ATP6AP2 functions as a V-ATPase assembly factor in the endoplasmic reticulum

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\textbf{ABSTRACT} ATP6AP2 (also known as the [pro]renin receptor) is a type I transmembrane protein that can be cleaved into two fragments in the Golgi apparatus. While in \textit{Drosophila} ATP6AP2 functions in the planar cell polarity (PCP) pathway, recent human genetic studies have suggested that ATP6AP2 could participate in the assembly of the V-ATPase in the endoplasmic reticulum (ER). Using a yeast model, we show here that the V-ATPase assembly factor Voa1 can functionally be replaced by \textit{Drosophila} ATP6AP2. This rescue is even more efficient when coexpressing its binding partner ATP6AP1, indicating that these two proteins together fulfill Voa1 functions in higher organisms. Structure–function analyses in both yeast and \textit{Drosophila} show that proteolytic cleavage is dispensable, while C-terminus-dependent ER retrieval is required for ATP6AP2 function. Accordingly, we demonstrate that both overexpression and lack of ATP6AP2 causes ER stress in \textit{Drosophila} wing cells and that the induction of ER stress is sufficient to cause PCP phenotypes. In summary, our results suggest that full-length ATP6AP2 contributes to the assembly of the V-ATPase proton pore and that impairment of this function affects ER homeostasis and PCP signaling.

\textbf{INTRODUCTION} The vacuolar-type H\textsuperscript{+}-ATPase (V-ATPase) consists of a proton pore (V0 sector) and an ATP hydrolysis domain (V1 sector). The biogenesis of the pump begins in the ER with the assembly of the V0 sector under the control of several chaperones (assembly factors). Once assembled, the V0 sector is transported to the Golgi apparatus where the preassembled V1 sector is added. The fully assembled V-ATPase acidifies the secretory and the endolysosomal pathway thereby providing the adequate pH for proteolytic processing, glycosylation, and protein degradation (Forgac, 2007).

The biochemical purification of the V-ATPase identified ATP6AP1 and ATP6AP2 as accessory subunits (Supek et al., 1994; Ludwig et al., 1998). Yet, more recent findings showed that the transmembrane domain of ATP6AP1 has some sequence homology with the yeast V-ATPase assembly factor Voa1 and that it can partially rescue the growth defect of a yeast strain that is both deficient for Voa1 and harbors mutations in the ER retrieval motif of the assembly factor Vma21 (voa1::H vma21QQ) (Jansen et al., 2016a). Interestingly, mutations in both ATP6AP1 and ATP6AP2 in humans cause a multisystem disorder with steatohepatitis, immunodeficiency, and psychomotor impairment (Jansen et al., 2016a; Rujano et al., 2017).

Unlike ATP6AP1, ATP6AP2 has been studied in many contexts and species (Peters, 2017). Work in \textit{Drosophila} has suggested that ATP6AP2 functions as an important factor in the planar cell polarity (PCP) pathway (Buechling et al., 2010; Hermle et al., 2010, 2013), a fundamental morphogenetic program that polarizes cells in the plane of the epithelium. The pathway is best understood in the \textit{Drosophila} pupal wing, where it orchestrates hexagonal cell packing.

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E18-04-0234) on July 11, 2018. Present addresses: \textsuperscript{1}Development, Aging and Regeneration Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037; \textsuperscript{2}Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris Sud, Université Paris-Saclay, 91198 Gif-sur-Yvette Cedex, France.

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Abbreviations used: ANOVA, analysis of variance; ATP, adenosine triphosphate; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRFP, monomeric red fluorescent protein.

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and the orientation of hairs toward the distal end of the wing. Current data indicate that there is a set of evolutionary conserved PCP core factors that share the capacity to control polarized hair formation and to localize asymmetrically in cells in so-called PCP domains (Simons and Mlodzik, 2008; Goodrich and Strutt, 2011; Devenport, 2014).

ATP6AP2 shows all the features of a PCP core protein, because 1) loss-of-function experiments cause PCP phenotypes in several Drosophila tissues and also other species (Buechling et al., 2010; Hermle et al., 2010, 2013; Schafer et al., 2015), 2) ATP6AP2 interacts physically with other PCP core proteins (Buechling et al., 2010; Hermle et al., 2010, 2013), and 3) shows an asymmetric localization at PCP domains (Hermle et al., 2013). The specific role of ATP6AP2 in PCP is further underscored by the observation that lack of the PCP core factor Flamingo (Fmi) leads to loss of ATP6AP2 at the PCP domains, while the overexpression of Fmi causes a strong accumulation of ATP6AP2 at the cell surface (Hermle et al., 2013).

The molecular basis for the functional diversity of ATP6AP2 is currently unclear. However, it might be linked to a well-described proteolytic cleavage event in the Golgi apparatus that produces an N-terminal and a C-terminal fragment (NTF and CTF, respectively) (Cousin et al., 2009; Zhu and Yang, 2018). While mosaic analysis in flies demonstrated that the NTF from the hemolymph or from the secretion by neighboring cells can localize to PCP domains (Hermle et al., 2013), it was the CTF that copurified with the V-ATPase (Ludwig et al., 1998). Although the knockdown of several V-ATPase subunits has been shown to cause PCP phenotypes (Hermle et al., 2010, 2013), none of the V-ATPase subunits so far examined shares the PCP-like localization pattern of ATP6AP2 or have been shown to be stabilized by Fmi, which may suggest that the V-ATPase function of ATP6AP2 is independent of its function in the PCP pathway.

Here we addressed whether ATP6AP2 also functions as a V-ATPase assembly factor. Using the yeast model, we show that Drosophila ATP6AP2 can rescue the impaired growth of voa1::H vma21QQ yeast cells. This rescue requires the full-length (FL) sequence and is improved by the coexpression of ATP6AP1. We also demonstrate that the FL protein, and not the cleavage fragments, is required for both PCP and V-ATPase functions in Drosophila. Both gain-of-function and loss-of-function of ATP6AP2 causes Xbp1-dependent ER stress. Altogether, the results suggest that ATP6AP2 participates in the assembly of the V-ATPase in the ER and that failure to do so impairs the PCP pathway.

RESULTS AND DISCUSSION
Rescue experiments in yeast
The de novo formation of the multisubunit V-ATPase requires the coordinated assembly of at least 13 different subunits, some of which are present in multiple copies in the complex. In yeast, the assembly of the V0 sector in the ER is facilitated by the assembly factors Vma21, Vma12, Vma22, Pkr1, and Voa1 (Ryan et al., 2008). Recently, mammalian orthologues have been identified for Vma21 (Vma21QQ), Vma12 (TMEM199), and Vma22 (CCDC115) and possibly Voa1 (voa1::H vma21QQ; Supplemental Figure S1). 

As the V-ATPase assembly function was shown to require the FL background, both NTFΔC rescued and CTFΔN failed to rescue embryonic lethality even when expressed in combination demonstrating that an intact FL protein is essential for survival (Figure 2A). By contrast, ΔC rescue and ΔN rescue animals died in the late larval stage (Figure 2A) and were thus less viable than ΔAKXX rescue animals, which previously were shown to survive until late pupal stages (Rujano et al., 2017). Both ΔC rescue and ΔAKXX rescue animals showed higher levels of NTF, suggesting that preventing retrograde transport leads to more exposure to Golgi-localized proteases (Supplemental Figure S2, A and B). Interestingly, the insertion of the CTF transgene in the wild-type background was not viable in homozygosity, indicating that it may cause dominant-negative effects. This is consistent with the apoptotic effects and the reduction of endogenous ATP6AP2 at the PCP domains observed on CTF overexpression (CTFΔN) in the wing epithelium (Supplemental Figure S3A).

To analyze the rescue efficiency of the deletion rescue constructs on the cellular level, we expressed them in ATP6AP2 mutant clones surrounded by mutant cells rescued by FLΔC (see schematic representation in Figure 2B). Expressing NTΔN rescue and ΔC rescue, and particularly, CTFΔN rescue clones led to significantly smaller clones compared with the corresponding FLΔC twin clone (unpublished data), suggesting suboptimal viability or growth (Figure 2C–J). Staining the cell outlines with anti-E-cadherin also revealed that cells expressing NTΔN rescue, CTFΔN, and ΔC rescue had smaller apical surfaces (Figure 2, D–F). In addition, E-cadherin levels were increased compared with the surrounding wild-type tissue and the FLΔC clones, which is consistent with a reduced lysosomal degradation of E-cadherin and impaired V-ATPase function as shown previously (Hermle et al., 2013). To assess potential PCP phenotypes in the clones, we analyzed actin-based
wing hairs. While CTF

rescue

clones were too small to properly address

hair polarity, NTF

rescue

and

Δ

C

rescue

clones showed PCP phenotypes

such as multiple wing hairs per cell and misalignment of hairs (Figure

2, G–J). Altogether, these results suggest that despite the strong co-

localization of the NTF with Fmi at PCP domains, which is not seen for

any of the V-ATPase-associated proteins analyzed so far (Hermle

et al., 2013), it is the uncleaved FL ATP6AP2 that is needed for both

V-ATPase and PCP functions.

The C terminus of ATP6AP2 mediates ER localization in

Drosophila wing cells

Next, we analyzed whether ATP6AP2 localized to the ER of wing

epithelial cells. Previous experiments had demonstrated that a pep-
tide antibody recognizing a sequence close to the N-terminus

mainly detected endogenous ATP6AP2 (or NTF) at the asymmetric

PCP domains at the cell surface at steady state (Hermle

et al., 2013). This antibody also detected an intracellular pool of ATP6AP2,

but this pool was too weak to address any colocalization with ER mark-
ers (not shown). By contrast, overexpression of ATP6AP2 using the

wing driver patched (ptc)-GAL4 showed a strong colocalization

with the ER marker protein disulfide-isomerase (PDI) (Figure 3A). Interest-

ingly, PDI was up-regulated in FL

OE

cells compared with wild-type

cells outside the ptc compartment, indicating ER stress (Figure 3D).

The overexpression of ATP6AP2

Δ

C

(ΔC

OE

) and

Δ

KKXX

(ΔKKXX

OE

) showed less ER localization and reduced PDI up-regulation (Figure

3, B and C), and the overall staining pattern was more vesicular

compared with FL

OE

, particularly for

Δ

C

OE

(Figure 3, A–D). Accord-

ingly, we found by Western blotting significantly higher amounts of

NTF for both

Δ

C

OE

and

Δ

KKXX

OE

compared with FL

OE

, again sug-

gesting increased exposure to Golgi-localized proteases (Figure

3E). Together, these results suggest that the cytoplasmic tail controls

ER localization and hence residence time in the Golgi, which in turn

affects proteolytic processing of ATP6AP2.

ATP6AP2 overexpression causes ER stress and UPR induction

PDI is commonly up-regulated in the context of ER stress and the

unfolded protein response (UPR). An important UPR branch is the
Ire1α/Xbp1 pathway, in which the homodimerizing Ser/Thr kinase Ire1α is able to activate the transcription factor Xbp1 (X-box binding protein 1) by removing one intron from its mRNA. The spliced version of Xbp1 is able to up-regulate ER chaperones, including PDI, by direct binding to stress element promoters in the nucleus (Shen et al., 2001; Yoshida et al., 2001).

To better understand the effect of FL^{OE} on ER homeostasis, we used the xbp1-EGFP reporter, in which EGFP is expressed in frame with Xbp1 only when ER stress-induced splicing occurs (Ryoo et al., 2007). While xbp1-EGFP was not visible on expression of the control protein mRFP (Figure 3F), FL^{OE} caused a high expression of xbp1-EGFP in nuclei of the epithelial cells (Figure 3G), confirming the
induction of the UPR. Interestingly, the expression of both FLOE and Xbp1 RNA interference (RNAi) with ptc-GAL4 caused early lethality, suggesting that Xbp1-dependent UPR is essential to counteract the ER stress induced by FLOE.

Overexpression of ATP6AP2 causes V-ATPase and PCP defects in a cytoplasmic tail-dependent manner

To test whether the ER stress caused by FLOE correlated with V-ATPase phenotypes, we used a construct expressing GFP-Lamp1 under the ubiquitous tubulin promoter (Pulipparacharuvil et al., 2005). In normal cells, the luminal GFP is targeted to the lysosomes for degradation, but this does not occur in cells with V-ATPase dysfunction (Hermle et al., 2013). We observed GFP-Lamp1 accumulation in intracellular vesicles in cells expressing FLOE but not with ΔC0E (Figure 4, A and B). This suggests that FLOE has dominant-negative effects on V-ATPase function, possibly by interfering with the precise stoichiometry required for proper V-ATPase function and/or assembly.

With regard to PCP phenotypes, we found that FLOE but not ΔC0E produced cells with multiple wing hairs and mild hair misalignment in adult wings (Figure 4, C and D). Immunostainings of pupal wings showed a slightly less polarized localization for the transmembrane
PCP factors Frizzled (Fz), Strabismus (Stbm), and Fmi in FL^OE cells compared with wild-type cells (Figure 4, E and G–I). Fmi showed an additional intracellular accumulation (Figure 4, E and G). Containing with the ER marker KDEL showed a partial overlap in the ER, indicating that a subset of Fmi molecules is retained in this compartment via FL^OE (Figure 4G). This effect was not seen with ΔC^OE, NTF^OE, or CT^OE (Figure 4F and Supplemental Figure S4, A and B). Together, the results suggest that FL^OE affects the PCP pathway, at least in part, by causing partial accumulation of Fmi in the ER.

**Lack of ATP6AP2 induces ER stress and ER stress is sufficient to cause PCP phenotypes**

As overexpression of membrane proteins may cause ER stress in an unspecified manner, we analyzed next whether ER stress was seen in cells lacking ATP6AP2. Indeed, we found increased levels of xbp1-EGFP and PDI on knockdown of ATP6AP2 (Figure 5, A and B). PDI up-regulation was suppressed by the coexpression of xbp1 RNAi, indicating that ER stress is xbp1 dependent (Figure 5C). Clones mutant for ATP6AP2 also showed an up-regulation of PDI (Figure 5D), providing additional evidence for the presence of ER stress in cells with too-high and too-low levels of ATP6AP2.

Finally, to address potential links between ER stress and PCP in ATP6AP2-deficient cells, we asked whether the induction of ER stress is sufficient to disrupt PCP. For this, we silenced the ER chaperone BiP, which caused an increase in PDI levels and Xbp1 splicing, as reported previously (Chow et al., 2015) (Figure 5, E and F). In the pupal and adult stage, cells showed severe packing defects, delayed hair growth, and substantial hair misorientation (Figure 5, G and H). Immunostaining for Fmi revealed loss of asymmetry, but no retention in the ER (Figure 5I). Compared with ATP6AP2 knockdown using the same driver (Hermle et al., 2010), these phenotypes obtained by BiP knockdown were thus very similar with respect to Fmi localization and hair orientation but more severe with respect to cell shape regulation and the timing of hair formation (Hermle et al., 2013).

**Conclusions**

In this study, we show that impairment of V-ATPase assembly caused by a Voa1 deficiency in yeast can functionally be rescued by the expression of fly ATP6AP2. Our data also demonstrate a role of ATP6AP2 in the ER of fly wing cells, indicating that the assembly function is conserved. In both systems, the rescues were dependent on the presence of the NTF and the short cytoplasmic tail containing the ER retrieval motif. As the NTF contains the binding domain for ATP6AP1 (Rujano et al., 2017), it could specifically be required for V0 assembly. However, the ER retrieval motif, which is absent in ATP6AP1, could be important to allow the return of ATP6AP2 and interacting proteins that have escaped to the Golgi. The retrieval motif is lacking, ATP6AP2 cleavage is increased (this study has been shown for Vma21 (Malkus et al., 2013)), suggesting that cleavage in the Golgi may also somehow be coupled with the addition of the V1 sectors in the Golgi. However, in the fly at least the rescue with an uncleavable form of ATP6AP2 did not produce any obvious phenotypes (Hermle et al., 2013; Rujano et al., 2017), suggesting that cleavage is not an essential aspect of ATP6AP2 function.

As unassembled V0 subunits have been shown to be targeted for ER-associated degradation (Hill and Cooper, 2000), prolonged.
Misassembly may be directly linked to both V-ATPase dysfunction and ER stress induction (Sekiya et al., 2017). The observation that BiP knockdown caused similar but also different wing phenotypes compared with ATP6AP2 knockdown suggests that part of the PCP phenotypes are ER stress dependent. As the decisive players in PCP are transmembrane proteins that travel through the early secretory pathway while undergoing posttranslational modifications, any disturbance in ER homeostasis could indeed influence signaling at the plasma membrane (Merte et al., 2010; Wansleeben et al., 2010).

This may be particularly important for the large protocadherin Fmi, which is modified extensively in the ER via glycosylation and autocatalytic cleavage at the G protein–coupled receptor proteolytic site (Usui et al., 1999; Promel et al., 2012). It can also be speculated that Fmi maturation and V-ATPase assembly could at least in the pupal wing epithelium be linked by common molecular factors in the early secretory pathway where the establishment of PCP requires constant turnover of Fmi in the acidic lysosomes (Strutt and Strutt, 2008; Strutt et al., 2011).

Altogether, we provide evidence that ATP6AP2 functions together with ATP6AP1 in the assembly of the V-ATPase in the ER and that ATP6AP2 can influence PCP signaling at the level of the ER. Additional work is needed to understand by which mechanisms ATP6AP2 can act as a chaperone for PCP proteins in conjunction to its role in V-ATPase assembly.
MATERIALS AND METHODS

Plasmids and yeast strain

Plasmids encoding ATP6AP2CTF, ATP6AP2ΔC, ATP6AP2ΔKKXX, or ATP6AP1CTF have the same architecture as previous constructs encoding human ATP6AP1 (Jansen et al., 2016a). Briefly, pRS316 vector containing HA-tagged Voa1 with genomic flanking sequence (pMR072) (Ryan et al., 2008) was modified by replacing S26–N265 of the Voa1 ORF with DmAtp6ap2CTF (D252–N320), resulting in pMR1503, or with DmAtp6ap1 E18–E379, resulting in pMR1701. These plasmids insert fragments were constructed by PCR amplifying yeast codon optimized D. melanogaster ds synthetic DNA sequence (gBlocks, Integrated DNA Technologies) with primers having 5’ and 3’ sequence complementary to pMR072 sequence adjacent to the targeted insertion site. D. melanogaster sequence fragments were joined by ligase free cloning (Li et al., 2011) to inverse amplified pMR072 with Voa1 S26–N265 omitted. pMR1608, encoding DmAtp6ap2CTF, was prepared from pMR1503 by inserting the N-terminal fragment (S18–R251, gBlock) 5’ of the D252 codon. pLG493 (pRS415 DmAtp6ap2CTF) and pLG494 (pRS415 DmAtp6ap2ΔKKXX) were generated by subcloning the DmAtp6ap2 insert with Voa1 flanking sequence, from pMR1503 and pMR1608, respectively, into pRS415. pLG496 (pRS316 DmAtp6ap2ΔCΔKKXX) was generated using pMR1608 as PCR template. pMR1608 was inverse amplified using KOD Hot Start DNA polymerase (Novagen) and primers designed to omit C-terminal sequence encoding KKDN and add an EcoRI restriction site immediately following stop codon. For each plasmid, the entire ORF was verified by DNA sequencing. These plasmids all have Voa1 5’UTR, the Voa1 signal sequence (M1–A24) plus the first amino acid after Voa1 signal cleavage (D25), an HA tag followed by the Dm sequence indicated, and Voa1 3’ UTR. Plasmids were transformed into voi1::H vma21QQ yeast (MRY5: MA Taura3-52 leu2-3,112 his4-519 ade6 pep4-3 gal2 voi1::HygR vma21QQ::HA) (Ryan et al., 2008).

Yeast growth assays and quinacrine staining

The ability of the yeast strain MRY5 transformed with DmAtp6ap2 and/or DmAtp6ap1 plasmids to grow at nonpermissive conditions was assayed using a previously described protocol with few exceptions (Jansen et al., 2016a). Liquid cultures of yeast strains with plasmids were grown at 30°C overnight in synthetic media plus dextrose (SD) and supplemented with amino acids (SD-aa) appropriate to each strain. Yeast mids were grown at 30°C overnight in synthetic media plus dextrose (SD) and supplemented with amino acids (SD-aa) and grown for an additional 6 h. The actively dividing yeast cells were assayed using a previously described protocol with few exceptions. Yeast growth assays and quinacrine staining

Generation of mutant clones

For the clonal analysis, the progeny was heat shocked for 60 min 2–6 d after egg laying, and pupae were staged for immunostaining. Two time points were analyzed: early for immunostaining (28 h after puparium formation [APF] at 25°C or 65 h APF at 18°C) and late for the analysis of pupal wing hairs (34 h APF at 25°C or 72 h APF at 18°C). Clones were recognized by lack of β-Gal staining or in the case of clones expressing the genomic rescue constructs (ATP6AP2>ATP6AP2 or others) in a null background by lack of expression of mRFP.

Immunostaining of pupal wings

Pupae were dissected in phosphate-buffered saline (PBS). After the pupal case was removed, pupae were fixed with fixation solution (paraformaldehyde 8% in PBS) for 1 h at room temperature (RT). Wings were carefully pulled out of the cuticle and transferred to an Eppendorf with PBS. After three washing steps with PBS–Triton (PBST: 0.1% Triton-X in PBS) wings were blocked for at least 30 min at RT in blocking solution (5% goat serum in PBST) and incubated overnight with primary antibodies diluted in blocking solution. The next day, wings were washed three times with PBST and incubated with fluorescein labeled secondary antibody for 1–2 h at RT. After three washing steps with PBST, the liquid was removed. For the mounting of the pupal wings, Roti-Mount Fluocare (Roth) was added, and wings were placed on a microscope slide with the help of a pipette. Finally, a coverslip was placed on top. Primary antibodies can be found in Supplemental Table S2. Images were taken using a Zeiss LSM 510 META UV confocal microscope or Zeiss LSM 700 confocal microscope. Image processing was performed using ImageJ and Adobe Photoshop CS5 software.

Mounting of adult wings

Adult wings were incubated in isopropanol for 15 min, mounted in Euparal (Roth), and viewed using a Zeiss Axioplan microscope. Nanoszoomer 2.0 was used to acquire the images (Hamamatsu). Analysis of the images was performed using NDPview software and Fiji.

Immunoblotting

For Western blots from pupae, white prepupae were collected and aged for 28 h at 25°C. After the removal of the case, three pupae of the targeted genotype were heat shocked for 60 min 2–6 d after egg laying, and pupae were staged for immunostaining. Two time points were analyzed: early for immunostaining (28 h after puparium formation [APF] at 25°C or 65 h APF at 18°C) and late for the analysis of pupal wing hairs (34 h APF at 25°C or 72 h APF at 18°C). Clones were recognized by lack of β-Gal staining or in the case of clones expressing the genomic rescue constructs (ATP6AP2>ATP6AP2 or others) in a null background by lack of expression of mRFP.

Fly genotypes

ATP6AP2ΔC and genomic rescue construct was described in Hermle et al. (2013). Note that the gene was then termed VhaPPR. The new gene name ATP6AP2 that now also can be found in Flybase is adapted to the mammalian nomenclature. For the transgenesis of rescue constructs and of the upstream activating sequence (UAS) overexpression constructs, attP landing sites at cytosome 51C1 and 86F8, respectively, were used (Bestgene). NTF and CTF comprise amino acids 1–248 and 252–320, respectively. See Supplemental Table S1 for a full list of the genotypes used in this study. Primer sequences for cloning into pattB and pUA5gt.attB, respectively, are available on request. New lines generated for this study are ATP6AP2>NTF, ATP6AP2>CTF, ATP6AP2>ΔC, UAS-ATP6AP2ΔC, and UAS-CTF. All the others can be found in Hermle et al. (2013) and Rujano et al. (2017).
method within a Mini-Protease 2 Cell chamber (BioRad). Membranes were incubated overnight with the desired primary antibodies at 4°C in Tris-buffered saline, 0.1% Tween 20 (TBS-T) and then incubated with the specific horseradish peroxidase–coupled secondary antibody for 1 h at RT (in 1% bovine serum albumin TBS-T). Bound secondary antibodies were visualized by the enhanced chemiluminescence method (Supersignal West Femto; ThermoScientific). Densitometric quantification of protein expression levels was performed using ImageJ software.

ACKNOWLEDGMENTS

We thank Hermann Steller, Helmut Krämer, Giorgios Pyrowolakis, and the Bloomington Stock Center for fly strains. We thank David Strutt as well as the Developmental Studies Hybridoma Bank for antibodies. We thank Magda Cannata Serio for critically reading the article. The work has been supported by the ATP-Avenir program, for the Fondation Bettencourt-Schuller (Liliane Bettencourt Chair of Developmental Biology), as well as state funding by the Agence Nationale de la Recherche (ANR) under the “Investissements d’avenir” program (ANR-10-IAHU-01) and a NEPHROFLY (ANR-14-ACHN-0013) grant to M.S. and by National Institutes of Health grant GM38006 to T.H.S.

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