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SHORT COMMUNICATION

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Specific phospholipid scramblases are involved in exposure of phosphatidylserine, an "eat-me" signal for phagocytes, on degenerating axons

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ABSTRACT

Axonal degeneration is a key pathological feature of several neurological disorders. Emerging evidence has suggested a pathological connection between axonal degeneration and autophagy, a lysosomal degradation pathway. We recently reported that GSK3B-mediated phosphorylation of MCL1 regulates axonal autophagy to promote axonal degeneration. GSK3B–MCL1 pathway affects ATP production locally in degenerating axons and the exposure of phosphatidylserine (PS), an "eat-me" signal for phagocytes, on degenerating axons, resulting in the failed engulfment of axonal debris *in vivo*. Here we showed that the PS exposure is accomplished by phospholipid scramblase activity. This finding provides a novel mechanism that local ATP production through autophagy promotes PS exposure on degenerating axons. In addition, it opens new perspectives for the understanding of axonal autophagy to regulate Wallerian degeneration.

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Axonal degeneration is recognized as a key pathological feature of many neurological disorders, including Alzheimer disease and Parkinson disease.[1,2] A typical form of pathological axonal degeneration is Wallerian degeneration, which has been observed in segments distal to the site of injury. Emerging evidence has suggested pathological connections between Wallerian degeneration and autophagy, a lysosomal degradation pathway. Previous reports have revealed increased expression of autophagy markers and formation of autophagosomes in degenerating axons.[3,4] However, the pathophysiological significance and regulation of axonal autophagy remain elusive.

We previously reported that glycogen synthase kinase 3B (GSK3B) is one of the critical mediators to regulate Wallerian degeneration.[5,6] Using Wallerian degeneration models *in vitro* and *in vivo*, we recently demonstrated that myeloid cell leukemia 1 (MCL1), a BCL2 family protein, negatively regulates axonal autophagy by binding to BECLIN1, a key regulator of autophagy, and also that GSK3B-mediated phosphorylation of MCL1 serves as an initiating signal to induce axonal autophagy.[7] Interestingly, the perturbation of axonal autophagy by inhibition of the GSK3B–MCL1 pathway affected the exposure of phosphatidylserine (PS), which serves as the "eat-me" signal for phagocytes, on degenerating axons, and resulted in the failed engulfment of axonal debris. These results raised the next question on the connecting mechanism between the local ATP production by autophagy and PS exposure in degenerating axons.

Dying cellular structures, including axonal debris, are rapidly cleared by phagocytes. In this process, phagocytes recognize "eat-me" signals, including PS on degenerate cells.[8] Autophagy has been implicated in PS exposure on apoptotic cells.[9,10] Using *in vitro* Wallerian degeneration experiments, we recently reported a significant increase in PS exposure on axons 3 h post-transection,[7] and reduction of autophagic activity by shRNAs for the autophagy genes including *atg5* or *atg7* prevented PS exposure, implicating autophagy in PS exposure.[7] PS exposure is known to be accomplished by the activity of phospholipid scramblases, including ATP-binding-cassette transporter 1 (ABC1), Transmembrane protein 16F (TMEM16F), and XK-related protein 8 (XKR8).[11-13] To demonstrate the involvement of phospholipid scramblase in PS exposure during axonal degeneration, we examined PS exposure under the RNAi-mediated down-regulation of a phospholipid scramblase in axons after transection *in vitro*. We found reduced PS exposure on axons expressing shRNAs against *abc1* and *xkr8*, but not *tmem16f* (Fig. 1B–C). These results suggest that ABC1

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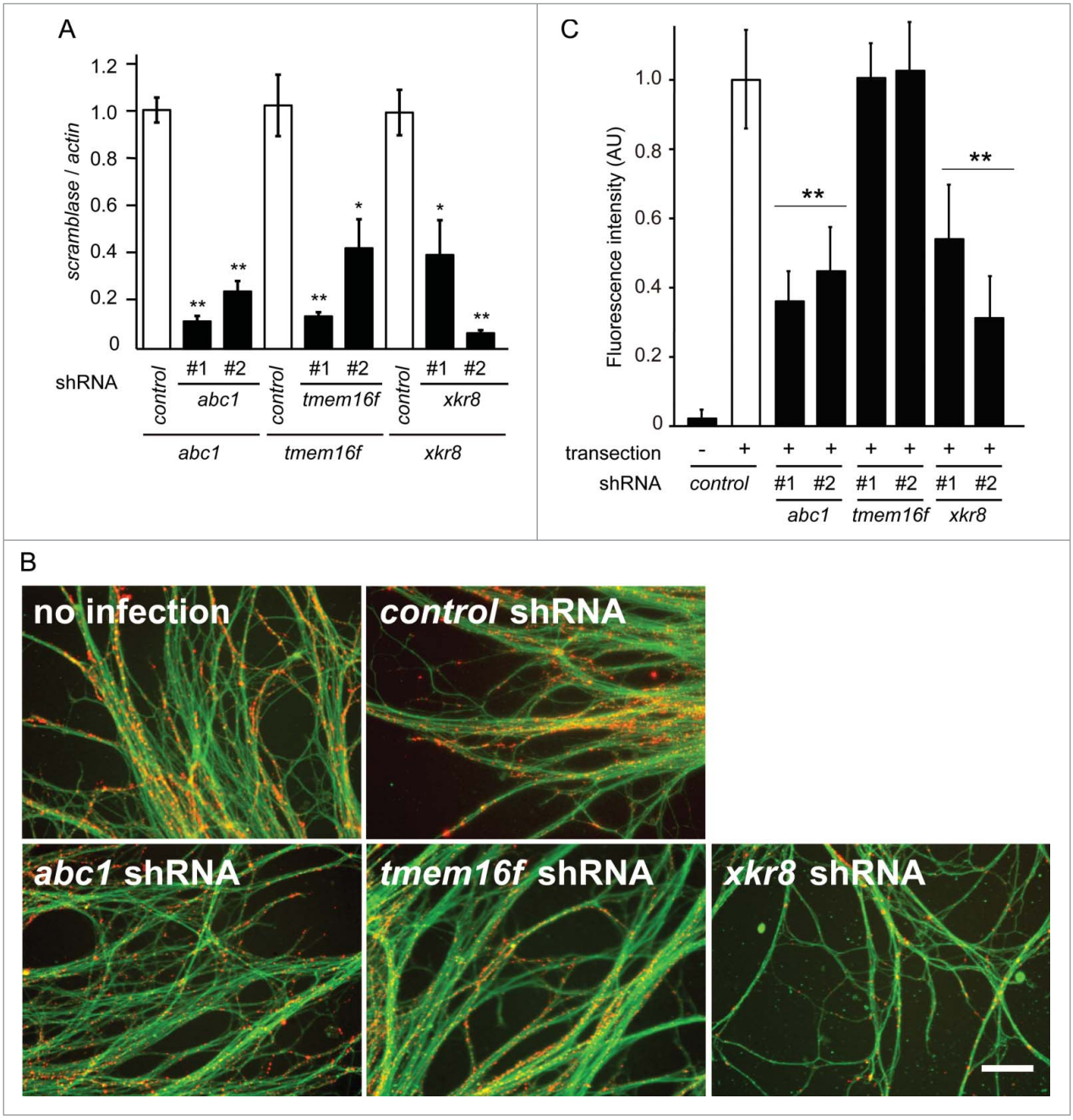

Figure 1. Scramblase activity is responsible for PS exposure on degenerating axons. (A) The shRNA-mediated downregulation of *abc1*, *tmem16f*, or *xkr8* expression was performed in cultured DRG neurons using lentivirus vectors. The downregulated expression of *abc1*, *tmem16f*, or *xkr8* by 2 independent shRNAs used in this study was confirmed by quantitative RT-PCR. The expression levels of each molecule normalized to β-*actin* are shown relative to that of the control (mean ± SEM, n = 5). (B, C) PS exposure on the transected axons was monitored using the specific probe Alexa594-labeled Annexin V (red). DTAF (green) was used to stain axons. Representative photomicrographs of the fluorescent signal in transected axons expressing the indicated molecules are shown in B. Scale bar = 25 μm. Quantified levels of fluorescent intensities for Annexin V normalized to DTAF relative to the level in the control (labeled as "no infection") are shown in (C)(mean ± SEM, n = 5). Note that PS exposure is reduced on axons expressing shRNAs for *abc1* or *xkr8*, but not *tmem16f*. Significant differences from the control (*, $P < 0.05$; **, $P < 0.01$) were determined by a one-way ANOVA with Tukey's post-hoc test.

and XKR8 are the phospholipid scramblases responsible for PS exposure on degenerating axons.

The precise role of autophagy during axonal degeneration has been debated.[13,14-16] We recently showed that upregulation of axonal autophagy is promotes degradation of axonal structures.[7] There, we demonstrated that axonal autophagy contributes to the local production of ATP, which is required to promote axonal degeneration, and also to the PS exposure on degenerating axons to recruit phagocytes to the injury site. Thus, our results provide a

novel significance of axonal autophagy to regulate Wallerian degeneration. Combined with the additional evidence we showed here, our data further support the model that axonal autophagy contributes to the local ATP production and subsequent PS exposure on degenerating axons through activation of the specific phospholipid scramblases.

Materials and methods

Animals

All animals were maintained in accordance with the guidelines of the National Center for Neurology and Psychiatry. The technical protocols for animal experiments in this study were approved by a review committee for Animal Resources in the National Center for Neurology and Psychiatry.

Dorsal root ganglia (DRG) explant culture and in vitro Wallerian degeneration

Murine DRG explants dissected from embryonic day (E) 13 C56BL/6J mice were cultured on poly-L-lysine (PLL) and laminin-coated 24-well plates in Neuro-medium (Miltenyi Biotec) containing 10% FBS and 25 ng/ml nerve growth factor (NGF) (Harlan Bioproducts). After 24 h, the culture medium was changed to Neuro-medium supplemented with 2% Neuro-Brew-21 (Miltenyi Biotec), 25 ng/ml NGF, and 1 mM Glutamine in addition to a mixture of 1 mM 5′-fluoro-2′-deoxyuridine and 1 mM uridine to remove non-neuronal cells. The *in vitro* Wallerian degeneration of axons was introduced by removing cell bodies at 10–14 d *in vitro* using a pipette tip.[5,6]

Viral vectors and infection

In experiments using lentiviral vectors, regarding specific short hairpin RNAs (shRNAs) against the phospholipid scramblase genes *abc1, tmem16f*, and *xkr8*, each target nucleotide sequence was designed using BLOCK-iT RNAi Designer (Thermo Fischer) and cloned into the pLKO.1 puromycin-resistant lentiviral vector. The target nucleotide sequences for each shRNA were as follows: *abc1* #1, 5′-GCAGCACAATTTGTCCCTTCC-3′; *abc1* #2, 5′-GGGACAGAATTGCCATCATTT-3′; *tmem16f* #1, 5′-GCTCCTCATACTCCTCTACCA-3′; *tmem16f* #2, 5′-GCTCAAGAGTTCACAGTATTT-3′; *xkr8* #1, 5′-GCTACCTGTATAGGTGTTTGC-3′; and *xkr8* #2, 5′-GCCATGTGATGCCCAGTAAGT-3′. Non-target control shRNAs in the pLKO.1 vector (Cat No. SHC002) was purchased from Sigma-Aldrich. Lentiviral packaging was performed using HEK293FT cells, as described previously.[17]

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured DRG neurons using the RNeasy MiniKit (Qiagen). Real-time quantitative RT-PCR was performed as described previously[3,4] using the Applied Biosystems Prism model 7300 sequence detection instrument and a standard SYBR green detection protocol. The sequences of the PCR primers using the SYBR green method are as follows: glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) forward, 5′-CCCCCAATGTATCCGTTGTG-3′; *gapdh* reverse, 5′-TAGCCCAGGATGCCCTTTAGT-3′; *abc1* forward, 5′- CTTCTTGGAAAACGATGGGGG-3′; and *abc1* reverse, 5′- TGTGGTGTTGGCAAATATGTCTT-3′; *tmem16f* forward, 5′-AGGAGCGCATCCCATTTACC-3′; *tmem16f* reverse, 5′-CGATCACAGAGGCGATGATGA-3′;*xkr8*forward,5′-GGCCGTTGTCCAGTACGTG-3′; and *xkr8* reverse, 5′-GCAAACACCTATACAGGTAGCC-3′. The samples were run in duplicate. The expression level for each sample of interest was normalized to that of *gapdh*.

Evaluation of PS exposure

PS exposure on transected axons of cultured DRG neurons was evaluated using Alexa594-labeled Annexin V (A13203, Thermo Fisher Scientific). Axons were co-stained with 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) (D-16, Life Technologies) for visualization. Three non-overlapping images per explant were randomly collected. More than 5 explants were examined for each experimental condition. To evaluate PS exposure on transected axons, the fluorescent intensities of each image were measured using ImageJ software (National Institutes of Health). Quantified levels of fluorescent intensities for PS normalized to DTAF were averaged and compared with the control.

Statistical analysis

Quantification data were shown as the mean ± SEM. Differences between groups were examined for significance using one-way ANOVA with Tukey's post hoc test.

Abbreviations

ANOVA	analysis of variance
CRMP2	collapsin response mediator protein 2
DRG	dorsal root ganglion
DTAF	5-(4,6-dichlorotriazinyl) aminofluorescein
GSK3B	glycogen synthase kinase 3B
MCL1	myeloid cell leukemia 1

PS	phosphatidylserine
SEM	standard error of mean
UPS	ubiquitin proteasome system
WT	wild-type

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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