Sequoia regulates cell fate decisions in the external sensory organs of adult *Drosophila*

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The adult *Drosophila* external sensory organ (ESO), comprising the hair, socket, neuron, sheath and glia cells, arises through the asymmetric division of sensory organ precursor cells (SOPs). In a mosaic screen designed to identify new components in ESO development, we isolated mutations in *sequoia*, which encodes a putative zinc-finger transcription factor that has previously been shown to have a role in dendritogenesis. Here, we show that adult clones mutant for *seq* exhibit a loss of hair cells and a gain of socket cells. We propose that the *seq* mutant phenotype arises, in part, owing to the loss of several crucial transcription factors known to be important in peripheral nervous system development such as D-Pax2, Prospero and Hamlet. Thus, *Sequoia* is a new upstream regulator of genes that orchestrates cell fate specification during development of the adult ESO lineage.

Keywords: D-Pax2; *Drosophila* PNS; Hamlet; Prospero; *Sequoia*
EMBO reports (2009) 10, 636–641. doi:10.1038/embor.2009.66

INTRODUCTION

Cell fate acquisition relies on the coordinated action of a combination of cell fate determinants and transcriptional regulators. An excellent model for uncovering new aspects of cell fate specification consists of the external mechanosensory bristles that are present on the thorax of the adult *Drosophila*, also known as external sensory organs (ESOs). ESOs can be screened readily for aberrations in their stereotypic pattern; furthermore, the ESO lineage and the mechanisms of its development are well characterized. Each ESO arises from the asymmetric divisions of a single sensory organ precursor cell (SOP; Hartenstein & Posakony, 1989). On division of each SOP, the cell fate determinants Numb and Neuralized are inherited by the p11b cell, but not by its sibling, the p11a cell (Rhyu *et al*, 1994; Le Borgne & Schweisguth, 2003). This activates *Notch* (*N*) signalling only in the p11a. After

two more asymmetric divisions, five cell types are specified: these are the 'external' hair and socket cells, and the 'internal' sheath, neuron and glial cells, the last of which undergoes apoptosis (Gho *et al*, 1999; Fichelson & Gho, 2003).

In addition to perturbations in *N* signalling that affect cell fate acquisition, other mutations are known to disrupt cell identity. For example, *hamlet* (*ham*) mutations cause a gain of external structures on the adult cuticle, as internal cells of the ESO lineage transform into external cells after specification (Moore *et al*, 2004).

In a forward genetic screen to identify novel genes involved in bristle development, we identified new alleles of *sequoia* (*seq*), a putative zinc-finger transcription factor originally identified for its role in dendrite development in the embryonic peripheral nervous system of *Drosophila* (Brenman *et al*, 2001). Our experiments suggest that *seq* functions in parallel or downstream from *N* signaling, and regulates the expression of crucial components in the development of the ESO lineage, including D-Pax2, Prospero (*Pros*) and *Ham*.

RESULTS

Mutations in *seq* cause a gain of socket cells in the ESO

To identify genes that regulate ESO development, we carried out an adult mosaic screen on chromosome 2R using *Ultrabithorax*-flipase (*Ubx*-FLP; see Methods). Complementation group 2R5 consists of five lethal alleles, all of which lead to a loss of hairs and a gain of socket cells in clones of mutant tissue on the adult thorax (Fig 1A and supplementary Fig 1 online).

All 2R5 alleles are embryonic lethal, and adult clones show similar phenotypes. We narrowed down the lethality by using deficiency and P element meiotic mapping (Zhai *et al*, 2003) within the distal 50-kb region of *Df(2R)Exel8057*. On sequencing, we found that all of the molecular lesions mapped to *seq* (Fig 1B). The alleles we used for this study—*seq*^{A41} and *seq*^{U5}—are embryonic lethal when trans-heterozygous to each other or to *Df(2R)Exel8057*. In addition, *Seq* is absent in homozygous mutant clones of *seq*^{A41} (supplementary Fig 2A–C online) and *seq*^{U5} (data not shown). Therefore, *seq*^{A41} and *seq*^{U5} are probably severe loss-of-function alleles or null alleles.

All five alleles isolated from our screen failed to complement a previously published allele of *seq*, *seq*²² (Brenman *et al*, 2001).

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Received 28 October 2008; revised 26 February 2009; accepted 13 March 2009; published online 15 May 2009

Fig 1 | Mutations in *seq* lead to the development of additional socket cells. (A) *Ubx*-FLP-induced *yellow*^{-/-} marked clones of *seq*^{U5} and (A') unmarked clones of *seq*²² on the adult notum. In marked clones, the wild-type bristles are *yellow*⁺ and the mutant *yellow*⁻. The most prominent phenotype seen in *seq* mutant clones is the loss of hairs and a gain of socket cells (arrowhead). (B) Linear representation of the Seq protein and the amino-acid changes in five new alleles and the previously described substitution in *seq*²². Asterisks represent stop codons. (C, C') A 26–28 APF clone of *seq*^{U5}. The anterior is up; green fluorescent protein (GFP) marks wild-type cells; cut (blue) marks the ESO cells; Su(H) (red/greyscale) marks the socket cells. (D) Pie charts represent the distribution of *seq*^{U5} mutant precursor clusters that contain 1, 2, 3 or 4 socket cells at approximately 28 APF (top chart) and approximately 38 APF (bottom chart). wild-type external sensory organ (ESO) clusters always only contain one Su(H)-positive cell. APF, hours after puparium formation; Seq, *sequoia*; ZF, zinc finger.

Surprisingly, in adult clones, *seq* has been reported not to affect cell fate acquisition in the ESO lineage (Brenman *et al*, 2001). However, clones of *seq*²², *seq*^{U5} or *seq*^{A41} showed identical phenotypes—namely bristle loss and additional sockets on the adult thorax—when the clones were induced by *Ubx*-FLP or *heat shock*-flipase (*hs*-FLP; for the alleles *seq*^{U5} and *seq*^{A41}; Fig 1A, supplementary Fig 3 online). Therefore, the ‘multiple sockets’ phenotype is not due to sensitization of the genetic background caused by different transgenes. Given that most alleles, including *seq*²², contain a stop codon and are severe loss-of-function alleles, we cannot explain this discrepancy.

We examined the development of the ESO lineage in *seq* mutant clones. At 28 hours after puparium formation (APF), 77% of *seq* mutants contained a normal complement of ESO-positive cells labelled by Cut, but two socket cells instead of one were labelled by Su(H) (Fig 1C,D, top chart). Intriguingly, at 38 APF, 95% of mutant clusters had more than one socket cell (Fig 1D). Similarly, in adult flies, 91% of the mutant sensory organs had more than one socket cell ($n=361$), although the exact number of socket cells within the mutant clusters on the adult cuticle could not be quantified because socket cells fuse during morphogenesis of the ESO (Shiomi *et al*, 1994; Manning & Doe, 1999).

In summary, our experiments indicate that *seq* controls cell fate acquisition in the ESO lineage. In the absence of *seq*, ESO clusters contain more socket cells than wild-type clusters, but have no

bristles. Therefore, we decided to examine whether socket cells develop at the expense of the hair cells in the lineage, during or even post-specification of the ESO lineage.

N signalling appears normal in *seq* mutants

A gain of socket cells is consistent with a pllB to pllA transformation in the ESO lineage owing to a gain of function of *N* signalling. Such a defect might arise through mis-localization of cell fate determinants such as Numb and Neuralized (Rhyu *et al*, 1994; Le Borgne & Schweisguth, 2003). However, both proteins localized correctly to the anterior cortex of both wild-type and *seq* mutant dividing pl cells (Fig 2A,A'; data not shown). In addition, the mitotic spindle aligned correctly with the Numb crescent, suggesting that only the anterior daughter cell inherits Numb in *seq* mutants (Fig 2A).

To confirm further whether *N* signalling occurs normally in *seq* mutants, we monitored the expression of Tramtrack (Ttk). In wild-type two-cell-stage precursors, Ttk is expressed as a result of *N* signalling only in pllA, whereas it is repressed in pllB, either through post-transcriptional degradation or through translational repression (Guo *et al*, 1995; Okabe *et al*, 2001; Pi *et al*, 2001). In *seq* mutant clones, Ttk is normally expressed in pllA (Fig 2B,B'). To corroborate further that pllA is specified correctly, we monitored the expression of Deadpan (Dpn; Gho *et al*, 1999). Dpn is also expressed normally in 100% of *seq* mutant two-cell-stage clusters ($n=22$; Fig 2C,C'). Thus, these data indicate that *N* signalling

Fig 2 | The gain of socket cells in *seq* mutant external sensory organ clusters is not due to a defect in *N* signalling. Sequoia acts in parallel or downstream from *N* signalling. The anterior is up and Senseless (Sens; red) marks the pI and pII stage precursor cells. (A,A') In both wild-type (WT) and *seq*^{U5} dividing pI cells, Numb (blue) localizes to the anterior cortex and the mitotic spindle (green in A') aligns with the Numb crescent in *seq* mutants. (B,B') In both wild-type (left cluster) and *seq* mutant (right cluster) pII stage clusters, Tramtrack 69 (Ttk69; blue/greyscale) is excluded from the anterior pIIb cell, but expressed in the posterior pIIa cell. Image is a single XY section. (C,C') In both wild-type (left cluster) and *seq* mutant (right cluster) pII stage clusters, Deadpan (Dpn; blue/greyscale) is expressed in the posterior pIIa cell. Sequoia is epistatic to downregulation from *N*, mediated by the overexpression of UAS-Numb-GFP in MARCM clones of *hsFLP*, *UAS-CD8::GFP*; *FRT42D seq*^{A41}/*FRT42D tub-GAL80*; *UAS-Numb-GFP/tub-GAL4* flies (D–G), or by inactivation of the *N*^{ts} allele at the restrictive temperature of 30 °C. (D) Composite panel of (E–G): (E) GFP marks the mutant *seq*^{A41} tissue that overexpresses UAS-Numb-GFP, (F) cut marks the cells in the mutant ESO cluster and (G) Su(H) marks the socket cells within the cluster. (H) Detail from the thorax of a *UbxFLP N*^{ts}/*Y*; *FRT42D seq*^{A41}/*FRT42D tub*^{iso} fly, between 17–29 APF at 30 °C. The asterisk indicates the abnormal bristle that is also apparent in *seq* mutants alone. The arrowheads indicate the sockets and the arrow points to double bristles, presumably as only *N* is being inactivated in the specific cluster. APF, hours after puparium formation; ESO, external sensory organ; GFP, green fluorescent protein; N, Notch; Seq, *sequoia*.

temperature, and observed the generation of socket cells within the clones (Fig 2H). Therefore, we conclude that *seq* acts in parallel to or downstream from *N*.

seq mutant ESO cells do not express D-Pax2

The lack of hair cells and a gain of socket cells in *seq* mutants phenocopy partial loss-of-function mutations in *D-Pax2*, which lead to the transformation of hair to socket cells (Kavaler *et al*, 1999). In addition, Seq is expressed dynamically from the pI stage onwards, throughout the development of the ESO lineage (supplementary Fig 4 online), in a manner similar to D-Pax2. Therefore, we monitored D-Pax2 expression in *seq* mutant ESOs. D-Pax2 was not expressed in 18–20 APF mutant clusters (Fig 3A,A'). At 24 APF, D-Pax2 was observed in 18% of mutant clusters (*n*=22), but was further decreased to 7.7% of mutant clusters at 26 APF (*n*=13). At 38 APF, 92% of mutant clusters did not express D-Pax2 (*n*=25) (data not shown). Thus, the absence of D-Pax2 expression in most *seq* mutant ESO cells might account for the early double socket phenotype and the loss of hair cells.

seq mutant ESO cells do not express Pros

To determine whether the internal lineage is affected in *seq* mutants, we followed the expression of Pros. Pros is first expressed in the pIIb cell and its progeny, the pIIb and the glial cell, and finally is restricted to the sheath cell (Manning & Doe, 1999; Reddy & Rodrigues, 1999). In *seq* mutant precursor cells, Pros was not expressed at the pIIb stage (data not shown). Pros expression could only be detected at low levels in the small glia at the 4–5-cell stage (Fig 3B,B'). By 38 APF, 97% of mutant clusters did not express Pros (*n*=72; data not shown).

occurs normally in *seq* mutants, and that the additional socket cell phenotype is not due to a pIIb to pIIa transformation.

We then examined the relationship of *seq* and *N* by using epistasis experiments. If *seq* is an upstream negative regulator of *N* and *N* is a positive regulator of socket cell development, then loss of *N* signalling should alleviate the 'multi-socket' phenotype of *seq* mutations. Alternatively, if *seq* acts in parallel to or downstream from *N*, then we should observe the *seq* 'multi-socket' phenotype when *N* activity is downregulated. We addressed this question by using two approaches. First, we overexpressed *numb*, which should lead to four neurons and a loss of *N* signalling (Wang *et al*, 1997). We observed that ESO cells, which are homozygous mutant for *seq*^{A41} and also overexpress Numb, acquired a socket fate, as assessed by the high levels of Su(H) expression (Fig 2D–G). Second, we created *seq* mutant clones in *N*^{ts} animals grown at a restrictive

Fig 3 | Mutations in *seq* lead to the loss of D-Pax2 and Pros in the external sensory organ lineage. (A,A',A'') An 18–20 APF clone of *seq*⁴⁴¹. Cut (red) marks the ESO cells and D-Pax2 (blue/greyscale). Note the complete loss of D-Pax2 in the mutant clone. (B,B',B'') A 26–28 APF *seq*^{U5} clone. Cut (red) marks the ESO cells; Pros (blue/greyscale) marks the sheath cells and is transiently expressed in the wild-type glial cells. Pros is expressed in the small glial cell in the mutant, but is not expressed in the sheath cell. (C,C',C'') A 26–28 APF *seq*^{U5} clone. Elav (red/greyscale) marks the neuron; Pros (blue) marks the sheath cell in wild-type ESO cells. These markers are absent or only transiently expressed in the glial cell in *seq* mutant ESO cells. (D,D',D'') A 26–28 APF *seq*^{U5} clone. Cut (red) marks ESOs; HRP (blue/greyscale) marks the neuronal membrane in wild type. The HRP pattern is severely disrupted in *seq* mutant ESO clusters. Green fluorescent protein (GFP) marks wild-type (WT) cells in all panels. APF, hours after puparium formation; Elav, embryonic lethal, abnormal vision; ESO, external sensory organ; HRP, horseradish peroxidase; Pros, Prospero; Seq, Sequoia.

In *pros* mutants, the internal lineage is incorrectly specified, leading to the loss of neuron and sheath cell fates (Manning & Doe, 1999; Reddy & Rodrigues, 1999). Therefore, we compared the expression of the neuronal markers Elav (embryonic lethal, abnormal vision) and HRP (horseradish peroxidase) in *seq* and wild-type cells. Elav was only transiently expressed at 26–28 APF in *seq* mutant cells (Fig 3C,C''). At 38 h APF, Elav was absent from all *seq* mutant ESO clusters ($n = 73$; data not shown). Furthermore, the normal pattern of the neuronal membrane marker HRP was severely disrupted in *seq* mutants (Fig 3D,D''), similar to the phenotype of *pros* mutations (Manning & Doe, 1999). In conclusion, these data suggest that pIIb and its progeny are not specified correctly and develop aberrantly in *seq* mutants.

Fig 4 | Ham is not expressed in *seq* mutant external sensory organ cells. (A,A',A'') A 26–28 APF *seq*^{U5} clone. Green fluorescent protein (GFP) marks the wild-type (WT) cells; cut (red) marks the ESO cells; Ham (blue/greyscale) marks the sheath and the neuron, where it is later restricted. Ham is not expressed in *seq* mutant ESO cells. (B,B',B'') A 26–28 APF *ham* clone. GFP marks the wild-type cells; cut (red) marks the ESO cells; Seq (blue/greyscale) marks the sheath and the neuron. Seq is expressed normally in *ham* mutant ESO cells. (C,C',C'') A 22–24 APF *pros*¹⁷ clone. GFP marks wild type; cut (red) marks ESO cells; Ham (blue/greyscale) marks the pIIb, neuron and sheath. Ham is expressed normally in *pros* mutant ESO cells. (D,D') A 24–26 APF *seq*^{U5} MARCM clone, in which UAS-Pros is only expressed in the *seq* mutant ESO lineage using *neur*-GAL4. Mutant clones are positively marked using UAS-CD8::GFP; Ham (red/greyscale) marks the sheath and neuron, to which its expression is later restricted. Note that in the MARCM clones, the expression of Pros is unable to induce expression of Ham in the absence of Seq. APF, hours after puparium formation; ESO, external sensory organ; Ham, Hamlet; Pros, Prospero; Seq, Sequoia.

seq mutant ESO cells do not express Ham

The loss of Pros cannot account for the increase in socket cells observed after specification (Fig 1), as *pros* mutants do not show this phenotype (Manning & Doe, 1999; Reddy & Rodrigues, 1999). However, mutations in *ham* lead to a post-specification cell fate transformation of the internal lineage to cells of the external lineage (Moore et al, 2004). Ham is normally expressed in pIIb, the sheath and neuron (Moore et al, 2004); however, Ham is not expressed at any stage of development in *seq* mutant precursor cells (Fig 4A,A''), whereas Seq is expressed normally in *ham* mutant clones (Fig 4B,B''). Hence, the loss of Ham in the

Fig 5 | The *seq* mutant phenotype can be modulated by the expression of D-Pax2 and Ham in the external sensory organ lineage. (A) *Ubx*-FLP-induced clones of *seq*. (B,B',C,C') Adult nota, in which the indicated UAS trans-genes are expressed using the ESO-specific *neur*-GAL4 driver in wild-type (B,C) or *Ubx*-FLP-induced *seq* mutant clones (B',C'). (B,B') Expression of *UAS-Pax2* in *seq* mutant clones can rescue the loss of hair cells. (C,C') Expression of *UAS-Ham* in *seq* mutant clones suppresses the gain of socket cells on the adult cuticle. (D,D',D'') Expression of *UAS-Ham* in *seq* mutant clones (marked by GFP) suppresses the loss of neurons (Elav: red; cut: blue). (E) Seq is an upstream regulator of the expression of D-Pax2, which regulates the shaft differentiation programme. In the internal branch of the lineage, Seq controls the expression of Pros and Ham. Seq acts in parallel to *N* signalling, opposing its activity in the determination of socket versus hair cells. However, it is also possible that *N* signalling regulates the function of Seq (dashed line). Elav, embryonic lethal, abnormal vision; ESO, external sensory organ; GFP, green fluorescent protein; Ham, Hamlet; N, Notch; Pros, Prospero; Seq, Sequoia.

aberrantly specified *seq* mutant internal lineage might account for the additional socket cells generated after specification.

To test whether the loss of Ham is due to the loss of Pros expression in *seq* mutant clusters, we monitored Ham expression in *pros* mutants, and found that Ham was expressed normally in pIIb and its daughter cells (Fig 4C,C'). Furthermore, in the absence of *seq*, Pros was unable to induce Ham expression (Fig 4D,D'). Hence, Pros is neither required nor sufficient for Ham expression and, therefore, Seq regulates Pros and Ham independently.

Suppression of *seq* by D-Pax2 and Ham

To test whether the loss of D-Pax2 and Ham expression might be responsible for the cell fate phenotypes in *seq* mutant ESOs, we investigated whether overexpressed D-Pax2 and Ham could partly rescue the phenotype in *seq* mutants. Overexpression of D-Pax2 led to the development of ESOs with two and three hair cells: 90% of adult ESOs had 1 hair, 9% had 2 hairs and 1% had 3 hairs ($n=224$; Fig 5B; Kavalier et al, 1999). Similarly, when D-Pax2 was overexpressed in the *seq* mutant ESO lineage, there was a consistent gain of hair cells and a loss of multiple socket structures (Fig 5B'): 89% of adult ESOs contained 1 hair, 11% had 2 hairs and none had 3 hairs ($n=264$), as opposed to the aforementioned 9% of *seq* mutant ESOs with a single hair cell. Overexpression of Ham in the ESO lineage causes a pIIa to pIIb conversion and the development of only sheath cells and neurons, which leads to balding of the adult cuticle (Fig 5C; Moore et al, 2004). When Ham was overexpressed in the *seq* mutant ESO lineage, the 'multi-socket' phenotype on the adult cuticle was replaced by a balding phenotype (Fig 5C'). Importantly, the expression of the neuronal marker Elav was rescued when Ham was overexpressed in *seq* mutants (Fig 5D,D''), as 90% of the

clusters at 24 APF contained 1–2 Elav-positive cells ($n=70$), in contrast to *seq* mutants (see above).

The data above suggest that overexpression of D-Pax2 or Ham in *seq* mutants can partly rescue the *seq* mutant phenotype. Therefore, in conjunction with our expression studies, we propose that Seq regulates the expression of D-Pax2, Ham and Pros. Interestingly, Seq behaves as a transcriptional activator when bound to an artificial promoter in cell culture (supplementary Fig 6 online), suggesting that it might regulate its downstream target genes at the transcriptional level.

DISCUSSION

In a genetic screen for bristle development defects, we identified new alleles of *seq*. At 24 APF, *seq* mutant ESO clusters consist of the correct number of cells, but they contain additional socket cells and an aberrantly specified internal lineage. Intriguingly, by 38 APF, the number of mutant precursor clusters that contain 2–4 socket cells is increased. The *seq* mutant phenotype, therefore, shares phenotypes that are associated with a loss of *D-Pax2* and *ham*—that is, the generation of socket cells during and after specification (Kavalier et al, 1999; Moore et al, 2004). Indeed, the expression of D-Pax2 and Ham is lost in *seq* mutant clones. Interestingly, the *seq* phenotype can be partly rescued by the exogenous expression of D-Pax2 and Ham, suggesting that these transcription factors can bypass the requirement for Seq in ESO development. Therefore, D-Pax2 and Ham function downstream from Seq in development of the ESO lineage.

Sequoia might directly or indirectly regulate the expression of Pax2, Pros, Ham and any other target gene that is essential for the correct specification and differentiation of the ESO lineage. Furthermore, unlike in adults, D-Pax2 and Pros in *seq* mutant embryos are not lost, as documented in supplementary Fig 7

online, indicating that context-specific differences in Seq function must exist.

Our study reveals the role of Seq in regulation of cell fate decisions in the ESO lineage. We propose that the Seq acts along with *N* signalling to determine the fate of various cells (Fig 5E). The multiple sockets in the *seq* mutant tissue are not due to a gain of *N* signalling at the two-cell stage. Instead, loss of D-Pax2 and Pros in *seq* reveals that both the external and the internal branches of the ESO lineage are affected from the early stages. In the external lineage, *N* signalling specifies the socket versus the hair cell. We observe that the mutant 'multi-socket' phenotype persists under conditions of simultaneous downregulation of *N* signalling and loss of *seq*, suggesting that Seq acts either in parallel or downstream from *N* at this stage. In the internal lineage, *seq* controls the expression of Ham, which 'locks' the specification of cell fates. Loss of Ham in *seq* might underlie the time-dependent aspect of the *seq* phenotype.

In conclusion, during development of the adult ESO lineage, Seq regulates the expression of multiple components, important for appropriate cell fate acquisition. Further studies on the regulation of Seq expression and mode of action on target genes, possibly at the transcriptional level, will reveal significant aspects of cell fate specification.

METHODS

Genetics. All crosses were performed on standard media at 25 °C, unless otherwise indicated. For the genetic screen, we used the *FLP/FRT* system (Xu & Rubin, 1993). Around 5,500 isogenized *y w*; *FRT42D* males were mutagenized using 15 mM EMS, mated en masse with *y w Ubx-FLP*; *FRT42D y+* females, and raised at 25 °C. The genotype used in this screen produces large mitotic clones on the notum marked by absence of the *y+* mutation. Mutants with interesting phenotypes were backcrossed three times to ensure germ-line transmission. Out of approximately 55,000 F1 flies scored, 163 lines with bristle phenotypes were established, which fell in 32 complementation groups with two or more alleles.

Image acquisition and processing. Images were acquired and processed according to Jafar-Nejad et al (2005).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank J. Brenman, C. Doe, E. Frei, Y.-N. Jan, F. Matsuzaki, A. Moore, the Bloomington Stock Center and the Developmental Studies Hybridoma Bank for providing reagents. Confocal microscopy was supported by the Baylor College of Medicine Intellectual and Developmental Disabilities Research Center. Scanning electron microscopy was supported by Institutional Core Grant #CA16672 to the High Resolution Electron Microscopy Facility, University of Texas M.D. Anderson Cancer Center. N.G. is supported by a long-term EMBO fellowship and the Howard Hughes Medical Institute. S.Y. is supported

by the Nakajima Foundation. H.J.B. is an investigator at the Howard Hughes Medical Institute.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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