Long non-coding RNA C2dat1 regulates CaMKIIδ expression to promote neuronal survival through the NF-κB signaling pathway following cerebral ischemia

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Increasing evidence has demonstrated a significant role of long non-coding RNAs (lncRNAs) in diverse biological processes. However, their functions in cerebral ischemia remain largely unknown. Through an lncRNA array analysis in a rat model of focal cerebral ischemia/reperfusion (I/R), we have identified CAMK2D-associated transcript 1 (C2dat1) as a novel I/R-induced lncRNA that regulated the expression of CaMKIIδ in murine models of focal cerebral ischemia. C2dat1 mRNA was upregulated in a time-dependent manner in mouse cortical penumbra after focal ischemic brain injury, which was accompanied by increased expression of CaMKIIδ at transcript and protein levels. The expression patterns of C2dat1 and CAMK2D were confirmed in mouse Neuro-2a cells in response to in vitro ischemia (oxygen-glucose deprivation/reoxygenation, OGD/R). Knockdown of C2dat1 resulted in a significant blockade of CaMKIIδ expression, and potentiated OGD/R-induced cell death. Mechanistically, reduced CaMKIIδ expression upon silencing C2dat1 inhibited OGD/R-induced activation of the NF-κB signaling pathway. Further analysis showed that the downregulation of IKKα and IKKβ expression and phosphorylation, and subsequent inhibition of IκBα degradation accounted for the inhibition of the NF-κB signaling activity caused by silencing C2dat1. In summary, we discovered a novel I/R-induced lncRNA C2dat1 that modulates the expression of CaMKIIδ to impact neuronal survival, and may be a potential target for therapeutic intervention of ischemic brain injury.

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Received 15.10.15; revised 19.2.16; accepted 23.2.16; Edited by A Verkhratsky
protein kinases with broad substrate specificity, and the holoenzyme is a homo- or heteromultimer that is assembled by 8–12 isoforms. CaMKII is activated by Ca$^{2+}$ influx through the activation of the glutamate receptors in neurons. Ischemic conditions can trigger massive release of glutamate leading to ‘glutamate excitotoxicity’ involving Ca$^{2+}$ overload, and subsequent neuronal cell death, which accounts for much of the neuronal damage after cerebral ischemia. It has been shown that focal ischemia CaMKII is quickly activated and then inactivated in a time- and location-dependent manner, and the extent of neuronal damage correlates with the degree of loss of CaMKII activity.14–16 Meanwhile, CaMKIIa knock-out mice display increased infarct volume than that of wild-type litter mates.17 The studies from the Hudson group have also showed that prolonged pharmacological inhibition of CaMKII promotes neuronal death by increasing neuronal vulnerability to glutamate,18,19 which is in contrast to short-term inhibition of CaMKII that protects neurons from excitotoxic insult.19 Other studies have also shown that acute inhibition of CaMKII before excitotoxic insults, such as glutamate challenge or hypoxia/hypoglycemia treatment, was neuroprotective in vivo and in vitro.20–24 The mechanisms underlying the paradoxical effects of CaMKII inhibition/depletion are not well understood. Moreover, despite extensive studies on the abundantly expressed CaMKIIα or CaMKIIβ isoform, the function of CaMKIIδ, one of the most ubiquitous CaMKII isoforms expressed both in neuronal and non-neuronal cells, remains largely unknown in the CNS.10,25

In this study, we report the discovery of a novel CAMK2D-associated lncRNA, CAMK2D-associated transcript 1 (C2dat1). This lncRNA was upregulated in murine ischemia/reperfusion (I/R) models and in mouse neuronal cells upon in vitro ischemia oxygen-glucose deprivation/reoxygenation (OGD/R). C2dat1 regulated the expression of CAMK2D/CaMKIIδ expression in response to OGD/R. C2dat1 induced CaMKIIδ expression promoted neuronal survival by activating the NF-κB signaling pathway. The neuroprotective role of C2dat1 may be exploited for therapeutic intervention of I/R-induced neuronal injury.

**Results**

*C2dat1* was a novel CAMK2D-associated lncRNA induced by I/R. After focal cerebral ischemia, massive cell death occurs in the ischemia core in an irreversible manner; however, in the surrounding penumbra the injury was less severe and may be reversible upon therapeutic intervention. We examined the IncRNA expression profiles of the rat brains subjected to transient focal ischemia via middle cerebral artery occlusion (MCAO) using microarray.26,27 MCAO induced time-dependent irreversible cell death in the ischemic core in 6–24 h after transient focal ischemia in rats and mice. We specifically determined changes of IncRNA expression profiles in the penumbra areas using microarray (Wu et al., unpublished results). In all, 1187 IncRNAs (360 up- and 827 downregulated) and 1894 mRNAs (1081 up- and 813 down-regulated) were found to be differentially expressed (>2-fold change, *P* ≤ 0.05). Eighty-one IncRNAs showed >5-fold changes (46 up- and 35 down-regulated) (Wu et al., unpublished results). A panel of differentially expressed IncRNAs was then selected for validation in mouse Neuro-2a (N2a) cells subjected to in vitro ischemia (OGD/R) (see Supplementary Table S2 for selected IncRNAs). The IncRNA detected by AK153573 was among a few validated IncRNAs that showed consistent changes in cellular and rat/mouse models of I/R, which we later named it CAMKIIδ-associated transcript 1 (C2dat1). The known sequence of C2dat1 overlaps in part with introns 13–15 and exon 14 of CAMK2D on mouse chromosome 3, implying that C2dat1 is a CAMK2D-associated IncRNA that may regulate CAMK2D gene expression (Figure 1a). Taken together, focal cerebral ischemia caused genome-wide alternations of IncRNA expression in ischemic penumbra, and C2dat1 as a novel I/R-induced IncRNA could have a role in the pathophysiology of ischemia.

**Focal ischemia caused the upregulation of C2dat1 and CAMK2D in mice.** The expression of C2dat1 and CAMK2D were assessed in a mouse model of I/R. Focal cerebral ischemia was induced in mice by MCAO for 1 h, followed by 24-h reperfusion.26,27 Brain tissues from the ischemic core and surrounding penumbra were obtained at different time points (6, 12 and 24 h) and subjected to RNA extraction and real-time RT-qPCR. Focal ischemia caused progressive cell death in the ischemic core and was visible at 12 and 24 h (Figure 1b). In the ischemic core, both C2dat1 and CAMK2D transcripts were abruptly upregulated at 6 h, and then gradually downregulated to basal levels at 24 h, in line with the massive neuronal death in this area (Figures 1e and f). In contrast, penumbra exhibited a time-dependent upregulation of C2dat1 and CAMK2D with an over sevenfold increase for C2dat1 and a nearly fourfold increase for CAMK2D at 24 h after ischemia (Figures 1c and d). In summary, focal ischemia induced a parallel increase in C2dat1 and CAMK2D mRNA in mouse model of I/R with differential patterns in ischemic core and penumbra regions.

**Focal ischemia caused the upregulation of CaMKIIδ in neurons at the peri-infarct region and in the primary cortical neuron cultures.** The expression of CaMKIIδ was examined in the ischemic core and the peri-infarct regions (penumbra) of mouse cerebral cortex after I/R by immunofluorescence staining. As shown in Figure 2a, CaMKIIδ was upregulated and colocalized with the neuronal marker microtubule-associated protein-2 (Map2) in neurons of peri-infarct (PI) region after I/R. Quantitative measurement of the fluorescence intensity of CaMKIIδ in the contralateral (CL) side, the ischemic core (Core) and PI region indicated that CaMKIIδ was upregulated in the PI region (Figure 2b). In contrast, CaMKIIδ was significantly downregulated in ischemic core after I/R (Figures 2a and c). Western blot analysis of the tissues from the ischemic region (ischemic core) and the CL side showed rapid and progressive decrease of CaMKIIδ expression in the ischemic core in response to I/R, whereas its levels in the CL side remained unchanged at all time points (Figures 2c and d). Next, the levels of CaMKIIδ were evaluated in the primary cortical neuronal cultures subjected to OGD/R. Consistent with the previous findings, we observed about twofold time-dependent
upregulation of CaMKIIδ in the primary neurons in response to OGD/R (Figures 2e and f). These data implied a potential role of CaMKIIδ in ischemic-associated biological processes in mice.

C2dat1 and CAMK2D/CaMKIIδ were upregulated in response to OGD/R in mouse neuronal cells. Using mouse neuroblastoma (N2a) cells, we further evaluated the expression of C2dat1 and CAMK2D/CaMKIIδ in response to

Figure 1  I/R-induced C2dat1 and CAMK2D transcription in mouse model of I/R. (a) Genomic locus of C2dat1. C2dat1 contains overlapping sequences from introns 13 to 15 and exon 14 of CAMK2D in sense direction. (b) Focal ischemia induced by MCAO resulted in progressive brain damage that was visible at 12 and 24 h. Representative coronal brain sections stained with TNN are shown. Right, the image showing the anatomical location of the ischemic core and penumbra in the cortex. 1, ischemic cortex; 2, ischemic penumbra. (c-f) Transcript levels of C2dat1 and CAMK2D in ischemic cortex and the surrounding penumbra in sham and ischemic tissues. C2dat1 (c and e) and CAMK2D (d and f) were measured by real-time RT-qPCR. Data are the mean ± S.E.M. of three independent experiments. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.
in vitro ischemia. It has been reported that N2a cells express glutamate receptors and exhibit glutamate receptor-mediated Ca\(^{2+}\) overload and excitotoxicity. Thus, excitotoxicity-induced cell death may have an important role in OGD/R-induced cell death in N2a cells. We first examined the effect of OGD/R on N2a cell survival. As shown in Figure 3a, OGD/R time-dependently induced cell death in N2a cells, which plateaued at about 40% cell death at 24 h post OGD. Second, cells were collected at different time points of reoxygenation and the levels of C2dat1 and CAMK2D transcripts were measured by real-time RT-PCR. OGD/R caused the upregulation of C2dat1 over time, which peaked at about 12 h and then declined within 48 h (Figure 3b).

A persistent and time-dependent upregulation of CaMKII\(\delta\) was also detected at protein level (~2-fold) (Figures 3d and e). Thus, OGD/R induced the upregulation of C2dat1 and CAMK2D/CaMKII\(\delta\) in mouse neuronal cells.

Knockdown of C2dat1 blocked the transcription and protein expression of CaMKII\(\delta\) and enhanced neuronal death. The intracellular localization of IncRNA is critical to its biological function. To evaluate the subcellular localization of C2dat1, N2a cells were fractionated into cytosolic and nuclear fractions. Levels of C2dat1 were measured by real-time RT-PCR. OGD/R caused the upregulation of C2dat1 over time, which peaked at about 12 h and then returned to baseline at about 36 and 48 h (Figure 3c).
nucleus of N2a cells at 12 h post OGD/R and at basal state as measured by RNA FISH. Thus, C2dat1 was a nucleus-localized lncRNA.

The overlapping sequences suggest that C2dat1 may regulate CAMK2D/CaMKIIδ expression. To test this hypothesis, a C2dat1 siRNA was designed to target the region of the lncRNA outside of the overlapped sequences with CAMK2D exon 14. N2a cells were transfected with the C2dat1-targeting siRNA (si-C2dat1), which resulted in a significant knockdown of C2dat1 transcript at basal state (data not shown) and 12 h post OGD/R (Figure 4c). Accordingly, knockdown of C2dat1 significantly blocked OGD/R-induced CAMK2D expression (Figure 4d). At protein level, knockdown of C2dat1 significantly decreased CaMKIIδ expression in N2a cells before and after OGD/R (Figures 4e and f). Note that, as there are no overlapping sequences between C2dat1 and other CaMKII isoforms (CaMKIIα, β, γ), it is unlikely that the expression of these CaMKII isoforms is regulated by C2dat1. In addition, it is known that each CAMK2 gene gives rise to multiple isoforms by alternative splicing, and at least 10 CAMK2D/CaMKIIδ isoforms have been described.31 Sequence analysis indicates that the exon 14 of CAMK2D that overlaps in part with C2dat1 encodes the variable domain of CaMKIIδ, and this regions of CAMK2D1/4, and CAMK2D4 aligns with that of C2dat1, suggesting that C2dat1 may selectively target CaMKIIδ1/4 and CaMKIIδ4. Using primers that selectively detect the transcripts of CAMK2D1/4, CAMK2D2/3 and CAMK2D5/9, we then examined the mRNA levels of CAMK2D isoforms in response to OGD/R with or without knockdown of C2dat1. Knockdown of C2dat1 abolished IR-induced expression of CAMK2D1/4, partially inhibited that of CAMK2D5/9, but had no effect on CAMK2D2/3, implying that C2dat1 could regulate the expression of CAMK2D1/4 selectively (Supplementary Figure S1). Taken together, C2dat1 regulated the expression of CAMK2D/CaMKIIδ in response to OGD/R in mouse neuronal cells.

C2dat1 promoted neuronal survival through the activation of the NF-κB signaling pathway. To gain insights into the signaling events downstream of C2dat1, we examined

![Figure 3](image-url)
several potential downstream signaling pathways involved in I/R-induced neuronal cell death including the NF-κB, Akt, ERK and p38 signaling pathways (Supplementary Figure S2), and the NF-κB signal pathway was consistently altered upon knocking down of C2dat1. The NF-κB pathway is one of the main pro-inflammatory signaling pathways that can be activated in response to a variety of stimuli. I/R induces the activation of NF-κB, which has an important role in the inflammatory responses associated with I/R. The pro-inflammatory activities induced by NF-κB often lead to neuronal cell death, however, pro-survival effects of NF-κB has also been reported. The dual role of the NF-κB signal pathway appears to be stimulus- and cell/tissue-type dependent. As shown in Figures 5a-c, in the neuronal N2a cells, the NF-κB signal pathway was activated at 12 and 24 h post OGD, evident by the increased phosphorylation of IKKa and IKKβ measured by the p-S176/180-IKKα/β antibody, followed by decreased IκB protein expression. Knockdown of C2dat1 inhibited CaMKIIδ expression, blocked the phosphorylation of IKKa and IKKβ at S176/180 and downregulated IKKa and IKKβ protein expression (Figures 5a and b). This resulted in the nearly complete blockade of IκBα degradation (Figures 5a and c) and inhibition of the NF-κB activity (Figures 5a and c).

The functional impact of C2dat1 induction on neuronal survival was determined by knocking down this IncRNA using RNAi. As shown in Figure 6a and Supplementary Figure S3A, knockdown of C2dat1 exacerbated OGD/R-induced neuronal cell death at 12 h post OGD, indicating that the ischemia-induced C2dat1/CaMKIIδ/NF-κB pathway was neuroprotective in N2a cells. Moreover, inhibition of the NF-κB signaling activity by an IKK inhibitor BAY11-7082 resulted in decreased IκB protein expression. Knockdown of C2dat1 inhibited CaMKIIδ expression, blocked the phosphorylation of IKKa and IKKβ at S176/180 and downregulated IKKa and IKKβ protein expression (Figures 5a and b). This resulted in the nearly complete blockade of IκBα degradation (Figures 5a and c) and inhibition of the NF-κB activity (Figures 5a and c).

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**Figure 4** C2dat1 was primarily localized in the nucleus of N2a cells and knockdown of C2dat1 caused the downregulation of CaMK2D mRNA and protein. (a) Majority of C2dat1 was in the nuclear fraction. N2a cells were fractionated to cytosol, nucleoplasm, and nucleus fractions. Levels of C2dat1 were quantified by real-time RT-PCR at basal state. (b) C2dat1 was detected in the nucleus of N2a cells post 12-h OGD/R by RNA FISH. (c and d) Knockdown of C2dat1 abolished OGD/R-induced CaMK2D expression. N2a cells were transfected with an si-C2dat1 and a non-targeting siRNA (si-NT) at 60 nM. Two days after transfection, cells were subjected to OGD for 3 h and then 12-h reoxygenation. The transcript levels of C2dat1 (c) and CAMK2D (d) were examined by real-time RT-qPCR. (e) Knockdown of C2dat1 blocked the upregulation of CaMKIIδ by OGD/R. N2a cells were similarly transfected with si-C2dat1 and si-NT. Following OGD/R treatment, the levels of CaMKIIδ were examined by western blot analysis. The α-tubulin was blotted as loading control. The experiments were repeated at least three times and data from a representative experiment are shown. (f) Quantitative analysis of the results from e. The above data in a, c, d and f are the mean ± S.E.M. of three independent experiments performed in triplicate.*P < 0.05; ****P < 0.0001; ns, not significant.
time-dependent cell death in response to OGD/R, implying that activation of the NF-κB signal pathway promotes the survival of N2a cells (Figure 6b and Supplementary Figure S3B). Similarly, knockdown of CaMKIIδ potentiated cell death induced by OGD/R in a time-dependent manner (Figures 6c and d). The signaling pathway through which C2dat1 regulates neuronal survival in response to ischemia is depicted in Figure 7. Taken together, our data implied that the NF-κB signal pathway was a major target of C2dat1-regulated CaMKIIδ in response to OGD/R in mouse neuronal cells and the activation of NF-κB promoted neuronal survival.

Discussion

**C2dat1 in regulation of CaMKIIδ.** Stroke as one of the leading cause of death and adult disability worldwide has incurred significant family and society burdens. Substantial efforts have been devoted to obtain more details of its cellular and molecular pathophysiology, yet, much remains to be elucidated. LncRNAs, as one of the most abundantly expressed ncRNAs, may have critical roles in stroke-related biological processes. In this study, we report the discovery of C2dat1, the first CAMK2D-associated IncRNA, induced by I/R in murine focal cerebral ischemic models, which may be neuroprotective during ischemia-induced neuronal injury. There have not been any reports of CaMKII-associated IncRNAs, and C2dat1 represents the first-ever CaMKII regulatory IncRNA identified thus far. C2dat1 was identified through an IncRNA array analysis of rat brain tissues subjected to transient focal ischemia. It contains overlapping nucleotide sequences with introns 13–15 and exon 14 of CAMK2D. C2dat1 upregulated the expression of CaMKIIδ in murine models of I/R and in vitro ischemia. This regulation is likely isotype specific as there are no overlapping sequences between C2dat1 and other CaMKII isoforms (CaMKIIα, β, γ). In light of the abundancy and significance of CaMKII in the CNS, the finding of an IncRNA that selectively targets CaMKIIδ to modulate neuronal survival will have profound biological and therapeutic implications.

There are five different categories of lncRNAs classified based on their positions relative to protein coding genes, including sense, antisense, bidirectional, intronic and intergenic. C2dat1 is a sense lncRNA with complimentary sequences with the exons and introns of CAMK2D. LncRNAs regulate transcription through multiple mechanisms, such as recruiting epigenetic complexes, directly modulating the transcriptional process as decoys, coregulators or interfere with RNA polymerase II activity. It may also act in post-transcriptional control by regulating mRNA processing and stability. Our data indicate that C2dat1 was upregulated in a transient time-dependent manner that peaked at 12 h, which correlated to the gradual elevation of CaMKIIδ protein in N2a cells subjected to OGD/R and in mouse penumbra under I/R. This kinetics of regulation implies a possible direct transcriptional control via targeting the CAMK2D gene. This can be further supported by the nuclear localization of C2dat1 as determined by RNA FISH. Although it remains to be determined how C2dat1 regulates CAMK2D expression (outside the scope of the current article), we will direct future
efforts to further elucidate the molecular basis underlying the regulation of CAMK2D by C2dat1 at the transcriptional level.

Roles of CaMKIIδ in neuronal survival. CaMKIIδ is one of the most ubiquitously expressed CaMKII isoforms in both neuronal and non-neuronal cells and a common component of the CaMKII holoenzyme. Its expression and function in the CNS are not well defined despite numerous studies on CaMKII and CaMKIIα. In our study, analysis of CaMKIIδ expression by Western blotting and immunostaining has led to the detection of abundant CaMKIIδ in N2a cells, primary neuronal cultures and mouse brain sections, implying that CaMKIIδ may be functionally important to neurons in the brain. I/R and OGD/R-induced gradual and persistent upregulation of CaMKIIδ at transcript and protein levels, which correlated to the increase of C2dat1. Knockdown of C2dat1 blocked the upregulation of CaMKIIδ, indicating that C2dat1 controls the expression of CaMKIIδ at the transcriptional level. As reduced CaMKIIδ by silencing C2dat1 potentiated OGD/R-induced neuronal cell death, we conclude that CaMKIIδ likely promotes neuronal survival in response to ischemic stress in the brain. Our findings agree with a previous study showing that CaMKIIδ expression was elevated in the survival neurons after traumatic brain injury in rat brain, and the increased expression is implicated in the apoptosis of the neuron and the recovery of motor functional outcome.34 It has been well documented that chronic loss/inactivation of CaMKII promotes neurotoxicity, whereas acute CaMKII inhibition is neuroprotective.17–24

Figure 6  C2dat1 promotes neuronal cell survival in response to OGD/R. (a) Knockdown of C2dat1 promoted OGD/R-induced cell death. Cell viability was measured by CCK-8 assay before and 12 h after OGD. (b) Inhibition of IKK by BAY11-7082 enhanced cell death induced by OGD/R in N2a cells. BAY11-7082 (1 μM) was added at the beginning of reoxygenation right after OGD for a total of 6 h. Cell viability was measured by CCK-8 assay. (c) Knockdown of CAMK2D potentiates OGD/R-induced cell death. N2a cells were transfected with a CAMK2D siRNA (si-CAMK2D-3), followed by OGD/R treatment for 0, 12 and 24 h. Cell viability was measured by CCK-8 assay. (d) CAMK2D siRNAs (si-CAMK2D-2 and -3) caused significantly knockdown of CAMK2D/CaMKIIδ. Cells were transfected for 48 h using 50 nM siRNAs, followed by immunoblotting for CaMKIIδ and α-tubulin. The data are the mean±S.E.M. of at least three independent experiments performed in triplicate:*P<0.05; **P<0.01; ****P<0.0001; ns, not significant

Figure 7  A diagram depicting the signaling mechanisms of C2dat1. I/R-induced C2dat1 upregulates the expression of CAMK2D at the transcriptional level, possibly through direct binding to the CAMK2D gene via the overlapping sequence. The upregulation of CAMK2D led to an increased CaMKIIδ protein expression and enhanced activity (I/R is known to activate CaMKII). The activated CaMKIIδ stimulates IKKα/β protein expression and their phosphorylation at S176/180, which resulted in IκBα degradation and subsequent NF-κB translocation to the nucleus, where it induces the transcription of multiple genes including Bcl-xL to promote cell survival.
mechanisms underlying these opposing effects of CaMKII inhibition on neuronal survival are complex. One hypothesis is that the neuroprotective effect of acute CaMKII inhibition is limited to the excitotoxic challenges as it could paradoxically prevent excitotoxicity-induced CaMKII inactivation-aggregation, as described by Ashpole et al.\textsuperscript{18} The diverse functions of CaMKII are also consistent with the dual actions of CaMKII signaling that activate CaMKII in different biological systems.\textsuperscript{35} The I/R-induced upregulation of CaMKII\textdelta may act independently or together with other isoforms to alter the function of the CaMKII holoenzyme, thereby affecting the neuronal injury and recovery process after ischemia.

The CaMKII\textdelta/NF-\textkappaB signaling pathway in neuronal survival. CaMKII downstream targets may promote excitotoxic cell death or neuronal survival.\textsuperscript{12} We have examined a number of potential downstream pathways, such as NF-\textkappaB, Akt, ERK and p38 signaling pathways, which are known to be involved in I/R-induced pathophysiology. Knockdown of \textit{C2dat1} led to the identification of the NF-\textkappaB signaling pathway as a primary target of CaMKII\textdelta in conferring neuroprotection in N2a cells. I/R-induced activation of the NF-\textkappaB signaling pathway was abolished by silencing \textit{C2dat1}, followed by downregulation of CaMKII\textdelta and inhibition of IKK/\beta phosphorylation and protein expression. The inhibition of the NF-\textkappaB signaling pathway resulted in increased OGD/R-induced cell death, further supporting the pro-survival effect of the NF-\textkappaB signaling pathway in N2a cells. The pro-survival effect of NF-\textkappaB has been demonstrated in MCAO-based ischemic model by other groups. For example, it has been shown that NF-\textkappaB participates in survival signaling following transient focal ischemia (MCAO paradigm) in p502/2 mice showed a clear neuroprotective role in the hippocampus and striatum, in which degenerating neurons were detected 4 days after 1 h of ischemia.\textsuperscript{36} Nonetheless, as a functionally divergent transcription factor, NF-\textkappaB has also been shown to contribute to neuronal cell death as part of the pro-inflammatory response, depending on the cerebral ischemia models and experimental approaches.\textsuperscript{36–38} Schneider et al.\textsuperscript{38} reported that NF-\textkappaB was activated after 2 h of MCAO followed by 20 h of reperfusion and contributed to I/R-induced neuronal damage. In the heart, CaMKII\textdelta has been implicated as a mediator of I/R-induced myocardial injury through the NF-\textkappaB pathway.\textsuperscript{39} It is also possible that the NF-\textkappaB pathway may exert alternative functions in primary neurons and mouse models of I/R as compared with undifferentiated N2a cells. In addition to the NF-\textkappaB pathway, other pro-survival signaling pathways may also be activated by CaMKII\textdelta, such as the phosphorylation and activation of nNOS, regulation of several ion channels, activation of ERK and CREB, and inhibition of GSK-3, Bad and HDAC5,\textsuperscript{13} which may have a role in mediating the neuroprotective function of \textit{C2dat1} during ischemia.

In summary, our study reveals a novel IncRNA \textit{C2dat1} that regulates CaMKII\textdelta expression in focal cerebral ischemia. \textit{C2dat1} promotes neuronal survival in response to I/R through upregulating CaMKII\textdelta expression. The identification of CaMKII\textdelta regulatory IncRNAs has shed light to the transcriptional control of CaMKII, one of the most important families of protein kinases for neurons, and may provide new opportunities to study or selectively target a specific CaMKII isoform (CaMKII\textdelta) in biological processes.

Materials and Methods
Reagents and antibodies. The iScript cDNA synthesis kit and Eva Green SMX 500R were obtained from Bio-Rad Laboratories (Richmond, CA, USA). Cell culture reagents and media were from American Type Culture Collection (ATCC, Rockville, MD, USA). Anti-CaMKII\textdelta antibody was purchased from Abcam (Cambridge, MA, USA) and the NF-\textkappaB Pathway Sampler Kit and Bcl-xL antibody were purchased from Cell Signaling (Danvers, MA, USA). GAPDH and a-tubulin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Goat anti-rabbit and goat anti-mouse HRP-conjugated secondary antibodies were from Promega (Madison, WI, USA).

Animals. Male C57BL/6J mice (8–10 weeks of age, 18–20 g weight) used for MCAO study were purchased from Guangdong Medical Laboratory Animal Center, Guangzhou, China. The use of animals under this protocol followed all guidelines set forth in the Guangdong Medical Laboratory Animal Center Institutional Animal Use Policy. Mice were housed in cages maintained in a regulated environment (12-h light/dark cycle), supplied with food and water without restriction. Mice used for preparation of primary cortical neuron culture and immunostaining were housed in a temperature-controlled room on a 12-h light/12-h dark cycle with standard mouse diet and water ad libitum at the University of Pittsburgh animal facility. The mice at ages 2–3 months were used for fluorescence immunostaining. All studies were in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals from the US Department of Health and Human Services and were approved by the University of Pittsburgh Medical Center Institutional Animal Care and Use Committee.

Mouse model of focal cerebral ischemia and reperfusion. Focal cerebral ischemia was induced by intraluminal MCAO using an intraluminal monofilament technique as described previously.\textsuperscript{26,27} Briefly, mice (18–20 g) were anesthetized with isoflurane (3% initially, 1% to 1.5% maintenance) in O\textsubscript{2} and N\textsubscript{2}O (1:3) and the rectal temperature was maintained at 37.0 ± 0.5 °C during surgery. After isolating the right common carotid artery (CCA), the right external carotid artery (ECA) and the right internal carotid artery (ICA), a 6-0 suture was tied at the origin of the ECA and at the distal end of the ECA. The left middle cerebral artery (MCA) was occluded by a silicone rubber coated monofilament insertion of a 6-0 nylon monofilament with a thin silicon coat (filament size 6-0, diameter 0.16 mm, length 25 mm; diameter with coating 0.20 ± 0.02 mm; coating length 5 mm), was pushed up the ICA to occlude the origin of the MCA. After 1-h occlusion, it was removed to allow reperfusion for 6, 12 or 24 h with the ECA tied permanently. Sham-operated mice underwent the similar operations to expose the carotid arteries without the suture insertion. After reperfusion, the mice were killed and the brains were removed quickly for biochemical analysis. Some brain tissues were snap frozen in isopentane for cryostat sectioning. Serial coronal sections (1 mm apart) were stained with 2, 3, 5-triphenyltetrazolium chloride monohydrate (TTC; Sigma-Aldrich, St. Louis, MO, USA) for assessing size of infarction. Tissues from the ischemic core and surrounding penumbra were isolated and snap frozen in liquid N\textsubscript{2} for western blotting.

Cell culture and OGD/R model. N2a cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Minimum Essential Medium Eagle (MEM) with Eagle’s salt and l-glutamine supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) at 37 °C with 5% CO\textsubscript{2}. The primary neuron culture was prepared as described previously.\textsuperscript{27} Cortical tissues were obtained from 14 to 16 day-old C57BL/6 mouse embryos. To mimic ischemic-like conditions in vitro, the growth medium was replaced with deoxygenated glucose-free Hank’s Balanced Salt Solution (Invitrogen). Cells were placed into a temperature-controlled (37 ± 1 °C) anaerobic chamber (Forma Scientific, Marietta, OH, USA) that contains a gas mixture composed of 5% CO\textsubscript{2} and 95% N\textsubscript{2} for 3 h (N2A)\textsuperscript{26} or 1 h (primary neuronal cells).\textsuperscript{27} After OGD exposure, the medium was replaced with glucose-containing growth medium containing 10% FBS and cultured under normoxic condition for different time points at 37 °C with 5% CO\textsubscript{2}. Control cell cultures were not deprived of oxygen and glucose, and always placed in normoxigenated glucose-containing DMEM.
Nuclear and cytoplasmic cell fractions. The nuclear and cytoplasmic fractions were prepared as described previously. Briefly, N2a cells were cultured in 150-mm dishes for 48 h. After OGD/R treatment, N2a cells were harvested and washed with PBS, and then resuspended in ice-cold hypotonic lysis buffer (HLB) (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 0.3% NP-40). To prepare cytoplasmic fraction, supernatant after the addition of sodium acetate and ethanol was precipitated at −20 °C overnight. To make nuclear/cytoplasmic fraction, pelleted nuclei were washed 3x with ice-cold HLB and resuspended in ice-cold modified 1× TBS for 30 min and then incubated with the following secondary antibodies (1:200) and DAPI (1:1000) and mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA; catalog #H1000) for imaging under confocal microscope.

**RNA FISH.** RNA FISH probe sets were designed by using software available through Stellaris Probe Designer (http://www.biosearchtech.com/stellarisdesigner/), which were labeled with the reporter dye Quasar 570 and synthesized by Biosearch Technologies (Petaluma, CA, USA). RNA FISH staining was performed according to standard Stellaris protocols (www.biosearchtech.com/stellarisprotocols) from the manufacturer. Briefly, OGD/R-treated N2a cells were fixed and hybridized with Stellaris RNA FISH probes. After hybridization, the N2a cells were counter-stained by DAPI and mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA; catalog #H1000) for imaging under confocal microscope.

**RNAi transfection.** N2a cells were seeded into six-well plates at a density of 3 × 10⁵ cells per well. The siRNAs at 50–75 nM final concentration were transfected into cells using DharmaFECT transfection reagent according to the manufacturer’s instructions for 48 h. The IncRNA C2dat1-targeted siRNA, CAMK2D-targeted siRNA and a control non-targeting siRNA (negative control) were obtained from GE Dharmacon (Lafayette, CO, USA). Two days after transfection, N2a cells were subjected to either control or OGD treatment, followed by reoxygencation under normoxic culture conditions for various times (0, 12 and 24 h).

**Preparation of samples and RNA extraction.** For N2a cells, total RNA was extracted using TRIzol LS Reagent according to the manufacturer’s instructions. For tissue RNA extraction, the ischemic penumbra and ischemic cortex were taken as experimental samples as described previously. The brain cerebral cortex was sectioned into three slices, section 1 is ischemic cortex; section 2 is ischemic penumbra and section 3 is the rest part of brain tissue. The section 1 and section 2 were used for RNA extraction, respectively. This was done to avoid mesial hemispheric structures, where the blood supply primarily by the anterior cerebral artery. RNA extraction was carried out with TRIzol LS Reagent according to the manufacturer’s instructions. RNA quality and quantity were measured by Synergy H1 Hybrid Reader (BioTek, Winooski, VT, USA). The value of OD260/280 is around 1.8 as a criterion of acceptable purity and RNA integrity was assessed using standard denaturing agarose gel electrophoresis. The total amount of RNA extracted did not differ significantly between the samples.

**Immunostaining and binary image analysis.** Fluorescence immunostaining on brain tissue sections was conducted as described previously. Briefly, mouse tissue sections were incubated with blocking solution containing either rabbit anti-CaMKII (1 : 100) or mouse anti-HIF-2α (1 : 100). Sections were washed with TBS for 30 min and then incubated with the following secondary antibodies (1 : 200): goat anti-rabbit Alexa Fluor 488-conjugated IgG for CaMKII (1 : 200) and goat anti-mouse Alexa Fluor 564 conjugated IgG for HIF-2α (1 : 100). The nuclei were stained with ToPro-3 (1 : 1000 in blocking solution) and mounted with Vectashield (Vector Laboratories). Brain sections stained with secondary antibody only were used as negative controls. The imaging method and the binary image analysis were described previously.

**Primers.** A list of real-time RT-qPCR primers used in this study and their respective sequences were described in the Supplementary Table S1.

**Real-time RT-qPCR.** One microgram of total RNAs was used to generate cDNA using the iScript cDNA synthesis kit. Real-time PCR was subsequently performed using the SsoFast EvaGreen Supermix on CFX96 Real-Time PCR Detection System (Bio-Rad, Richmond, CA, USA) to analyze the expression of C2dat1 and CAMK2D transcripts using the primers listed in Supplementary Table S1. Data were normalized using GAPDH as control.

**Western blot analysis.** Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 10% glycerol, 1% Triton X-100, 5 mM EGTA, 20 μM leupeptin, 1 mM AEBSF, 1 mM NaVO₃, 10 mM NaF and 1 x protein inhibitor cocktail). Western blot analysis was carried out as previously reported. Briefly, 20 μg of proteins were separated on SDS-PAGE and transferred onto nitrocellulose membranes. After blocking, the membranes were incubated with the appropriate primary antibody at 4 °C overnight. After washing, the membranes were incubated with secondary antibody at room temperature for 1 h. Proteins were detected with the enhanced chemiluminescence (ECL) kit. The images were captured on X-ray film and the band intensity was analyzed by Image J software (NIH, Bethesda, MD, USA).

**Cell survival assay.** Cell survival was assayed by Cell Counting Kit-8 (Dojin Laboratories, Kumamoto, Japan), according to the manufacturer’s instructions. N2a cells were plated at a density of 1 × 10⁵ cells per well in 24-well plates. After siRNA transfection and/or drug treatment, CCK-8 solution containing a highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfolphenyl)-2H-tetrazolium, monosodium salt] was added to cells in each well, followed by incubation for 1–4 h. Cell proliferation/viability was determined by measuring the OD at 450 nm. Percent over control was calculated as a measure of cell viability.

**Statistical analysis.** All statistical analysis was done using GraphPad Prism IV software (GraphPad Software, La Jolla, CA, USA). A P value < 0.05 was considered statistically significant (P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not significant).

**Conflict of Interest**

The authors declare no conflict of interest.

**Acknowledgements.** This work was supported in part by the National Institutes of Health grant RO1CA142580 (Wang), R21NS096946 (Wang), NIH NS308118 (Sun), Overseas Hong Kong and Macao Scholars Collaborative Research Fund of NSFC in China (grant# 81328020, Deng and Wang), and National Nature Science Foundation of China grant NSFC81370449 (Ji).
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