Tip60- and sirtuin 2-regulated MARCKS acetylation and phosphorylation are required for diabetic embryopathy

Penghua Yang1, Cheng Xu1, E. Albert Reece1,2, Xi Chen1, Jianxiang Zhong1, Min Zhan3, Deborah J. Stumpo4, Perry J. Blackshear4,5 & Peixin Yang1,2

Failure of neural tube closure results in severe birth defects and can be induced by high glucose levels resulting from maternal diabetes. MARCKS is required for neural tube closure, but the regulation and of its biological activity and function have remained elusive. Here, we show that high maternal glucose induced MARCKS acetylation at lysine 165 by the acetyltransferase Tip60, which is a prerequisite for its phosphorylation, whereas Sirtuin 2 (SIRT2) deacetylated MARCKS. Phosphorylated MARCKS dissociates from organelles, leading to mitochondrial abnormalities and endoplasmic reticulum stress. Phosphorylation dead MARCKS (PD-MARCKS) reversed maternal diabetes-induced cellular organelle stress, apoptosis and delayed neurogenesis in the neuroepithelium and ameliorated neural tube defects. Restoring SIRT2 expression in the developing neuroepithelium exerted identical effects as those of PD-MARCKS. Our studies reveal a new regulatory mechanism for MARCKS acetylation and phosphorylation that disrupts neurulation under diabetic conditions by diminishing the cellular organelle protective effect of MARCKS.

1Department of Obstetrics, Gynecology & Reproductive Sciences, University of Maryland School of Medicine, Baltimore 21201 MD, USA. 2Department of Biochemistry & Molecular Biology, University of Maryland School of Medicine, Baltimore 21201 MD, USA. 3Department of Epidemiology and Public Health, University of Maryland School of Medicine, Baltimore 21201 MD, USA. 4Signal Transduction Laboratory, National Institute of Environmental Health Sciences, Research Triangle Park, Durham, NC 27709, USA. 5Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, NC 27710, USA. Correspondence and requests for materials should be addressed to P.Y. (email: pyang@fpi.umaryland.edu)
Neurulation is a process occurring during early embryonic development in which the developing neuroepithelium is folded into the neural tube, which is the primitive form of the central nervous system (CNS). Failed neural tube closure leads to neural tube defects (NTDs), which are severe structural birth defects affecting offspring mortality and morbidity1–3. The high glucose (HG) level in maternal diabetes induces NTD formation in both humans and animal models4–7. Mitochondrial dysfunction and endoplasmic reticulum (ER) stress in the developing neuroepithelium have been demonstrated to be critically involved in NTD formation in diabetic pregnancies8–12. However, the mechanism underlying cellular organelle stress in diabetic embryopathy is unclear. During neurulation, neuroepithelial cells, which are essentially neural stem cells, undergo rigorous proliferation and migration to acquire the competence for neural plate elevation, neural fold convergence, extension, and closure. Therefore, neuroepithelial cells may possess mechanisms for the protection of their organelles to ensure cellular homeostasis. Maternal diabetes may disrupt these mechanisms, leading to cellular organelle stress and NTD formation.

Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) is required for neurulation, and deleting the Marcks gene results in NTDs, mainly exencephaly13. MARCKS protein modification is critical for its biological function. Protein kinase C (PKC) phosphorylates MARCKS, which converts MARCKS from a membrane-bound protein to a cytoplasmic protein14. PKCs mediate the cellular stress response because deleting one of the PKC isoforms, i.e., the Prkcd gene, abolishes maternal diabetes-induced cellular organelle stress in embryos during the neurulation stage15. This evidence suggests that PKC triggers cellular stress by suppressing the protective effects of MARCKS on cellular organelle. However, the predicted cellular organelle protective effect of MARCKS has never been previously demonstrated.

Lysine acetylation is another protein modification that influences the biological action of proteins. The crosstalk between acetylation and phosphorylation in amino acid residues of the same protein has been revealed in transcription factors16,17. The acetylation of a transcription factor can positively or negatively regulate the phosphorylation of the same protein, leading to increased or decreased activity of that transcription factor. Although MARCKS is not a transcription factor, it may be regulated by acetylation. In a large proteomic study, the lysine residue lysine 172 of human MARCKS was identified as an acetylation site18. Lysine 172 of MARCKS is adjacent to four serine residues that are often modulated by phosphorylation19. If MARCKS is regulated by acetylation, it could be interesting to determine whether MARCKS acetylation impacts its phosphorylation.

Since its discovery, histone acetylation has become a well-known euchromatin marker of the activation of gene transcription20. Various enzymes involved in histone acetylation, i.e., histone acetyltransferases (HAT), and histone deacetylation (HDAC), i.e., histone deacetylases, have been discovered. Currently, it is recognized that these histone enzymes also acetylate or deacetylate nonhistone proteins21. HATs and HDACs are substrate-specific22. If MARCKS is acetylated, a specific HAT should pair with an HDAC to regulate MARCKS acetylation. Among the HATs acetylating nonhistone proteins, Tat-interactive protein 60 (Tip60) is activated by cellular stress23 and induces DNA damage responses24, which are manifested in diabetes-induced NTDs, suggesting that Tip60 may mediate the cellular stress response in diabetic embryopathy by acetylating protein substrates. In contrast, the seven sirtuin HDAC family members (SIRT1-7) suppress cellular stress by deacetylating nonhistone proteins17,25. Therefore, we hypothesize that maternal diabetes induces cellular organelle stress by decreasing SIRT expression, thereby increasing the acetylation of their protein substrates.

Here, we report that MARCKS is regulated by acetylation through Tip60 and SIRT2. We also show that maternal diabetes-induced MARCKS acetylation is required for its phosphorylation, which disables the protective effects of MARCKS on mitochondrial and the ER, leading to cellular organelle stress and NTD formation. Preventing the phosphorylation of MARCKS under hyperglycemic conditions either with PD-MARCKS or SIRT2 overexpression restores the protective effects of MARCKS on the neuroepithelium. Thus, we provide mechanistic insight into the essentiality of MARCKS in neurulation.

Results

MARCKS acetylation is required for its phosphorylation. Hyperglycemia in maternal diabetes increases histone acetylation in the developing embryo26. Because acetylation in a lysine residue of MARCKS has been detected in human tissues in a global acetylome analysis18, we sought to determine whether HG in vitro or maternal diabetes in vivo induces MARCKS acetylation. In the mouse neural stem cell line C17.2, HG levels mimic the key adverse effects of maternal diabetes, including cellular stress, apoptosis, and histone acetylation, in neuroepithelial cells9,26–28. The HG levels triggered MARCKS acetylation in a dose-dependent and time-dependent manner in the C17.2 cells (Fig. 1a, b), whereas mannitol, which is used for the osmotic control of glucose, had no effect on MARCKS acetylation (Supplementary Figure 1A). Consistent with the effect of HG observed in vitro, maternal diabetes increased MARCKS acetylation during the neurulation stage in embryos in vivo (Fig. 1c).

The acetylation site of human MARCKS is lysine 172 (K172)18, the site of mouse MARCKS acetylation has not been identified to date. Based on the homologous amino acid sequence alignment between the human and mouse MARCKS sequences, lysine 165 (K165) is the putative acetylation site in mouse MARCKS (Supplementary Figure 1B)29. To determine whether HG levels induce MARCKS acetylation in K165, Lysine 165 was mutated into Alanine (A) to prevent acetylation, serving as a dominant negative MARCKS (DN-MARCKS), or into Glutamine (Q) to mimic acetylation, thus serving as a constitutively active form of MARCKS (CA-MARCKS) (Supplementary Figure 1B). The ectopic overexpression of DN-MARCKS blocked the HG-induced acetylation (Fig. 1d), suggesting that DN-MARCKS in the A mutation at K165 competes with endogenous wild-type (WT) MARCKS for acetyltransferase. The blockade of MARCKS acetylation by DN-MARCKS inhibited the HG-induced MARCKS phosphorylation (Fig. 1d). CA-MARCKS mimicked the HG level and induced MARCKS phosphorylation (Fig. 1e). Furthermore, the DN-MARCKS overexpression blocked the constitutively active PKCα-induced MARCKS acetylation and phosphorylation (Fig. 1f). These results suggest that HG levels induce MARCKS acetylation at K165 and that this acetylation is a prerequisite for MARCKS phosphorylation.

Phosphorylated MARCKS dissociates from the mitochondrion. Maternal diabetes induces mitochondrial abnormalities in neuroepithelial cells in the developing embryo; however, the underlying mechanism is unclear. Because MARCKS phosphorylation plays a key role in MARCKS cycling from phospholipid membranes, such as the plasma membrane, to the cytoplasm30, we hypothesize that MARCKS protects neuroepithelial cells from mitochondrial abnormalities and that phosphorylated MARCKS induced by hyperglycemia dissociates from the mitochondrial membrane and does not have a protective effect against the...
hyperglycemic insult exerted by mitochondria-bound MARCKS on cells. To test this hypothesis, mitochondria were purified from wild-type (WT) and MARCKS-PD (phosphorylation dead) transgenic embryos.

Under nondiabetic conditions, both WT MARCKS and MARCKS-PD were enriched in the mitochondrion (Fig. 2a). Maternal diabetes in vivo or HG in vitro significantly reduced the amount of MARCKS in the mitochondrial fraction (Fig. 2a, b). The MARCKS-PD overexpression rescued the MARCKS loss in the mitochondria (Fig. 2a, b). p-MARCKS was exclusively located in the cytosolic fractions (Fig. 2a, b). The immunofluorescence staining confirmed that under normal glucose (NG) or non-diabetic conditions, MARCKS was colocalized with the mitochondrial membrane marker Tom20, whereas p-MARCKS...
induced by HG or maternal diabetes did not overlap with Tom20 (Fig. 2c–e). MARCKS-PD diminished the HG-induced or maternal diabetes-induced p-MARCKS signals and restored the colocalization of MARCKS and Tom20 (Fig. 2c–e). The proximity ligation assay confirmed the interaction between MARCKS and Tom20 under the nondiabetic or NG conditions (Fig. 2f, g), whereas MARCKS-PD restored the interaction between MARCKS and Tom20 disrupted by maternal diabetes or HG (Fig. 2f, g).

To determine the effect of MARCKS-PD on maternal diabetes-induced or HG-induced mitochondrial abnormalities, the structures of the mitochondria were examined by electron microscopy. Maternal diabetes significantly increased the number of defective mitochondria (Fig. 3a), which is consistent with our previous findings. Under the diabetic conditions, the overexpression of MARCKS-PD significantly reduced the number of defective mitochondria (Fig. 3a). The activation of pro-apoptotic members of the Bcl-2 family indicates mitochondrial abnormalities. The mitochondrial enrichment in Puma, Bim, Bak, and Bik was increased in the embryos exposed to maternal diabetes (Fig. 3b, c). The increased mitochondrial translocation of these four Bcl-2 family members by maternal diabetes was abrogated in the embryos with the MARCKS-PD overexpression (Fig. 3b, c). Similarly, MARCKS-PD inhibited the HG in vitro-induced mitochondrial translocation of these four Bcl-2 family members (Fig. 3d, e). Because MARCKS is predominantly expressed in neural tissues, these findings collectively suggest that mitochondrial abnormalities induced by maternal diabetes occurs in the neuroepithelium and that MARCKS protects the neuroepithelium from mitochondrial abnormalities.

Phosphorylated MARCKS detaches from the ER. ER stress is another form of cellular organelle stress involved in the pathogenesis of diabetic embryopathy. We hypothesize that MARCKS is present in the ER membrane and that its dissociation from the ER membrane after phosphorylation caused by maternal diabetes leads to ER stress. The colocalization signal of calnexin, which is an ER membrane marker, and MARCKS in the neuroepithelia of embryos from diabetic dams was significantly lower than that in the embryos from the nondiabetic dams (Fig. 4a). MARCKS-PD overexpression in the neuroepithelium reversed the maternal diabetes-suppressed calnexin-MARCKS colocalization (Fig. 4a). HG levels in vitro also reduced MARCKS localization in the ER, and MARCKS-PD restored the calnexin-MARCKS colocalization under the HG conditions (Fig. 4b). The proximity ligation assay confirmed that MARCKS binds the ER membrane under NG or nondiabetic conditions (Fig. 4c, d). MARCKS-PD prevented the dissociation of MARCKS from the ER induced by HG in vitro and maternal diabetes in vivo (Fig. 4c, d).

To further investigate whether maternal diabetes-disrupted MARCKS localization in the ER causes ER stress in the developing embryo, the unfolded protein response (UPR) pathways IRE1α and PERK were evaluated. The phosphorylation of IRE1α and JNK1/2 was induced by maternal diabetes. MARCKS-PD diminished the phosphorylation of these molecules (Fig. 4e, f). XBP1, which is a downstream effector of IRE1α, was also activated by maternal diabetes and inactivated in the MARCKS-PD transgenic embryos (Fig. 4g). The PERK pathway, including PERK, eIF2α, and CHOP, was activated by maternal diabetes, and MARCKS-PD abrogated the activation of the PERK pathway (Fig. 4h). Similarly, the HG levels activated the IRE1α and PERK pathways, which were suppressed by the MARCKS-PD overexpression in vitro (Supplementary Figure 2A, B). These results indicate that MARCKS can regulate the ER activity status through phosphorylation and that maternal diabetes-induced MARCKS phosphorylation leads to ER stress.

MARCKS-PD reduces maternal diabetes-induced NTD formation. Subsequently, we assessed the functionality of maternal diabetes-induced MARCKS phosphorylation using a transgenic (Tg) mouse line with MARCKS-PD overexpression in the developing neuroepithelium (Fig. 5a). As expected, maternal diabetes increased the phosphorylation of MARCKS in the wild-type embryos (Fig. 5b). However, the phosphorylation of MARCKS was diminished in the MARCKS-PD overexpressing embryos from the diabetic dams (Fig. 5b). The MARCKS phosphorylation impeded its cellular organelle protective effect and led to mitochondrial abnormalities and ER stress, which are critically involved in maternal diabetes-induced embryonic anomalies. Thus, we examined the effect of MARCKS-PD on NTD formation. The NTD rate in the MARCKS-PD overexpressing embryos was significantly lower than that in the wild-type embryos from the diabetic dams and was comparable to that in the embryos from the nondiabetic dams (Fig. 5c, d and Supplementary Table 1). Thus, MARCKS-PD ameliorated maternal diabetes-induced NTDs. Caspase cleavage-triggered neuroepithelial cell apoptosis is observed in diabetes-induced NTDs. MARCKS-PD ablated caspase 3 and caspase 8 cleavage in embryos exposed to diabetes (Fig. 5e, f) and reduced the number of apoptotic neuroepithelial cells in embryos from diabetic dams compared to that in embryos from nondiabetic dams (Fig. 5g).

NTDs can result from genetic insults that cause premature neurogenesis. However, our previous study demonstrated that maternal diabetes delays neurogenesis in the developing neuroepithelium as evidenced by the absence of the expression of TuJ1 (class III β-tubulin), which is a marker of terminal neuronal differentiation, from E8.75 to E9.0 and the enhanced expression of Sox1, which is a marker of neural stem cells. Consistent with our previous findings, TuJ1-expressing neurons were absent, whereas Sox1 expression was enhanced in the E8.75 neuroepithelia from wild-type embryos exposed to maternal diabetes (Fig. 5i). In contrast, TuJ1-expressing neurons were present in the floor plate of the E8.75 MARCKS-PD transgenic embryos from the diabetic dams, and the expression of Sox1 was lower than that in the wild-type embryos from the diabetic dams (Fig. 5i). These results suggest that MARCKS-PD
overexpression in the neuroepithelium restores maternal diabetes-delayed neurogenesis.

**Tip60 interacts with and acetylates MARCKS.** MARCKS acetylation was prerequisite to its phosphorylation (Fig. 1d–i). Subsequently, we sought to identify the acetyltransferase responsible for MARCKS acetylation. Among the histone/protein acetyltransferases that acetylate nonhistone proteins, Tip60 is activated by cellular stress and induces DNA damage responses, which are manifested in diabetes-induced NTDs, suggesting that Tip60 may mediate the cellular stress response in diabetic embryopathy by acetylating protein substrates. A co-immunoprecipitation (CoIP) assay was performed to evaluate the interaction between MARCKS and the three most common protein acetyltransferases, i.e., Tip60, Gcn5, and p300, using embryonic tissue extracts. Maternal diabetes enhanced the interaction between MARCKS and Tip60, whereas MARCKS-
Gcn5 and MARCKS-p300 complexes were not observed (Fig. 6a, Supplementary Figure 3A). Similarly, HG levels increased the formation of MARCKS-Tip60 complexes (Supplementary Figure 3B). These data suggest that Tip60 may be responsible for MARCKS acetylation. The Tip60 siRNA knockdown abrogated the HG-induced MARCKS acetylation (Fig. 6b). Moreover, ectopic Tip60 overexpression mimicked the HG levels and induced MARCKS acetylation (Fig. 6c). Finally, recombinant Tip60 directly acetylated recombinant MARCKS in a noncell system (Fig. 6d, e). Thus, Tip60 is a MARCKS acetyltransferase.
we tested the functionality of SIRT2 overexpression in vivo. We observed that SIRT2 negatively regulates MARCKS acetylation, SIRT2 alleviates maternal diabetes-induced NTDs, and forebrain commissures, and failure of fusion of the cerebral hemispheres. Recent studies have attempted to unravel the functionality of MARCKS in CNS development. MARCKS has a spectrum of neural defects, including exencephaly, omphalocele, and forebrain commissures, and failure of fusion of the cerebral hemispheres. Recent studies have attempted to unravel the functionality of MARCKS in CNS development. MARCKS has a spectrum of neural defects, including exencephaly, omphalocele, and forebrain commissures, and failure of fusion of the cerebral hemispheres. Recent studies have attempted to unravel the functionality of MARCKS in CNS development. MARCKS has a spectrum of neural defects, including exencephaly, omphalocele, and forebrain commissures, and failure of fusion of the cerebral hemispheres. Recent studies have attempted to unravel the functionality of MARCKS in CNS development. MARCKS has a spectrum of neural defects, including exencephaly, omphalocele, and forebrain commissures, and failure of fusion of the cerebral hemispheres. Recent studies have attempted to unravel the functionality of MARCKS in CNS development. MARCKS has a spectrum of neural defects, including exencephaly, omphalocele, and forebrain commissures, and failure of fusion of the cerebral hemispheres.
been demonstrated to be involved in neurite initiation and outgrowth\(^4\), modulate radial glial placement and expansion\(^4\), and promote axon development\(^4\). Abnormalities induced by MARCKS deficiency can be rescued, at least partially, by the following two types of MARCKS mutants: the phosphorylation-dead site mutant MARCKS-PD (serines to asparagines)\(^1\) and the nonmyristoylated MARCKS mutant\(^4\). These findings suggest that membrane-associated MARCKS is required for, while cytoplasmic phosphorylated MARCKS may be detrimental to, CNS development.

Under diabetic conditions, hyperglycemia activates PKC\(\alpha\) and PKC\(\delta\), leading to NTD formation in the developing embryo\(^2\). Both PKC\(\alpha\) and PKC\(\delta\) can induce MARCKS phosphorylation\(^5\). Increased MARCKS phosphorylation is implicated in the etiology of NTD.
Alzheimer’s disease (AD)\textsuperscript{31}, which is another neurological disorder. Amyloid beta, which is the toxic species responsible for our pathological cause, causes MARCKS phosphorylation through PKC\textsuperscript{31}. Our original hypothesis was that MARCKS phosphorylation is the pathological cause of the failure of neural tube closure in embryos derived from diabetic mothers. We observed that NTDs induced by maternal diabetes are inhibited by MARCKS-PD, supporting the causal role of MARCKS phosphorylation in failed neural tube closure in diabetic pregnancies.

MARCKS is associated with the cell plasma membrane and the intracellular membranes of mucin granules\textsuperscript{52}. MARCKS binds the membranes of mitochondria and the ER, and the phosphorylation of MARCKS caused by hyperglycemia causes it to dissociate from these intracellular membranes in embryos during the neurulation stage. Indeed, one proteomic study revealed enrichments from these intracellular membranes in embryos during the neurulation stage. We observed that NTDs induced by maternal diabetes are inhibited by MARCKS-PD, supporting the causal role of MARCKS phosphorylation in failed neural tube closure in diabetic pregnancies.

MARCKS likely maintains the integrity of and protects cellular organelle membranes from stress. During the neurulation stage, cellular organelles in embryos have been shown to be vulnerable to stress insults\textsuperscript{11,55}. Phosphorylated MARCKS mimics the effect of MARCKS deficiency\textsuperscript{56,57}. MARCKS phosphorylation leads to the loss of its function in protecting vascular endothelial cells from oxidative stress\textsuperscript{56}. Thus, phosphorylated MARCKS is an inactive form of MARCKS. MARCKS phosphorylation under diabetic conditions abrogates its protection of cellular organelles.

MARCKS deficiency and phosphorylation mediate oxidative stress-induced vascular endothelial cell abnormalities\textsuperscript{56,57}. Non-phosphorylatable MARCKS abolishes, whereas pseudophosphorylated MARCKS mimics, PKC-induced neuron dysfunction\textsuperscript{58}. This evidence suggests that MARCKS protects cells from stress, and its deficiency or phosphorylation causes cellular stress. Maternal diabetes-phosphorylated MARCKS dissociates from the mitochondrial membrane and the ER, leading to mitochondrial abnormalities and ER stress, which are two causal events in diabetic embryopathy\textsuperscript{11,27}.

Mitochondria are essential for embryogenesis\textsuperscript{59}. Both the inadequate function of mitochondrial folate metabolism and the lack of key mitochondrial redox genes result in NTDs\textsuperscript{60,61}. The overexpression of the mitochondrial antioxidant superoxide dismutase 2 ameliorates maternal diabetes-induced NTDs\textsuperscript{62}. Here, we demonstrate that mitochondrial abnormalities due to MARCKS phosphorylation causes NTDs in diabetic pregnancies. MARCKS-PD blocks MARCKS phosphorylation-induced mitochondrial abnormalities by binding the mitochondrion and ER, leading to NTD reduction under diabetic conditions.

Normal ER function is required for neural tube closure. ER stress activates UPR signaling. The active form of XBP1, which is a major downstream effector of IRE1\textalpha, induces NTDs in Xenopus\textsuperscript{63,64}, suggesting that the IRE1\textalpha-ER stress pathway is highly relevant to diabetes-induced NTDs. ER stress is present in the neuroepithelia of embryos exposed to maternal diabetes, and the ER stress inhibitor 4-phenylbutyric acid reduces HG-induced NTDs in cultured embryos\textsuperscript{11}. We showed that MARCKS-PD inhibits maternal diabetes-induced ER stress, supporting the protective effect of unphosphorylatable MARCKS on the ER.

MARCKS acetylation is required for its phosphorylation. The inhibition of MARCKS acetylation by SIRT2 blocks maternal diabetes-induced ER stress and NTD formation. Thus, maternal diabetes-induced MARCKS acetylation and subsequent phosphorylation disrupt the association between MARCKS and the ER and mitochondrion, leading to cellular organelle stress.

The phosphorylation and myristylation of MARCKS are well-recognized post-translational modifications that are primarily involved in the binding of MARCKS to or release from plasma membranes\textsuperscript{39}. The four serine residues in the effector domain of MARCKS, which have negative charges, bind the negatively charged phospholipids of the plasma membrane\textsuperscript{65}. The N-terminal myristoyl group of MARCKS acts second by binding to the plasma membrane and facilitating a relatively stable interaction\textsuperscript{55}.

MARCKS on lysine residue 165 (K165) is important for its phosphorylation by PKC in neural cells. The phosphorylation sites in MARCKS PSD (phosphorylation site domain) are Serine 152 (S152), S156, S160 and S163. The acetylation residue K165 is adjacent to these four serine residues. K165 acetylation introduces another negative charge that may neutralize the positive charge in the nearby amino acid residues, leading to a MARCKS protein with a more open structure, particularly in the PSD. This structural change may render PKC more accessible to the four serine sites and potentiate these sites to becoming phosphorylated. However, directly investigating the physical function of acetylation on phosphorylation is very challenging. Future studies may explore these structural changes in the MARCKS protein upon acetylation.

We revealed that acetylation, which is another form of post-translational modification, regulates MARCKS phosphorylation under HG conditions. Protein function and subcellular localization are regulated by phosphorylation and acetylation\textsuperscript{66}. Phosphorylation plays a key role in cellular signaling, whereas acetylation functions in regulating protein expression and improving protein stability\textsuperscript{66}. In some cases, the acetylation of proteins, especially transcription factors, enhances their transcriptional activity by increasing their DNA binding affinity, protein stability and phosphorylation sensitivity\textsuperscript{67}. Similar to proteins, which become more sensitive to phosphorylation after they are first acetylated, acetylation is a prerequisite for MARCKS phosphorylation. Indeed, the MARCKS acetylation constitutive active isoform induces its phosphorylation under NG conditions. Moreover, the MARCKS acetylation dominant negative isoform blocks MARCKS phosphorylation increased by HG. Since the subcellular localization of MARCKS is controlled by its phosphorylation, MARCKS acetylation and
resultant phosphorylation leads to a dissociation from cellular organelle membranes.

Following acetylation, PKC and Protein Phosphatase 2A (PP2A) are responsible for the phosphorylation/dephosphorylation of MARCKS; however, the enzymes responsible for the acetylation/deacetylation of MARCKS are unknown. This study identified Tip60 as a MARCKS acetyltransferase and SIRT2 as a MARCKS deacetylase. Maternal diabetes in vivo and HG in vitro do not increase Tip60 expression but enhance its activity. In contrast, both SIRT2 expression and activity are down-regulated by maternal diabetes. Further investigation indicated that restoring SIRT2 expression attenuated maternal diabetes-
induced NTDs, suggesting that SIRT2-induced MARCKS deacetylation exerts a protective effect on neuroepithelial cells through the inhibition of MARCKS phosphorylation.

In summary, MARCKS acetylation, which is reversibly regulated by Tip60 and SIRT2, is a prerequisite for MARCKS phosphorylation, which is equivalent to loss of function mutations leading to NTDs, and mediates the teratogenicity of maternal diabetes in NTD induction by causing cellular organelle stress. Because MARCKS is broadly involved in an array of neurological disorders, identifying avenues, such as acetylation and phosphorylation, to modulate MARCKS activity and restore its functionality is fundamentally important.

**Methods**

**Mice.** The procedures used for the animal experiments were approved by the University of Maryland Baltimore Institutional Animal Care and Use Committee. The MARCKS-PD-transgenic (M-Tg)19 mice (C57BL/6J) were originally obtained from Dr. Perry J. Blackshear at the Signal Transduction Laboratory, National Institute of Environmental Health Sciences. The nestin promoter driven SIRT2 transgenic (SIRT2-Tg) mice were generated on a C57BL/6J background at the Institute of Environmental Health Sciences. The nestin promoter driven SIRT2 transgenic (SIRT2-Tg) mice were obtained from the Jackson Laboratory (Stock #: 012772, Bar Harber, ME).

**Model of maternal diabetes-induced NTDs.** We31,12,37,35-37 and other researchers47,69 have used a rodent model of Streptozotocin (STZ)-induced diabetes in research investigating diabetic embryopathy. Eight- to ten-week old female mice were intravenously injected with 65 mg/kg STZ, daily into the tail vein over two days to induce diabetes. Diabetes was defined as 12-hour fasting blood glucose concentrations greater than or equal to 14 mM, which usually occurred 3–5 days after the STZ injections. We did not detect any differences in embryonic development between the STZ-insulin-treated and non-STZ-treated mice8, suggesting that STZ had no residual toxic effect in our animal model. The male M-Tg mice and SIRT2-Tg mice were bred with female diabetic mice. As previously described33,35, the embryos were harvested at E8.75 (200 PM at E8.5) for the biochemical and molecular analyses. At E10.5, the embryos were examined under a Leica MZ16F stereo microscope (Leica, Bannockburn, IL) to identify the NTDs.

**Pathogenic maternal diet treatment.** Eight- to ten-week-old SIRT2 heterozygous (SIRT2+/−) female mice were fed a Purina5015 (LabDiet, St. Louis, MO) or control (Purina5001, LabDiet, St. Louis, MO) diet for 1 week prior to mating with the SIRT2 heterozygous (SIRT2+/−) male mice. To identify the NTDs, the embryos were examined at E10.5 under a Leica MZ16F stereo microscope (Leica, Bannockburn, IL).

**Whole embryo cultures.** The SIRT2 heterozygous (SIRT2+/−) female and male mice were mated overnight. The day on which a vaginal plug was observed was designated E0.5. At E8.5 (8 AM), mouse embryos with an intact visceral yolk sac were dissected from the uterus into PBS (Invitrogen, La Jolla, CA). Then, three embryos were cultured in 4 ml of culture medium containing 2.66 ml of rat serum, 1.34 ml of Tyrode’s salt solution (Cat#: T2397, Sigma, St. Louis, MO), 100 units/ml of penicillin and 100 µg/ml of streptomycin with or without 1 µM ROPA (Resi-niferoid-9,13,14-orthoarylbenzyl) (Abcam, Cambridge, MA) at 38 °C in a roller bottle system. The embryos were cultured for 36 h under the following conditions: 5% O2–5% CO2–90% N2.

**TUNEL assay.** An ApopTag Red In Situ Apoptosis Detection Kit (Catalog No: S7165, Millipore) was used to detect apoptosis33. Ten-micrometers frozen embryonic sections were fixed with 4% PFA in PBS and incubated with TUNEL reaction agents. The percentage of apoptotic cells was obtained in three separate experiments by dividing the number of TUNEL positive cells by the total number of cells in a microscopic field and then multiplying by 100.

**Cell culture and transfection.** C17.2 mouse neural stem cells, which were originally obtained from the ECACC (European Collection of Cell Culture, Catalogue No.: 07062902, Salisbury, UK), are newborn mouse cerebellar progenitor cells transformed with retroviral v-myec12,35. The C17.2 cells were cultured under NG or HG conditions and then transfected with scramble control siRNA or Tip60-siRNA (Invitrogen, La Jolla, CA) using Lipofectamine (Invitrogen, Carlsbad, CA) according to the protocol from the manufacturer. The C17.2 cells were transfected with pMARCS-PD, pMARCS-Ca, pMARCS-DN, pTip60, pSIRT2 and pPKCa-Ca using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol from the manufacturer.

**Immunoprecipitation (IP) and immunoblotting.** In total, 300 mg protein from C17.2 cells or five to six embryos pooled per litter, a protease inhibitor cocktail (Sigma-Alrich, St. Louis, MO), lysis buffer (Cell Signaling Technology, Danvers, MA), Protein A/G Magnetic bead slurry (New England BioLabs, Ipswich, MA), and 1 mg rabbit anti-MARCKS antibody (Santa Cruz Biotechnology, Dallas, TX) were used for IP. The mitochondria were isolated from the C17.2 cells or embryos using a Pierce mitochondria isolation kit (Thermo Fisher Scientific, Waltham, MA). For immunoblotting, equal amounts of protein (30 or 50 mg) from C17.2 cells or one whole embryo per dam were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Billerica, MA). Precision Plus Protein Standard (2 mg: Bio-Rad Laboratories, Hercules, CA) were loaded onto one lane of the gel. The membranes were incubated in 5% nonfat milk for 45 min, followed by incubation with the primary antibodies in 5% nonfat milk for 18 h at 4 °C. The detailed antibody information is provided in Supplementary Table 5. Following the primary antibody incubation, the membranes were exposed to goat anti-rabbit or anti-mouse secondary antibodies. To ensure that equivalent amounts of protein were loaded among the samples, the membranes were stripped and probed with a mouse antibody against β-actin at a dilution of 1:5000 (Abcam, Cambridge, UK) or prohibitin (1:2000) (Calbiochem, Billerica, MA). The signals were detected using a SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Fisher Scientific, Waltham, MA). All experiments were repeated three times using independently prepared cell or tissue lysates. All uncropped immunoblotting images were present in Supplementary Fig. 4.

**Immunofluorescence.** Embryos at E8.5 were fixed in 4% paraformaldehyde (PFA) overnight, followed by embedding in optimum cutting temperature medium (OCT) compound (Sakura Finetek, Torrance, CA). Then, 10-µm cryosections were pretreated with citrate buffer and blocked in 5% bovine serum albumin in PBST (0.1% Triton X-100 in PBS) for 1 h. The following antibodies were used as primary antibodies: MARCKS (1:200), TOM20 (1:200), Connexin (1:200), Sox1 (1:200), Tuj1 (1:200) and GFP (1:200). Normal rabbit or mouse IgG at the same dilutions as the antigens were used as controls. After washing with PBS, the sections were incubated with the secondary antibodies and then the sections were counterstained with DAPI and mounted with aqueous mounting medium (Sigma, St Louis, MO). The images were captured under a confocal microscope (Carl Zeiss, Ober- cohen, Germany).

**Hematoxylin-eosin staining.** E10.5 embryos were collected for a morphological examination. First, the embryos were fixed in methacarn (methanol, 60%; chloroform, 30%; and glacial acetic acid, 10%), embedded in paraffin, and cut into 5-µm sections. After deparaffinization and rehydration, all specimens underwent hematoxylin and eosin (H&E) staining using a standard procedure. All embryo sections were photographed under a Nikon Ni-U microscope (Nikon, Tokyo, Japan), and the NTDs were examined.

**Electron microscopy.** The structures of the mitochondria were examined by transmission electron microscopy (EM) at our University EM core facility. Thick sections (1 µM) were cut and visualized at 100× magnifications to identify the neuropathology of the E8.5 embryos. Thin sections (80 nm) of the identified
PCR primers for XBP1 were as follows: forward, 5′-GAACCAGGAGTTAAGAAC and reverse, 5′-AGGCAACAGTGTCAGAGTCC-3′, using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The mRNA was extracted from E8.5 embryos by TRIzol (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA. Detection of XBP1 mRNA splicing occurred, a 205-bp band was produced. If XBP1 splicing occurred, a 205-bp band was produced. If no XBP1 mRNA splicing occurred, a 205-bp band and a 179-bp main band were produced. All primer sequences were listed in Supplementary Table 6.

Detection of XBP1 mRNA splicing. The mRNA of XBP1 was extracted from E8.5 embryos by TRIzol (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The PCR primers for XBP1 were as follows: forward, 5′-GAACCAGGAGTTAAGAAC and reverse, 5′-AGGCAACAGTGTCAGAGTCC-3′. If no XBP1 mRNA splicing occurred, a 205-bp band was produced. If XBP1 splicing occurred, a 205-bp band and a 179-bp main band were produced. All primer sequences were listed in Supplementary Table 6.

Statistical analysis. The statistical differences in the two group comparisons were determined by Student’s t-test, and those in the comparisons of more than two groups were determined by one-way ANOVA using SigmaStat 3.5 software. In the comparisons of more than two groups, the Tukey test was used to analyze the data. * Indicates significant difference (P < 0.05) compared to the other group or groups.

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neuroepithelium were cut and viewed under an electron microscope (Joel JEM-1200EX; Tokyo, Japan) at a high-power resolution (6 and 15 K) for the identification of the cellular organelle structures.

Detection of XBP1 mRNA splicing. The mRNA of XBP1 was extracted from E8.5 embryos by TRIzol (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The PCR primers for XBP1 were as follows: forward, 5′-GAACCAGGAGTTAAGAAC and reverse, 5′-AGGCAACAGTGTCAGAGTCC-3′. If no XBP1 mRNA splicing occurred, a 205-bp band was produced. If XBP1 splicing occurred, a 205-bp band and a 179-bp main band were produced. All primer sequences were listed in Supplementary Table 6.
ANOVA analyses, Tukey tests were used to estimate the significance of the results. Significant differences between the groups in the NTD incidences were analyzed by the Bonferroni test. For all immunoblotting experiments, one embryo from one dam in each group was used for one run. Each experiment was repeated three times with three embryos from different dams each group. For immunoprecipitation experiment, five or six embryos from one dam in one experimental group were combined and used for one run, each experiment was repeated three times with three different litters from different dams per group. All quantification data were indicated as means ± standard derivation.

Data availability
The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information Files or from the corresponding author on reasonable request. The data were also deposited in https://figshare.com/7361126 [https://doi.org/10.6084/m9.figshare.7361126.v1].

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Author contributions

P.Y., C.X., and X.C. researched data. P.Y. conceived the project, designed the experiments, and wrote the manuscript. E.A.R., M.Z., D.J.S., and P.J.B. participated in data analyses and reviewed the manuscript. J.Z. helped data analysis and reviewed the manuscript.

Additional information

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