PCR and gene expression analysis**

mRNA of the hippocampus tissues was extracted by TRIzol reagent (Life Technologies) and cDNA was reverse transcribed using SuperScript III Reverse Transcriptase (Life Technologies). Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies) was used for Real-time PCR. Transcript expression is presented as average Ct. Primer sequences for Cks1 mRNA are TACGACGACGAGGAGTTCGAAT (Forward) and ACCAGCTTGGCTATGTCCTTGGG (Reverse). The fold changes for Cks1 transcript from hippocampus of wildtype mice relative to that of Cks1−/− mice was calculated with the 2^−ΔΔCt method. (Huggett J et al 2005)

**Immunoprecipitation and Western Blotting**

Hippocampal extracts were made from homogenized Cks1−/− and wild-type brains in lysis buffer containing 5 mM HEPES (pH 7.3), 200 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 0.5% Triton X-100, and 5 % glycerol with protease inhibitors (Roche). Primary hippocampal neurons were made from E14 embryos (see below). Anti-p27 antibody (sc-528, Santa Cruz Biotechnology) was firstly cross-linked to Protein A/G agarose beads (Thermofisher) using dimethyl pimelimidate dihydrochloride (Sigma). Extracts were incubated with antibody-conjugated beads at 4 °C overnight. After extensive washing, the immunoprecipitates were separated by SDS-PAGE for Western blot analysis with anti-p27 antibody (#610241, BD Transduction Laboratories) and anti-RhoA antibody (sc-418, Santa Cruz Biotechnology). For active RhoA pull-down, a GSTRhotekin-RBD column was used according to the manufacturer’s protocol (#16116, Thermofisher). Cofilin was detected using the following antibodies: anti-cofilin (#5175, Cell Signaling), anti-Ser3 cofolin (#3313, Cell Signaling).

**Culture of primary hippocampal neurons and Immunostaining**

The primary hippocampal neurons were derived from the E14 embryos of WT and Cks1−/− mice. The neurons were seeded on 24-well plates with coverslips coated with poly-L-lysine
with Neurobasal Medium (Life Technologies) and B27 supplement (50 X). Immunostaining was then performed on the primary neurons after culture for 14 days. For neurons immunostaining, cells were rinsed with phosphate-buffered saline (PBS) twice and fixed for 30 min in a solution of 4 % paraformaldehyde, pH 7.4. Coverslips were then rinsed three times in PBS and permeablized with ice-cold methanol for 90 seconds. Permeabilization solution was removed and washed three times with PBS. Anti-cofilin antibody (#5175, Cell Signaling) and anti-beta III tubulin antibody [TUJ-1] (ab14545, Abcam) were added together with a donkey serum blocking solution in PBS and set to incubate overnight at 4 °C. Coverslips were then rinsed three times in PBS for 10 min. Samples were further incubated for 1 hour in anti-rabbit Alexa Fluor 488-conjugated secondary antibodies and anti-mouse Alexa Fluor 647-conjugated secondary antibodies (Invitrogen). The slips were washed finally with PBS for three times. Images were taken with Carl Zeiss LSM 510 Meta/ Axiocam. Cofilin aggregations were counted with ImageJ software with puncta analyzer plug-in (National Institutes of Health). Details of the quantification method using this plug-in have been described previously, and each of the image backgrounds was subtracted (rolling ball radius = 50) in order to detect discrete puncta (cofilin aggregates) without introducing background noise (Ippolito DM and Eroglu C 2010.)

**Statistical tests and analyses**

Significance was analyzed either with the Mann-Whitney test or t-test, and for the multiple parameter comparisons with one way ANOVA and post hoc Bonferroni or Tukey’s test. Parametric distribution of data was tested with Shapiro-Wilk test.
Results

CKS1 is involved in establishment of long-term memory in adult hippocampus

During investigation of CKS1 in the developing murine cortex, we recently observed that apart from controlling cell cycle exit during neurogenesis, CKS1 was also actively expressed in mature neurons (Frontini M et al. 2012). We therefore examined Cks1 expression in the adult brain by using β-galactosidase activity as a marker in heterozygous mice where one copy of Cks1 was disrupted by a LacZ insertion cassette. Cks1 expression was detected in various regions of the brain (Fig. 1A), including the hippocampus (Fig. 1B). Adult hippocampal expression of Cks1 was further confirmed with quantitative real-time PCR (RT-PCR) of RNA extracted from the hippocampi of wild-type mice (Fig. 1C). Given the well-established role of CKS1 in dividing cells, we expected to see Cks1 expression in areas where adult neurogenesis occurs (Drew LJ et al. 2013), but not in post-mitotic neurons. Surprisingly, in addition to the dentate gyrus, Cks1 expression was detected in the CA1, CA2 and CA3 areas and particularly in the stratum pyramidale.

To determine whether the Cks1 expression has significance in brain function, we compared the behavior of Cks1 knockout (Cks1^{-/-}) mice with wild-type littermates. Although Cks1^{-/-} mice were physically smaller than wild-type mice, possibly related to the accumulation of p27 (Spruck C et al. 2001), they did not show statistically different performance on the standardized SHIRPA protocol (Supplementary Table 1), and no motor deficits were observed.

Given the prominent CKS1 expression in the adult hippocampus, we tested Cks1^{-/-} mice and their wild-type littermate controls in a novel object recognition (NOR) task. Cks1^{-/-} mice displayed a statistically significant (P < 0.05) decrease in preference for a novel object (Fig. 2A). To further investigate if this was due to a hippocampal defect, hippocampus-dependent spatial learning and memory were examined using Barnes circular maze (Patil SS et al. 2009). Performance was measured by the time (primary latency) and distance (primary path length) taken for an animal to reach an escape hole from the open surface of a Barnes maze arena. Mice were initially given 3 learning trials, 15 minutes apart every day for 4 days. After 12 test trials, animals were tested on the 5th day to see whether they remembered the route to the escape hole without further training. If acquisition of new memory was normal, Cks1^{-/-} mice
and their wild-type control animals would be expected to demonstrate a similar degree of reduction in latency. However, the $Cks1^{-/-}$ mice spent a significantly longer time ($P < 0.01$, $t$-test) and used a longer path, very close level of significant difference the wild-type ($P = 0.05$), to reach the escape hole, reflecting a defect in establishing long-lasting memory ($n = 18$ mice in both groups) (Fig. 2B). We next performed the Barnes maze experiment on a separate cohort of mice, but providing the mice 1 trial per day for six days (i.e. a retention time of 24 hours) instead of 3 temporarily closely spaced trials each day (i.e. a retention time of 15 minutes each). Similarly, $Cks1^{-/-}$ mice exhibited a significant impairment in memory as reflected by the longer primary latency and longer primary path length taken by them to reach the escape hole on days 4-6 and 5-6, respectively ($P < 0.05$ for both, $t$-test) compared to wild-type littermates (Fig. 2C). To support the contention that wildtype mice and $Cks1^{-/-}$ mutants are distinct in their learning ability and not their performance in the Barnes maze per se, we tested an independent group of mice in the Barnes maze. Our results confirmed that wildtype mice ($n = 6$) and $Cks1^{-/-}$ mutants ($n = 6$) have no performance difference the first time they encounter the maze (primary latency, $P = 0.64$; primary path length $p = 0.70$). Collectively, our results highly suggested that CKS1 in the adult hippocampus is required for normal acquisition and consolidation of memory.

**CKS1 is required for late long-term potentiation and dendritic spine maturation**

We suspected $Cks1^{-/-}$ mice would have difficulties to establish long-term potentiation (LTP), which is considered to be the cellular substrate of hippocampal memory formation (Bliss TV and GL Collingridge 1993). Studying field excitatory postsynaptic potential (fEPSP) in acute hippocampal slices, we observed that both WT and $Cks1^{-/-}$ mice establishing early ($< 60$ minutes from theta-burst stimulation, post-TBS) LTP (Fig. 3A1-2). The recordings were made with standard extracellular solution without added drugs. At 60 minutes post-TBS, the fEPSP potentiation was significant from baseline (15 min) in the wild-type mice hippocampal Schaffer collateral pathway ($1.38 \pm 0.08$, $n = 14$, $P < 0.01$) as well as in the $Cks1^{-/-}$ ($1.26 \pm 0.05$, $n = 15$, $P < 0.01$) (at 50-60 min from TBS). Although average early LTP was moderately smaller in the $Cks1^{-/-}$ mice, there was significant difference between the two genotypes ($P = 0.18$, $t$-test). In recordings following the fEPSP for 2 hours post-TBS, we found that the late LTP was compromised in $Cks1^{-/-}$ mice ($1.10 \pm 0.03$, $n = 6$) as compared with the littermate controls ($1.65 \pm 0.14$, $n = 6$) (Fig. 3A2) ($P < 0.01$ comparing baseline-
normalized fEPSPs between the groups at 110-120 min post-TBS, n = 6 and 6, t-test). Thus, the inability of Cks1−/− mice to establish late LTP may at least partially explain the memory defects we observed on the NOR experiment and the Barnes maze.

Late LTP requires protein synthesis and consolidation of synaptic plasticity in postsynaptic sites (Bramham CR 2008). LTP establishment also involves growth of dendritic spines, the actin-based membrane protrusions where the majority of excitatory synapses reside. To investigate whether the density of excitatory synapses in CA1 pyramidal cells were altered, we recorded glutamatergic miniature EPSCs (mEPSCs) generated by stochastic release of synaptic transmitter vesicles in the presence of tetrodotoxin, 1 µM (and the GABA_A blocker, picrotoxin, 100 µM) (Fig. 3B1-2). Pyramidal cells in Cks1−/− mice showed significantly lower mEPSC frequency (0.39 Hz, n = 12) than those of wild-type littermates (1.04 Hz, n = 11) (P < 0.01, t-test) (Fig. 3B2). In addition, we found that mEPSC amplitudes were moderately higher in the Cks1−/− mice (13. 8 ± 0.5 pA) than in control cells (11.5 ± 0.6 pA, P < 0.01, t-test). These findings suggest that hippocampal CA1 pyramidal cells in the Cks1−/− mice have lowered density of afferent glutamatergic synapses and the average strength of a quantal current is moderately stronger than in the wild-type hippocampus.

During learning, neuronal activity is known to facilitate the growth of dendritic spines that results in an increase in spine head area-to-length ratio (hence formation of the so-called mushroom spines from filopodia or thin spines). This type of structural plasticity is commonly associated with establishment of late LTP (Bosch M and Y Hayashi 2012). Hence, we studied whether dendritic spine maturation differed between Cks1−/− and wild-type mice. We visualized and analyzed dendrites of pyramidal cells (which we previously made electrophysiological recordings from and filled them with neurobiotin) in the hippocampal CA1 area with confocal microscopy. We found that Cks1−/− pyramidal cell apical dendrites were deficient in mushroom spines, which represent the mature spine form (Fig. 3C1). The relative proportion of mushroom spines in dendrites was significantly lower in Cks1−/− pyramidal cells (21 ± 4 %, n = 8, P < 0.01) than in wild-type pyramidal cells (38.6 ± 6%) (Fig. 3C2). Moreover, to assess the presence of dendritic synapses, we examined the expression levels of synaptophysin and PSD95 (pre-synaptic and post-synaptic markers, respectively) in the primary hippocampal neurons from E14 embryo of the Cks1−/− and the wild-type mice using immunoblotting. As shown in figure 3D1-2, the expression levels of synaptophysin and
PSD95 were significantly lower in the $CksI^{−/−}$ neurons, suggesting that fewer synapses were present in the $CksI$ null neuronal dendrites. All these results supported the notion that CKS1 contributes to formation of dendritic spines and in its absence, the establishment of late LTP and long-term memory, development of mushroom-shape spines and functional dendritic synapses are compromised.

**CKS1 controls phosphorylation of cofilin through destabilization of p27 and activation of RhoA kinase**

Dendritic spine maturation requires active actin cytoskeleton remodeling (Calabrese B et al. 2006). Cofilin, an actin cytoskeleton severer, is essential in this process. Previous studies have shown that expression of a constitutively active non-phosphorylatable coflin inhibited dendritic spine maturation (Shi Y et al. 2009). To examine whether the inactive phospho-Ser3 form of coflin in hippocampal extract of $CksI^{−/−}$ mice was reduced, an antibody that specifically recognizes the phospho-Ser3 form of cofilin was used. Immunoblotting showed that phospho-Ser3 coflin was significantly reduced in hippocampus of $CksI^{−/−}$ mice (Fig. 4A1-3). Similarly, the level of phospho-Ser3 coflin was also markedly lower in $CksI^{−/−}$ primary hippocampal neurons than that of the wild type (Fig. 4A4-6). Because previous studies have shown that non-phosphorylated coflin aggregation induces synaptic loss in hippocampal neurons (Cichon J et al. 2012), we investigated if there was also increased coflin aggregation in $CksI^{−/−}$ primary hippocampal neurons (Fig. 4B1). The percentage of $CksI^{−/−}$ primary hippocampal neurons with coflin aggregates ($59.47 ± 2.58\%$, $n > 200$) was significantly higher ($P < 0.0001$) than that in wild-type neurons ($11.33 ± 2.25\%$, $n > 200$) (Fig. 4B2). Neuron-specific class III β-tubulin, a neural specific marker, was used to outline the normal neuronal morphology.

Ser3 phosphorylation of cofilin is mediated by GTPase RhoA, which is in turn negatively regulated by p27 (Kawauchi T et al. 2006; Belletti B et al. 2010). Given that $CksI^{−/−}$ mice accumulate high levels of p27, we hypothesized that RhoA was inhibited due to increased RhoA bound to p27, resulting in decreased cofilin phosphorylation in the brain of $CksI^{−/−}$ mice. To test this, we examined RhoA binding to p27 in primary hippocampal neurons of wildtype and $CksI^{−/−}$ mice. Immunoprecipitation experiments showed increased amount of RhoA bound to p27 in the $CksI^{−/−}$ background (Fig. 4C1-3). To confirm if the increased binding to p27 resulted in suppression of RhoA activity in $CksI^{−/−}$ mice, we employed a
Rhotekin Rho binding-domain column to enrich for active GTP-bound Rho kinases. We found that Cks1−/− primary hippocampal neurons indeed harbored less active RhoA than the wild-type littermate controls (Fig. 4D1-2). To further validate the effect of CKS1 on p27-RhoA axis, Cks1 was knocked down with siRNA in primary hippocampal neurons from wildtype mice (Fig. 5). Consistent with the findings in Cks1−/− mice, knocking down Cks1 in wildtype neurons resulted in reduced amount of CKS1 (Fig. 5A), increased p27 level and p27-RhoA binding (Fig. 5B1-3), inhibition of RhoA (Fig. 5C1-2), and increase in the formation of coflin aggregates (Fig. 5D and E). Taken together, these findings suggested that CKS1 is required for Ser3 phosphorylation of coflin and for preventing coflin aggregates formation.

**Decreasing p27 level by PIN1 inactivation reduces coflin aggregate formation in Cks1−/− hippocampal neurons**

Cofilin aggregation and rod-like aggregate formation are associated with the development of neurodegenerative diseases, such as Alzheimer’s disease (AD) (Bamburg JR et al. 2010). The rods have been shown to be co-localized with phosphorylated tau and responsible for phosphorylated tau accumulation in striated neuropil threads (Whiteman IT et al. 2009; Whiteman IT et al. 2011), a characteristic of tau pathology in the early stage of AD brain. The peptidyl-prolyl-isomerase PIN1 has been shown to compete with CKS1 for interaction with p27 and suppression of PIN1 activity is associated with destabilization of p27 (Zhou W et al. 2009). As shown in figure 6A, the coflin aggregates gradually disappeared with treatment of increasing concentration of PiB, an inhibitor of PIN1, in primary hippocampal neurons from Cks1−/− mice. PiB treatment reduced the p27 level, decreased the interaction between p27 and RhoA, and increased phosphorylation of coflin (Fig. 6B1-7). Similarly, in wildtype primary hippocampal neurons with Cks1 knocked down by siRNA, PiB treatment also resulted in diminished number of coflin aggregates (Fig. 6C). In addition, it decreased binding between p27 and RhoA, and increased phosphorylation of coflin (Fig. 6D1-7). Treatment with PINTIDE (Fig. 7A-C), a very specific PIN1 inhibitory phosphopeptide, also showed similar biochemical results, confirming the effect of PIN1 inhibition in reversing the coflin aggregates formation (Fig. 7D1-4). Taken together, the results suggested that regulation of p27 level via PIN1 and CKS1 determines the activity of coflin and the formation of coflin aggregates.
Discussion

CKS proteins are generally regarded as cell cycle regulators. Although CKS1 does not bind the brain specific cyclin-dependent kinase, CDK5 directly (Pines J 1996), it may bind indirectly as a complex in the brain (Veeranna et al. 2000). Here we describe a post-mitotic role for CKS1 in facilitating dendritic spine maturation in the hippocampus. We showed that CKS1 is actively expressed in the adult brain. Cks1\(^{-/-}\) mice exhibit impaired learning of hippocampus-dependent tasks, implying that CKS1 is required for the establishment of memory. Indeed, electrophysiological studies showed that the absence of CKS1 seriously compromised establishment of LTP.

In our model, we ascribe the phenotypes we saw in the Cks1\(^{-/-}\) mice to decreased RhoA kinase activity due to increased binding to p27. In order to establish long-lasting LTP, inactivation of coflin by Rho kinase-mediated phosphorylation is required to increase F-actin content within dendritic spines (Fukazawa Y et al. 2003). RhoA, through its effector RhoA kinase ROCK, activates LIM kinase (LIMK) that in turn phosphorylates coflin (Maekawa M et al. 1999). Accordingly, a number of chemical agents, which interfere with actin polymerization, specifically block late LTP (Rex CS et al. 2009). In the context of the hippocampus, we postulate that Cks1\(^{-/-}\) partially phenocopies the S3A coflin mutant. The coflin S3A mutant is non-phosphorylatable on serine 3, hence resistant to inhibitory phosphorylation. Over-expression of the S3A mutant results in an increase in the active form of coflin and an inability to mature dendritic spines to the mushroom form. Also, elevated coflin activity under certain conditions has been shown to contribute to enhanced AMPA receptor trafficking during synaptic potentiation (Gu J et al. 2010). This may explain the increased amplitude seen in the Cks1\(^{-/-}\) background. Of note, coflin phosphorylation is decreased, but not absent in the Cks1\(^{-/-}\) mouse. This is expected, as coflin phosphorylation is under control of multiple signaling pathways (Pontrello CG and IM Ethell 2009). A study has showed that p27 promotes microtubule polymerization and negatively regulates myosin II activity (Godin JD et al. 2012). Inhibition of myosin IIb has been shown to destabilize mushroom spines and inhibit excitatory synaptic transmission (Ryu J et al. 2006; Koskinen M et al. 2014). Further investigation is required to see whether this also contributes to the Cks1\(^{-/-}\) phenotype.
The implication of CKS1 in memory formation is manifold. First, this suggests an evolution redundancy in the use of cellular mechanisms that control cytoskeleton remodeling within and without the mitotic cycle. Secondly, this implies that when neurons exit the mitotic cycle, certain components of the cell cycle machinery remain, but take on different roles. Cyclin E has been shown to play a similar role in post-mitotic neurons (Odajima J et al. 2011). Our work therefore adds to the increasing repertoire of cell cycle proteins that play non-cell cycle dependent roles in neurons and in neurodegeneration (van Leeuwen LA and JJ Hoozemans 2015).

In summary, our results on this Cks1<sup>−/−</sup> murine model demonstrate that CKS1 has a cell-cycle independent role in adult hippocampus contributing to memory consolidation, pyramidal cell dendritic spine maturation and late LTP. Inhibition of CKS1 facilitates formation of cofilin aggregates through the p27-RhoA axis (Fig. 8), yet, the exact role of CKS1 in the pathogenesis of human neurodegenerative diseases remains to be determined.
Acknowledgements
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Author contributions
Y.M.N. performed the in-vitro and primary neuronal culture experiments, and microscopy. A.K. and K.L. performed the histology and electrophysiology study, and analyzed the data. L.J., A.S., S.E.W., D.N.S., and A.M.C. performed the mouse behavior studies and analyzed the data. S.L., A.M., D.D., W.G., and P.W.H., provided technical support. Y.M.N., E.T. and V.Y. wrote the manuscript. A.M.C., K.L., E.T., and V.Y. supervised the project.

Conflicts of interest
The authors declare that they have no conflict of interest
Figure legends

Figure 1  CKS1 is expressed in the adult mouse brain including hippocampus
A) In mice heterozygous for the Cks1 knockout cassette that harbors a LacZ insertional cassette for gene disruption (Cks1+/−), β-galactosidase activity acts as a marker for Cks1 gene expression. Above: Prominent Cks1 expression in the entorhinal cortex, particularly in the layer 2-3 (coronal section of a 4-month old male). Below: Confocal fluorescence images showed that neurons expressing Cks1 were mature neurons as they also expressed the neuronal marker NeuN. Coronal section stained with an anti-β-galactosidase antibody (β-gal Cks1+/−), anti-NeuN antibody (NeuN) and image overlap (merged). Scale 200 µm. L1 indicates layer 1.
B) The β-galactosidase activity was detected in various brain areas including the cerebellum showing staining in Purkinje cells and the hippocampal CA1-CA3 area and the dentate gyrus. Scale 200 µm.
C) Real-time PCR of Cks1 transcript in hippocampus of wildtype (WT) and Cks1−/− mice. The average Ct obtained in Cks1−/− mice was undetermined and was set as 40 for calculation. The fold change ($2^{-ΔΔCt}$) was calculated relative to Cks1−/− (P < 0.0001, t-test). Data (n = 5) were expressed as mean ± SEM. GAPHD was used as internal control.

Figure 2  CKS1 is required for hippocampus-dependent learning and long-term memory
A) Cks1−/− mice were deficient in novel object recognition. Average novel object preference index (PI, mean ± SD) in at least 4 animals of each genotype (*P < 0.05, t-test).
B-C) Cks1−/− showed impaired learning in the Barnes maze test. (B) Wild-type and Cks1−/− males were trained and assessed with a retention time of 15 min, having 3 trials per day for 4 days (trials 1-12) and tested on the 5th day to study whether they remembered the route to the escape hole without further training, as shown in a scheme on top. Animals were individually video-tracked using the EthoVision system. Left: time (mean ± SD) taken for an animal to reach the escape hole on day 5 was plotted as primary latency (n = 18 in each group, **P < 0.017, t-test). Right: the total distance travelled as primary path length (n = 18 in each group, P = 0.05, t-test). (C) Cks1−/− animals also showed a defect in memory on a training protocol as in (B), but having trials with 24 hour intervals for six days (scheme on top). Left: wild-type
mice showed significant change in the primary latency from the training day 4 onwards (compared to day 1, black asterisks **P < 0.01, one way ANOVA with Bonferroni test), whereas *CksI*<sup>-/-</sup> mice showed difference from day 5 onwards (black asterisk *P < 0.05). The genotypes were significantly different at days 4-6 (grey asterisk *P < 0.05). Right: Correspondingly, the wild-type showed significant change in primary path length from day 5 onwards (compared to day 1, black asterisks **P < 0.01, one way ANOVA with Bonferroni test) whereas in the *CksI*<sup>-/-</sup> mouse population this was not significantly changed from day 1 in any of the days. The day 5-6 results were significantly different between the wild-type and the *CksI*<sup>-/-</sup> mice (grey asterisk *P < 0.05) (n = 6 in each group). In addition, analysis of the pooled data (from day 1-6) by repeated measure ANOVAR showed a statistical significance between wildtype and *CksI*<sup>-/-</sup> animals: primary path length, P < 0.026; primary latency, P < 0.045.

**Figure 3**  
**Impaired long-term potentiation and dendritic spine maturation in *CksI*<sup>-/-</sup> mice**

A) *CksI*<sup>-/-</sup> mice exhibited impaired late-phase LTP. (A1) Averaged field potential EPSP (fEPSP) from a sample experiment in wild-type (WT) and in *CksI*<sup>-/-</sup> mouse during baseline (bl), and at a time point of early (60 min) and late LTP (120 min) elicited by theta-burst stimulation (TBS). (A2) Mean ± sem of fEPSP slope (baseline-normalized) in the hippocampal Schaffer collateral – CA1 pathway in WT and *CksI*<sup>-/-</sup> mice. Arrow indicate LTP induction with TBS (wild-type, grey symbols; *CksI*<sup>-/-</sup>, open symbols). In both genotypes early phase LTP (up to 60 min) was observed (t-test). Yet, slices from *CksI*<sup>-/-</sup> mice failed to establish late phase LTP (from 60 – 120 mins) (n = 6 in both groups, t-test). P < 0.05 between the groups at 60-120 min (t-test).

B) Pyramidal cells in *CksI*<sup>-/-</sup> mice showed decreased frequency of quantal miniature EPSCs (mEPSCs). (B1) Sample traces from individual recordings showing glutamatergic mEPSCs in CA1 area pyramidal cells (at -60 mV) in a wild-type and a *CksI*<sup>-/-</sup> mouse hippocampal slice. (B2) Mean ± se of mEPSC frequency (left) and amplitude (right) in all studied cells. The mEPSC frequency was robustly decreased in *CksI*<sup>-/-</sup> cells (t-test). In addition, mEPSC amplitude showed moderate increase (t-test).

C) Decreased mushroom spine density in *CksI*<sup>-/-</sup> pyramidal cells. (C1) Confocal images of CA1 area pyramidal cell dendrites showing the three spine types in the wild-type and *CksI*<sup>-/-</sup> mice. Characteristic ‘thin’ (T), ‘stubby’ (S) and ‘mushroom’ (M) spines were indicated by
arrowheads. Scale 1 µm. Semi-automated scoring of the spine types was carried out using the NeuronStudio software. (C2): Mean ± se of the density of the spine types (t-test).

D) Levels of PSD95 and synaptophysin were lower in primary hippocampal neurons derived from Cks1−/− mice than wild type controls. (D1) Band intensities in Western Blot -experiments were normalized using β-actin as loading control. (D2) Histograms showing relative intensity of PSD95 (left) and synaptophysin (right) in wild-type controls and Cks1−/− mice neurons. Data were expressed as mean ± SEM (n = 5).

Figure 4 CKS1 controls the phosphorylation of cofilin at Ser3 site and the formation of cofilin aggregates by limiting binding of p27 to RhoA in primary hippocampal neurons

A) The level of cofilin phosphorylated at Ser3 both in Cks1−/− hippocampal extracts and in primary hippocampal cells was lower than that in the WT hippocampal controls. (A1) Western blot of hippocampal extract using β-actin as loading control. (A2) Histogram showing relative intensity of total cofilin in hippocampal extracts. Data were expressed as mean ± SEM (n=9). (A3) Histogram showing relative amount of p-cofilin (Ser3) in hippocampal extract. Data were expressed as mean ± SEM (n=9). (A4) Western blot of primary hippocampal neurons with β-actin as loading control. (A5) Relative intensity of total cofilin in primary hippocampal neurons. Data were expressed as mean ± SEM (n=9). (A6) Relative amount of p-cofilin (Ser3) in primary hippocampal neurons. Data were expressed as mean ± SEM (n=9). B) The percentage of primary hippocampal neurons with aggregates was significantly higher in the Cks1−/− genotype than in wild-type derived neurons. (B1) Confocal immunofluorescence images illustrate aggregates in WT and Cks1−/− neurons. Scale 50 µm. (B2) Plot shows % of cells associated with the aggregates.

C) (C1-C2) Immunoprecipitation of p27 showed that more RhoA was bound to p27 in Cks1−/− primary hippocampal neurons than in the WT. (C3) Histogram showing amount of RhoA interacting with p27 in primary hippocampal neurons. Data were expressed as mean ± SEM (n=9).

D) A GST-rhotekin column was used to pull-down active RhoA in WT or Cks1−/− primary hippocampal neurons. (D1) By using antibody specific to RhoA, more active RhoA was detected in WT primary hippocampal neurons than in Cks1−/− mice cells. Input showed the same amount of total RhoA used in the RhoA pull-down assay for WT and Cks1−/−. (D2)
Histograms showing the amount of active RhoA in primary hippocampal neurons. Data were expressed as mean ± SEM (n=9).

Figure 5  
*Cks1* knocked down in WT primary hippocampal neurons leads to increased cofilin aggregates

Wild type primary hippocampal neurons were treated with either scrambled control siRNA (siCtrl) or siRNA against *Cks1* (si*Cks1*). The effects of *CKS1* on p27, RhoA and cofilin were examined with immunoblotting. The results of these experiments were similar to the results observed in *Cks1*−/− primary hippocampal neurons.

A) The expression of *CKS1* in wild-type primary hippocampal neurons was down-regulated by si*Cks1*, resulting in decreased p-cofilin. (A1) Western blot of using β-actin as loading control. (A2-A4) Histograms showing relative intensity of CKS1, cofilin and p-cofilin (Ser3) in primary neuron extracts. Data were expressed as mean ± SEM (n =9).

B) Immunoprecipitation of p27 showed that more RhoA was bound to p27 in the cells treated with si*Cks1*. (B1-B2) Western blot with β-actin as loading control. (B3) Histograms showing amount of RhoA interacting with p27 in primary neuron extract. Data were expressed as mean ± SEM (n =9).

C) From active RhoA pull-down, more active RhoA was present in the primary hippocampal neurons treated with siCtrl. (C1) Western blot showing active and total RhoA. (C2) Histograms showing relative intensity of active RhoA in primary neuron extracts. Data were expressed as mean ± SEM (n =9).

D) Wild-type primary hippocampal neurons treated with siCtrl or si*Cks1*. Confocal fluorescence micrographs showing DAPI and immunohistochemical reaction against cofilin and tubulin. Scale 50 µm. (E) The percentage of cofilin aggregates was significantly higher in the neurons treated with si*Cks1* than siCtrl (t-test). Scale 50 µm.

Figure 6  
Inhibition of PIN1 diminished cofilin aggregates through lowering p27 levels

A) PiB treatment effectively reduced cofilin aggregates in the *Cks1*−/− primary hippocampal neurons in a dose dependent manner compared control (DMSO) (* P < 0.05, *** P < 0.001, one way ANOVA with Tukey's test). Confocal images showing cofilin and tubulin staining in the different conditions. Scale 50 µm.
B) In Cks1−/− primary hippocampal neurons, PiB treatment decreased p27 level and increased phosphorylation of coflin via controlling the activity of RhoA. (B1) Western blot showing p27 and p-cofilin (Ser3) levels in primary hippocampal neuron extract. (B2) Histogram showing relative intensity of p27 compared to control (DMSO) (* P < 0.05, *** P < 0.001, t-test). There is highly significant difference between PiB treatment with 4 µM and 8 µM PiB (one way ANOVA with Tukey's test) (n =9). (B3) Histogram shows relative intensity of p-cofilin (Ser). (n = 9). (B4) This was associated with less RhoA binding with p27 (Western blot). (B5) More active RhoA was observed in PiB treated cells (Western blot). (B6) Histogram showing the relative (compared to control treatment with DMSO) amount of RhoA interacting with p27 in Cks1−/− primary hippocampal neurons. (n = 9). (B7) Amount of active RhoA in Cks1−/− primary hippocampal neurons. (n = 9). C) PiB treatment effectively reduced coflin aggregates in wild-type primary hippocampal neurons treated with siCks1 (* P < 0.05, ** P < 0.01, *** P < 0.001, one way ANOVA with Tukey's test). Confocal images showing coflin and tubulin staining as in (A). Scale 50 µm.

D) Similarly, in wild-type primary hippocampal neurons with Cks1 knocked-down by siRNA, (D1-D3) the PiB treatment decreased p27 level and increased phosphorylation of coflin (D4-D7) via controlling the activity of RhoA, causing less RhoA binding with p27 and more active RhoA in PiB treated cells. Data were expressed as mean ± SEM (n =9).

Figure 7 PIN1 inhibitory phosphopeptide decreased p27 levels and activated RhoA in Cks1−/− neurons

In Cks1−/− -derived neurons treatment of PIN1 inhibitory phosphopeptide (PINTIDE) A-B) decreased the p27 levels, and increased coflin phosphorylation and C) increased activated RhoA, demonstrated with immunoprecipitation, GST-rhotekin pull-down and immunoblotting.

D) Treatment with PINTIDE) of primary hippocampal neurons from Cks1−/− mice. Histograms demonstrate that the increased concentration of PINTIDE D1) reduced the p27 level, D2) increased phosphorylation of coflin, and D3) decreased the interaction between p27 and RhoA, D4) resulting in increased amount active RhoA. Data were expressed as mean ± SEM (n =9).

Figure 8 CKS1-mediated control of RhoA and coflin phosphorylation via p27
Cks1, via regulating p27 ubiquitylation and degradation, fine tunes RhoA activity, and hence cofilin phosphorylation during this process. In the absence of Cks1, p27 level is increased. As a result, there is less active RhoA and decreased in cofilin phosphorylation, leading to formation of cofilin aggregation and impairment of dendritic spine maturation.
References


Fig. 1

A

β-gal Cks1+/− neocortex

β-gal Cks1+/−

NeuN

merged

B

cerebellum

hippocampus

Cks1 transcript expression

<table>
<thead>
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<th></th>
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</tr>
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<tr>
<td>Cks1 transcript</td>
<td>16000</td>
<td>10000</td>
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** ****
Fig. 2

A

\[ PI = \frac{\text{Time on new object (s)}}{\text{Time on familiar + new object}} \]

B

day 1 | day 2 | day 3 | day 4
---|---|---|---
trials 1 | 2 | 3 | 4
---|---|---|---
10 | 11 | 12 | 13

C

day 1 | day 2 | day 3 | day 4 | day 5 | day 6
trials 1 | 2 | 3 | 4 | 5 | 6

**Primary latency (s)**

- WT
- Cks1\(^{-/-}\)

**Primary path length (cm)**

- WT
- Cks1\(^{-/-}\)

\( P < 0.05 \)

\( P < 0.01 \)

\( P = 0.06 \)

\( P < 0.05 \)

\( P < 0.01 \)

\( \bullet \)

\( \bullet \bullet \)

\( \bullet \bullet \bullet \)
Fig. 3

**A1**

WT bl  60 min  120 min

Cks1⁻/⁻ bl  60 min  120 min

TBS

**A2**

T

WT (n = 6)

Cks1⁻/⁻ (n = 6)

P < 0.05

**B1**

WT

Cks1⁻/⁻

**B2**

P < 0.01

**C1**

WT  Cks1⁻/⁻

**C2**

Spines per 100 μm

WT (n = 8)

Cks1⁻/⁻ (n = 11)

P < 0.01

**D1**

WT  Cks1⁻/⁻

β-actin

PSD95

Syn.phys.

**D2**

Amount of PSD95

Amount of syn.phys.
**A1** Hippocampal extract

WT  Cks1\(^{-/-}\)

\[
\begin{array}{l}
\beta\text{-actin} \\
\text{Cofilin} \\
p\text{-Cofilin (Ser-3)}
\end{array}
\]

**A2** Amount of Cofilin

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\[P = 0.065\]

**A3** Amount of p-Cofilin (Ser3)

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<tbody>
<tr>
<td>Relative intensity</td>
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<td>1</td>
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</tbody>
</table>

\[P < 0.001\]

**A4** Primary hippocampal neurons

WT  Cks1\(^{-/-}\)

\[
\begin{array}{l}
\beta\text{-actin} \\
\text{CKS1} \\
\text{Cofilin} \\
p\text{-Cofilin (Ser-3)}
\end{array}
\]

**A5** Amount of Cofilin

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\[P = 0.299\]

**A6** Amount of p-Cofilin (Ser3)

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<tr>
<td>Relative intensity</td>
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\[P < 0.001\]

**B1**

WT coflin  Cks1\(^{-/-}\) coflin

\[
\begin{array}{l}
\beta\text{-III-tubulin} \\
\beta\text{-III-tubulin}
\end{array}
\]

**C1** Primary hippocampal neurons

\[
\begin{array}{l}
\text{Input} \\
\text{WT} \\
\text{Cks1\(^{-/-}\)}
\end{array}
\]

**C2** IP of p27

\[
\begin{array}{l}
\text{IgG} \\
\text{WT} \\
\text{Cks1\(^{-/-}\)}
\end{array}
\]

**C3** RhoA with p27

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<td>Relative intensity</td>
<td>0</td>
<td>1</td>
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</tbody>
</table>

\[P < 0.0001\]

**D1**

\[
\begin{array}{l}
\text{Input} \\
\text{WT} \\
\text{Cks1\(^{-/-}\)}
\end{array}
\]

**D2** Active RhoA

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<tr>
<td>Relative intensity</td>
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\[P < 0.0001\]

**B2**

\[
\begin{array}{l}
\%\text{ of cells in cofilin aggregates}
\end{array}
\]

\[P < 0.001\]

\[
\begin{array}{l}
\%\text{ of cells in cofilin aggregates} \\
\text{WT} \\
\text{Cks1\(^{-/-}\)}
\end{array}
\]

\[P < 0.001\]
**Fig. 5**

**A1** Primary neurons extract

- siCtrl
- siCks1

- β-actin
- CKS1
- Cofilin
- p-Cofilin (Ser-3)

**A2** Amount of Cks1

- WT + siCtrl
- Cks1<sup>−/−</sup> + siCks1

- Relative intensity

- P < 0.0001

**A3** Amount of Cofilin

- WT + siCtrl
- Cks1<sup>−/−</sup> + siCks1

- Relative intensity

- P = 0.31

**A4** Amount of p-Cofilin (Ser3)

- WT + siCtrl
- Cks1<sup>−/−</sup> + siCks1

- Relative intensity

- P < 0.0001

**B1** Input

- siCtrl
- siCks1

- p27
- RhoA

**B2** IP of p27

- IgG
- siCtrl
- siCks1

- p27
- RhoA

**B3** RhoA with p27

- WT + siCtrl
- Cks1<sup>−/−</sup> + siCks1

- Relative intensity

- P < 0.0001

**C1** Primary neurons extract

- Active RhoA pull-down

- siCtrl
- siCks1

- Active RhoA
- Total RhoA

**C2** Active RhoA

- WT + siCtrl
- Cks1<sup>−/−</sup> + siCks1

- Relative intensity

- P < 0.0001

**D**

- siCtrl DAPI
- siCks1 DAPI

- siCtrl cofillin
- siCks1 cofillin

- β-III-tubulin

**E**

- % of cells in cofilin aggregates

- siCtrl
- siCks1

- P < 0.0001
Fig. 6

A  Cks1−/− primary hippocampal neurons

% of cells in cofilin aggregates

B1  Input

DMSO  4µM PiB  8µM PiB

β-actin  p27  p-Cofilin (Ser-3)

B2  Amount of p27

Relative intensity

Cks1−/−  DMSO  Cks1−/−  4µM PiB  Cks1−/−  8µM PiB

*  ***

P < 0.0001

B3  Amount of p-Cofilin (Ser3)

Relative intensity

Cks1−/−  DMSO  Cks1−/−  8µM PiB

***

P < 0.0001

B4  IP of p27

IgG  DMSO  8µM PiB

p27  RhoA

B5  Active RhoA pull-down

DMSO  8µM PiB

Active RhoA

Total RhoA

B6  RhoA with p27

Relative intensity

Cks1−/−  DMSO  Cks1−/−  8µM PiB

*  ***

P < 0.0001

B7  Active RhoA

Relative intensity

Cks1−/−  DMSO  Cks1−/−  8µM PiB

P < 0.0001
WT primary hippocampal neurons with siCks1

D1 Input

\[
\text{Input} \quad \text{DMSO} \quad 4\, \mu M \quad 8\, \mu M \quad \Pi B
\]

D2 Amount of p27

\[
\text{Relative intensity} \quad \begin{array}{c}
\text{WT+siCks1} \\
\text{WT+siCks1} \\
\text{WT+siCks1}
\end{array}
\]

\[
\text{DMSO} \quad 4\, \mu M \quad 8\, \mu M \quad \Pi B
\]

D3 Amount of p-Cofilin (Ser-3)

\[
\text{Relative intensity} \quad \begin{array}{c}
\text{WT+siCks1} \\
\text{WT+siCks1} \\
\text{WT+siCks1}
\end{array}
\]

\[
\text{DMSO} \quad 4\, \mu M \quad 8\, \mu M \quad \Pi B
\]

D4 IP of p27

\[
\beta\text{-actin} \quad \text{IgG} \quad \text{DMSO} \quad 8\, \mu M \quad \Pi B
\]

D5 Active RhoA pull-down

\[
\text{WT+siCks1} \quad \text{WT+siCks1} \\
\text{DMSO} \quad 8\, \mu M \quad \Pi B
\]

D6 RhoA with p27

\[
\text{Relative intensity} \quad \begin{array}{c}
\text{WT+siCks1} \\
\text{WT+siCks1} \\
\text{WT+siCks1}
\end{array}
\]

\[
\text{WT} \quad \text{WT} \quad \text{WT}
\]

D7 Active RhoA

\[
\text{Relative intensity} \quad \begin{array}{c}
\text{Cks1}\text{--/} \\
\text{Cks1}\text{--/}
\end{array}
\]

\[
\text{DMSO} \quad 8\, \mu M \quad \Pi B
\]

E1 Amount of p27

\[
\text{Relative intensity} \quad \begin{array}{c}
\text{Cks1}\text{--/} \\
\text{Cks1}\text{--/} \\
\text{Cks1}\text{--/}
\end{array}
\]

\[
\text{Ctrl peptide} \quad \text{PINTIDE} \quad \text{PINTIDE}
\]

E2 Amount of p-Cofilin (Ser3)

\[
\text{Relative intensity} \quad \begin{array}{c}
\text{Cks1}\text{--/} \\
\text{Cks1}\text{--/} \\
\text{Cks1}\text{--/}
\end{array}
\]

\[
\text{Ctrl peptide} \quad \text{PINTIDE} \quad \text{PINTIDE}
\]
Fig. 7

**A**  
*Cks1−/−*-derived neurons

<table>
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<td>β-actin</td>
<td>![β-actin Image]</td>
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</tr>
<tr>
<td>p27</td>
<td>![p27 Image]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Cofilin (Ser-3)</td>
<td>![p-Cofilin Image]</td>
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</tbody>
</table>

**B**  
IP of p27

<table>
<thead>
<tr>
<th></th>
<th>IgG control peptide</th>
<th>50µg/ml PINTIDE</th>
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<tbody>
<tr>
<td>p27</td>
<td>![p27 Image]</td>
<td></td>
</tr>
<tr>
<td>RhoA</td>
<td>![RhoA Image]</td>
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</table>

**C**  
Active RhoA pull-down

<table>
<thead>
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<th></th>
<th>50µg/ml control peptide</th>
<th>50µg/ml PINTIDE</th>
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<tbody>
<tr>
<td>Active RhoA</td>
<td>![Active RhoA Image]</td>
<td></td>
</tr>
<tr>
<td>Total RhoA</td>
<td>![Total RhoA Image]</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 8

With Cks1 | No Cks1
---|---
Active RhoA | Inactive RhoA

Favors | Favors
---|---
Cofilin | Cofilin

Cofilin aggregates

Impaired dendritic spine maturation