

Proline-, glutamic acid-, and leucine-rich protein 1 mediates estrogen rapid signaling and neuroprotection in the brain

Gangadhara R. Sareddy^{a,1}, Quanguang Zhang^{b,1}, Ruimin Wang^{b,c}, Erin Scott^b, Yi Zou^d, Jason C. O'Connor^{e,f}, Yidong Chen^d, Yan Dong^b, Ratna K. Vadlamudi^{a,2}, and Darrell Brann^{b,g,2}

^aDepartment of Obstetrics and Gynecology, University of Texas Health Science Center, San Antonio, TX 78229; ^bDepartment of Neuroscience and Regenerative Medicine, Medical College of Georgia, Georgia Regents University, Augusta, GA 30912; ^cNeurobiology Institute of Medical Research Center, North China University of Science and Technology, Tangshan, People's Republic of China 063000; ^dDepartment of Epidemiology & Biostatistics, University of Texas Health Science Center, San Antonio, TX 78229; ^eDepartment of Pharmacology, University of Texas Health Science Center, San Antonio, TX 78229; ^fSouth Texas Veterans Health System, Audie L. Murphy VA Hospital, San Antonio, TX 78229; and ^gCharlie Norwood Veterans Affairs Medical Center, Augusta, GA 30904

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17- β estradiol (E2) has been implicated as neuroprotective in a variety of neurodegenerative disorders. However, the underlying mechanism remains unknown. Here, we provide genetic evidence, using forebrain-specific knockout (FBKO) mice, that proline-, glutamic acid-, and leucine-rich protein 1 (PELP1), an estrogen receptor coregulator protein, is essential for the extranuclear signaling and neuroprotective actions of E2 in the hippocampal CA1 region after global cerebral ischemia (GCI). E2-mediated extranuclear signaling (including activation of extracellular signal-regulated kinase and Akt) and antiapoptotic effects [such as attenuation of JNK signaling and increase in phosphorylation of glycogen synthase kinase-3 β (GSK3 β)] after GCI were compromised in PELP1 FBKO mice. Mechanistic studies revealed that PELP1 interacts with GSK3 β , E2 modulates interaction of PELP1 with GSK3 β , and PELP1 is a novel substrate for GSK3 β . RNA-seq analysis of control and PELP1 FBKO mice after ischemia demonstrated alterations in several genes related to inflammation, metabolism, and survival in PELP1 FBKO mice, as well as a significant reduction in the activation of the Wnt/ β -catenin signaling pathway. In addition, PELP1 FBKO studies revealed that PELP1 is required for E2-mediated neuroprotection and for E2-mediated preservation of cognitive function after GCI. Collectively, our data provide the first direct *in vivo* evidence, to our knowledge, of an essential role for PELP1 in E2-mediated rapid extranuclear signaling, neuroprotection, and cognitive function in the brain.

17- β estradiol | PELP1 | extranuclear | neuroprotection | cognition

The steroid hormone, 17 β -estradiol (E2), exerts multiple actions in the brain, including regulation of synaptic plasticity, neurogenesis, reproductive behavior, and cognition. E2 has also been implicated to serve as an important neuroprotective factor in a variety of neurodegenerative disorders, including stroke and Alzheimer's disease (1–3). The main source of E2 synthesis in females is the ovary. Circulating E2 levels are known to fluctuate during development and puberty, as well as during the menstrual cycle. After menopause, however, circulating E2 levels decline precipitously (4). Relative to men, women are “protected” against stroke until the years of menopause. However, after menopause, women exhibit a significantly higher disability and fatality rate compared with men (5–7). Intriguingly, the higher risk and poorer outcome of stroke in postmenopausal women parallels the falling E2 levels that occur after menopause (7, 8). Furthermore, exogenous administration of E2 significantly reduces the infarct volume in cortex and hippocampus after focal and global cerebral ischemia (GCI) in various animal models, and female animals exhibit reduced neural damage compared with young adult males after brain injury (1, 9–11). Taken as a whole, these studies suggest E2 functions as an important neuroprotective factor. However, the molecular mechanisms by which E2 exerts its neuroprotective effects remain unclear.

E2 signaling is thought to be primarily mediated by the classical estrogen receptors, estrogen receptor alpha (ER α) and estrogen receptor beta (ER β). Both subtypes are expressed in the brain and have been shown to mediate various neural actions of E2, including neuroprotection (4, 12–14). For example, ER genomic actions have been shown to contribute to increased expression of the antiapoptotic gene, *bcl-2*, and the antiapoptotic prosurvival factor, *survivin*, which can contribute to E2 neuroprotective effects (11, 15, 16). Recent evidence suggests that E2 also exerts “rapid signaling” in the brain via interaction with extranuclear ER, which can lead to regulation of kinase activation and ion channels (17, 18). ER “extranuclear” signaling has been suggested to contribute to E2 neuroprotective effects by facilitating the rapid activation of extracellular signal-regulated kinases (ERKs) and prosurvival serine threonine kinase Akt (4, 19–22). Furthermore, E2 also interacts with nonclassical ERs such as G protein-coupled estrogen receptor, which can also contribute to activation of ERK and PI3K (4, 23). Transcriptional activity of E2-bound ERs is regulated by several coregulatory proteins that associate with the ER in response to E2 binding (24). Association of the coregulatory

Significance

Ever since the rapid extranuclear signaling effects of 17 β -estradiol (E2) were first identified in the brain decades ago, it has remained an enigma as to how these nondclassical effects are achieved. Using a forebrain-specific knockout animal model, the current study demonstrates that a recently cloned estrogen receptor coregulator protein, Proline-, glutamic acid-, and leucine-rich protein 1 (PELP1), is critical for mediating E2 regulation of rapid extranuclear signaling, as well as E2-induced neuroprotection and cognitive function in the hippocampus after ischemic injury. Our studies also identified PELP1 as a novel interacting protein and a substrate of glycogen synthase kinase-3 β (GSK3 β). Finally, PELP1 was also shown to mediate E2 genomic effects to regulate genes involved in inflammation, metabolism, and survival after ischemic injury.

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¹G.R.S. and Q.Z. contributed equally to this work.

²To whom correspondence should be addressed. Email: dbrann@gru.edu and vadlamudi@uthscsa.edu.

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proteins with ER leads to formation of an “ER signalosome” that facilitates ER genomic and extranuclear actions in a tissue-dependent manner. However, the composition of the ER-signalosome that mediates the beneficial effects of E2 in the brain is not completely clear.

Work by our group led to the cloning of proline-, glutamic acid-, and leucine-rich protein 1 (PELP1) and its characterization as a coregulator of ER (25, 26). PELP1 is a multidomain scaffold protein that serves as a platform for various protein–protein interactions (27). For example, PELP1 can interact with ERs, using the nuclear receptor interaction motif LXXLL; Src kinase, using SH2 domains; and PI3K, using PXXP motifs (28). The PELP1 C-terminal region is unique and contains a 70-aa glutamic acid-rich domain that facilitates PELP1 interactions with histones (29). Recent studies in cancer cells revealed that PELP1 also interacts with and modulates the activity of several chromatin-modifying enzymes such as histone deacetylase 2 (HDAC2), lysine (K)-specific demethylase 1A (KDM1A), and coactivator-associated arginine methyltransferase 1 (CARM1) (27, 30), and thus may contribute to E2-mediated genomic actions by facilitating the recruitment of chromatin modifiers to ER target genes. PELP1 is widely expressed in neuronal tissues, and its expression is modulated by E2 (31). However, the role of PELP1 in mediating the signaling, neuroprotective, and cognitive effects of E2 in the brain remains unknown. In addition, the factors that interact with and modulate PELP1 in the brain remain unidentified.

To address these deficits, in the present study, we developed the first PELP1 knockout model, to our knowledge, that conditionally depletes PELP1 in the forebrain. Our studies discovered that E2-mediated neuroprotection and rapid extranuclear signaling are lost in PELP1 knockout mice compared with floxed control mice. PELP1 antisense oligonucleotide knockdown studies in the rat further confirmed the role of PELP1 in mediating E2 extranuclear signaling and neuroprotection. RNA-seq studies identified several genes uniquely regulated by PELP1 under conditions of ischemia, and they represent pathways related to neuronal survival, apoptosis, and inflammation. PELP1 forebrain-specific knockout (FBKO) mice exhibited defects in spatial learning and memory. Mechanistic studies demonstrated that the PELP1 signalosome contains ER α , Src, and p85, and discovered that although PELP1 is a novel substrate of glycogen synthase kinase-3 β (GSK3 β) kinase, phosphorylation of PELP1 by GSK3 β regulates PELP1's stability. Collectively, these findings demonstrate a key role for PELP1 in mediating E2 rapid signaling, neuroprotection, and cognitive function in the brain.

Results

Characterization of PELP1 FBKO Mice. To examine the functional role of PELP1 in the regulation of E2 signaling, we generated conditional PELP1 FBKO mice using a site-specific Cre/loxP recombination system. A transgenic mouse that expresses a floxed allele of PELP1 was custom-generated and preliminarily characterized by Lexicon Pharmaceuticals Incorporated. In this model, exon 1 of PELP1 was flanked by loxP sites; the strategy is depicted in Fig. 1A. The targeted PELP1 allele was confirmed with Southern blot analysis of EcoRV- and PstI-digested genomic DNA, using 5' internal and 3' external probes (Fig. 1B). PELP1 loxP/loxP mice were bred with Cre mice that expressed Cre under the control of the CaMKII α promoter, which is specifically expressed in excitatory neurons of the forebrain. The CaMKII α -Cre transgene is not expressed until after birth, thereby allowing normal embryonic development of the transgenic mice. Mice were genotyped by using PCR on genomic DNA of tail biopsies to confirm the genotype of floxed and Cre alleles (Fig. 1C). PELP1 FBKO mice are viable, with no obvious gross immunological, reproductive, or neurological abnormality or phenotype. To confirm knockout effectiveness of PELP1 in the CaMKII α -Cre mice, we used genomic PCR of forebrain samples (Fig. 1D), as well as immunohisto-

chemistry (Fig. 1E) and Western blot analysis (Fig. 1F and G) for PELP1. As shown in Fig. 1D, deletion of the PELP1 exon 1 resulted in generation of a unique PCR band because of the expression of CaMKII α -Cre in the forebrain compared with FLOX control mice. Immunohistochemical analysis of various regions of the forebrain further confirmed the efficient knockdown of PELP1 in the hippocampus and cortex, with no effect noted in the hindbrain (Fig. 1E). In addition, Western blot analysis showed the efficient knockdown of PELP1 in the forebrain (hippocampus and cerebral cortex) of PELP1 FBKO mice compared with FLOX control and PELP1 FBKO heterozygote mice (61% knockdown in hippocampus, 42% knockdown in cortex with no change in cerebellum) (Fig. 1F and G). The residual amount of PELP1 in the hippocampus and cortex may be attributable to the expression of PELP1 in glia, interneurons, and a small percentage of excitatory neurons that lack Cre expression. PELP1 knockdown was specific to the forebrain, as PELP1 levels did not change in the cerebellum (hindbrain) in PELP1 FBKO versus FLOX control mice.

E2-Induced Rapid Extranuclear Signaling Is Attenuated in PELP1 FBKO Mice.

We previously showed that E2, through its cognate receptors ER α and GPR30, activates rapid extranuclear signaling to offer immediate protection to neurons after an insult (19, 23). To evaluate the role of PELP1 in the E2-mediated extranuclear signaling *in vivo*, ovariectomized placebo- and E2-implanted FLOX control and PELP1 FBKO mice were subjected to GCI, and the hippocampi were isolated after 10 min, 30 min, and 3 h reperfusion. As shown in Fig. 2A and C, Western blot analysis demonstrated that in FLOX control mice, E2 significantly increased the phosphorylation of survival signaling kinases including pERK1/2 and pAkt. Phosphorylation of ERK1/2 was increased at 10 min and 30 min after reperfusion, with an increase in pAkt observed at 10 min, 30 min, and 3 h after reperfusion. However, E2 was unable to induce these survival signals in PELP1 FBKO mice (Fig. 2B and D). We previously showed that E2 robustly attenuates phosphorylation/activation of the proapoptotic factor JNK after GCI insult (19, 23). We thus examined the role of PELP1 in this effect, using the PELP1 FBKO mice. As shown in Fig. 2A and C, although E2 significantly decreased the phosphorylation of JNK in FLOX control mice at 3 h reperfusion, this effect was lost in PELP1 FBKO mice (Fig. 2B and D), suggesting PELP1 is critical for E2 regulation of JNK activation. Immunofluorescence analysis also demonstrated that E2 significantly increased the phosphorylation of p-Src, p-ERK1/2, and p-Akt in hippocampal CA1 regions of FLOX control mice at 30 min reperfusion. However, in PELP1 FBKO mice, this E2-mediated increase was attenuated (Fig. S1A and B). These results suggest that PELP1 is essential for the activation of rapid E2-mediated extranuclear signaling under conditions of cerebral ischemia. In addition, use of an alternative knockdown approach (central administration of PELP1 antisense oligonucleotides in the rat) further confirmed the critical role of PELP1 in mediating E2 regulation of rapid signaling (Akt and JNK activation) after GCI (Fig. S2).

PELP1 is a scaffolding protein that contains several protein–protein interaction motifs that interact with SH2 and SH3 domains present in Src and PI3 kinases. To determine whether PELP1 forms a scaffold complex with Src, PI3 kinase, and ER α in the hippocampus, and whether E2 enhances this effect, we examined the *in vivo* association of these proteins at 30 min after GCI in wild-type mice treated with placebo or E2, using the Duolink II *in situ* proximity ligation assay detection kit. The results revealed that there is low PELP1 interaction with ER α , Src, and PI3K in the placebo group after GCI, whereas, in contrast, the E2-treated group showed robust PELP1 interaction with ER α , Src, and PI3K 30 min after ischemia (Fig. S3). Taken together, these results suggest PELP1 is needed for the optimal activation of E2-mediated extranuclear signaling under conditions of ischemic damage.

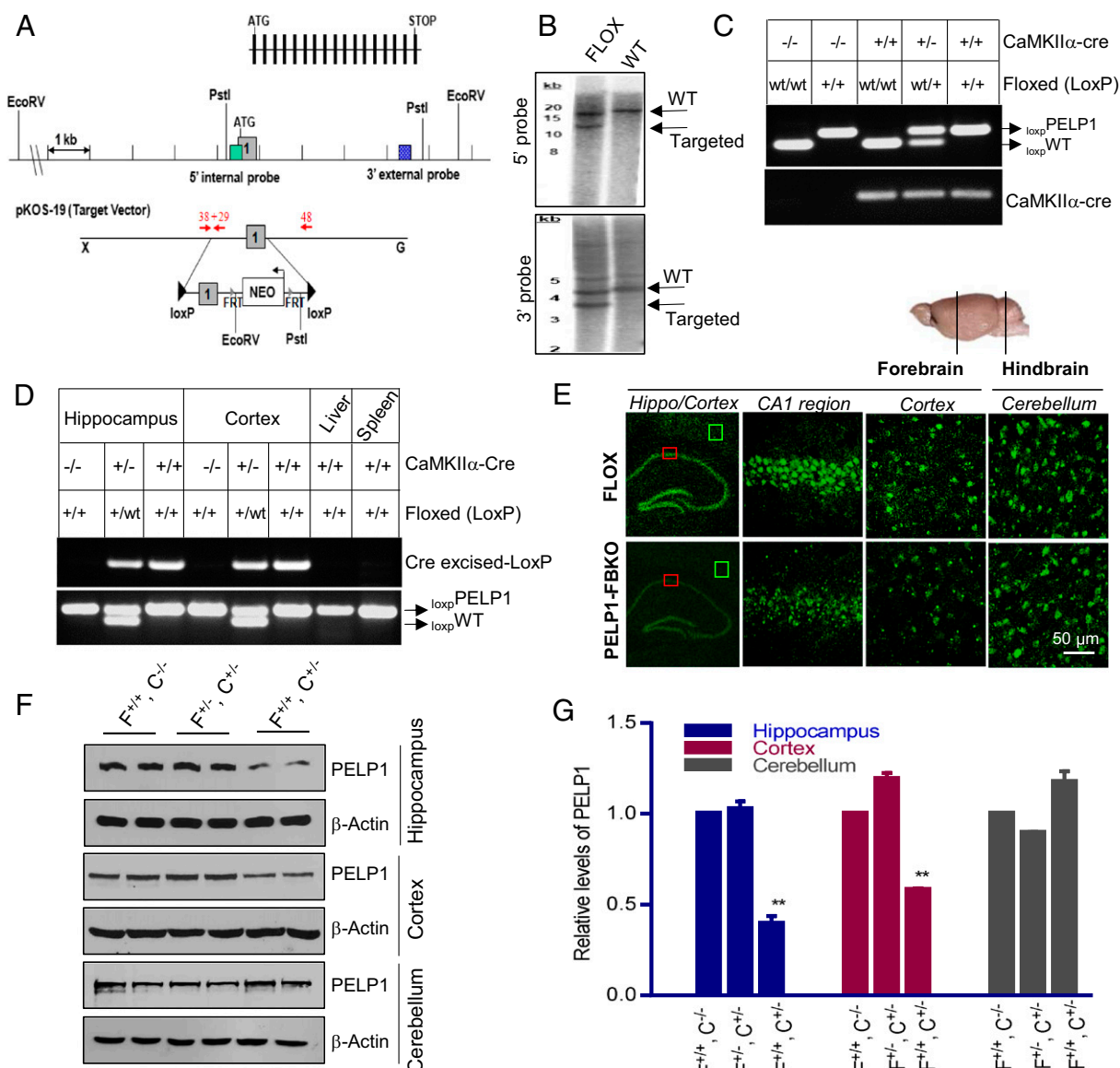


Fig. 1. Generation and characterization of PELP1 FBKO mice. (A) Schematic representation of endogenous (WT) and recombined locus mPELP1. (B) Confirmation of targeted clones by Southern blotting of genomic DNA. (C) Tail DNA PCR confirmation of bitransgenic PELP1 FBKO mice expressing CaMKII α -Cre and loxPPELP1. (D) Confirmation of excision of floxed region in hippocampus and cortex of FLOX control and PELP1 FBKO mice using genomic PCR. (E) Immunohistochemical analysis of coronal sections of hippocampus, cortex, and hindbrain of control and PELP1 FBKO mice. (F) Lysates of hippocampus, cortex and cerebellum collected from control (PELP1 loxP/loxP), heterozygous (PELP1 loxP^{+/-}, CaMKII α Cre^{+/-}), and homozygous PELP1 FBKO (PELP1 loxP/loxP, CaMKII α -Cre^{+/-}) mice were subjected to Western blotting with PELP1 antibody. β -actin was used as a loading control. (G) The data were quantified using Image J software and normalized to β -actin. C, Cre; F, FLOX. Bar graphs represent mean \pm SEM. ** $P < 0.01$.

E2-Mediated GSK3 β Inhibition Is Attenuated in PELP1 FBKO Mice. Previous work suggests GSK3 β is one of the key mediators of cell death in several neurodegenerative models, including cerebral ischemia and Alzheimer's disease, and that inhibition of GSK3 β improves functional recovery after cerebral ischemia (17, 32–34). GSK3 β phosphorylates many substrate proteins to regulate their stability (35, 36). In turn, GSK3 β activity is inhibited by several kinases, including the Akt, Wnt, and MAPK pathways (37). It is known that the prosurvival kinase, Akt, can phosphorylate GSK3 β at its N terminus, leading to its inactivation. Because we showed that PELP1 status modulates Akt phosphorylation, we examined whether PELP1 might play a role in regulating GSK3 β phosphorylation, using FLOX control and PELP1 FBKO hippocampi, after GCI. As shown in Fig. 3 A and C, GSK3 β phosphorylation is initially elevated at 10 min after GCI in the hippocampus of FLOX

control mice, followed by a robust fall in its phosphorylation at 30 min and 3 h, indicating a transient ischemia-induced inhibition of GSK3 β after GCI. Interestingly, E2 treatment did not alter GSK3 β phosphorylation at 10 min after GCI, but significantly increased its phosphorylation at 30 min after GCI compared with the placebo control, suggesting E2 maintains a longer period of GSK3 β inhibition. However, this regulatory effect of E2 on GSK3 β phosphorylation is lost in PELP1 FBKO mice (Fig. 3 B and D). GSK3 β is one of the key components in the Wnt signaling pathway. It negatively regulates prosurvival signaling by phosphorylating β -catenin, which leads to its degradation. We previously showed that E2 significantly up-regulates the β -catenin pathway after GCI (11). As shown in Fig. 3 A and C, Western blot analysis revealed that E2 substantially reduced the phosphorylation of β -catenin after GCI compared with placebo in FLOX control mice.

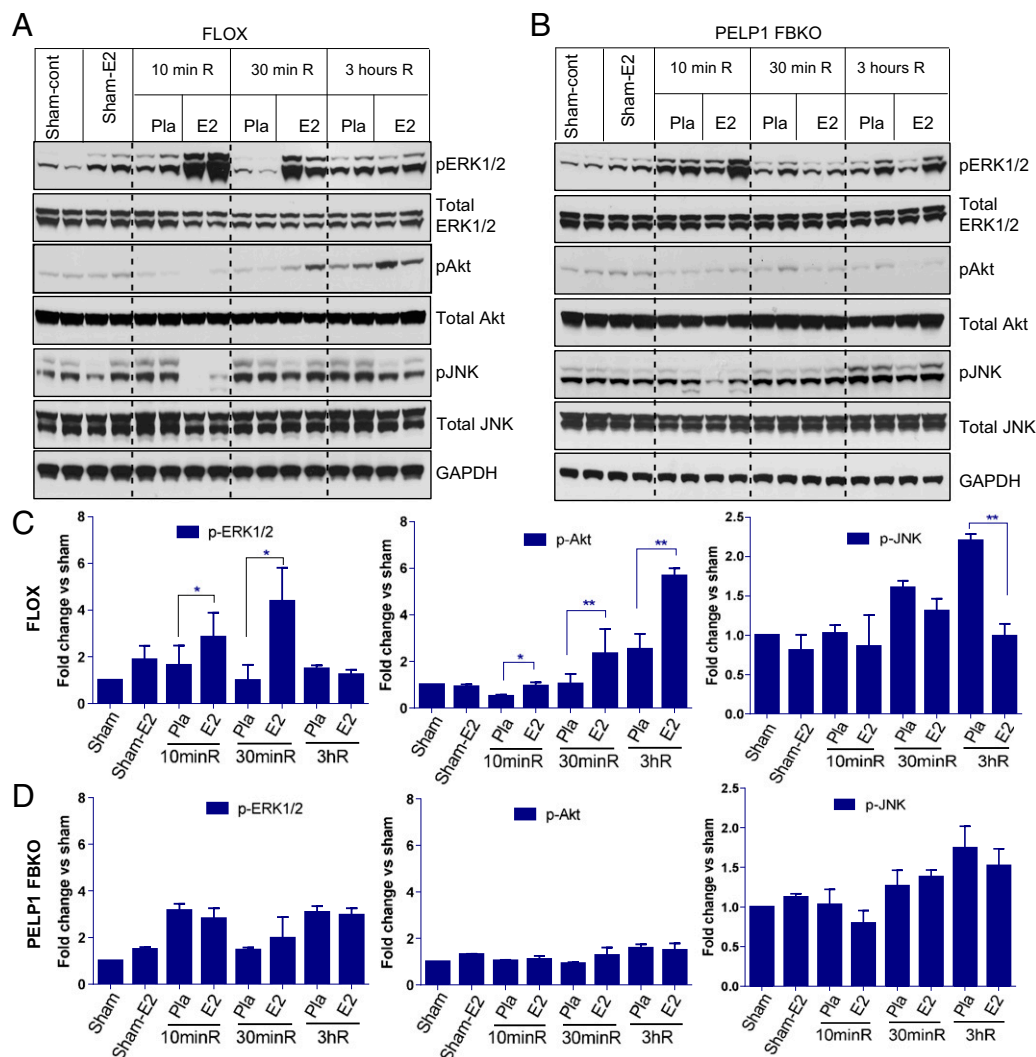


Fig. 2. E2 activation of extracellular survival signaling and inhibition of proapoptotic JNK signaling is significantly attenuated in PELP1 FBKO mice. (A–D) Hippocampal lysates obtained from sham, sham E2, and at 10 min, 30 min, and 3 h after GCI/reperfusion from placebo and E2-treated ovariectomized female FLOX control mice (A and C) and PELP1 FBKO mice (B and D) were subjected to Western blotting. Blots were probed with phospho- and total ERK1/2, Akt, and JNK antibodies. GAPDH was used as internal control. Pla = placebo; R = reperfusion. Bar graphs represent mean \pm SEM. * $P < 0.05$; ** $P < 0.01$ versus sham. $n = 4$ –5 mice per group.

However, the ability of E2 to attenuate β -catenin phosphorylation was lost in PELP1 FBKO mice (Fig. 3 B and D). Interestingly, after GCI, phosphorylation of β -catenin was increased more in PELP1 FBKO mice (two- to threefold increase vs. sham) compared with FLOX control mice (1.5–1.7-fold increase vs. sham) (Fig. 3 C and D). Immunohistochemical analysis further demonstrated that E2 increases phosphorylation of GSK3 β and decreases phosphorylation of β -catenin in the hippocampal CA1 regions of FLOX control mice at 30 min after reperfusion (Fig. S4A). However, no substantial changes in the phosphorylation of GSK3 β and β -catenin were observed between E2 and placebo groups in PELP1 FBKO mice (Fig. S4B). These results suggest that PELP1 is essential for E2-mediated GSK3 β inactivation and further activation of β -catenin signaling under conditions of cerebral ischemia.

PELP1 is a Novel Substrate of GSK3 β . Emerging evidence suggests that PELP1 functions as multifunctional scaffolding protein that interacts with many transcriptional factors, including ER α and chromatin-modifying factors. However, the components of the PELP1 interactome under conditions of cerebral ischemia remain unknown. To identify PELP1-interacting proteins, we performed mass spectrometry analysis of PELP1 immunoprecipitates obtained

from hippocampal lysates that were subjected to GCI (24 h reperfusion). We found several cytosolic PELP1 binding proteins that play an important role in neuronal survival and cell death as novel PELP1 interacting proteins (Fig. 4A). Interestingly, GSK3 β is one of the proteins that interacted with PELP1 during cerebral ischemia. Because of its established role in cerebral ischemia, we focused on the PELP1–GSK3 β interaction in subsequent studies. We confirmed the binding of PELP1 to GSK3 β by performing an immunoprecipitation assay using a PELP1 antibody and by performing a GST pull-down assay using GST–PELP1. Results from both assays confirmed that GSK3 β specifically interacts with PELP1 (Fig. 4B). To map the region of PELP1 binding, we performed GST pull-down assays by using various PELP1–GST deletion fragments (Fig. S5). The results showed that GSK3 β binds to the 400–600-aa region of PELP1 (Fig. 4C). Minor interaction of GSK3 β with the 1–400-aa region of PELP1 was also observed. Close examination of the amino acid sequence of PELP1 revealed several GSK3 β phosphorylation consensus S/TXXXXS/T motifs. To examine whether PELP1 is a novel substrate of GSK3 β , we performed an *in vitro* kinase assay using both GST–PELP1 and various GST-deletion fragments of PELP1. Our results demonstrated that PELP1 indeed is phosphorylated by GSK3 β (Fig.

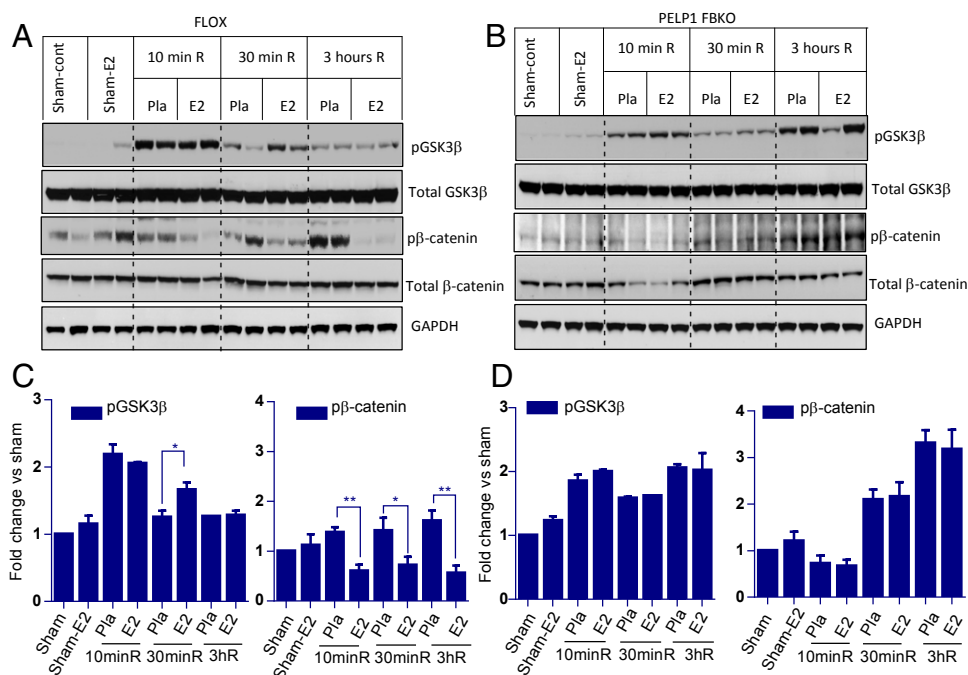


Fig. 3. E2-mediated enhancement of p-GSK3 β and reduction of p- β -catenin is significantly attenuated in PELP1 FBKO mice. (A–D) Hippocampal lysates obtained from sham, sham E2, and at 10 min, 30 min, and 3 h after GCI/reperfusion from placebo and E2 treated ovariectomized female FLOX control mice (A and C) and PELP1 FBKO mice (B and D) were subjected to Western blotting to detect changes in the levels of p-GSK3 β and p- β -catenin. GAPDH was used as an internal control. Representative blots show the attenuation of E2-mediated effects in PELP1 FBKO mice. *Pla* = placebo; *R* = reperfusion (C and D). Bar graphs represent mean \pm SEM. * P < 0.05; ** P < 0.01 versus sham. n = 4–5 mice per group.

4D). Mapping studies revealed that GSK3 β phosphorylates PELP1 at two different residues (Thr⁷⁴⁵ and Ser¹⁰⁵⁹; Fig. 4D). The proposed locations for GSK3 β binding and phosphorylation

sites in the PELP1 protein are illustrated in Fig. S6A. Collectively, these studies identified PELP1 as a novel interacting protein and a substrate of GSK3 β .

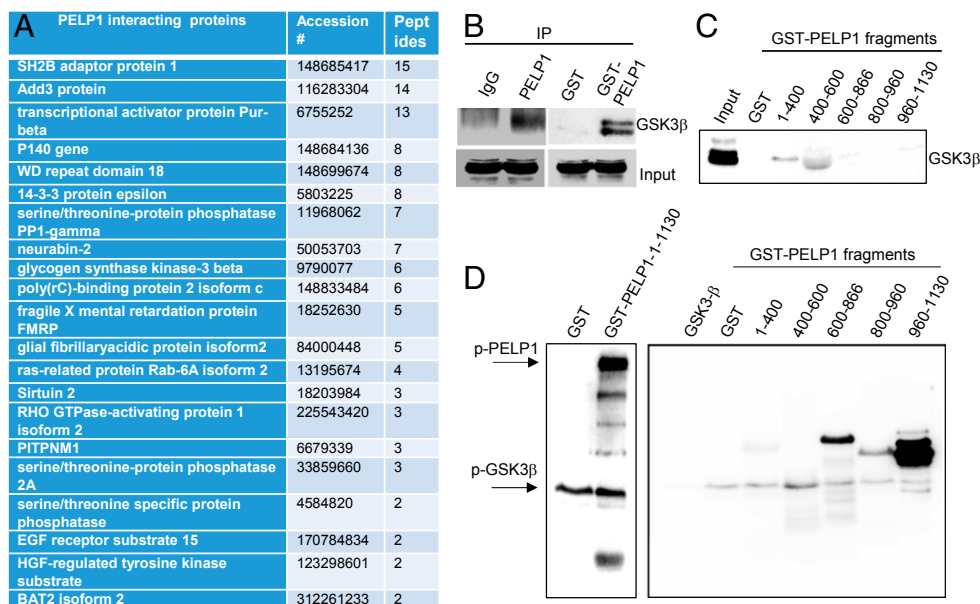


Fig. 4. PELP1 is a novel substrate of GSK3 β . Hippocampal lysates collected 24 h after GCI reperfusion were subjected to immunoprecipitation with PELP1 or IgG antibody. Immunoprecipitates were pulled down by adding dynabeads protein A. Immunoprecipitates were subjected to SDS/PAGE, followed by mass spectrometry analysis. (A) List of unique PELP1-interacting proteins. (B) Forebrain lysates were subjected to immunoprecipitation or GST pull-down assay, and PELP1 interaction with GSK3 β was confirmed by Western blotting. (C) Forebrain lysates were used in pull-down assays using GST or various deletion fragments of PELP1, and interaction of PELP1 deletions with GSK3 β was analyzed by Western blotting. (D) GST-tagged PELP1 deletions and full-length GST-PELP1 were incubated with purified GSK3 β enzyme in kinase buffer with [³²P] ATP and 100 μ M cold ATP for 30 min at 30 $^{\circ}$ C, and the phosphorylation of PELP1 fragments was analyzed by PhosphorImager.

GSK3 β -Mediated Phosphorylation of PELP1 Leads to Its Degradation.

To further demonstrate the effect of E2 on the interaction of GSK3 β and PELP1, we examined their interaction under the conditions of GCI (placebo), as well as after E2 treatment, using an *in vivo* DuoLink proximity ligation assay. The results from this assay demonstrated that GCI significantly increases the interaction of PELP1 with GSK3 β in the hippocampal CA1 region compared with sham, whereas E2 treatment significantly decreased their interaction (Fig. 5A). Earlier published studies demonstrated that GSK3 β phosphorylates and regulates the stability of many of its substrates. Because GSK3 β activity is altered after ischemia, and because PELP1 is phosphorylated by GSK3 β *in vitro*, we tested whether GSK3 β phosphorylation of PELP1 also regulates its stability. Although the mapping studies using human PELP1 identified two GSK3 β sites, only Thr⁷⁴⁵ is conserved in mouse. Therefore, to demonstrate that GSK3 β phosphorylates PELP1 *in vivo*, we generated a novel, affinity-purified phospho-Thr⁷⁴⁵ PELP1 antibody. Antibody specificity was confirmed by competing with blocking peptide that is specific to the Thr⁷⁴⁵ site (Fig. S6 B and C). To better understand the functional relevance of phosphorylation of PELP1 by GSK3 β , we inhibited the GSK3 β activity, using lithium chloride in two different cell models (glioblastoma, U87, and neuroblastoma, SH-SY5Y) and analyzed the levels of phospho-Thr⁷⁴⁵ PELP1. Western blot analysis of total

lysates indicated that lithium chloride treatment significantly reduced the phosphorylation of PELP1, and total PELP1 levels were simultaneously stabilized (Fig. 5B). To examine whether such regulation occurs *in vivo*, we studied the total PELP1 levels at 6 d reperfusion after GCI in the hippocampus by immunofluorescence analysis. The results showed that PELP1 levels were significantly reduced in the placebo group; however, E2 significantly restored the PELP1 levels in hippocampus (Fig. 5C). Collectively, these results suggest that PELP1 is a novel substrate of GSK3 β and that phosphorylation of PELP1 by GSK3 β at Thr⁷⁴⁵ leads to its degradation, and that E2 has a protective effect of restoring the PELP1 levels by blocking GSK3 β activation.

PELP1 Is Needed for Optimal Activation of E2-Regulated Genes After GCI.

E2-mediated neuroprotective effects involve both nuclear and extranuclear functions of ER (4). PELP1 functions as coregulator of ER and can modulate both extranuclear and nuclear functions of ER in cancer cells (25, 28). To examine the role of PELP1 in E2-ER-mediated transcription in the hippocampus under conditions of GCI, we performed global transcriptome analysis. E2-treated FLOX control, and PELP1 FBKO mice were subjected to GCI, followed by 24 h reperfusion, and the isolated RNA was used for RNA-seq analysis. The genes that showed at least twofold changes ($P < 0.01$) were chosen for analysis. Overall, 229 genes were differentially expressed in PELP1 FBKO mice compared with FLOX control mice, and among those, 167 genes were up-regulated and 62 genes were down-regulated. A representative heat map is shown in Fig. 6A. The complete list of differentially expressed genes is available at the Gene Expression Omnibus database under accession number GSE72136. To further explore the biological significance of differentially expressed genes in PELP1 FBKO mice, we performed pathway analysis using ingenuity pathway analysis software. The top five networks that were significantly altered in PELP1 FBKO mice include tissue development and function, cellular assembly and organization, neurological disease, and inflammatory disease (Figs. S7A and S8). Some of the top ingenuity pathway analysis canonical pathways that were significantly altered in PELP1 FBKO mice include neuroinflammation/neuronal death (including chemokine signaling, inhibition of matrix metalloproteases, granzyme A signaling) and Wnt pathway. Other pathways regulated by PELP1 were related to activation of FXR/RXR, LXR/RXR, and ketogenesis (Fig. 6B). Further analysis of molecular and cellular functions of PELP1-regulated genes revealed that they are involved in cellular movement, cell-to-cell signaling interaction, cell death, survival, and DNA repair (Fig. S7B). We used quantitative RT-PCR (qRT-PCR) to validate the expression of some of the top differentially expressed genes involved in inflammation, cell death, survival, cellular function, and signaling, as well as some of the important Wnt pathway target genes in FLOX and PELP1 FBKO mice (Fig. 6C). The results suggested that the β -catenin pathway is one of the major pathways activated by E2 after GCI, which is attenuated in PELP1 FBKO mice.

E2-Mediated Neuroprotection Is Compromised in PELP1 FBKO Mice.

We, and others, have shown previously that E2 exerts neuroprotective functions after global cerebral ischemia (38, 39). We therefore examined the effect of PELP1 knockdown on E2-mediated neuroprotection in the hippocampal CA1 region after GCI. Tissue sections collected at 6 d after GCI were subjected to cresyl violet and NeuN staining. Fig. 7A shows the representative images of the cresyl violet staining and NeuN staining in the hippocampal CA1 region at 6 d reperfusion after GCI in placebo- and E2-treated ovariectomized FLOX control and PELP1 FBKO mice. Sham animals are included as nonischemic controls. The results showed that in FLOX control animals, GCI (placebo) resulted in a significant reduction in the number of surviving neurons, as evidenced by the nonviable cells with condensed, pyknotic, and shrunken

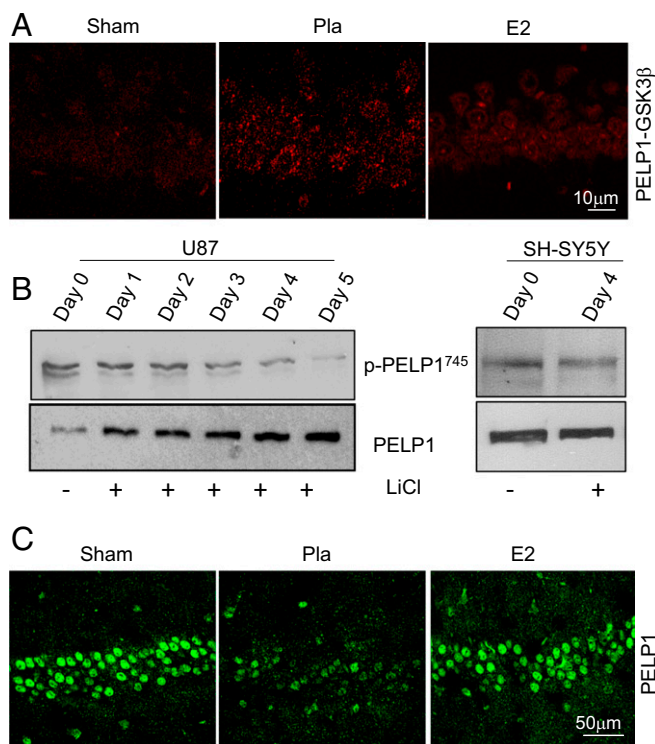


Fig. 5. GSK3 β phosphorylation of PELP1 results in reduced stability of PELP1. (A) Tissue sections derived from sham, placebo, and E2-treated mice at 24 h after GCI were blocked in 5% (vol/vol) goat serum for 1 h and incubated overnight with appropriate primary antibodies at 4 °C. Slides were then incubated with DuoLink PLA Rabbit MINUS and PLA Mouse PLUS proximity probes for 1 h at 37 °C. Ligation and amplification were carried out using the DuoLink detection reagent kit according to manufacturer's protocol. Images were captured using a confocal microscope. (B) Glioblastoma (U87) and neuroblastoma (SH-SY-5Y) cells were treated with GSK3 β inhibitor lithium chloride, and lysates were collected at the indicated periods. Lysates were subjected to Western blot analysis with ⁷⁴⁵P-PELP1 and total PELP1 antibodies. (C) Coronal brain sections collected from sham, placebo, and E2-treated FLOX mice 6 d after GCI reperfusion were subjected to immunofluorescence for PELP1 antibody.

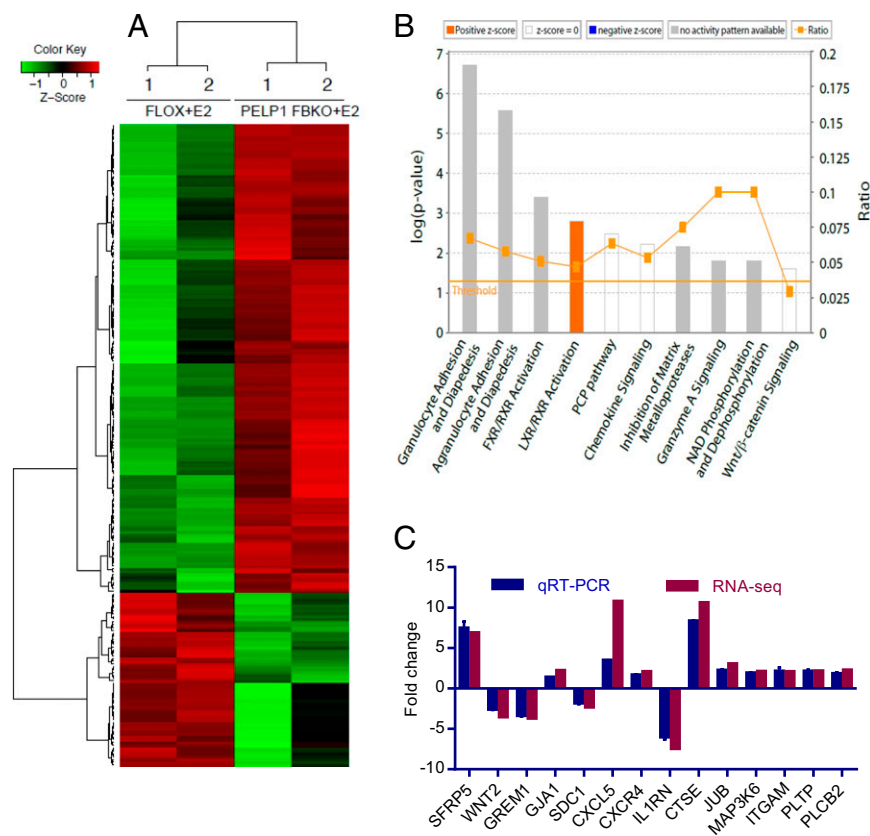


Fig. 6. PELP1 FBKO mice have alterations in E2-induced transcriptome. Total RNA was isolated from ovariectomized FLOX control mice and PELP1 FBKO mice (implanted with E2 mini pumps) that were subjected to GCI and killed at 24 h reperfusion. Hippocampi samples were subjected to RNA-seq analysis, as detailed in Methods. Differential expression analysis was performed by Dseq, and significant genes with at least twofold change were chosen for analysis. (A) Representative heat map of differentially expressed genes from two groups. (B) Differentially expressed genes with greater than twofold change in expression were further analyzed by ingenuity pathway analysis. The top canonical pathways are shown. (C) Selected differentially expressed genes were further validated by quantitative RT-PCR; RNA-seq expression values are plotted side by side for comparison.

nuclei in the CA1 region compared with the sham control group. However, E2 treatment exerted significant neuroprotection in the CA1 region after GCI, as evidenced by dense rounded neuron cells similar to CA1 pyramidal cells. These results were further confirmed by NeuN positive staining (Fig. 7 *A* and *B*). Interestingly, PELP1 FBKO mice had increased sensitivity to GCI damage, as evidenced by a decreased number of surviving neurons in PELP1 FBKO (Pla) mice compared with FLOX (Pla) control mice (Fig. 7 *A* and *B*). Furthermore, in PELP1 FBKO mice, E2-mediated neuroprotection is markedly attenuated, as evidenced by a decreased number of surviving neurons and NeuN-positive cells in the hippocampal CA1 region (Fig. 7 *A* and *B*). In addition, use of an alternative knockdown approach (central administration of PELP1 antisense oligonucleotides in the ovariectomized rat) further confirmed the critical role of PELP1 in mediating E2 neuroprotection after GCI (Fig. S9). Taken together, these results demonstrate that PELP1 is critical for the ability of E2 to induce neuroprotection after GCI.

E2-Mediated Cognitive Function Is Lost in PELP1 FBKO Mice. To investigate the importance of PELP1 in E2-mediated cognitive functional recovery after GCI, spatial learning and memory were tested in the Barnes maze, as previously described (40). Both FLOX control and PELP1 FBKO mice were subjected to sham, placebo + GCI, or E2 + GCI treatment, and cognitive testing was initiated 24 after sham/GCI and continued for 4 d, with a probe trial on day 5 after reperfusion. As shown in Fig. 7C, primary latency to locate the correct escape hole during the

probe trial was significantly increased by GCI in both FLOX control and PELP1 FBKO mice. E2 treatment rescued the GCI-induced deficit only in FLOX control mice, whereas no difference was observed between placebo and E2 treatment in PELP1 FBKO mice. Further, as shown in Fig. 7D, FLOX control mice subjected to GCI spent less time in the quadrant of the maze where the escape hole was located compared with sham. E2 treatment significantly rescued this deficit during the probe trial, whereas E2-mediated effect was abolished in PELP1 FBKO mice. However, in the open field test, we did not find any significant differences in distance traveled between PELP1 FBKO and FLOX control mice after GCI, suggesting motor deficits were not responsible for the differences in performance on the Barnes maze (Fig. S10). Finally, central administration of PELP1 antisense oligonucleotides in the ovariectomized rat further confirmed the PELP1 FBKO results, as PELP1 antisense (AS) knockdown animals showed a loss of E2-induced cognitive enhancement after GCI as measured, using the Morris water maze (Fig. S11 *A–D*). In contrast, missense (MS) oligonucleotides did not affect the ability of E2 to enhance cognitive function after GCI. Collectively, these results suggest a critical role for PELP1 in mediating the cognitive-enhancing effects of E2 after cerebral ischemia.

Discussion

PELP1 was initially identified by our group as a coactivator of ER α in cancer cells, and it was suggested that PELP1 functions as a scaffolding protein to facilitate both genomic and extranuclear functions of ER. Further work revealed that PELP1 is

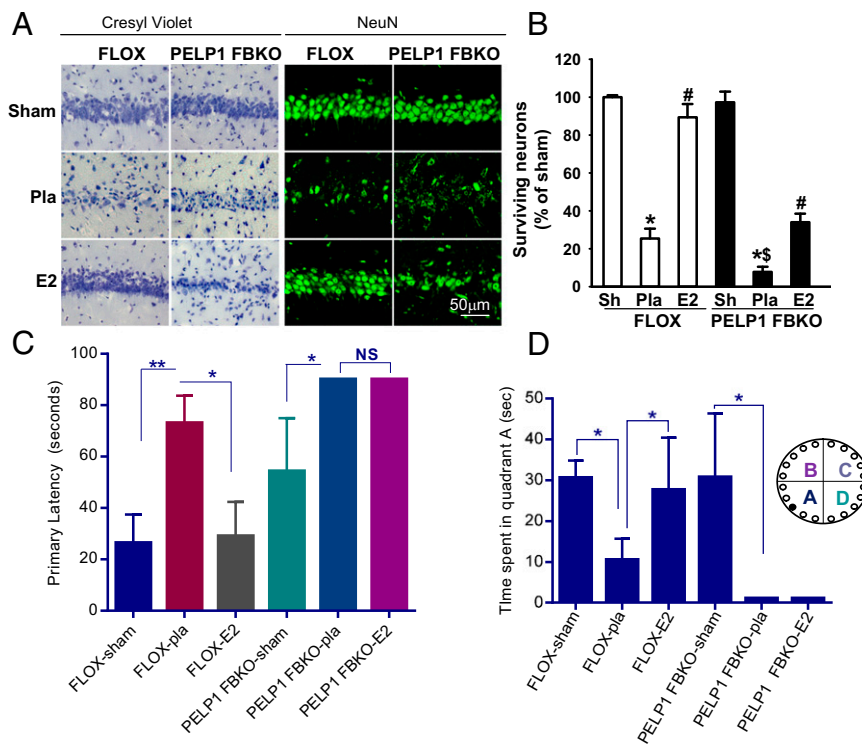


Fig. 7. PELP1 FBKO mice display diminished E2-induced neuroprotection: Coronal brain sections collected 6 d after GCI-reperfusion from sham, placebo, and E2-treated female ovariectomized FLOX control and PELP1 FBKO mice were subjected to cresyl violet and NeuN immunostaining, as described in *Methods*. (A) Representative image of hippocampal CA1 region showing significant neuroprotection with E2 treatment, as evident from the significant increase in the number of surviving neurons compared with placebo in FLOX control mice. (B) Surviving neuronal counts of NeuN-positive neurons are shown. * $P < 0.05$ versus sham; # $P < 0.05$ versus placebo; $^{\$}P < 0.05$ versus FLOX placebo. (C) Spatial learning and memory were assessed in sham, placebo, and E2-treated female ovariectomized FLOX and PELP1 FBKO mice that were subjected to GCI, using the Barnes maze test, as described in *Methods*. (D) Time spent in each quadrant was quantified for all treatment groups. Data analysis indicated that PELP1 FBKO mice exhibited impaired E2-mediated spatial learning and memory. Data represent mean \pm SE, $n = 4-7$ mice per group. * $P < 0.05$ and ** $P < 0.01$.

expressed in many tissues, with the highest expression noted in the brain (25, 26). Despite it being highly expressed in the brain, virtually nothing is known regarding the role and importance of PELP1 in this key tissue. The current study provides the first *in vivo* molecular evidence, to our knowledge, that PELP1 is essential for optimal neuroprotective functions of E2. Our study implicates an important role of PELP1 in E2-mediated neuroprotective actions, including its role in rapid extranuclear signaling; anti-inflammatory effects; induction of survival signaling, including Wnt/ β -catenin pathway; facilitation of E2 mediated neuroprotection; and improvement in cognitive function. Collectively, these findings suggest PELP1 is essential for optimal E2-mediated functions in the brain under conditions of ischemic injury.

E2 has been implicated to regulate multiple functions in the brain, including synaptic plasticity, adult neurogenesis, reproductive behavior, and cognition. Work from many laboratories, including our own, has provided abundant evidence that E2 serves as a neuroprotective factor in neurodegenerative disorders, including cerebral ischemia (4). Recent studies demonstrated that E2 mediates rapid extranuclear signaling by activating the neuroprotective ERK and PI3K signaling pathways, as well as the inhibition of proapoptotic JNK signaling (17, 22, 23); however, the mechanism or mechanisms by which E2 facilitates these activities are poorly understood. We previously showed that PELP1 is a multidomain scaffold protein that interacts with ERs, using the nuclear receptor interaction motif LXXLL; Src kinase, using SH2 motifs; and PI3K, using PXXP motifs. In the current study, proximity ligation assays confirmed complex formation of PELP1 with ER α , Src, and PI3K in the hippocampus after GCI, which was markedly enhanced by E2 treatment. The importance of this in-

teraction was confirmed using a conditional PELP1 FBKO mouse model, which showed that PELP1 plays a key role in E2's ability to induce robust activation of p-ERK and p-Akt signaling pathways, and to inhibit activation of the JNK signaling pathway in the hippocampus after GCI. It is important to note that antisense oligonucleotide knockdown of PELP1 in the rat further confirmed the PELP1 FBKO results by also demonstrating a critical role for PELP1 in E2-mediated kinase pathway activation, neuroprotection, and cognitive recovery after GCI.

GSK3 β has been implicated in the pathology of many neurodegenerative disorders (41), and inhibition of GSK3 β has been shown to be neuroprotective (11, 42). We previously showed that GSK3 β inhibition leads to a reduction in Tau phosphorylation and regulates the stability of β -catenin by phosphorylation (11). In the current study, after GCI, we observed that E2 increased phosphorylation of GSK3 β on residue Ser⁹, which is known to lead to its inactivation (43). However, E2's ability to modulate phosphorylation of GSK3 β is lost in PELP1 FBKO mice. GSK3 β modulates the Wnt/ β -catenin pathway via regulating the stability of β -catenin by phosphorylating at Ser^{33/37} and Thr⁴¹ after priming by CK1 α at Ser⁴⁵. It has been shown that E2-mediated β -catenin stability occurs through an ER α -PI3K-Akt-GSK3 β signaling cascade (44, 45). In this study, we found that E2 reduces the phosphorylation of β -catenin after GCI reperfusion in E2-treated FLOX control mice; however, in PELP1 FBKO mice, E2-mediated attenuation of β -catenin phosphorylation was abolished. These findings suggest that PELP1 scaffolding function is critical for formation of an ER α -PI3K-Akt-GSK3 β - β -catenin multimolecular complex that leads to the activation of further downstream signaling (44, 46). PELP1 FBKO mice also had a

higher induction of β -catenin phosphorylation in the hippocampus after GCI compared with FLOX control mice. This finding may explain, in part, our observation of a greater sensitivity of PELP1 FBKO mice to ischemic damage after GCI.

Mass spectrometry data revealed that PELP1 interacts with several proteins under conditions of GCI/reperfusion. These studies identified for the first time, to our knowledge, that GSK3 β is one of the novel PELP1-interacting proteins. As a kinase, GSK3 β phosphorylates several proteins in vivo, including β -catenin, and regulates a wide range of cellular processes such as cell growth, differentiation, and survival. GSK3 β phosphorylates on the consensus sequence Ser/ThrXXXSer/Thr-P, where X is any residue (35, 36). PELP1 contains several GSK3 β phosphorylation consensus sites, and our deletion studies identified that PELP1 is phosphorylated by GSK3 β at hThr⁷⁴⁵ (m⁷⁵¹) and hSer¹⁰⁵⁹. Because Thr⁷⁴⁵ is conserved among many species, including mice, we generated a phospho-specific antibody for hThr⁷⁴⁵ (mThr⁷⁵¹). Using this unique reagent (p745), we confirmed that E2 significantly reduced the phosphorylation of PELP1 at p745 by modulating GSK3 β activation. Accordingly, proximity ligation assays demonstrated that the in vivo interaction of PELP1 and GSK3 β was reduced after E2 treatment. Furthermore, our results also demonstrated that inhibition of GSK3 β results in decreased phosphorylation of PELP1 at p745 and stabilization of PELP1 in vitro. We also observed reduction in PELP1 levels in the hippocampus after 6 d of GCI/reperfusion, which was reversed by E2 treatment. Finally, global profiling of phosphoproteins in the developing brain also identified phosphorylation of PELP1 at Thr⁷⁴⁵ in brain tissue, further confirming the physiological relevance of p745 of PELP1 in the brain (47). Collectively, these results establish PELP1 as a novel substrate of GSK3 β and suggest the possibility of an autocrine loop involving E2-ER α -GSK3 β signaling in modulating the levels of PELP1 at the site of neuronal damage.

Work in cancer cells has shown that PELP1 is an important coactivator of ER α that facilitates ER α target gene expression by modulating the activities of several chromatin modifiers, including histone acetyl transferase (creb-binding protein/p300) (26, 29), histone deacetylases (HDAC2) (48), and histone demethylase (KDM1A) (30). PELP1 is widely expressed in neuronal tissues, but little is known about PELP1-regulated genes and pathways in the brain. Our studies using global RNA-sequencing revealed several pathways that are directly or indirectly regulated by PELP1 in the presence of E2. The top PELP1-regulated pathways include several immunological, inflammatory, metabolic pathways and survival pathways. Interestingly, canonical and noncanonical Wnt pathways were also differentially expressed in FLOX control and PELP1 FBKO mice and are known to play vital roles in E2-mediated neuronal survival (11, 49). In an earlier RNA-seq study using in vitro breast epithelial model cells, we found that PELP1 has the potential to regulate multiple signaling pathways, including

oxidative stress response, DNA repair, protein ubiquitination, and p53 (50). Our current RNA-seq results using PELP1 FBKO mouse tissues also confirm PELP1's ability to modulate multiple pathways in the brain after ischemic injury, thus extending the list of PELP1-regulated genes to include immunological, inflammatory, and metabolic pathways.

Postischemic inflammation in the brain plays a key role in tissue injury and repair (51, 52). Several studies showed that apart from its neuroprotective functions, E2 also exhibits immunomodulatory effects (53, 54). The results from RNA-seq analysis conducted in this study showed that inflammatory pathways are among the top enriched pathways that are differentially expressed between E2-treated FLOX control and PELP1 FBKO mice after GCI. Robust activation of chemokines and cytokines contributes either to cerebral damage or neuroprotection, depending on their concentration and time of release (55–58). Previous studies showed that E2 could inhibit production of the neutrophil chemoattractants CXCL1, CXCL2, and CXCL3, and secretion of proinflammatory cytokines, such as IL-1 β , IL-6, and TNF α (53, 54). Our RNA-seq analysis demonstrated that several chemokines, including CXCL5 and CXCR4, were up-regulated, whereas ccl9, ccl2, ccl7, CXCL10, and MMP12 were down-regulated in PELP1 KO mice. In addition, we also found that interleukin-1 receptor antagonist (IL1RN) was down-regulated in PELP1 FBKO mice. However, additional detailed studies are needed to better understand the role of PELP1 in modulating genes involved in inflammation in the ischemic brain. In conclusion, the current study provides the first direct in vivo evidence, to our knowledge, of an essential role for PELP1 in E2-mediated rapid extranuclear signaling, genomic signaling, neuroprotection, and cognitive function in the brain. As such, it significantly advances our understanding of how E2 exerts its important actions in the brain and sets the stage for future studies to further unlock the mysteries of this key hormone.

Materials and Methods

Details regarding animals, PELP1 FBKO mouse generation, antibodies and reagents, two-vessel occlusion global cerebral ischemia animal model, E2 replacement, histology, immunohistochemistry, mass spectrometry, immunoprecipitation, confocal analysis, Western blot analysis, RNA Seq and qRT-PCR, Barnes maze, and statistical analysis are contained in *SI Materials and Methods*. All procedures used were approved by the University of Texas Health Science Center at San Antonio (UTHSCSA) Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines for animal research.

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