Brief Communications

# Foxo3a Transcriptionally Upregulates AQP4 and Induces Cerebral Edema Following Traumatic Brain Injury

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**Increased cranial pressure due to development of edema contributes significantly to the pathology of traumatic brain injury (TBI). Induction of an astrocytic water channel protein, Aquaporin 4 (AQP4), is known to predominantly contribute to cytotoxic edema following TBI. However, the mechanism for the increase in AQP4 following 24 h of TBI is poorly understood. Here we show that transcriptional activation of a ubiquitously expressed mammalian forkhead transcription factor, Foxo3a, induces cerebral edema by increasing the AQP4 level in the controlled cortical impact model of TBI in mice. TBI stimulates nuclear translocation of Foxo3a in astrocytes and subsequently augments its binding to AQP4 promoter in pericontusional cortex. Nuclear accumulation of Foxo3a is augmented by a decrease in phosphorylation at its Ser256 residue due to inactivation of Akt after TBI. Depletion of Foxo3a in mice rescues cytotoxic edema by preventing induction of AQP4 as well as attenuates memory impairment after TBI in mice.**

## Introduction

Increased intracranial pressure (ICP) following traumatic brain injury (TBI), caused by development of brain edema, contributes to poor outcomes associated with TBI. Imbalance in water homeostasis can explain the pathologies of edema. Several studies have shown that an astrocyte-specific protein, Aquaporin 4 (AQP4), plays an important role in formation of edema in brain (Vizuete et al., 1999; Bloch and Manley, 2007). It was demonstrated that TBI leads to a significant increase in AQP4 mRNA expression at the site of injury compared with sites distant from the sites of injury in brain. Although the functional role of AQP4 in TBI has not been well defined, it was shown that AQP4 knock-out mice are less susceptible to cerebellar edema formation after brain injury (Manley et al., 2000). Reducing the level of AQP4 leads to resolution of brain edema and better neurobiological outcomes following injury in brain. Overexpression of AQP4 accelerates cytotoxic brain swelling after TBI in mice (Yang et al., 2008). AQP4 can also be upregulated by induction of proinflammatory cytokines such as IL-1$\beta$ following TBI in mice (Laird et al., 2010).

In neurons, activation of Foxo3a can protect against excitotoxic insults (Mojsilovic-Petrovic et al., 2009) or trigger neuronal death. Activation of Akt phosphorylates Foxo3a at Ser256 residue and prevents nuclear translocation, subsequently inhibiting transcriptional activation (Arden, 2004; Yang et al., 2005). Mutation of phosphorylation sites of Foxo3a (Foxo3aTM) causes a significant increase in nuclear translocation and transcriptional activity of Foxo3a (Brunet et al., 1999).

In the present study we show that TBI leads to transcriptional activation of Foxo3a, which stimulates induction of AQP4 at the site of injury after 24 h following TBI. Depletion of Foxo3a in mice prevents augmentation of AQP4 in brain and reduces cerebral edema that subsequently improves neurological outcome after TBI.

## Materials and Methods

In this study we used 8- to 10-week-old C57BL/6 male mice unless mentioned otherwise.

*Biochemical studies.* Primary astrocyte cultures were obtained from cerebral cortices of 1- to 2-d-old C57BL/6 mice (of either sex) as described previously (Laird et al., 2010). Primary astrocytes were treated with IL-1$\beta$ (10 ng/ml) overnight. With or without treatment with IL-1$\beta$, cells were lysed with lysis buffer and supernatant was used for Western blotting using anti-Foxo3a, anti-phospho-Akt, anti-phospho Foxo3a, anti-AQP4, and anti-actin antibodies. Antibodies were obtained either from Cell Signaling Technology or Santa Cruz Biotechnology. For *in vivo* studies a 1 mm micropunch was used to collect tissue from the pericontusional cortex or from the corresponding contralateral hemisphere. For confocal microscopy, serial coronal sections (12 $\mu$M) were prepared from the pericontusional cortex using a cryostat microtome, and sections were processed as described previously (Laird et al., 2010). Total RNA was isolated (SV RNA Isolation kit; Promega) and reverse transcription (RT)-PCR was performed as described previously (Laird et al., 2010). For chromatin immunoprecipitation (ChIP) assays, we used a chromatin immunoprecipitation assay kit purchased from Millipore and followed the instructions from the supplier. Briefly, primary astrocytes were stimulated with or without IL-1$\beta$. After sonication, lysates containing soluble chromatin were incubated overnight with an anti-Foxo3a antibody or with normal rabbit IgG. DNA–protein immunocomplexes were precipitated with protein A-agarose beads, washed, and eluted. The eluates were used as templates in PCR using the primers 5′-TTCTCTTCAA TC-3′ and 5′-AATTGTCCCTGTAC-3′. The expected DNA fragment

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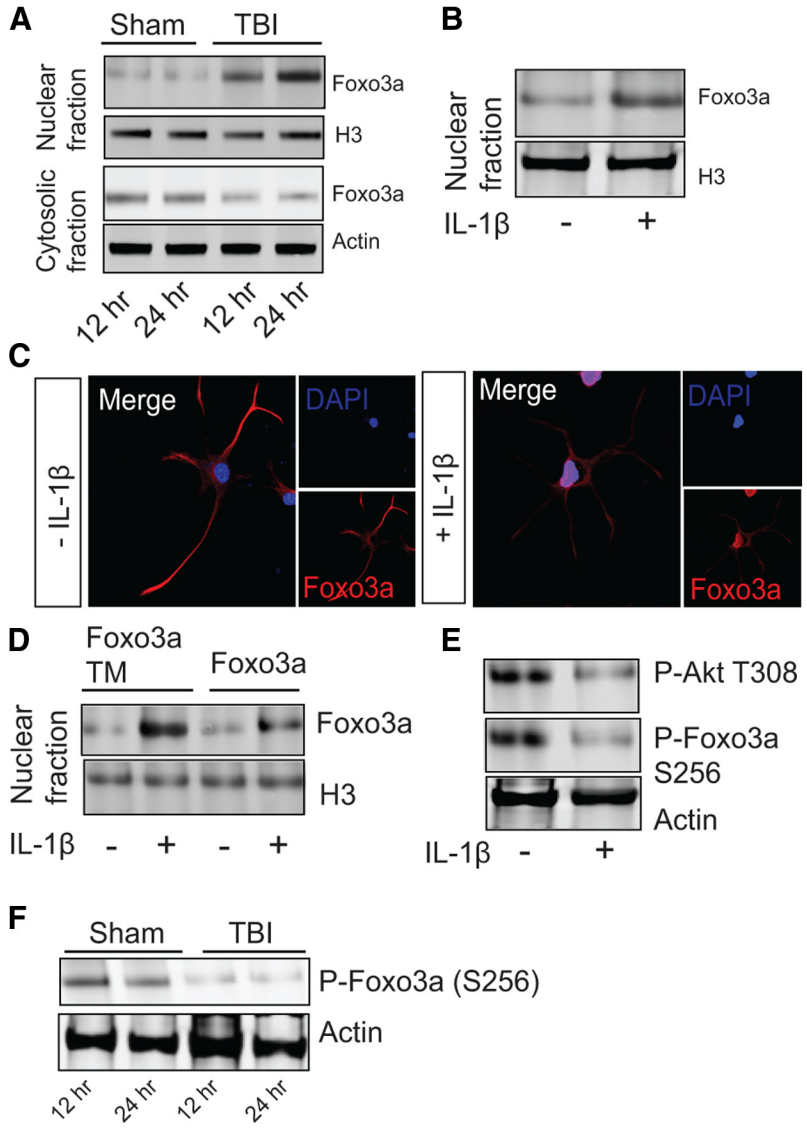


**Figure 1.** Nuclear translocation of Foxo3a was increased after TBI. ***A***, Cytosolic and nuclear levels of Foxo3a were measured in ipsilateral cortex after 12 and 24 h following TBI. ***B***, Nuclear fraction of Foxo3a was measured in primary astrocytes after treatment with IL-1$\beta$ by Western blot hybridization. ***C***, Confocal microscopic analysis nuclear translocation of Foxo3a after treatment with or without IL-1$\beta$. ***D***, Overexpression of Foxo3a M causes an increase in nuclear accumulation of Foxo3a compared with overexpression of wild-type Foxo3a. ***E***, Phosphorylation levels of both Akt and Foxo3a were measured in primary astrocytes after treatment with IL-1$\beta$ using Western blot hybridization. ***F***, Phosphorylation of Foxo3a (S256) was measured after 12 and 24 h following sham or TBI.

was 178 bp in length and amplified the AQP4 promoter region, which encompassed the Foxo3a binding site. Primary astrocytes were transfected with Altogen and various DNA constructs to overexpress either FOXO3a wild-type or Foxo3aTM according to the manufacturer's protocol. EMSA was performed using an Odyssey Infrared EMSA kit (LICOR Biosciences) according to the manufacturer's instructions using protocol published previously (Das et al., 2011). Approximately 25 $\mu$g of nuclear extracts or tissue extracts were incubated with 100 fmol of IR-dye labeled probe in binding buffer. The probe and nuclear proteins were incubated for 30 min at room temperature. DNA–protein complexes were resolved on 4.5% nondenaturing acrylamide gels. Gels were then scanned directly in an Odyssey scanner (LICOR Biosciences) to visualize DNA–protein interaction and image was saved as a gray color. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay and measurement of PI/DAPI-stained nuclei were performed to measure toxicity of cells after administration of lentiviral particles of either Foxo3a or control RNAi as per our method (Sen et al., 2009; Farook et al., 2013).

*Stereotaxic injection of lentivirus particles into intact mice.* The lentiviral particles of Foxo3a RNAi were purchased from Santa Cruz Biotechnology and injected into mouse brain as previously described. Briefly, after anesthesia, the skull was exposed and 5.0 $\mu$l ($10^7$–$10^8$ pfu/ml) of viral particles of Foxo3a RNAi ($n = 9$) were slowly injected into the cortex (anterior 0.5 mm, lateral 3.5 mm from bregma, and ventral 1.0 mm relative to dura). Seven days after viral infection, depletion of Foxo3a in coronal sections was detected by Western blot with anti-Foxo3a antibody. On day 8, TBI was performed as described previously and below (Kimbler et al., 2012).

*TBI procedures.* The procedure is based on the protocol described previously (Laird et al., 2010). Briefly, 8- to 10-week-old C57BL/6 mice (male) were impacted at 4.5 m/s with a 20 ms dwell time and 1.0 mm depression using a 3 mm diameter convex tip, mimicking a moderate TBI. Sham-operated mice underwent the identical surgical procedures, but were not impacted. Body temperature was maintained at 37°C using a small animal temperature controller throughout all procedures (Kopf Instruments).

*Assessment of cerebral edema.* Brain water content (measure of cerebral edema) was quantified using the wet-dry method (Hewett et al., 2006; Laird et al., 2010). Briefly, brain water content was estimated in a 3 mm coronal tissue section of the ipsilateral cortex (or corresponding contralateral cortex), centered on the impact site. Tissue was immediately weighed (wet weight), then dehydrated at 65°C. The sample was reweighed 48 h later to obtain a dry weight. The percentage of water content in the tissue samples was calculated using the following formula: [(wet weight − dry weight)/wet weight] ×100.

*Neurobehavioral tests.* The Y-maze test of spontaneous alternation was used to evaluate hippocampus-dependent spatial learning (Lloyd et al., 2008). Testing was performed daily by a blinded observer until day 30 of recovery. Each animal started in the vertical arm of the Y-maze. If the animal selected a different arm on the second run in the maze, it was scored as alternating. The percentage alternation over the duration of testing was calculated for each animal.

*Statistical analysis.* The effects of treatments were analyzed using a one-way ANOVA followed by Dunnett's *post hoc* test. Results are expressed as mean ± SEM. A $p < 0.05$ was considered to be statistically significant. For Y-maze study we used two-way ANOVA.

## Results

### TBI leads to increase in nuclear translocation of Foxo3a

To monitor whether TBI has any influence on translocation of Foxo3a into the nucleus, we performed cytosolic and nuclear fractionation of both sham and TBI-induced mice. We found that there was a significant increase in nuclear level of Foxo3a after 24 h following TBI (Fig. 1*A*). It is known that increased CSF levels of the proinflammatory cytokine, IL-1$\beta$, clinically correlate with edema and neurological demise after TBI (Hayakata et al., 2004; Hutchinson et al., 2007). It was also shown that intracerebral administration of IL-1$\beta$ induced edema by increasing AQP4

expression (Ito et al., 2006). Thus, to mimic injury situation in brain we treated primary astrocytes with IL-1β and monitored the nuclear level of Foxo3a in primary astrocytes after treatment by both Western blot (Fig. 1*B*) and confocal microscopy analysis (Fig. 1*C*). We found that treatment with IL-1β causes an ~1.5-fold increase in nuclear level of Foxo3a in both cases. Mutation of phosphorylation sites of Foxo3a (Foxo3aTM) causes a significant increase in nuclear translocation and transcriptional activation of Foxo3a (Brunet et al., 1999). To see whether nuclear translocation of Foxo3a depends on its phosphorylation status, we overexpressed Foxo3aTM as well as wild-type Foxo3a in primary astrocytes and treated with IL-1β. It was observed that overexpression of Foxo3aTM causes enrichment of Foxo3a in the nucleus compared with overexpression of Foxo3a in primary astrocytes upon treatment with IL-1β (Fig. 1*D*). The phosphorylation level of both Akt and Foxo3a were also measured in primary astrocytes treated with IL-1β by Western blot hybridization. We found that treatment with IL-1β causes a decrease in phosphorylation of both Akt and Foxo3a to the extent of >80 and 85%, respectively (Fig. 1*E*). The status of phosphorylation of Foxo3a in both sham and TBI-induced mice was also measured by Western blot hybridization using pericontusional cortex after TBI in mice. We found that the phosphorylation level of Foxo3a at S256 was significantly decreased to >80% after TBI (Fig. 1*F*). Our data suggest that a decrease in phosphorylation of Foxo3a leads to an accumulation of Foxo3a following TBI.

**Foxo3a transcriptionally upregulates AQP4 level after TBI**

To study whether Foxo3a transcriptionally regulates the AQP4 level in nucleus, we scanned for a consensus Foxo3a binding site [(G/A)TAAA(T/C)A](Tsai et al., 2007; Hedrick et al., 2012) on the AQP4 promoter. We found that a consensus DNA-binding site for Foxo3a (ATAAACA) is present in the promoter region of AQP4 at position −249 (Fig. 2*A*). Binding of Foxo3a to AQP4 promoter sequence was determined by EMSA assay using extract from pericontusional cortex isolated after 24 h following TBI in mice (Fig. 2*B*), and it was further confirmed by supershift assay (Fig. 2*C*). DNA binding of Foxo3a to AQP4 was evidenced by EMSA assay *in vitro* by using primary astrocytes after treatment with IL-1β (Fig. 2*D*). To further confirm whether Foxo3a binds the AQP4 promoter *in vivo*, we performed ChIP assay in primary astrocytes treated with or without IL-1β. It was observed that binding of Foxo3a to the AQP4 promoter was significantly increased after IL-1β treatment (Fig. 2*E*). The increase in Foxo3a binding to the AQP4 promoter was further evidenced by an increase in mRNA (Fig. 2*F*) and protein level (Fig. 2*G*) of AQP4 in primary astrocytes after treatment with IL-1β.

To further confirm whether Foxo3a has any direct influence on the AQP4 level, we depleted Foxo3a in primary astrocytes before treating with IL-1β. However, administration of lentiviral RNAi particles of Foxo3a does not alter expression level of any off-target protein such as actin; however, protein level of Foxo3a was reduced to the extent of 85% compared with control RNAi-treated cells as evidenced by Western blot (Fig. 2*H*) and confocal microscopy analysis (Fig. 2*J*). These data indicate that transfection efficiency of lentiviral particles of Foxo3a RNAi is almost 85% in astrocytes. Depletion of Foxo3a in cells does not either induce toxicity, measured by PI/DAPI staining (Fig. 2*I*), or cause alteration in morphology compared with administration of control lentiviral RNAi particles, which was measured by GFAP staining (Fig. 2*J*). We found that treatment with IL-1β (10 ng/ml) causes an increase in mRNA level of Foxo3a. However, in cells lacking Foxo3a, the mRNA level of AQP4 was not increased significantly following TBI (Fig. 2*K*). The protein level of AQP4 in primary astrocytes was also measured after depleting Foxo3a inside cells both by Western blot (Fig. 2*L*) and confocal microscopy analysis (Fig. 2*M*). In both cases, treatment with IL-1β causes an increase in AQP4 level by >1.8-fold. However, depletion of Foxo3a causes a significant decrease in AQP4 level. To monitor whether phosphorylation of Foxo3a affects the mRNA level of AQP4, we overexpressed either wild-type Foxo3a or Foxo3aTM construct, which is unable to be phosphorylated by Akt, in primary astrocytes. Cells were then treated with IL-1β, and the mRNA level of AQP4 was measured by RT-PCR analysis. It was observed that overexpression of Foxo3aTM induces the mRNA level of AQP4 three times more than cells overexpressing wild-type Foxo3a (Fig. 2*N*) and occurs in a dose-dependent manner. These data suggest that Foxo3a transcriptionally upregulates AQP4.

**Depletion of Foxo3a *in vivo* reduces induction of AQP4 level and brain water content following TBI**

To characterize the influence of Foxo3a on cerebral edema after TBI, we depleted Foxo3a in mice before performing the controlled cortical impact model of TBI in mice. Administration of lentiviral particles of Foxo3a RNAi does not have any influence on off-target protein level of actin (Fig. 3*A*) but causes depletion of Foxo3a level >90% as evidenced by Western blot (Fig. 3*A*) and confocal microscopy (Fig. 3*B*). These data indicate that transfection of lentiviral particles of Foxo3a RNAi is >90% *in vivo*. Depletion of Foxo3a *in vivo* does not alter either morphology of cells (Fig. 3*B*) or toxicity of cells as measured by TUNEL assay (Fig. 3*C*). We found that the TBI-induced increase in brain water content (cerebral edema) was significantly reduced (1.8-fold) in mice that overexpressed RNAi particles of Foxo3a compared with mice that overexpressed control RNAi in brain (Fig. 3*D*). Since the water content in brain is regulated by AQP4 level, we measured the protein level of AQP4 in pericontusional cortex 24 h after TBI in mice depleted with Foxo3a by both Western blot hybridization (Fig. 3*E*) and confocal microscopy analysis (Fig. 3*F*). It was observed that TBI leads to an increase in AQP4 level in pericontusional cortex. However, depletion of Foxo3a significantly reduces the increase in AQP4 >2.5-fold following TBI. To study whether the increase in protein level of AQP4 is due to an increase in its transcriptional level, mRNA levels of AQP4 were measured after TBI in mice depleted of Foxo3a (Fig. 3*G*). It was observed that induction of the mRNA level of AQP4 after TBI was reduced in mice depleted of Foxo3a in brain. Since induction of cytotoxic edema leads to long-term impairment in memory function in mice, we were interested to see whether reduction of edema by depletion of Foxo3a has any influence on memory function. We therefore performed a Y-maze analysis after 30 d of TBI mice. We found that depletion of Foxo3a in mouse brain improves the percentage of spontaneous alteration compared with control TBI mice (Fig. 3*H*). Our data suggest that depletion of Foxo3a reduces cerebral edema and also improves hippocampal-dependent memory impairment following TBI.

## Discussion

Brain edema and ensuing increased ICP following TBI contribute to increased patient mortality and long-term disability (Katayama et al., 1990; Aldrich et al., 1992). Increased pericontusional expression of AQP4 correlates with the development of cellular edema after TBI in humans and rodents (Hu et al., 2005; Guo et al., 2006). However, it is important to mention that the role of AQP4 in both cellular and vasogenic edema is controversial. In-

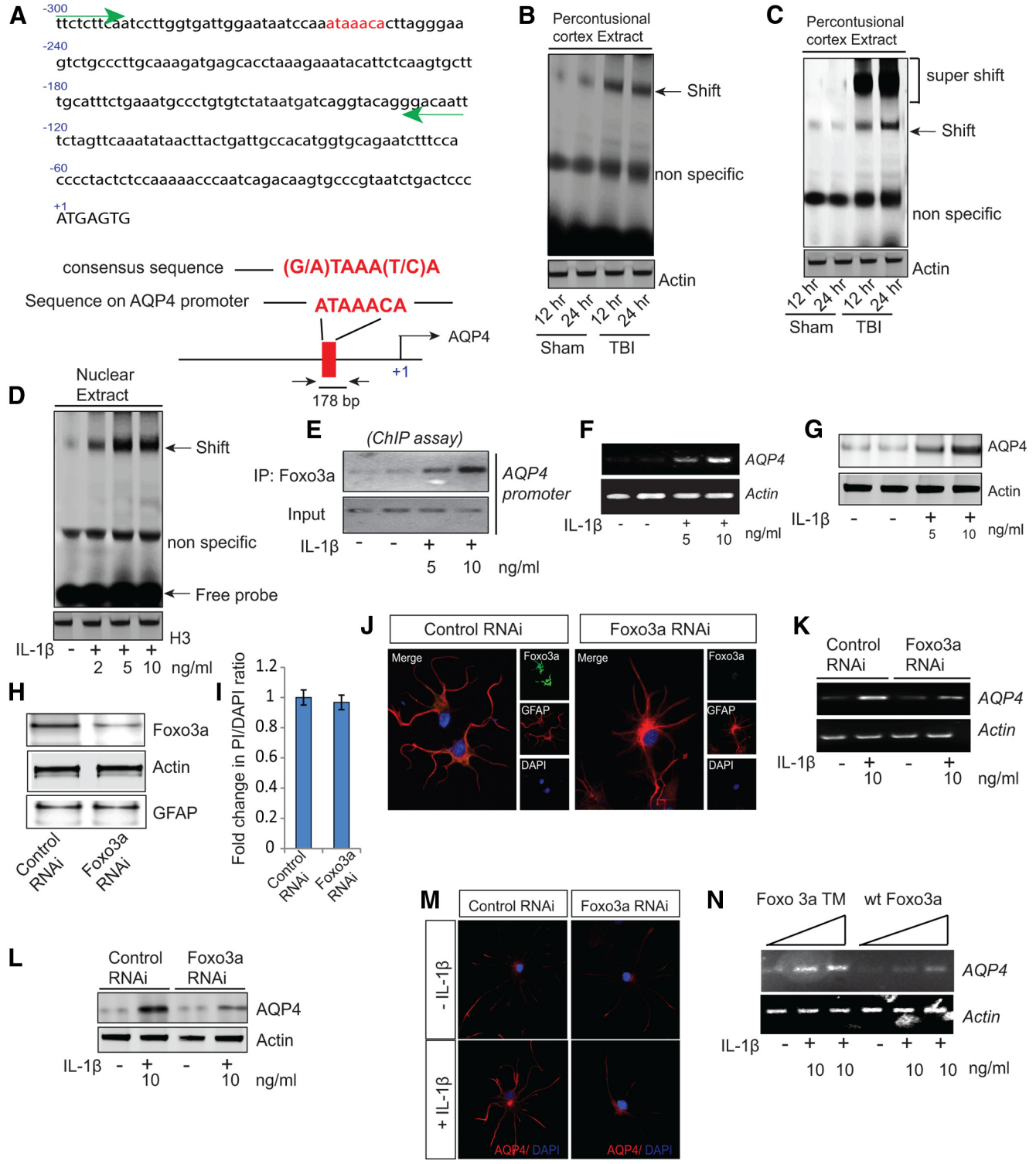


**Figure 2.** Foxo3a transcriptionally regulates AQP4 following TBI. ***A***, Sequence of promoter of AQP4. Foxo3a-binding residues are indicated in red. Green arrowhead indicates the position of primers for ChIP assay. Schematic representation of Foxo3a-binding site on AQP4 promoter. ***B***, EMSA assay to determine transcriptional activity of Foxo3a on AQP4 promoter. ***C***, DNA binding of Foxo3a on AQP4 promoter was confirmed by supershift assay using anti-Foxo3a antibody in EMSA assay. ***D***, DNA binding of Foxo3a on AQP4 promoter was monitored by EMSA assay after treatment with IL-1$\beta$ in a concentration-dependent manner. ***E***, ChIP analysis to identify the binding of Foxo3a on AQP4 promoter. ***F***, ***G***, Protein level (***F***) and mRNA level (***G***) of AQP4 were measured in primary astrocytes after treatment with IL-1$\beta$. ***H***–***J***, Depletion of Foxo3a does not have any influence on either protein level of actin (***H***), toxicity to cells as determined by ratio of PI-stained nuclei to DAPI-stained nuclei (***I***), or morphology of cells, monitored by confocal microscopy (***J***). ***K***, ***L***, Depletion of Foxo3a by RNAi in primary astrocytes reduces both mRNA level (***K***) and protein level (***L***) of AQP4, which was measured by RT-PCR analysis. ***M***, Confocal microscopic analysis of AQP4 after depletion of Foxo3a in primary astrocytes. ***N***, Overexpression of either Foxo3aTM or wild-type Foxo3a regulates mRNA level of AQP4, which was measured by RT-PCR analysis.

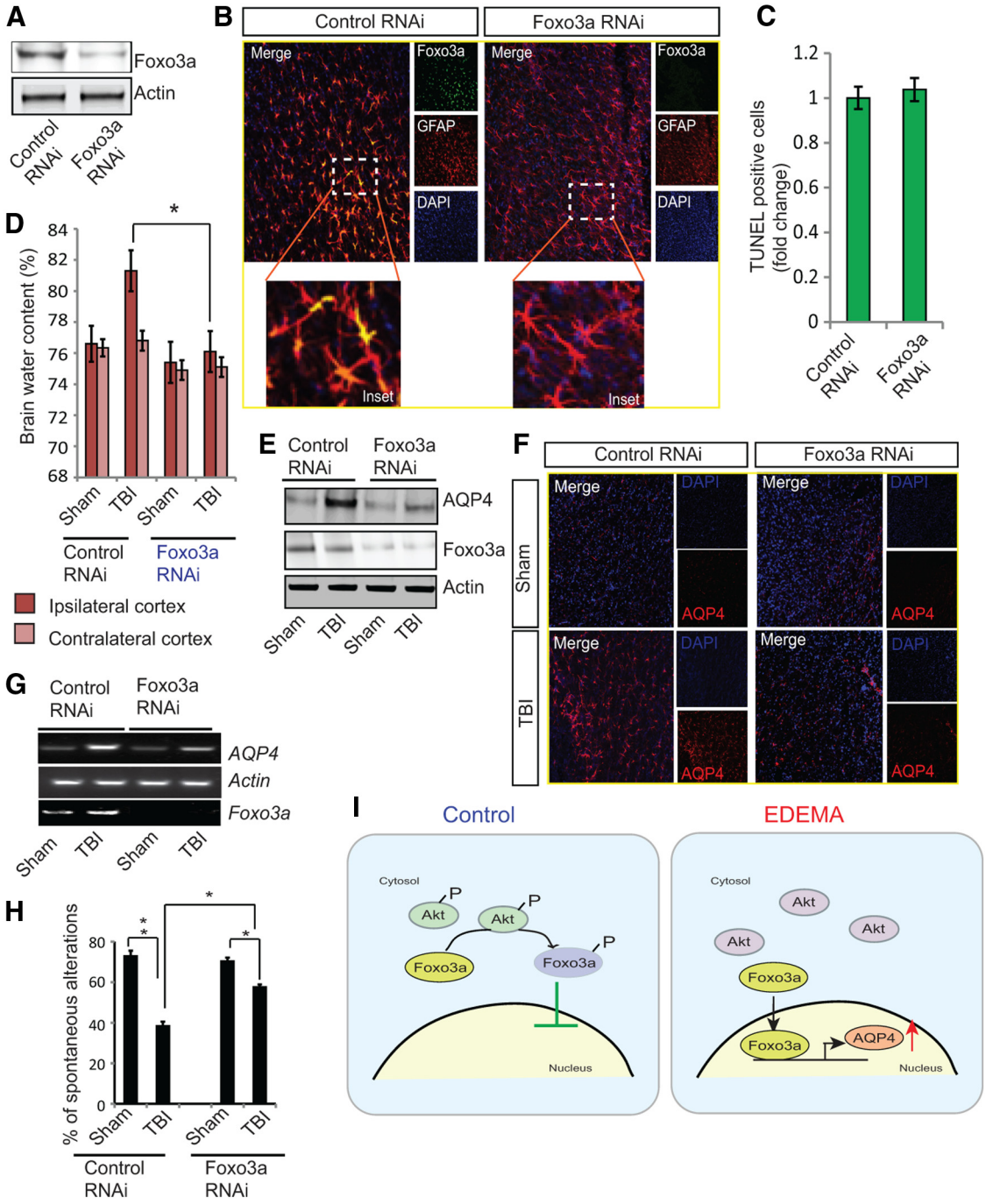


**Figure 3.** Depletion of Foxo3a reduces induction of AQP4 and cerebral edema in mouse model of TBI. ***A–C***, Depletion of Foxo3a does not have any influence on either protein level of actin (***A***), morphology of cells, monitored by confocal microscopy (***B***), or toxicity to cells as determined by TUNEL assays (***C***). ***D***, Depletion of Foxo3a causes reduction of TBI-induced increase in brain water content in ipsilateral cortex. $^*p < 0.01, n = 4–5$, one-way ANOVA, mean ± SEM. ***E***, Protein level of AQP4 was measured by Western blot hybridization following 24 h of TBI after depletion of Foxo3a in mice. ***F***, Confocal microscopic analysis of AQP4 level in ipsilateral cortex after 24 h of TBI. ***G***, Knockdown of Foxo3a in mice causes a reduction of mRNA level of AQP4 after 24 h following TBI as measured by RT-PCR analysis. ***H***, Depletion of Foxo3a improves percentage of spontaneous alteration after TBI in mice. $^*p < 0.05, n = 7–9$, two-way ANOVA, mean ± SEM. ***I***, Schematic representation of how Foxo3a induces edema following TBI. In the control, Foxo3a remains in the cytosol due to an increase in its phosphorylation by Akt. However, in TBI, the phosphorylation level of Foxo3a was decreased, leading to an increase in nuclear accumulation of Foxo3a. In the nucleus, Foxo3a transcriptionally upregulates AQP4 and subsequently increases the brain water content after TBI.

creased expression of AQP4 within the pericontusional cortex was associated with development of cellular edema and disruption of blood–brain barrier (Vizuete et al., 1999; Guo et al., 2006; Laird et al., 2010). In contrast, decreased expression of AQP4 aided in vasogenic edema resolution and improved neurological outcome (Dietrich et al., 1999; Kiening et al., 2002). Although both cellular and vasogenic edema contribute to brain swelling, we mainly focused on cellular edema because cellular edema predominates after TBI both in rodents and human patients (Ito et al., 1996; Unterberg et al., 2004; Kleindienst et al., 2006).

In the present study we have shown that transcriptional activation of Foxo3a directly regulates induction of AQP4 in pericontusional region following TBI (Fig. 3*I*). In control, Foxo3a remains in phosphorylated form and resides in cytosol. However, following

TBI, phosphorylation of Foxo3a was decreased significantly due to decrease in phosphorylation of Akt inside cells. Dephosphorylated Foxo3a accumulates in nucleus and transcriptionally upregulates AQP4 following TBI that may leads to cerebral edema. The function of Foxo3a in AQP4 expression was demonstrated through RNA interference to downregulate Foxo3a expression *in vivo* as well as in primary astrocytes. Furthermore, the binding of Foxo3a on AQP4 promoter was confirmed by ChIP analysis. These data indicate that the control of AQP4 expression by Foxo3a is crucial for water homeostasis. Like other cells, activation of Foxo3a in astrocytes is also regulated by activation of Akt. Consistent with other investigators we have shown that TBI leads to decrease in phosphorylation and activation of Akt (Wang et al., 2013).

In this study we have also shown that depletion of Foxo3a attenuates induction of AQP4 in ipsilateral cortex that leads to reduction of brain water content induced by TBI. Cerebral edema is known to be responsible for impairment in cognitive impairment after brain injury (Vallée et al., 1997; Wu et al., 2006). Thus, identification of Foxo3a inhibitor may represent a potent therapeutic agent that exerts multiple beneficial effects following TBI.

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