Casein kinase 1 regulates REST and protects neuronal cells from global ischemia

博士論文

Casein kinase 1 regulates REST and protects neuronal cells from global ischemia

（カゼインキナーゼ 1 による REST の調節および全脳虚血に対する神経保護に関する研究）

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I. Abstract

REST/NRSF (Repressor Element-1 (RE1) Silencing Transcription Factor/Neuron-Restrictive Silencer Factor) is a gene-silencing factor that is widely expressed during embryogenesis and plays a strategic role in neuronal differentiation. Recent studies indicate that REST can be activated in differentiated neurons during a critical window of time in postnatal development and in adult neurons in response to neuronal insults such as seizures and ischemia. However, the mechanism by which REST is activated in neurons is, as yet, unknown. Here I show that REST is controlled at the level of protein stability via β-TrCP-dependent, ubiquitin-based proteasomal degradation in differentiated neurons under physiological conditions and identify CK1 as an upstream effector that regulates REST cellular abundance. CK1 associates with and phosphorylates REST at two neighboring, but distinct motifs within the carboxy-terminus of REST critical for binding of β-TrCP and targeting of REST for proteasomal degradation. I further show that global ischemia in vivo triggers a decrease in CK1 and increase in REST in selectively-vulnerable hippocampal CA1 neurons. Administration of the CK1 activator pyrvinium pamoate by in vivo injection immediately after ischemia restores CK1 activity, suppresses REST expression and rescues neurons destined to die. My results identify a novel and previously unappreciated role for CK1 as a brake on REST stability and abundance and reveal that loss of CK1 is causally-related to ischemia-induced neuronal death. These findings point to CK1 as a potential therapeutic target for the amelioration of hippocampal injury and cognitive deficits associated with ischemic stroke.

II. Background

The transcriptional repressor REST is a gene silencing transcription factor that represses an array of coding and noncoding neuron-specific genes including synaptic vesicle proteins, neuroreceptors and channels, and microRNAs[1-4]. REST is widely expressed during embryogenesis but declines in expression during neuronal differentiation. REST downregulation is critical to acquisition of the neural phenotype. In mature neurons REST is quiescent, but can be activated in hippocampal neurons during normal postnatal development, driving the switch from immature to mature NMDA receptors[5] and in selectively-vulnerable hippocampal neurons by ischemic insults[6-8].

In cancer cells and pluripotent stem cells, REST is bi-directionally regulated by β-TrCP-dependent, ubiquitin-based proteasomal degradation[9-11] and HAUSP-dependent deubiquitination[12]. In the transition from neural progenitors to neurons, REST is downregulated by transcriptional repression and protein degradation[1]. REST harbors two neighboring, but distinct, noncanonical degron motifs, in its carboxy-terminus[13]. Phosphorylation of serine residues within these motifs enables binding by the E3 ubiquitin ligase β-TrCP, priming REST for ubiquitin-based proteosomal degradation. Whereas the mechanism by which REST expression is regulated in stem cells and cancer cells is established, the upstream kinase that phosphorylates REST is, as yet, unknown.

CK1 is a serine/threonine kinase that is evolutionary conserved and plays a pivotal role in membrane transport, cell division, DNA repair, and activation of transcription

factors such as β-catenin and p53[14, 15]. CK1 has seven isoforms, each encoded by a unique gene. CK1δ and ε are abundantly expressed in hippocampus, where they regulate NMDAR function[16] and are activated by group I mGluRs[17]. A prevailing view is that CK1 family members are constitutively active.

The present study was undertaken to identify the responsible kinase for degradation of REST and characterize the mechanism by which REST is activated in adult neurons in response to neuronal insults. Here I identify CK1 as the upstream kinase that associates with and phosphorylates serine residues at carboxy-terminus in REST and targets REST for β-TrCP-dependent, ubiquitin-based proteasomal degradation. Global ischemia in vivo triggers a decrease in CK1, thereby increasing REST in CA1 neurons. A single, acute injection of the CK1 activator pyrvinium after the ischemic episode affords robust neuroprotection in a clinically-relevant model of stroke. My results point to CK1 as a brake on REST expression and reveal that loss of CK1 in response to ischemic insults is causally-related to neuronal death.

III. Aim of Study

1. To identify the kinase which phosphorylates REST and induces degradation of REST.

2. To investigate the impact of the kinase which is identified in Aim 1 on neuronal cell death in CA1 after global ischemia.

IV. Methods

1. Cell culture, drug treatment and transfection.

HeLa and N2A cells were plated on culture dishes or plates maintained in DMEM supplemented with 10% FBS in an incubator with an atmosphere of 10% CO_2 at 37 ºC. Cells were grown to 70-80% confluency for drug treatment or transfection. To monitor the impact of CK1 and other kinases on REST, cells were treated with the CK1 activator pyrvinium pamoate (Sigma), the CK1 inhibitors D4476 (Tocris), the PKA inhibitor H89 (Sigma) or the PI3 kinase inhibitor LY294002 (Sigma) at indicated concentrations for 4 h. Pyrvinium pamoate is reported to be a highly selective activator of CK1, ~tenfold more potent as an activator of purified CK1 vs. its action as an inhibitor of Wnt signaling at sub-nanomolar concentrations[18], but see [19]. To monitor the impact of CK1 on the rate of protein degradation, HeLa cells were treated with pyrvinium (10 μM) or D4476 (10 μM) for 2 h prior to application of the protein synthesis inhibitor cycloheximide (100 μg/ml, Calbiochem). At indicated times, whole cell lysates were prepared and subjected to Western blot analysis. Immunoblots were probed with antibodies to REST or β-actin antibody. Cells were transfected with various constructs using Lipofectamine 2000 (Invitrogen).

2. Primary cultures of hippocampal neurons and oxygen-glucose deprivation (OGD).

Primary cultures of hippocampal neurons were prepared from embryonic (E18) rats as described[8]. Cultures were maintained 14 days in vitro (DIV) prior to experiments. To monitor the impact of CK1 on REST, neurons were treated with pyrvinium (10 μM), D4476 (10 μM), or clasto-lactacystin β-lactone (lactacystin, 2.5 μM, Calbiochem) for 24 h. To induce OGD, primary cultures of hippocampal neurons were exposed to serum-free, glucose-free medium saturated with 95% N_2/5% CO_2

(30 min at 37°C) in an airtight, anoxic chamber (Billups-Rothenberg). Cultures were returned to oxygenated, glucose-containing medium under normoxic conditions for 24 h and were used for western blotting analysis.

3. Plasmids.

Myc-CK1 and shCK1 constructs were kind gifts from Dr. Hiroyuki Inuzuka. HA-REST construct was obtained Dr. Michele Pagano. HA-REST mutants were generated using the PfuTurboDNA Polymerase (Stratagene) according to the manufacturer's instructions.

4. shRNAs and lentiviral vectors.

CK1 ε shRNA, β-TrCP1/2 and nontargeting (NT) shRNA, a silencer-resistant sequence that does not target any known eukaryotic gene sequence, were engineered in the pcDNA^{TM} 6.2-GW/EmGFP shRNA expression vector (Invitrogen): CK1 ε shRNA (5'- TAC ATG AGC ACA TAG CCC AGG -3'), β-TrCP1 (5'- AAC TCT TCC AGA ATG CTC CAC-3') or (5'- TAT GAA ATC CCT CTG CAG CAT -3'), β-TrCP2 (5"- TCA TAC TGG AGG CAG AGG ACA -3') and NT shRNA (5'-AAA TGT ACT GCG CGT GGA GAC-3'). For RNAi-mediated silencing of CK1 ε or β-TrCP in cultured hippocampal neurons, shRNA was cloned from the pcDNA^{TM} 6.2-GW/EmGFP expression vector into a self-inactivating lentiviral vector pRRLsin.cPPT.CMV.eGFP.Wpre[8, 20]. High-titer vesicular stomatitis virus-pseudo-typed lentiviral stocks were produced in HEK-293T cells as described [8, 20]. In brief, cells were transfected with pRRL.PPT.hCMV.GFP.shRNA.Wpre transfer construct, pMDLg/pRRE and pRSV-REV packaging constructs, and the envelope protein construct pMD2.G by means of calcium phosphate. Titers were determined by

infecting HEK-293T cells with serial dilutions of concentrated lentivirus. eGFP fluorescence was evaluated by flowcytometry at 48 h after transduction; Titers were 1 × 109 transducing units. The efficacy of CK1 ε and β-TrCP shRNA was evaluated by Western blot 7 d after transduction of primary hippocampal neurons prepared from the brains of E18 Sprague Dawley rats.

5. Western blots and immunoprecipitation.

Western blot analysis was performed as described[5, 21]. In brief, cells were washed with cold PBS and lysed in buffer containing (in mM): 25 Tris-HCl, pH 7.4, 150 NaCl, 1 EDTA, 1X protease inhibitor cocktail (Sigma), 0.1% SDS and 0.5% Na deoxycholate. For the tissue samples, hippocampi were rapidly dissected and transverse slices of dorsal hippocampus (1 mm) were cut with a McIlwain tissue chopper. The CA1 subfield was rapidly microdissected and lysed in buffer supplemented with a 1% cocktail of protease inhibitors (Sigma). Protein concentrations were measured by means of the bicinchoninic acid (BCA) assay (Pierce). Equal aliquots of protein (50 μg) were subjected to SDS-PAGE (4-20%), transferred to a nitrocellulose membrane and processed for incubation with antibody (anti-REST, 1:1000, Millipore; anti-CK1 δ, 1:500, Santa Cruz, anti-CK1 ε, 1:1000, BD Bioscience; anti-β-TrCP, 1:1000, Cell signaling; anti-ubiquitin, 1:1000, Cell signaling; anti-myc, 1:1000, Cell signaling; anti-Flag, 1:2000, Millipore; anti-HA, 1:1000, Covance; anti-β-actin, 1:50000, Sigma). Membranes were washed and reacted with enhanced chemiluminescence reagent (ECL; Amersham Biosciences). For immunoprecipitation, 500-1000 μg lysates were incubated with the appropriate antibody (1-10 μg) overnight at 4 ° C followed by one hour-incubation with Protein G sepharose beads (Millipore). Immuno-complexes were washed five times

with IP buffer containing (in mM): 25 Tris-HCl, pH 7.4, 150 NaCl, 1 EDTA, 1 EGTA and 0.5% Triton X-100) before being resolved by SDS-PAGE and immunoblotted with indicated antibodies. Band densities were normalized to β-actin. Mean band densities for samples from experimental animals were normalized to the corresponding samples from sham animals.

6. qRT–PCR.

Cells were washed twice with cold PBS and RNA was extracted using TRIzol (Invitrogen). RNA concentration was measured by means of a Nanodrop (NanoDrop Technologies). Aliquots of RNA (1 μg) were reverse-transcribed to cDNA with Superscript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was performed with TaqMan probes (Applied Biosystems) for REST (Ref: Hs00958503_m1); GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Ref: Hs02758991_g1) served as an endogenous reference. Reactions were run in triplicate in a StepOnePlus real-time PCR system (Applied Biosystems). mRNA abundance was calculated by means of the comparative Ct method at a threshold of 0.02. Relative expression ratios were calculated by normalization of values for experimental samples to those of the corresponding sham controls by means of the relative expression software tool (REST©) on the basis of the group mean for the target transcript versus reference GAPDH transcript[22)].

7. In vitro phosphorylation assay.

To assess phosphorylation of REST, N2A cells were transfected with HA-conjugated WT and mutant REST using Lipofectamine 2000. Forty-eight hours post-transfection, cells were rinsed with ice-cold PBS and pelleted by centrifugation and lysed in lysis buffer. Lysates (750 μg) were immunoprecipitated

with anti-HA agarose conjugate (Sigma). Immunoprecipitated HA-REST was incubated with recombinant active CK1 δ (New England BioLabs) in a reaction mix containing 1X assay buffer (50 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM EGTA, 0.4 mM DTT, and 1X Protease Inhibitor Cocktail (Sigma)), 60 μM Mg-ATP, 0.3 μCi γ -^{32}P-ATP (3000 Ci/mmol) (Perkin Elmer) and 1 unit/μl CK1 δ at 30˚C for 30 min. The reaction was stopped by the addition of ice-cold radioimmune assay buffer containing (in mM): 10 Tris-HCl, pH 7.4, 75 NaCl, 20 EDTA, 10 EGTA, 20 Na -pyrophosphate, 50 NaF, 20 glycerol-2-phosphate, and 1 p-nitrophenylphosphate, and stripped with loading buffer (Invitrogen). Samples were run on 3-8% Tris-acetate gels (Invitrogen) for subsequent autoradiography and Western blotting.

8. Animals.

Age-matched adult male Sprague-Dawley rats weighing 150-200 gram (Charles River Laboratories, Inc.) were maintained in a temperature- and light-controlled environment with a 12:12 h light/dark cycle and were treated in accordance with the principles and procedures of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Protocols were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

9. Global ischemia.

Animals were subjected to transient global ischemia by the four-vessel occlusion paradigm as described[8, 21)]. For sham surgery, animals were subjected to the same anesthesia and surgical conditions, except that the carotid arteries were not occluded. Body temperature was maintained at 37.5 ± 0.5 °C by external warming until recovery from anesthesia. Animals that failed to show complete loss of the righting reflex and dilation of the pupils 2 min after occlusion was initiated and the

rare animals that exhibited obvious behavioral manifestations (abnormal vocalization when handled, generalized convulsions, loss of > 20% body weight by 3-7 d or hypoactivity) were excluded from the study.

10. Drug injections in vivo.

Lactacystin (1 μg/μl, dissolved in DMSO) or DMSO was administered to rats by stereotaxic injection directly into the right hippocampal CA1 (3.0 mm posterior and 2.0 mm lateral to bregma, 4.0 mm below the skull surface) using a 28-gauge needle and a Hamilton syringe at a flow rate of 0.2 μl/min (total 4 μl). The needle was left in place for an additional 5 min and then gently withdrawn. The CA1 subfield was rapidly microdissected at 24 h after injection and used for Western blot analysis (see below). Pyrvinium (1 mM, dissolved in DMSO) or DMSO was administered to animals immediately after ischemia or sham surgery by intracerebroventricular (icv) injection. For icv injection, a 28 gauge needle attached to a Hamilton syringe was stereotaxically lowered into the right lateral ventricle at a flow rate of 1 μl/min (total 5 μl) to a position defined by the following coordinates relative to bregma: 0.92 mm posterior, 1.2 mm lateral, 3.6 mm below the skull surface.

11. Histology and Fluoro-Jade labeling.

Neuronal cell loss was assessed by histological examination of brain sections at the level of dorsal hippocampus from animals killed at 7 days after sham surgery or global ischemia. Animals were anesthetized with halothane and transcardially perfused using 0.9% saline with heparin followed by ice-cold 4% paraformaldehyde in PBS (0.1 M, pH 7.4). Brains were removed and immersed in fixative. Coronal sections (20 μm) were cut at the level of the dorsal hippocampus with a cryotome and processed for staining with toluidine blue or Fluoro-Jade C (Millipore). The

number of dying pyramidal neurons per 250-mm length of the medial CA1 region from 4 ~ 6 rats per group (8 sections per rat) was counted under a fluorescence microscope at x 40 magnification.

12. Statistical analysis.

For qRT-PCR, significance was assessed by means of the randomization test (Pfaffl et al., 2002) and a Student's t-test (unpaired, two-tailed). For Western band densities and radioactive band densities, significance was assessed by means of the Student's t-test (unpaired, two-tailed). $P < 0.05$ was considered to be statistically significant.

V. Results

1. CK1 as a responsible kinase of REST for β-TrCP-dependent, ubiquitin-based proteasomal degradation.

(1) CK1 regulates REST protein stability.

I first sought to identify the upstream kinase for phosphorylation of residues critical to recognition of REST by β-TrCP, a member of the F-box family of E3 ligases, which initiates ubiquitination of many transcription factors and other target proteins. Toward this end, I used the protein kinase prediction algorithm Motif Scan set at low stringency[23]. Among the protein kinases delineated by Motif Scan, Casein Kinase 1 (CK1) was predicted to phosphorylate REST at S1027, a residue within the motif which was shown to be recognized by β-TrCP. CK1 phosphorylates sites within or near the degron motifs of several other proteins, priming them for ubiquitin-based proteasomal degradation in a β-TrCP-dependent manner, including the transcription factor β-catenin[24], CDC25A, a phosphatase involved in cell division[25] and UHRF1 (ubiquitin-like, with PHD and RING finger domains), an epigenetic protein involved in maintenance of DNA methylation during DNA replication[26].

To examine a possible role for CK1 in regulation of REST protein abundance, I used a combination of pharmacological and genetic approaches. HeLa cells express REST at high levels and are readily amenable to genetic manipulation. Application of the CK1 activator pyrvinium to HeLa cells decreased REST protein abundance in a dose-dependent manner (Fig. 1a). CK1 inhibitors D4476 (Fig. 1b) and IC261 (data not illustrated) each increased REST expression. As expected, D4476 blocked the pyrvinium-induced increase in REST (Fig. 1c). These findings indicate that CK1

activity negatively regulates REST expression. To examine whether CK1 abundance is causally related to REST protein abundance, I genetically manipulated CK1 and assessed cellular levels of REST protein. Overexpression of Myc-tagged CK1 isoforms $\alpha 1$, $\alpha 2$, δ, and ε (but not $\gamma 1$, $\gamma 2$, or $\gamma 3$) decreased REST protein abundance, as assessed by Western blot analysis (Fig. 1d). Consistent with this, expression of shRNA constructs targeting CK1 δ (~60% knockdown), CK1 ε (~40% knockdown) or a chained construct containing both shRNAs and thereby targeting CK1 δ and ε (50% knockdown of CK1 δ; 60% knockdown of ε), but not NT shRNA, increased REST protein abundance (Fig. 1e). Collectively, these findings indicate that CK1 regulates REST protein expression in HeLa cells, but do not address whether CK1 regulates REST in neurons. To address this issue, I next examined the impact of CK1 on REST protein abundance in hippocampal neurons in vitro. Whereas activation of CK1 by pyrvinium decreased REST expression in neurons, inhibition or shRNA-mediated knockdown of CK1 increased REST (Fig. 1f). To evaluate a possible role for other kinases in regulation of REST protein levels, I examined the effect of inhibitors of other kinases on REST protein levels. Neither H89, an inhibitor of protein kinase A (Fig. 2a) nor LY294002, an inhibitor of phosphoinositide 3-kinase (Fig. 2b), significantly altered REST protein levels, indicating specificity of CK1 in regulation of REST expression.

The results thus far document a role for CK1 in REST protein expression, but do not address the mechanism by which CK1 may exert its effect. A reduction in protein could arise as a consequence of reduced protein synthesis and/or enhanced protein degradation. To examine whether CK1 regulates REST at the level of transcription, I examined the impact of the CK1 activator pyrvinium and the inhibitor D4476 on REST mRNA by quantitative RT-PCR (qRT-PCR). Neither pyrvinium nor D4476

detectably altered REST mRNA expression (Fig 2c). Moreover, neither overexpression of myc-tagged CK1 α1, α2, δ, ε, γ1, γ2, or γ3 (Fig. 2d), nor shRNA-mediated knockdown of CK1 δ, ε or δ and ε (Fig. 2e), detectably altered REST mRNA, consistent with regulation at the post-transcriptional level. I next examined whether CK1 regulates REST at the level of protein stability. Toward this end, I treated cells with the protein synthesis inhibitor cycloheximide (100 μg/ml) in the absence or presence of the CK1 activators pyrvinium or CK1 inhibitor D4476 and measured REST protein abundance over time. This protocol affords a measure of constitutive protein degradation. In the presence of cycloheximide, levels of REST protein declined. Whereas pyrvinium increased (Fig. 3a), D4476 attenuated (Fig. 3b) the rate of REST protein degradation. Collectively, these data indicate that CK1 negatively regulates REST abundance at the level of protein stability.

(2) CK1 associates with and phosphorylates REST, thereby promoting β-TrCP -mediated ubiquitination.

β-TrCP recognizes and binds phosphorylated serine residues in canonical and non-canonical degron motifs within target proteins, directing them to ubiquitin-based proteasomal degradation[26, 27]. Humans and rats both express two β-TrCP genes, β-TrCP1 and β-TrCP2. Whereas both proteins are functional in humans, rats express a short, truncated β-TrCP2, which lacks both the D-domain and the F-box like domain present in β-TrCP1 and is therefore nonfunctional. I therefore focused on β-TrCP1. REST contains two neighboring, but distinct, degron motifs which serve as β-TrCP binding sites, one containing E1009 and S1013[9] and the other containing S1024, S1027 and S1030[11] (Fig. 4a). Whereas it is known that phosphorylation of these serine residues is critical for recognition by β-TrCP, the

kinase that phosphorylates these residues in REST is, as yet, unknown. To address this issue, I first examined whether CK1 physically associates with REST. CK1 ε physically associated with endogenous REST, as assessed by co-immunoprecipitation (Fig. 4b).

To identify residues phosphorylated by CK1, I used site-directed mutagenesis to mutate serine residues in and around the two degron motifs singly or in combination to the non-phosphorylatable residue alanine. WT and mutant HA-REST constructs were expressed in N2A cells and immunoprecipitated with an antibody directed to HA. N2A cells are a neuron-like cell that express very low levels of full-length REST and are therefore suitable for evaluation of mutant REST constructs. Immunocomplexes were subjected to an in vitro phosphorylation assay in the presence and absence of recombinant CK1 δ. CK1 δ induced striking phosphorylation of WT REST (Fig. 5a-c). Several single point mutations (S1013A, S1024A and S1030A) reduced CK1-dependent phosphorylation of REST (Fig. 5a), consistent with the notion that loss of a single serine residue is sufficient to reduce REST phosphorylation. Importantly, the double REST mutant E1009A/S1013A and triple REST mutant S1024A/S1027A/S1030A shown by others to abolish β-TRCP binding[9, 11], also abolished CK1-dependent phosphorylation of REST (Fig. 5b). Moreover, two additional constructs, the double REST mutant, S1013A/S1030A, and the quadruple mutant, S1013/S1024A/S1027A/S1030A, each markedly reduced CK1-dependent phosphorylation (Fig. 5c). Importantly, CK1 increased association of β-TrCP with REST (Fig. 6a, upper) and REST ubiquitination, as assessed by immunoprecipitation (Fig. 6a, lower). Moreover, expression of two shRNA constructs targeting β-TRCP1/2 (β-TRCP1/2 shRNA-1, ~60% knockdown; β-TRCP1/2

shRNA-2, ~30% knockdown), but not NT shRNA, markedly upregulated REST in hippocampal neurons (Fig. 6b), demonstrating an inverse causal relation between β-TRCP and REST. Collectively, these data demonstrate that CK1 phosphorylates serine residues contained within the two degron motifs of REST, increases association of β-TrCP with REST and promotes ubiquitination of REST.

(3) REST is targeted to the proteasomal degradation pathway in neurons.

The results thus far demonstrate that CK1 is necessary and sufficient for phosphorylation of serine residues in the degron motifs within the REST carboxy-terminal domain, association of β-TrCP with REST and REST ubiquitination, but do not examine a role for proteasomal degradation of REST. Whereas β-TRCP-dependent proteasomal degradation in regulation of REST in cancer cells and pluripotent and neural stem cells is well-established[26, 27], its role in regulation of REST in neurons is as yet unclear. To address this issue, I first examined the impact of the proteasomal inhibitor lactacystin on REST abundance in the hippocampal CA1 under physiological conditions. Lactacystin injected directly into the CA1 of control animals increased REST abundance and enhanced global protein ubiquitination in the CA1, consistent with a role for proteasomal degradation in maintenance of REST at low, constitutive levels in adult neurons under physiological conditions (Fig. 7a).

I next examined a role for proteasomal degradation in regulation of REST abundance in insulted neurons. Neuronal insults such as global ischemia increase REST protein expression and induce silencing of target genes such as GluA2. This, in turn, leads to an increase in GluA2-lacking, Ca^{2+}-permeable AMPARs in selectively vulnerable hippocampal neurons, events causally related to neuronal

death[6, 8, 28]. This is significant in that GluA2-lacking AMPARs are implicated in ischemia-induced neuronal death. Toward this end, I induced oxygen-glucose deprivation (OGD), an in vitro model of global ischemia, in hippocampal neurons in vitro. Under control conditions, application of the proteasomal inhibitor lactacystin did not detectably alter β-TRCP, but increased REST and decreased the REST target gene GluA2. OGD induced upregulation of REST and downregulation of GluA2. In neurons subjected to OGD, application of lactacystin did not alter β-TRCP or further alter REST or GluA2 (Fig. 7b). These findings demonstrate that REST is upregulated by ischemic insults and degraded by proteasomal pathway in hippocampal neurons.

2. CK1 as a therapeutic target after global ischemia.

(1) Global ischemia decreases CK1 and promotes upregulation of REST in the CA1.

To examine a role for CK1 and ubiquitin-based proteasomal degradation in upregulation of REST in the hippocampal CA1 in a clinically-relevant model of ischemic stroke, I subjected animals to global ischemia in vivo. Global ischemia induced by four-vessel occlusion (10 min) in rats causes selective, delayed neuronal death and delayed cognitive deficits (reviewed in [21]). Pyramidal neurons in the hippocampal CA1 are particularly vulnerable. Histological evidence of degeneration is not observed until 2-3 days after ischemia, affording a substantial window of time for molecular studies. I evaluated CK1δ, CK1ε, β-TrCP, Cul1 and HAUSP expression in the CA1 of animals subjected to global ischemia or sham operation. Cul1 is a component of the SCF (SKP1-CUL1-F-box protein) ubiquitin ligase complex[27]. HAUSP is a deubiquitylase known to stabilize REST[12]. Under basal conditions, CK1δ, CK1ε, β-TrCP, Cul1 and HAUSP were robustly expressed in the CA1 of adult rats. By contrast, REST was detectable, but at very low levels.

Global ischemia mildly decreased β-TrCP and markedly reduced CK1 δ and CK1 ε protein abundance, with little or no change in Cul1 or HAUSP. By contrast, ischemia markedly increased REST protein abundance in the CA1 (Fig. 8a,b).

(2) CK1 activation protects against ischemia-induced neuronal death

To examine whether loss of CK1 is causally related to ischemia-induced upregulation of REST and neuronal death, I examined the ability of CK1 activation to repress REST and rescue CA1 neurons from ischemia-induced neuronal death. Toward this end, I injected the CK1 activator pyrvinium icv immediately after induction of global ischemia in vivo. Whereas rats subjected to global ischemia exhibited robust neuronal death in the CA1 7 d after insult, as assessed by histology and Fluoro-Jade staining (Fig. 9a,b), neuronal death was significantly reduced in rats injected with pyrvinium immediately following ischemia. To investigate a possible mechanism for the CK1 activator-induced neuroprotection, I analyzed the impact of pyrvinium on REST and GluA2 protein levels in the CA1 of rats subjected to global ischemia. Pyrvinium rescued the ischemia-induced increase in REST and decrease in GluA2 (Fig. 10a,b), consistent with a model whereby CK1 promotes targeting of REST for proteasomal degradation, and unsilencing of GluA2. These findings demonstrate an inverse causal relation between CK1 and neuronal death of CA1 neurons and implicate CK1 as a putative target for therapeutic intervention in ischemic stroke.

VI. Discussion

In this study I identify CK1 as an upstream kinase that regulates REST cellular abundance and show that REST is regulated at the level of protein stability via β-TrCP-dependent, ubiquitin-based proteasomal degradation in differentiated neurons under physiological conditions. CK1 associates with and phosphorylates REST at two neighboring, but distinct motifs within the carboxy-terminus of REST essential to binding by β-TrCP (the degron motifs), thereby priming REST for ubiquitin-based proteasomal degradation. Activation or overexpression of CK1 promotes REST ubiquitination and degradation in a β-TrCP-dependent manner. Inhibition or RNAi-mediated knockdown of CK1 promotes REST stability and increases REST cellular levels. These findings establish an inverse causal relation between CK1 and REST abundance. I further show that ischemia triggers a decrease in CK1 and an increase in REST in selectively vulnerable CA1 pyramidal neurons. This is significant in that activation of REST is causally linked to ischemia- and seizure-induced neuronal death. A single, acute injection of the CK1 activator pyrvinium to rats immediately after an ischemic episode restores decreases REST and rescues CA1 neurons destined to die. Collectively, these results identify a novel and previously unappreciated role for CK1 as a brake on REST stability and abundance and reveal that loss of CK1 is causally related to neuronal death in a clinically-relevant model of ischemic stroke. This is the first demonstration that CK1 directly phosphorylates REST in any cell type and that REST is regulated by proteasomal degradation in mature neurons.

Findings in the present study are consistent with a model whereby REST is normally

quiescent, but can be re-activated in differentiated neurons during postnatal development in an experience-dependent manner[5] and in response to neuronal insults such as ischemia[6, 8] and seizures [29, 30]. Under physiological conditions, CK1 (the upstream effector) maintains REST at low, constitutive levels in differentiated neurons. CK1 associates with and phosphorylates REST at specific serine residues within the two degron motifs, enabling recognition by the E3 ligase β-TrCP (Fig. 11). Upon binding, β-TrCP poly-ubiquitinates the substrate, priming it for ubiquitin-based proteasomal degradation. Ischemic insults blunt CK1 and β-TrCP, inducing upregulation of REST in selectively-vulnerable hippocampal neurons. Upon activation, REST binds to the RE1 element within the promoter of target genes important to synaptic function such as GluA2 and orchestrates the assembly of the corepressor complex (mSin3A and CoREST, HDACs 1 and 2 and G9a). The corepressor complex promotes epigenetic remodeling of core histone proteins at the promoter of target genes and represses transcription of genes important to synaptic plasticity and neuronal survival.

The present study sheds light on the mechanism by which CK1 regulates REST cellular levels. CK1 associates with REST and phosphorylates serine residues in the two degron motifs, enabling binding of β-TrCP and activation of the ubiquitin-based proteasomal degradation pathway. Importantly, both the double mutant E1009A/S1013A and the triple mutant S1024A/S1027A/S1030A shown by others to abolish β-TRCP binding[9, 11], also abolishes CK1-dependent phosphorylation of REST, consistent with a cooperative/synergistic interaction between the two motifs. Interestingly, loss of several individual serine residues within or in close proximity to the motifs is sufficient to reduce CK1-dependent phosphorylation of REST. My

findings do not, however, rule out the possibility that not only CK1, but also other protein kinases, phosphorylate serine residues within the degron motifs, or other serine residues in REST and thereby regulate cellular abundance of REST. Moreover, I cannot rule out the possibility that other signaling pathways regulated by CK1, as for example the Wnt-β-catenin pathway, may contribute to the neuroprotective effects of pyrvinium. Further study will be necessary for detecting other kinases which regulates REST protein abundance and other pathways involved in neuroprotection caused by pyrvinium pamoate.

Findings in the present study have broad implications for amelioration of the neurodegeneration and cognitive deficits associated with ischemic stroke. Importantly, dysregulation of REST and its target genes are implicated not only in ischemic stroke, but also in the pathogenesis of medulloblastomas, epilepsy, Huntington's disease and SMCX, a form of X-linked mental retardation[2, 3, 29]. Thus, my findings implicate CK1 as a therapeutic target not only for ischemic stroke but other brain disorders that involve dysregulation of REST in neurons.

VII. Conclusion

The present study demonstrates CK1 directly phosphorylates REST, causing REST degradation, and CK1 would be a new therapeutic target in global ischemia.

VIII. References

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IX. Figures

Figure 1.

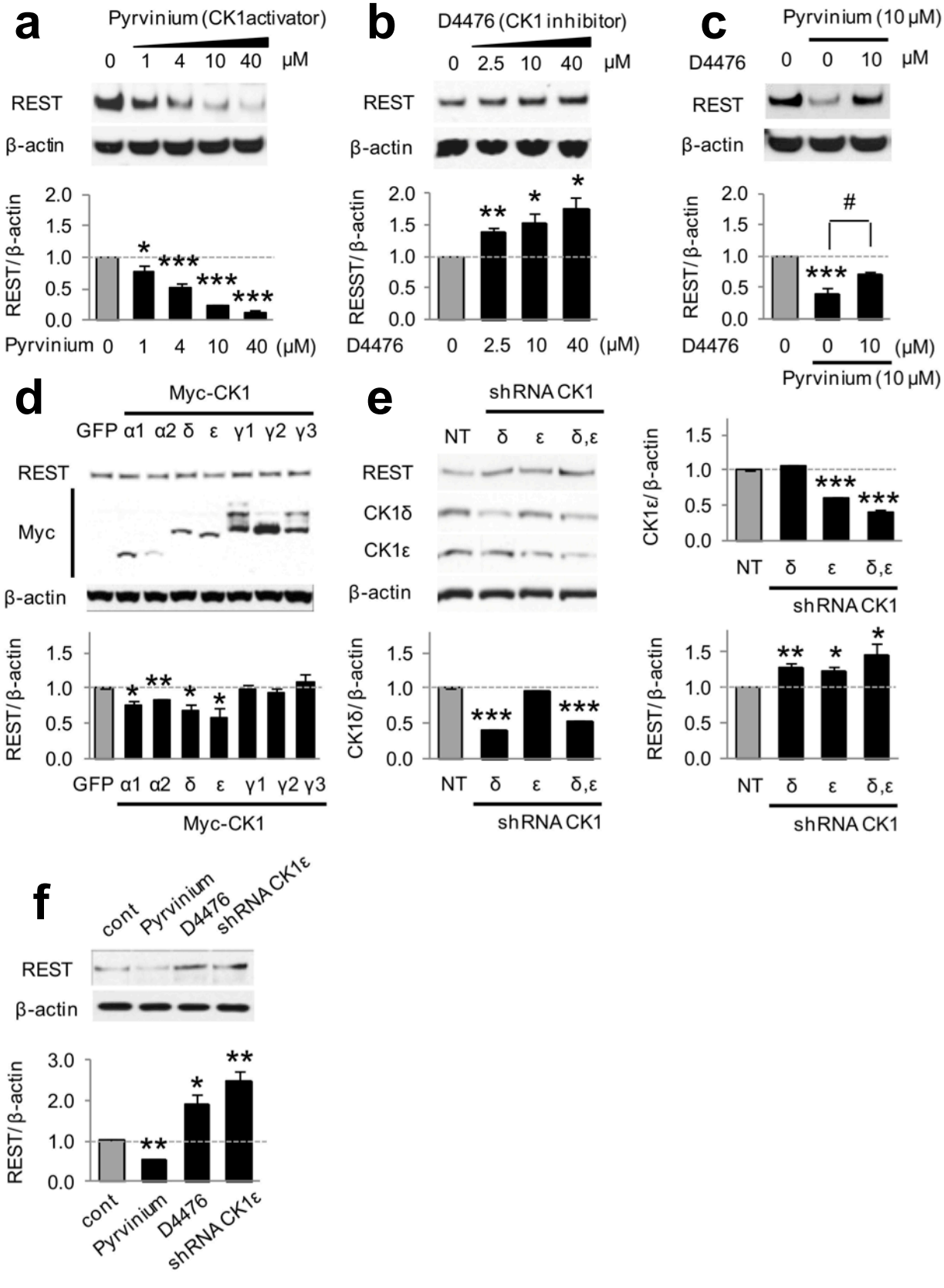


Figure 2.

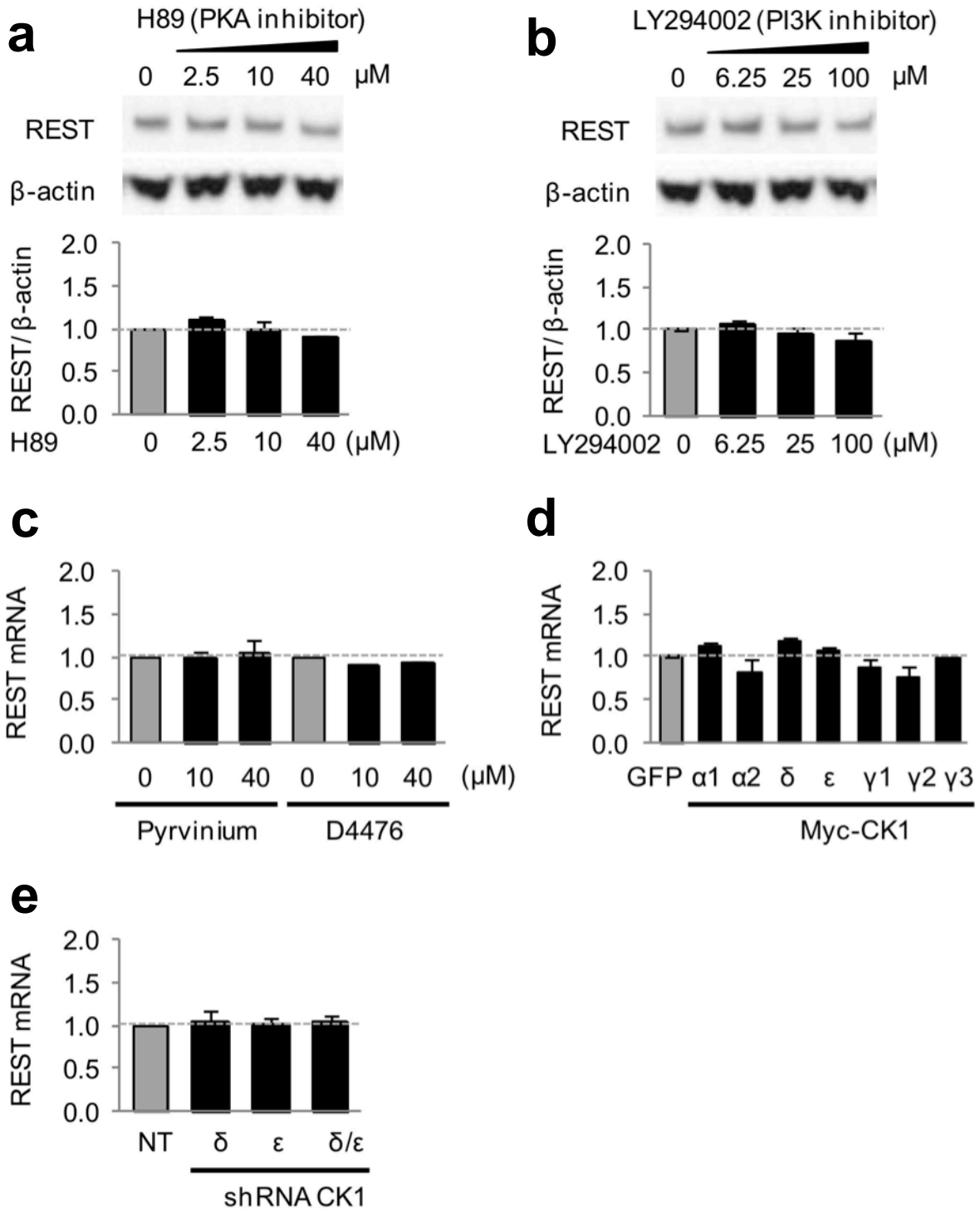

a
H89 (PKA inhibitor)
0 2.5 10 40 μM
REST
β-actin
REST/β-actin
2.0
1.5
1.0
0.5
0.0
H89 0 2.5 10 40 (μM)
b
LY294002 (PI3K inhibitor)
0 6.25 25 100 μM
REST
β-actin
REST/β-actin
2.0
1.5
1.0
0.5
0.0
LY294002 0 6.25 25 100 (μM)
c
REST mRNA
2.0
1.5
1.0
0.5
0.0
0 10 40 0 10 40 (μM)
Pyrvinium
D4476
d
REST mRNA
2.0
1.5
1.0
0.5
0.0
GFP α1 α2 δ ε γ1 γ2 γ3
Myc-CK1
e
REST mRNA
2.0
1.5
1.0
0.5
0.0
NT δ ε δ/ε
shRNA CK1

Figure 3.

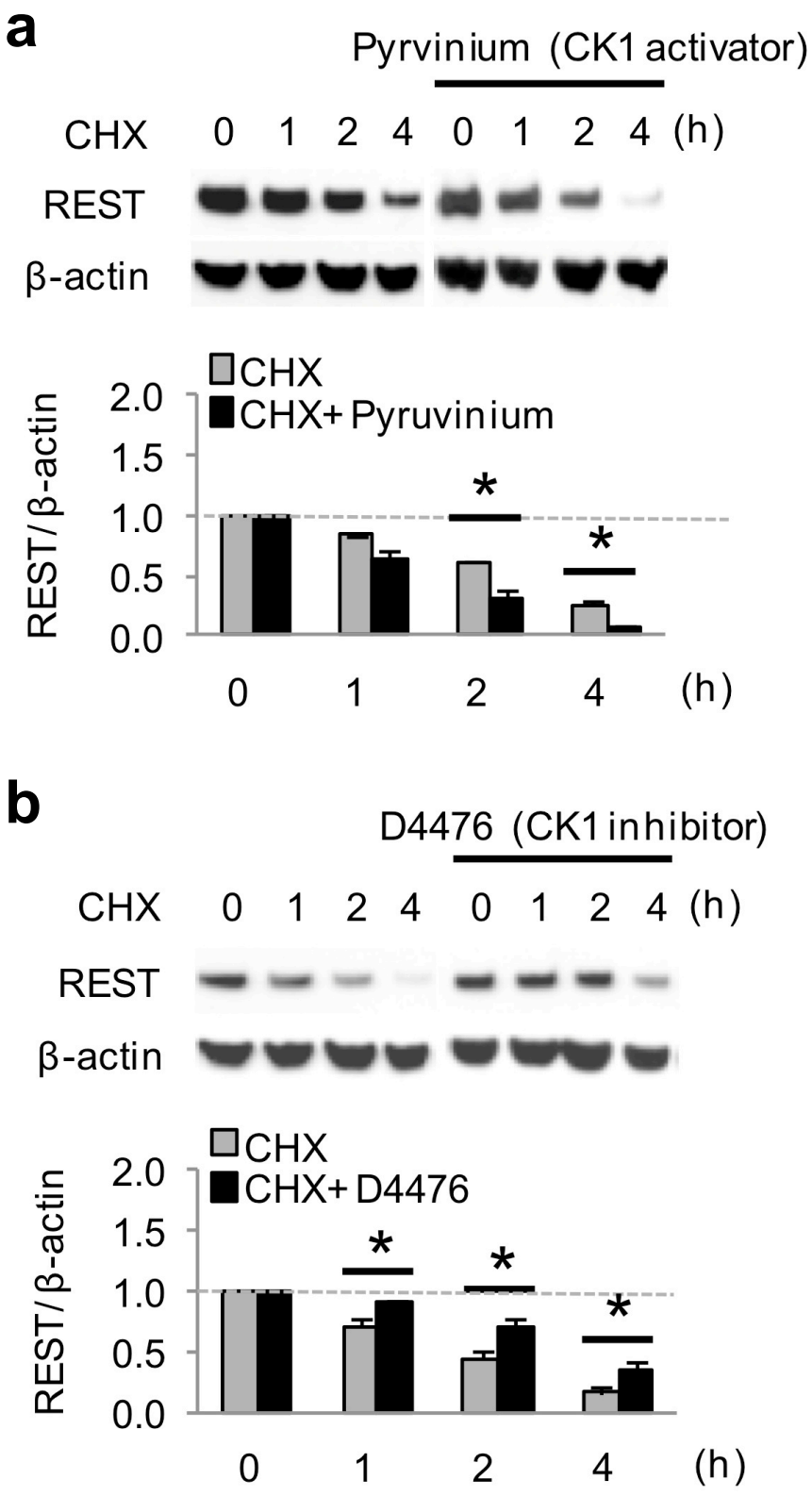
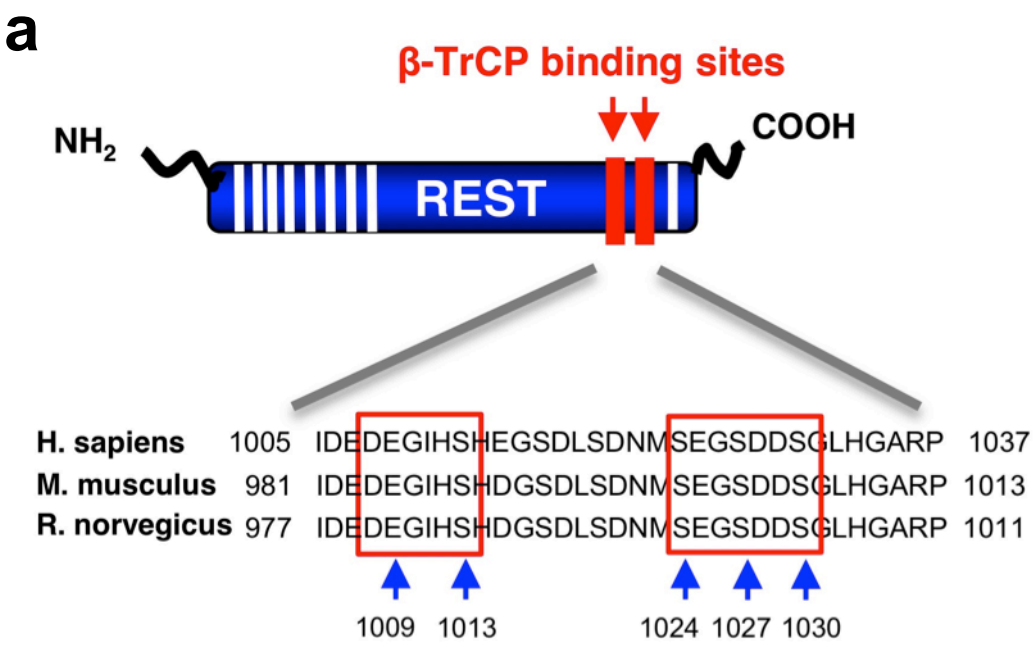

a
Pyrvinium (CK1 activator)
CHX 0 1 2 4 0 1 2 4 (h)
REST
β-actin
CHX
CHX+ Pyruvinium
REST/β-actin
2.0
1.5
1.0
0.5
0.0
*
*
0 1 2 4 (h)
b
D4476 (CK1 inhibitor)
CHX 0 1 2 4 0 1 2 4 (h)
REST
β-actin
CHX
CHX+ D4476
REST/β-actin
2.0
1.5
1.0
0.5
0.0
*
*
*
0 1 2 4 (h)

Figure 4.


a
β-TrCP binding sites
NH2
COOH
REST
H. sapiens 1005 IDEDEGIHSHEGSDLSDNMSEGSDDSGLHGARP 1037
M. musculus 981 IDEDEGIHSHDGSDLSDNMSEGSDDSGLHGARP 1013
R. norvegicus 977 IDEDEGIHSHDGSDLSDNMSEGSDDSGLHGARP 1011
1009 1013
1024 1027 1030

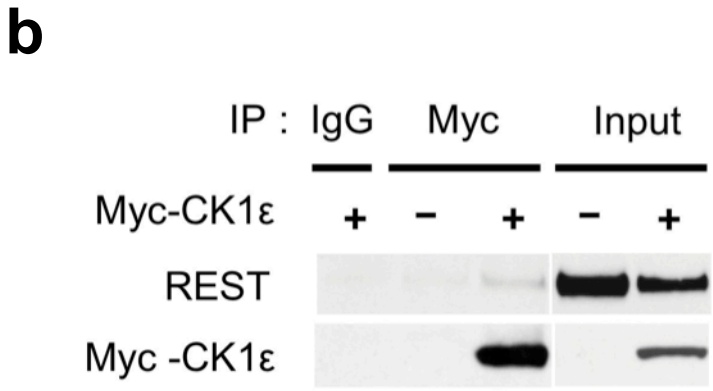

b
IP : IgG Myc Input
Myc-CK1ε + − + − +
REST
Myc -CK1ε

Figure 5.

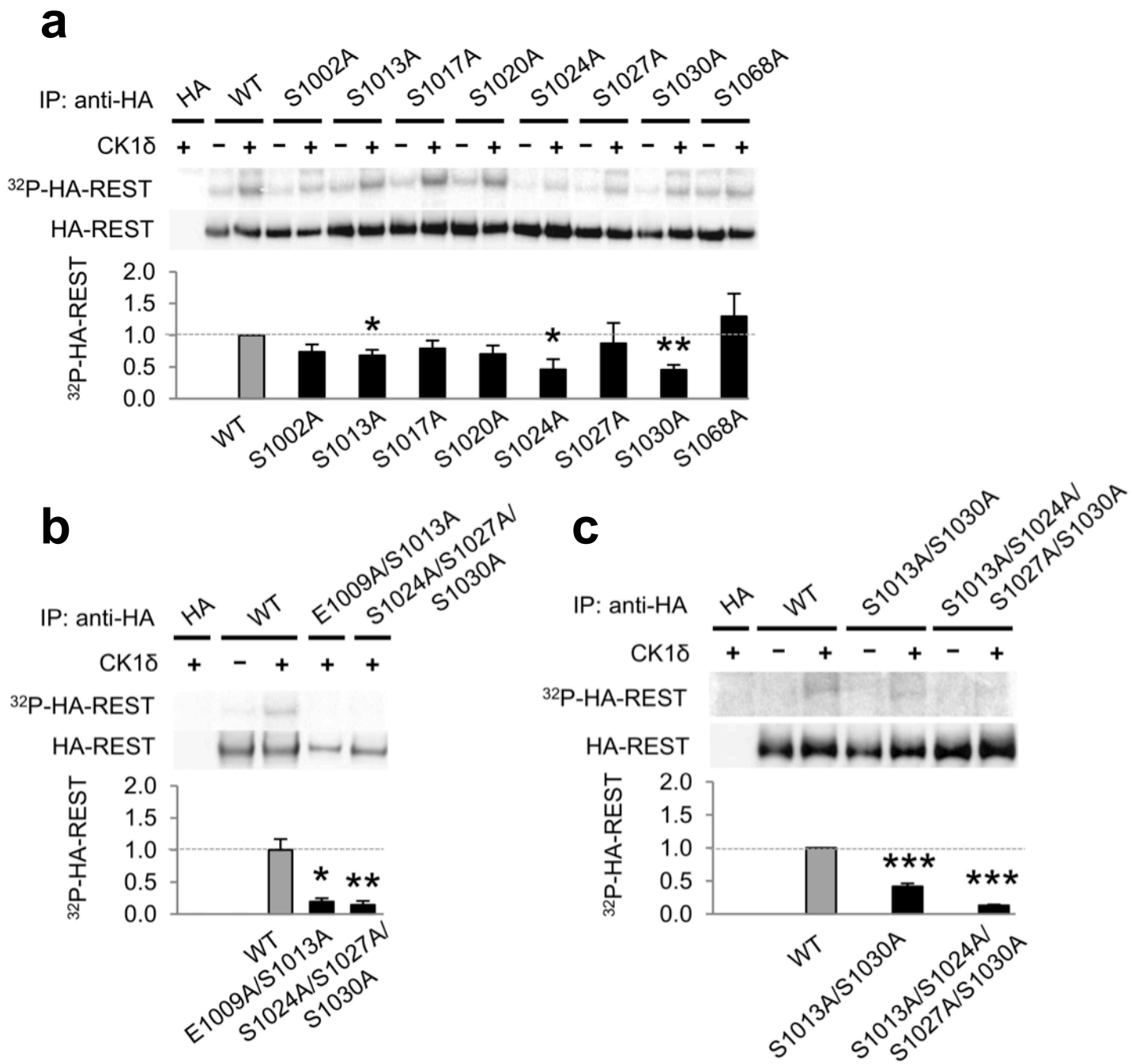

a
IP: anti-HA
HA
WT
S1002A
S1013A
S1017A
S1020A
S1024A
S1027A
S1030A
S1068A
CK1δ
32P-HA-REST
HA-REST
32P-HA-REST
2.0
1.5
1.0
0.5
0.0
*
*
**
WT
S1002A
S1013A
S1017A
S1020A
S1024A
S1027A
S1030A
S1068A
b
IP: anti-HA
HA
WT
E1009A/S1013A
S1024A/S1027A/
S1030A
CK1δ
32P-HA-REST
HA-REST
32P-HA-REST
2.0
1.5
1.0
0.5
0.0
*
**
WT
E1009A/S1013A
S1024A/S1027A/
S1030A
c
IP: anti-HA
HA
WT
S1013A/S1030A
S1013A/S1024A/
S1027A/S1030A
CK1δ
32P-HA-REST
HA-REST
32P-HA-REST
2.0
1.5
1.0
0.5
0.0
***
***
WT
S1013A/S1030A
S1013A/S1024A/
S1027A/S1030A

Figure 6.

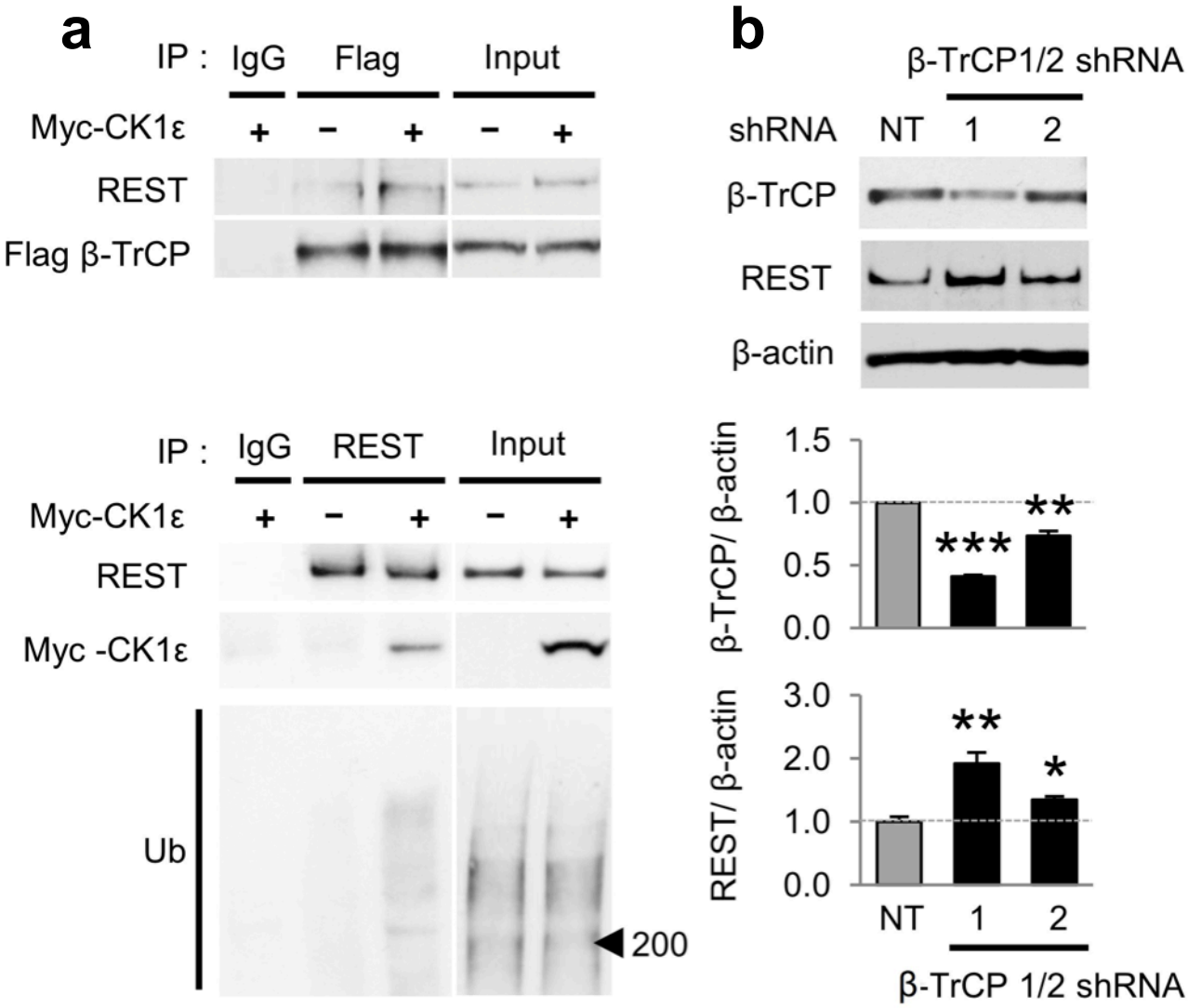

a
IP : IgG Flag Input
Myc-CK1ε + − + − +
REST
Flag β-TrCP
IP : IgG REST Input
Myc-CK1ε + − + − +
REST
Myc -CK1ε
Ub
200
b
β-TrCP1/2 shRNA
shRNA NT 1 2
β-TrCP
REST
β-actin
β-TrCP/ β-actin
1.5
1.0
0.5
0.0
***
**
REST/ β-actin
3.0
2.0
1.0
0.0
**
*
NT 1 2
β-TrCP 1/2 shRNA

Figure 7.

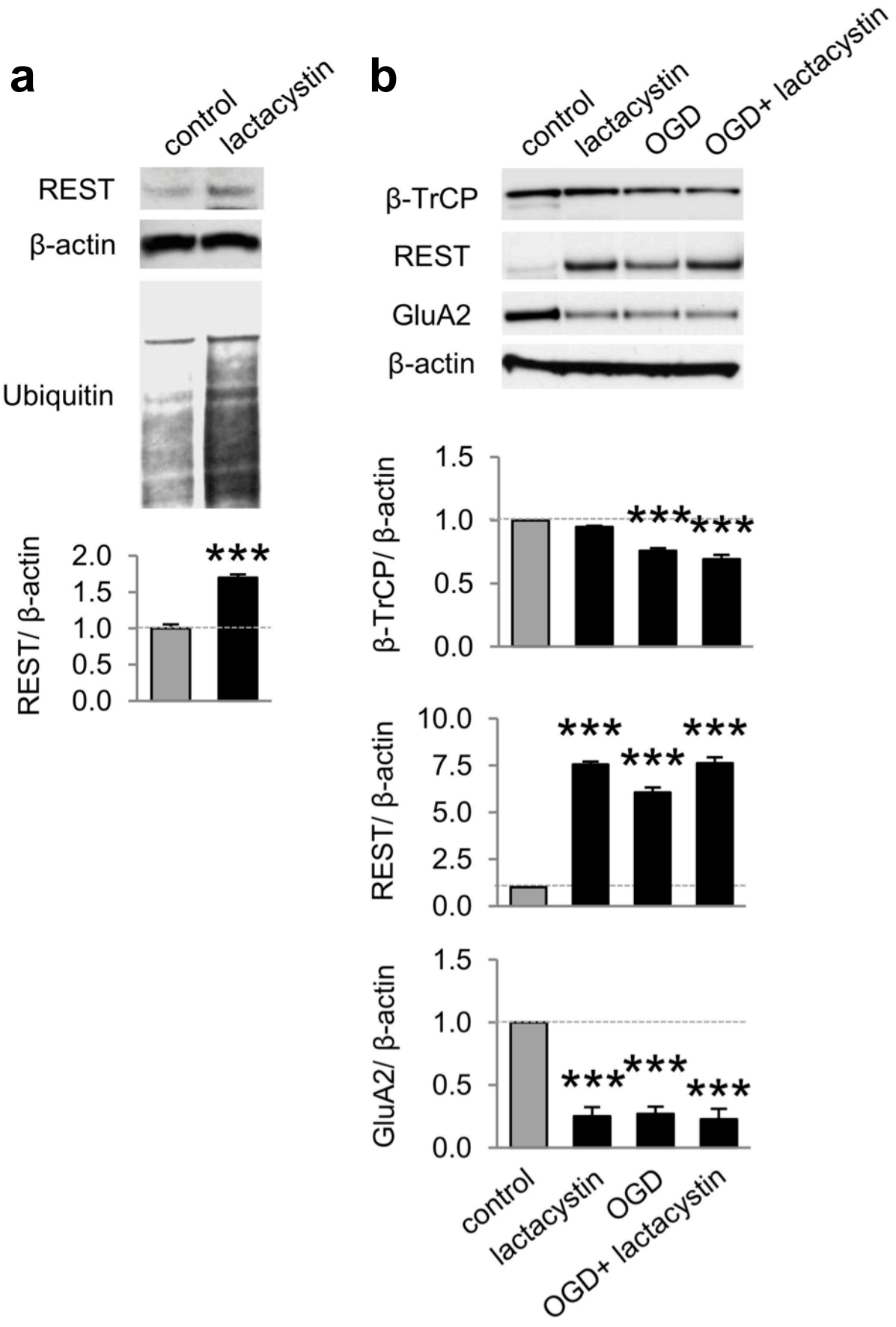

a
control
lactacystin
REST
β-actin
Ubiquitin
REST/ β-actin
2.0
1.5
1.0
0.5
0.0
***
b
control
lactacystin
OGD
OGD+ lactacystin
β-TrCP
REST
GluA2
β-actin
β-TrCP/ β-actin
1.5
1.0
0.5
0.0
***
***
REST/ β-actin
10.0
7.5
5.0
2.5
0.0
***
***
***
GluA2/ β-actin
1.5
1.0
0.5
0.0
***
***
***
control
lactacystin
OGD
OGD+ lactacystin

Figure 8.

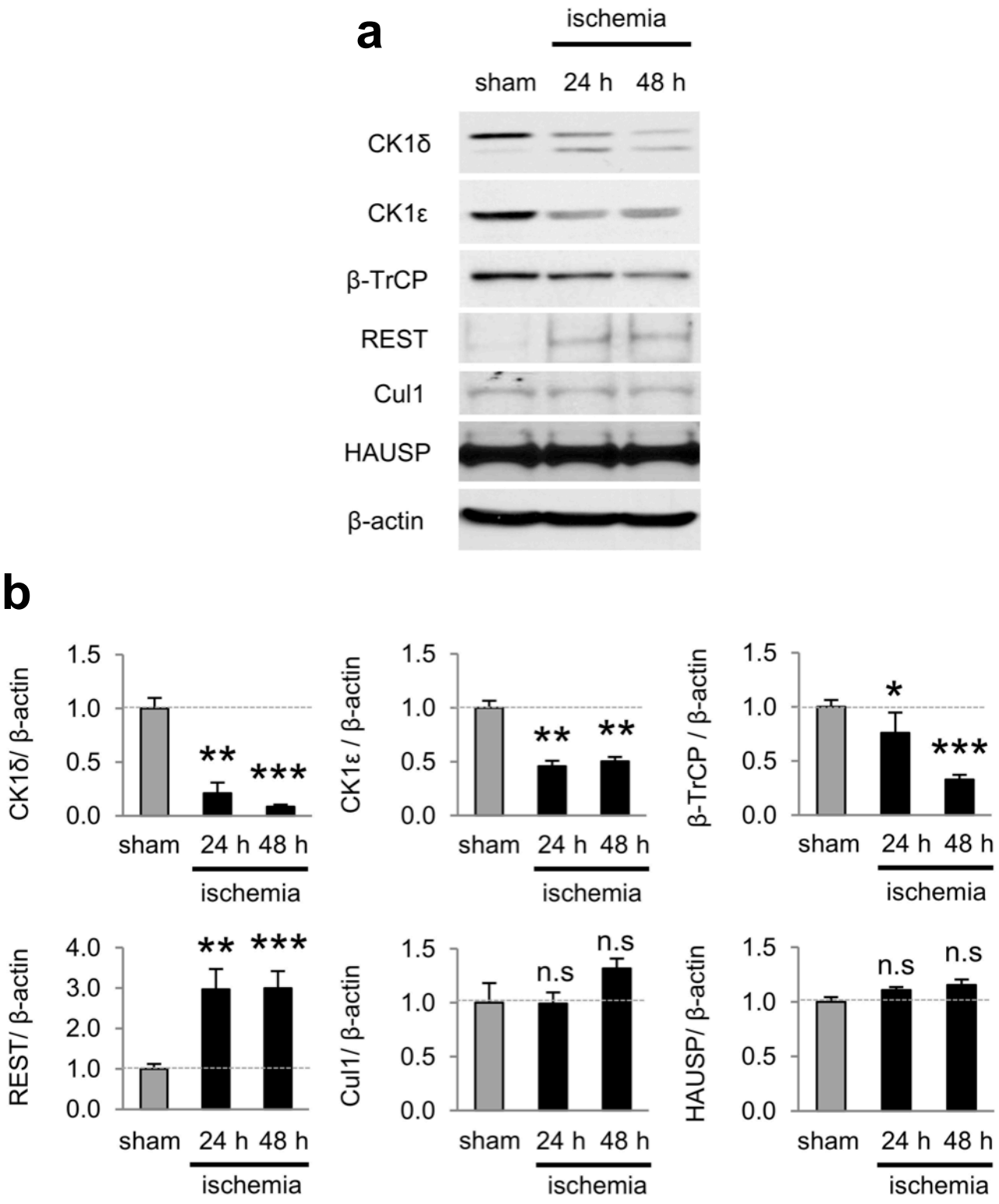

a
ischemia
sham
24 h
48 h
CK1δ
CK1ε
β-TrCP
REST
Cul1
HAUSP
β-actin
b
CK1δ/ β-actin
CK1ε / β-actin
β-TrCP / β-actin
REST/ β-actin
Cul1/ β-actin
HAUSP/ β-actin
sham
24 h
48 h
ischemia
n.s

Figure 9.

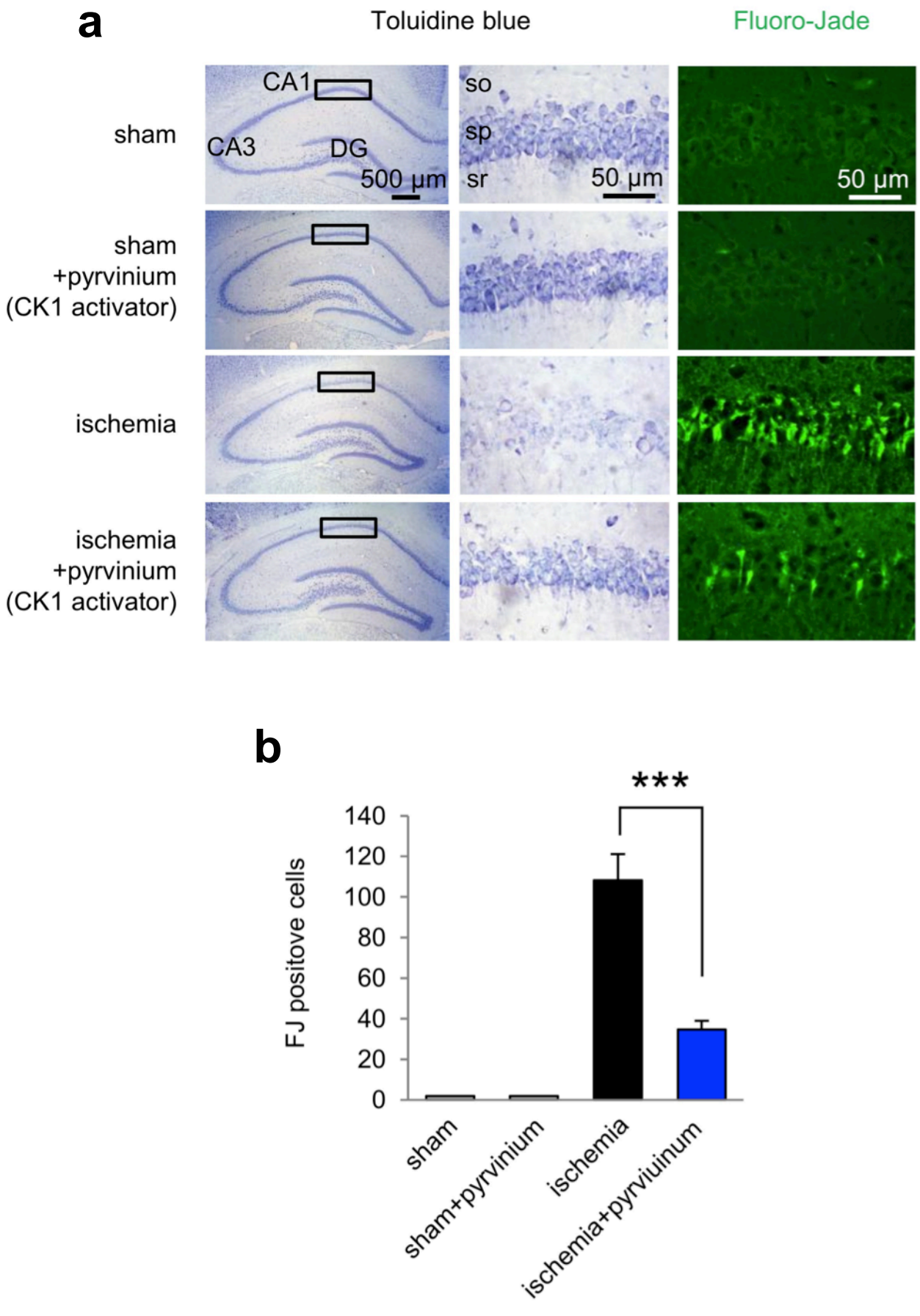

a
Toluidine blue
Fluoro-Jade
sham
sham
+pyrvinium
(CK1 activator)
ischemia
ischemia
+pyrvinium
(CK1 activator)
CA1
CA3
DG
500 μm
so
sp
sr
50 μm
50 μm
b
***
FJ positove cells
140
120
100
80
60
40
20
0
sham
sham+pyrvinium
ischemia
ischemia+pyrviuinum

Figure 10.

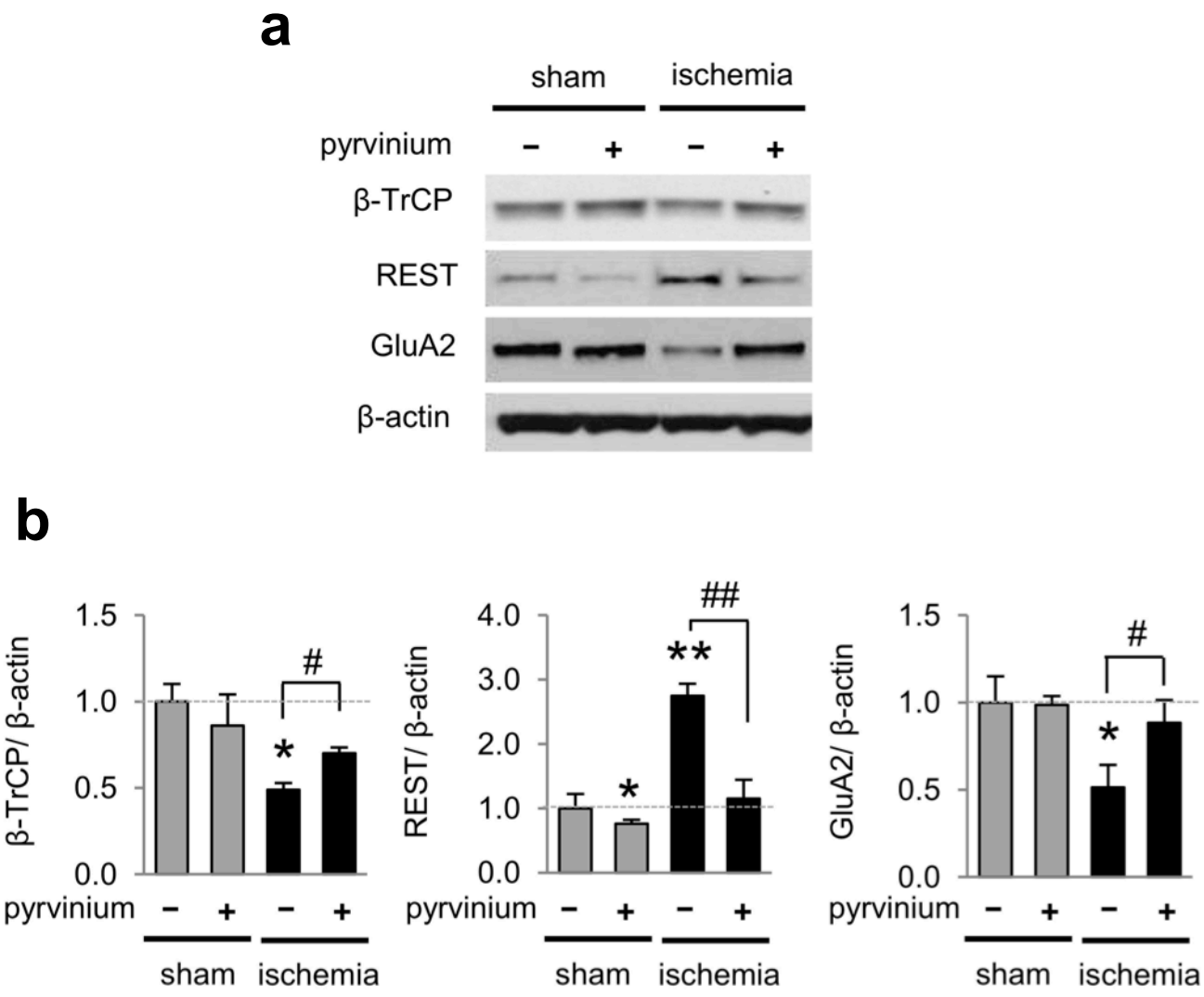
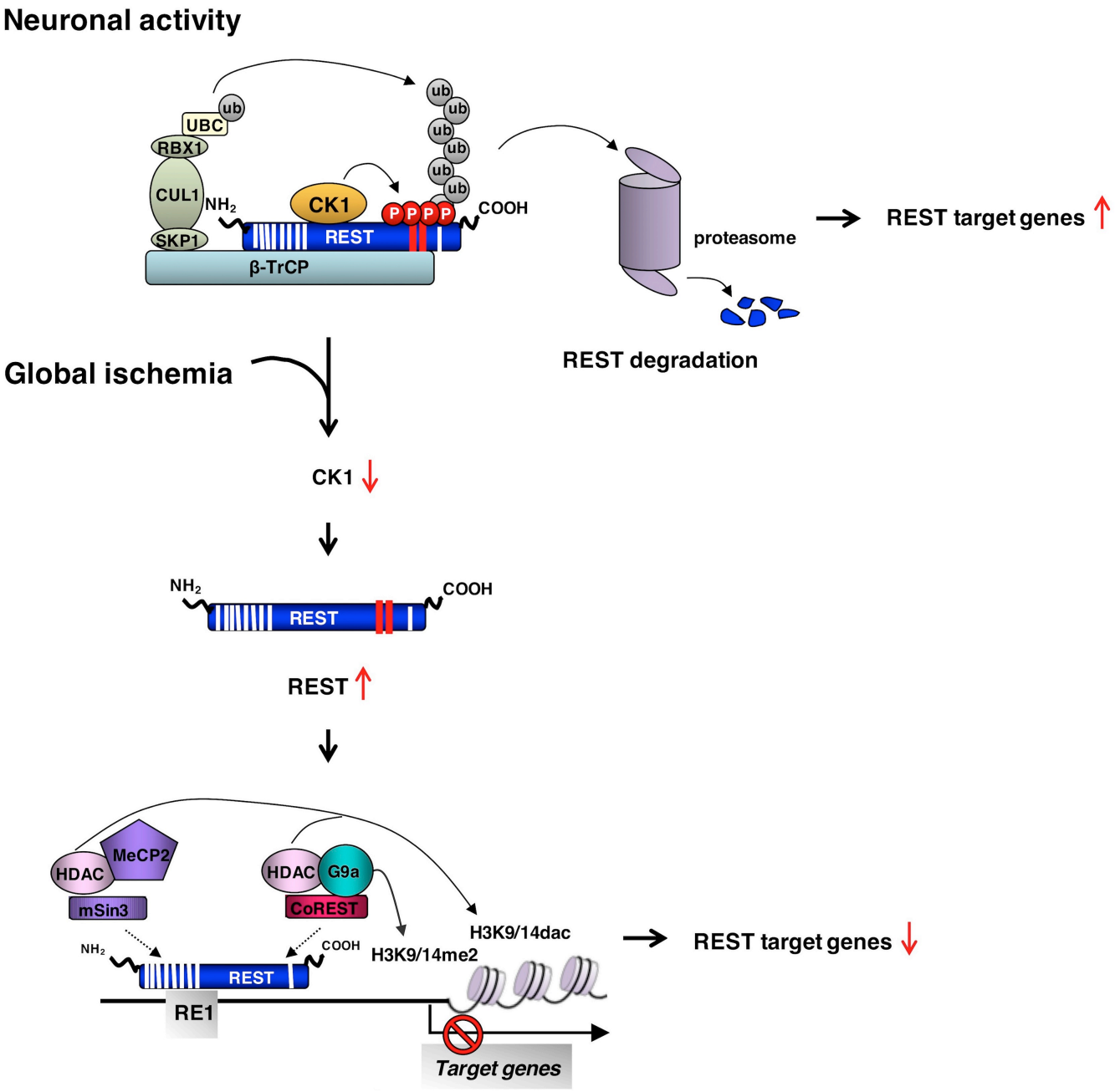

a
sham
ischemia
pyrvinium
– + – +
β-TrCP
REST
GluA2
β-actin
b
β-TrCP/ β-actin
1.5
1.0
0.5
0.0
#
*
pyrvinium
– + – +
sham
ischemia
REST/ β-actin
4.0
3.0
2.0
1.0
0.0
##
**
*
GluA2/ β-actin
1.5
1.0
0.5
0.0
#
*

Figure 11.


Neuronal activity
ub
UBC
RBX1
CUL1
SKP1
NH2
CK1
REST
COOH
P P P P
β-TrCP
proteasome
REST target genes
REST degradation
Global ischemia
CK1
NH2
REST
COOH
REST
HDAC
MeCP2
mSin3
HDAC
G9a
CoREST
NH2
REST
COOH
H3K9/14me2
H3K9/14dac
RE1
Target genes
REST target genes

X. Figure Legends

Figure 1. CK1 regulates REST protein abundance.

(a) Upper, representative Western showing that the CK1 activator, pyrvinium, reduces REST protein abundance in HeLa cells in a dose-dependent manner. Lower, summary data. (b) Upper, representative Western showing that the CK1 inhibitor D4476 increases REST protein abundance in a dose-dependent manner. Lower, summary data. (c) Pyrvinium reverses the increase in REST elicited by D4476. Lower, summary data. (d) Upper, representative Western blot showing overexpression of CK1 α1, α2, δ, or ε, but not γ1, γ2, or γ3, decreases REST protein abundance. Lower, summary data. (e) Upper, representative Western blot showing that two shRNAs targeting different sequences in CK1δ and CK1ε, but not NT shRNA, increases REST protein abundance in HeLa cells. Lower and right, summary data. (f) Upper, representative Western blot showing that pyrvinium decreases and D4476 and shRNA to CK1ε increases REST protein levels in cultured hippocampal neurons Lower: summary data. n = 3 per treatment group/ 3 independent experiments. Here and in Figs. 2-10, data represent mean ± SEMs. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$.

Figure 2. CK1 post-transcriptionally regulates REST abundance.

(a,b) Upper, representative Western blot showing REST protein abundance is unchanged in HeLa cells treated with the PKA inhibitor H89 (a) and the PI3 kinase (PI3K) inhibitor LY294002 (b). Lower, summary data. (c-e) REST mRNA levels were unchanged in HeLa cells after treatment with pyrvinium or D4476(c), myc-CK1 (d), or shRNA to CK1 (e). n = 3 per treatment group/ 3 independent experiments.

Figure 3. CK1 post-translationally regulates REST degradation.

(a,b) To monitor the impact of CK1 on the rate of protein degradation, HeLa cells were treated with pyrvinium (10 μM, a) or D4476 (10 μM, b) for 2 h prior to application of the protein synthesis inhibitor cycloheximide (100 μg/ml). At indicated times, whole cell lysates were prepared and immunoblots probed with REST or β-actin antibody. n = 3 per treatment group/ 3 independent experiments. *, $p < 0.05$.

Figure 4. CK1 associates with REST.

(a) Diagram illustrates serine residues within two noncanonical degron motifs in the carboxy-terminus of REST and critical to recognition by β-TrCP. (b) Co-immunoprecipitation of recombinant Myc from HeLa cells expressing Myc-CK1 ε and probed for REST showing that Myc-CK1 ε associates with endogenous REST.

Figure 5. CK1 directly phosphorylates serine residues in carboxy-terminus of REST.

(a) Immunoprecipitation of HA from N2A cells expressing WT or mutant HA-REST constructs was incubated in the presence or absence of recombinant CK1 δ and γ-^{32}P-ATP. Upper, autoradiogram depicting phospho-HA-REST shows that single-point mutations S1013A, S1024A and S1030A significantly alter CK1-dependent phosphorylation of REST. Lower, summary data. Values for phosphorylated HA-REST are normalized to values for HA-REST. (b) Immunoprecipitation of HA from N2A cells expressing WT HA-REST, double mutant HA-REST (E1009A/S1013A), and triple mutant HA-REST (S1024A/S1027A/S1030A) and was incubated in the presence or absence of recombinant CK1 δ and γ-^{32}P-ATP, Upper, autoradiogram depicting phospho-HA-REST shows that the double and triple HA-REST mutants

abolish CK1-dependent phosphorylation. Lower, Summary data. (c) Immunoprecipitation of HA from N2A cells expressing WT HA-REST, double mutant HA-REST (S1013A/S1030A), or quadruple mutant HA-REST (S1013A/S1024A/S1027A/ S1030A) with an antibody to HA was incubated in the presence or absence of recombinant CK1 δ and γ-^{32}P-ATP. Upper, autoradiogram depicting phospho-HA-REST shows that the double and quadruple HA-REST mutants significantly decrease CK1-dependent phosphorylation of REST. Lower, summary data. n = 3 per treatment group/ 3 independent experiments. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$.

Figure 6. CK1 associates with REST, increases β-TrCP binding and REST ubiquitination.

(a) Upper, Immunoprecipitation of Flag from HeLa cells expressing myc-CK1 ε, HA-REST and Flag-β-TrCP was subjected to Westerns probed for HA-REST and shows that CK1 increases interaction between REST and β-TrCP. Lower: Immunoprecipitation of HA from HeLa cells expressing HA-REST and Myc-CK1 ε was subjected to Westerns and probed for HA-REST, Myc-CK1 ε and ubiquitin and shows that CK1 binds REST and promotes ubiquitination of REST. (b) Upper, Representative Western blot showing that two different sequences of shRNA-mediated knockdown of β-TrCP1/2 (β-TrCP1/2 shRNA-1, β-TrCP1/2 shRNA-2) in hippocampal neurons increase REST expression. Lower, summary data. n = 3 per treatment group/ 3 independent experiments. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$.

Figure 7. REST is targeted to the proteasomal degradation pathway in neurons.

(a) Upper, representative Western blot showing that the proteasome inhibitor lactacystin injected in vivo increases REST abundance and global protein ubiquitination in the hippocampal CA1. Lower, summary data. (b) Upper, representative Western blot showing that application of the proteasome inhibitor lactacystin applied to hippocampal neurons in vitro does not detectably alter β-TRCP, but induces upregulation of REST and downregulation of the REST target gene GluA2 under control conditions. Oxygen-glucose deprivation (OGD) decreases β-TRCP, increases REST and decreases GluA2 in hippocampal neurons in vitro. Administration of lactacystin to neurons subjected to OGD does not further alter REST or GluA2. Lower, summary data. n = 3 per treatment group in 3 independent experiments. ***, $p < 0.001$.

Figure 8. Global ischemia decreases CK1 and upregulates REST in CA1.

(a) Representative Western blot showing that global ischemia induces a decrease in CK1 δ, CK1 ε, and β-TrCP and increase in REST, but does not detectably alter Cul1 or HAUSP protein expression in the CA1, assessed at 24 and 48 h after ischemia. (b) Summary data. n = 4 - 6 animals per group. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$.

Figure 9. The CK1 activator pyrvinium protects CA1 neurons from ischemia-induced cell death in vivo.

(a) Representative images of toluidine blue and Fluoro-Jade staining of CA1 pyramidal neurons in rats 7 d after global ischemia or sham surgery, followed by icv injection of the CK1 activator pyrvinium (1 mM) or vehicle. Scale bars: low magnification, 500 μm; high magnification, 50 μm. (b) Quantification of dying CA1 pyramidal neurons as assessed by Fluoro-Jade staining. Pyrvinium rescues

ischemia-induced neuronal death in the CA1. n = 5 - 6 animals per group. ***, $p < 0.001$.

Figure 10. The CK1 activator pyrvinium inhibits REST upregulation and restores GluA2 level in CA1 after global ischemia.

(a) Representative Western blot showing that injection of pyrvinium essentially restores the ischemia-induced increase in REST and decrease in the REST target GluA2. (b) Summary data. n = 4 - 6 animals per group. **, ## $p < 0.01$, *,# $p < 0.05$.

Figure 11. Model depicting a hypothetical mechanism by which CK1 regulates REST abundance in differentiated neurons under physiological and pathological conditions. Under physiological conditions, CK1 binds and phosphorylates REST at sites within two neighboring, but distinct degron motifs. The phosphorylated degrons (phospho-degrons) are critical to recognition of REST by the E3 ubiquitin protein ligase β-TrCP, which ubiquitinates and targets REST for proteasomal degradation. Global ischemia reduces CK1 and β-TrCP abundance, which lead to an increase in REST in the hippocampal CA1. REST binds to the RE1 element within the promoter of target genes such as GluA2 and orchestrates the assembly of mSin3A and CoREST, HDACs 1 and 2, G9a and MeCP2. The REST-corepressor complex promotes epigenetic remodeling of core histone proteins at the promoter of target genes and represses transcription of synaptic proteins. Modified with permission from (Frescas and Pagano, 2008; Noh et al., 2012).

XI. Acknowledgment

I thank Fabrizio Pontarelli and Adrianna Latuszek-Barrantes for excellent technical assistance, and Yoshihide Sehara, Kirsty Sawicka and Jack-Christophe Cossec for insightful scientific discussion. K. Sawicka is a distinguished scientist and taught me fundamentals of science, including not only useful techniques but also design, analysis and interpretation of study. I also thank Jee-Yeon Hwang, Michael Gertner and R. Suzanne Zukin for editing this work. This work was supported by National Institutes of Health grant NS46742 (R.S.Z.), a McKnight Foundation Brain Disorders Award (R.S.Z.), and the generous support of the F.M. Kirby Foundation.