Spen is required for pigment cell survival during pupal development in Drosophila

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Apoptosis is required during development to eliminate superfluous cells and sculpt tissues; spatial and
timed control of apoptosis ensures that the necessary number of cells is eliminated at a precise time in a
given tissue. The elimination of supernumerary pigment or inter-ommatidial cells (IOCs) depends on
cell–cell communication and is necessary for the formation of the honeycomb-like structure of the
Drosophila eye. However, the mechanisms occurring during pupal development and controlling apoptosis
of superfluous IOC in space and time remain unclear. Here, we found that split-ends (spen) is required for
IOC survival at the time of removal of superfluous IOCs. Loss of spen function leads to abnormal removal
of IOCs by apoptosis. We show that spen is required non-autonomously in cone cells for the survival
of IOCs by positively regulating the Spitz/EGFR pathway. We propose that Spen is an important survival
factor that ensures spatial control of the apoptotic wave that is necessary for the correct patterning and
formation of the Drosophila eye.

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Introduction

Programmed cell death (PCD) or apoptosis is essential in many metazoans to compensate for excess proliferation and maintain cellular homeostasis (Bergmann and Steller, 2010; Nagata and Golstein, 1995). Apoptosis is also required to remove superfluous cells to sculpt complex tissues during development (Mollereau, 2009; Mollereau and Ma, 2014). The apoptotic removal of about 2000 retinal pigment cells allows for instance the formation of the perfectly organized honeycomb like structure of the Drosophila eye (Cagan and Ready, 1989; Wolff and Ready, 1991). The Drosophila eye is composed of about 800 ommatidia, each ommatidium contains eight photoreceptor cells, four cone cells, two primary pigment cells and is surrounded by an hexagon of 12 inter-ommatidial cells (IOCs) or lattice cells, including three bristle, six secondary, and three tertiary pigment cells (Cagan and Ready, 1989). Retinal cells’ differentiation begins at the third instar larval stage with sequential recruitment and differentiation of the eight photoreceptor cells that is followed by differentiation of cone cells (Mollereau and Domingos, 2005; Treisman, 2013). At early pupal stages, primary pigment cells (PP) differentiate and form a concentric ring around the four cone cells above the photoreceptor cell cluster (Carthew, 2007). As the ring of PP cells closes, IOCs are rearranged around the ommatidium to form an array. This population of IOCs contains two to three more cells that can be accommodated in the retinal lattice (Cagan and Ready, 1989; Reiter et al., 1996; Wolff and Ready, 1991). Most superfluous cells are eliminated in an apoptotic wave that takes place around 28 h after puparium formation (APF) (Cordero et al., 2004; Miller and Cagan, 1998). The other IOCs differentiate as secondary (2’s) and tertiary (3’s) pigment cells. This apoptotic death is strictly dependent on Hid, Dronc and Dark and uses Cytochrome-c-d for on time elimi-
nation of IOCs (Mendes et al., 2006; Yu et al., 2002 ). Moreover, cell–cell communications control spatial elimination of IOCs and ensure that the exact final number of IOCs is reached in each ommatidium. Previous studies have indeed proposed that cone cells and/or primary pigment cells promote non-autonomously the survival of IOCs (Miller and Cagan, 1998; Monserrate and Brachmann, 2007). The EGFR/RAS pathway acts as a survival pathway that antagonizes Notch and Hid-induced apoptosis of IOCs (Miller and Cagan, 1998; Yu et al., 2002). However, monitoring of EGFR/Ras/MAPK and Notch pathways failed to show any differential activation at the time of death in IOCs (Monserrate and Brachmann, 2007). These results suggest that additional factors integrate the survival and death signals for IOC elimination.

The gene split-ends (spen) encodes several Spen isoforms which all contain three RNA recognition motifs (RRMs) and a Spen paralog

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and ortholog C-terminal domain (SPOC) (Chen and Rebay, 2000; Kuang et al., 2000; Rebay et al., 2000; Wiellette et al., 1999). Spen is a nuclear protein with pleiotropic functions, which has been implicated in cellular processes such as neuronal, glial cell fates, survival and axon guidance (Chen and Rebay, 2000; Kuang et al., 2000), regulation of cell cycle (Lane et al., 2000), epithelial planar polarity (Mace and Tugores, 2004) and repression of head identity in the developing embryo (Wiellette et al., 1999). To regulate these pleiotropic functions, it was proposed that Spen promotes or interferes at the transcriptional level with signaling pathways such as Notch, EGFR/Ras/MAPK and Wingless (Wg). In the developing Drosophila eye, Spen is both a negative and a positive regulator of Notch signaling and EGFR/Ras/MAPK, respectively. Spen fine-tunes Notch and EGFR/Ras/MAPK at the morphogenetic furrow to ensure proper recruitment and differentiation of photoreceptor cells (Dorozque et al., 2007).

In this study, we examined the role of Spen in the process of IOC elimination during pupal stage. We found that loss of Spen function induced the loss of extra IOCs compared to wild type retina. The loss of IOCs occurred by apoptosis in Spen mutant and could be rescued by the expression of the effector caspase inhibitor p35. Our results indicate that Spen has a survival role at the time of the elimination of superfluous IOCs. By performing a statistical analysis of mosaic ommatidia in Spen mutant, we showed that Spen is required in cone cells for survival of IOCs. Moreover, we found that Spen abnormally accumulates and dpERK is reduced in Spen mutant clones. We propose that Spen is required for Spitz activation in cone cells and optimal EGFR activation to control the elimination of IOCs, allowing the precise organization of the hexagonal array.

Material and methods

Drosophila genetics

All crosses and development staging were done at 25 °C unless otherwise noted. The following mutant fly stocks were obtained from the Bloomington stock center: GMR-Gal4, FRT40A w++; UAS-GFP, UAS-nds-GFP, UAS-whiteRNAi. The ubi-Gal80ts line and the method are as described in Napoleton et al. (2011). The lz-Gal4 line is a GIFT of Graeme Mardon Flores et al. (2000). For RNA interference, different Gal4 carrying lines were crossed with the UAS-RNAi line and different Gal4 carrying lines were crossed with the UAS-RNAi line and the method are as described in Napoleton et al. (2011). The lz-Gal4 line is a GIFT of Graeme Mardon Flores et al. (2000). For RNA interference, different Gal4 carrying lines were crossed with the UAS-RNAi line against spen (G4 line #48846 from VDRC, one off-target reported). Another line UAS-spenRNAi line (GIFT from K. Cadigan Lab, Chang et al., 2008) also induced IOC loss when expressed with GMR-Gal4 (data not shown). The FRT40A spen5/Cyo line was obtained from bloomington (BL#8734). The spen5 was described previously by Kuang et al. (2000). Whole-eye mutant clones were generated using the yw FRT40A GMR-hid Cl; ECFU line (Stowers and Schwarz, 1999). The induction of mitotic clones was done either with T155-FLP (Bloomington #5074) or ey-FLP (GIFT from Graeme Mardon). Flip-out Gal4 clones were obtained by crossing female flies, carrying the hs-FLP22 and the AyGal4, UAS-GFP transgenes Ito et al. (1997), with UAS-spen homozygous males. To minimize spontaneous recombination caused by heat-shock-independent FLP expression, the flies were kept at 18 °C, except when heat-shocked. 24 h APF (corresponding to ~12 APF at 25 °C) HS-FLP22; act > y > > Gal4, UAS-GFP; UAS-spen heterozygous flies were heat-shocked for 20 min at 29 °C. After the heat shock the animals continued their development at 18 °C and were at 84 h APF which corresponds approximately to 42 APF at 25 °C.

Fluorescent imaging of interommatidial cells

CO2- anesthetized adult flies were placed in a 35 mm cell culture dish half-filled with 1% agarose and covered with water at 4 °C as described in (Gambis et al., 2011; Dourlen et al., 2013; Pichaud and Desplan, 2001). Retinas were observed using an upright confocal fluorescent microscope (LSM700; Zeiss) equipped with a 40 × water immersion lens. Water is used to neutralize the cornea. Excitation wavelength was 554 nm to observe pigment autofluorescence.

Transmission electron microscopy

Transmission electron microscopy was performed as previously described (Dourlen et al., 2012). Briefly, eyes were dissected and fixed overnight at 4 °C in 1.5% glutaraldehyde, 1% paraformaldehyde and 0.1 M PIPES buffer (pH 7.4). After washing, eyes were post-fixed at room temperature in 1% OsO4, 0.1 M PIPES (pH 7.4). After dehydration, eyes were infiltrated with increasing concentrations of epoxy resin (EMbed 812 from EMS) in propylene oxide and samples were mounted in pure resin into silicone embedding molds. Polymerization was performed at 60 °C for 2 days. Ultrathin sections of 60 nm were stained with lead citrate and examined with a transmission electron microscope (Philips CM120) operating at 80 kV.

Generation of anti-Spen polycyclonal antibody

Rabbits were immunized with two peptides of Spen, according to manufacturer procedures (SpeedRabbit Eurogentec, France); peptides 1 (aa 238–252); PEKSISNPTPRT and 2 (aa 259–273); NVESQPKLSNETPQ. Prior to purification, ELISA determined the titer of each serum for each peptide. For removing unspecific antibodies, the sera were then affinity purified for specific IgG.

Immunostainings

Staged pupal retinas were dissected in PBS and fixed for 15 min in 4% paraformaldehyde at room temperature as in Domingos et al. (2004a). Immunostaining was carried out in PBT (PBS + 0.1% Triton-X100). The following antibodies were used overnight at 4 °C: anti-spen (1/1000), anti-Arm (1/500, N27A1-c, DSHB), anti-β-Galactosidase (1/500, MP Cappell), anti-Dcp-1 (1/500, Cell Signaling Technology Asp216), anti-dpERK (1/100, Cell Signaling Technology) and anti-Spitz (DSHB) (Schweitzer et al., 1995). After washing, samples were stained with the following appropriate secondary antibodies: anti-rabbit (alexa 405 or alexa 488 1/500, Invitrogen), anti-rat (alexa 633 1/500, Invitrogen), and anti-mouse (alexa 546 or alexa 405 1/500, Invitrogen). Samples were mounted in DAPI mounting media (Vectashield, AbCys). Fluorescent images were obtained using Zeiss 710, 780 and Leica Sp5 confocal microscopes. Apoptosis activity was evaluated by counting the number of Dcp-1 dots staining with ImageJ software.

Interommatidial cell count

IOCs were counted after dissection of pupal eye disk at 42 APF labeled with anti-Arm or after visualization with the neutralization of cornea method in the adu. At 42 APF, wild type retina contained 12 IOCs per ommatidium including six 2’s, three 3’s and three bristle cells. In the adult, pigment autofluorescence only allowed identifying 9 IOCs/ommatidium (six 2’s and three 3’s) as the bristle cells were not distinguishable with the cornea neutralization method. At least ten retinas with a minimum of 50 ommatidia were counted for each genotype unless specified. Unpaired student t-test was used to compare independent IOC counting one with another (Figs. 1, 3 and S4). A non-parametric Kruskal–Wallis test (ANova) was used to compare two or more IOC countings that are independent and may have different sample sizes (Fig. 5).
Fig. 1. spen is required for IOC survival (a–d) are optical view of Drosophila adult eye of the indicated genotypes. (a) GMR-GAL4 > UAS-GFP (GMR > GFP), (b) GMR > spenRNAi, (c) GMR > spen, (d) GMR > spen; spenRNAi. (e–h) show inter-ommatidial cells (IOCs) visualized by the cornea-neutralization method of the precedent genotypes in adult Drosophila eyes. The overexpression of spen almost fully rescues the loss of IOCs resulting from the inactivation of spen by RNAi (compare f and h). (i–k) IOCs are visualized with the cornea neutralization method. (l) Iz > GFP; (j) Iz > spenRNAi; (k) Iz > spen. Spen overexpression (k) leads to an increased number of IOCs compared to control (i). (l and m) are the quantifications of the different conditions presented before: (l) is the quantification of IOCs following the modulation of spen using the GMR-GAL4 driver, (m) is the quantification of IOCs of the panels (i–k), corresponding to the Iz-GAL4 driver. (n and o) are whole mount of pupal eye disk 42 h APF labeled with GFP (n) and anti-Arm (o). Spen5 mutant clones lack GFP. A yellow line delineates the border of the clone. (p) is the quantification of IOC number in wild-type (yw; FRT40A/FRT40GMR-hidCL; EGUF) and whole eye mutant retina for spen (yw; FRT40A spen5/FRT40GMR-hidCL; EGUF; n=5). Student t-test *p < 0.01, **p < 0.001, ***p < 0.0001.

Scanning electron microscopy

3 days old flies were collected, proboscis removed and head put 30 s in 2/1000 dish detergent (IJN, #507482, Groupe PROP, Paris, France) in cacodylate 0.1 M buffer to reduce hydrophobicity. Heads were then fixed in cacodylate 0.1 M buffer supplemented with glutaraldehyde 2%, for 2 days at 4 °C and rinsed in the cacodylate 0.1 M buffer. Heads were desiccated using the critic drying point technique, metallized using a Baltec MED020 and heads observed with a FEI QUANTA 250 FEG. Images were captured using Adobe Photoshop software.

Results

Spen is required for IOC survival

During pupal retinal development, superfluous IOCs are eliminated by apoptosis allowing the precise organization of ommatidia lattice in a crystal-like structure. To test the role of spen in cell death, we first examined phenotype of retina in which spen was silenced by expression of spen RNAi using GMR-GAL4 and lozenge-GAL4 (Iz-GAL4) (Figs. 1 and S1). The GMR driver is expressed in all differentiating retinal cells during third instar and pupal development. Iz expression is more restricted; it was shown to control the differentiation of a subset of retinal cells, i.e. R1, R6 and R7 photoreceptor cells, cone cells and the elimination of IOCs (Daga et al., 1996; Wildonger et al., 2005).

In agreement with this, we found liz > GFP expressed in few photoreceptor cells, in all cone cells and IOCs at pupal stage (Fig. S2). Spen silencing using GMR driver led to ommatidia with missing PRs as previously reported in spen mutant retina (Fig. S1b), (Doroquez et al., 2007). This phenotype was attributed to a role of spen in photoreceptor cell differentiation. We also noticed that retina with silenced spen exhibited a reduction in the number of IOCs compared to wild type retina, suggesting that spen has a survival role during pupal development (Figs. 1f, j and S1e). To further comfort the requirement of spen for IOC survival, we examined in mosaic mutant and counted IOC number in whole eye mutant clone for spen, using a spen null allele (spen5) (Kuang et al., 2000). As for spen silencing, spen mutant retina exhibited a reduced number of IOCs (Figs. 1n–p and S1g and h). Importantly, we could distinguish secondary and tertiary pigments as well as correct number of primary pigment or cone cells supporting that the differentiation of accessory cells underwent normally in spen mutant clones.

Consistent with a survival role of spen, its over-expression with Iz-GAL4 led to an increase of IOCs compared to wild type retina (Fig. 1f and h). The ectopic expression of spen using the GMR driver induced a rough eye phenotype and completely disorganized ommatidial pattern (Fig. 1c and c'). This is likely due to the strong expression of spen with the GMR driver that interfered with photoreceptor differentiation during third larval instar. Importantly the re-expression of spen concomitantly with spen RNAi was sufficient to rescue the rough eye phenotype and the number of IOC close to wild type level, demonstrating the specificity of the spen RNAi transgenic line (Fig. 1a–d, f, h and n). To further explore the consequences of spen over-expression, we used a heat inducible flip-out cassette construct at early pupal stage. We only found extra IOCs in areas where spen was over-expressed in multiple retinal cells including cone, pigment cells and IOCs. However we did not observe extra IOCs when spen was expressed in single retinal cells regardless of the subtype (Fig. 2).

Spen inhibits apoptosis in IOCs at pupal stages

Next, to determine if the loss of IOCs in spen mutant is due to excess apoptosis, we expressed the effector caspase inhibitor p35 concomitantly with spen RNAi in retina (Fig. 3). We found that p35 rescued the number of IOC after spen silencing (Fig. 3c, d and e) suggesting that the loss of spen induces ectopic apoptosis of IOCs. In agreement with this, cleaved Dcp-1 staining was increased in spen mutant clones (GFP−) at 28–30 h APF (Fig. 3g and h). Interestingly, retina co-expressing p35 and spen RNAi exhibited more IOCs than with p35 alone (Fig. 3e). About 5 extra IOCs per ommatidium were counted when p35 and spen RNAi were coexpressed which is similar to dark or dronc mutant clones in which IOC death is fully inhibited (Mendes et al., 2006). The extra IOCs in spen mutant clones with p35 could be due to a defect in IOCs rearrangement. Indeed, earlier reports have showed that defects in rearrangement at early pupal stage led to the accumulation of extra IOCs as reported in irregular chiasmaC-roughest or echinus mutant retina (Reiter et al., 1996; Wolff andReady, 1991). This suggests that spen in addition to its pro-survival role, may also regulate the rearrangement of IOCs for their proper positioning and elimination.

Next we asked if the role of spen in IOC survival is due to its role during pupal stage or an indirect consequence of its earlier role in third instar larval eye disk. To exclude the later, we conditionally silenced spen during pupal stage using the GAL4/GAL80 system. We found that spen silencing starting at 5 h APF was sufficient to induce extra IOCs loss compared to wild type (Fig. 4). This supports that the loss of IOCs after spen silencing is due to the lack of survival function of spen rather than the indirect consequence of spen function in retinal cell differentiation at larval stage.

![Fig. 2. spen clonal expression is associated with increased number of IOCs. We expressed spen in single cells using the Flip-out GAL4 clones in animals carrying the hs-FLP22 and the AyGAL4, UAS-GFP transgenes. hs-FLP22; Act > y+ > GAL4,UAS− GFP/UAS-spen; heterozygous pupae were heat shocked at 29 °C as described in the material and method and dissected at 42 h APF. Anti-armadillo immunostained and spen over-expressing cells identified by co-expression of GFP (green). Stars (•) are pointed to additional IOCs.](image-url)
Spen is required in cells for IOC survival and for Spitz/EGFR signaling. It was proposed that the elimination of superfluous IOCs is the result of death and life signal coming from neighboring cone and/or primary pigment cells during pupation (Miller and Cagan, 1998; Monserrate and Brachmann, 2007). Thus, we asked about the cellular specificity of spen function in IOC survival. We observed that the enhancer trap PZspen<sup>03350</sup> is ubiquitously expressed in primary pigment, cone cell and IOC at the time of death during pupation (Fig. S3a–c). We also generated an antibody against Spen that

![GMR-GAL4](image1)

Fig. 3. Inhibiting apoptosis rescues IOC loss in spen mutant. (a–d) IOCs are visualized with the cornea neutralization method. (a) GMR-GFP, (b) GMR-p35, (c) GMR-sp<sup>spenRNAi</sup>, (d) GMR-p35;sp<sup>spenRNAi</sup>, (e) quantification of the number of IOCs in (a–d). Statistical analysis is a Student T-test, **p < 0.001 ***p < 0.0001. Note that inhibiting apoptosis leads to extra IOCs (b and e). Inhibition of apoptosis when spen is silenced rescues the loss of IOCs. (f,g) Dcp-1 staining (red) is increased in spen<sup>5</sup> mutant clone, 28 h after pupariation formation (APF). (h) is the quantification of Dcp-1 staining, comparing wild-type tissue with mutant tissue, Mann–Whitney test, *p < 0.01.

![GAL4/GAL80<sup>Tts</sup>; lucRNAi](image2)

**Fig. 4.** Spen is required for IOC survival during pupation (a–d) Optical view of adult Drosophila eyes. (e–h) show IOCs visualized with the cornea neutralization method. The following genotypes are presented: (a–b and d–e) GMR-GAL4/ubi-GAL80<sup>Tts</sup>; UAS-luciferase<sup>RNAi</sup>, (c–d and f–g) GMR-GAL4/ubi-GAL80<sup>Tts</sup>; UAS-sp<sup>spenRNAi</sup>. (a, c, d and f), flies have been raised at 18 °C from embryo to adulthood. (b, d, e, and g), flies have been kept at 18 °C and then switched at 30 °C at 5 h APF. The expression of spen<sup>RNAi</sup> during pupation is sufficient to induce a rough eye and IOCs loss (d and g).
confirmed its ubiquitous expression in pupal retina that was found both nuclear and cytoplasmic (Fig. S3d–f). The ubiquitous expression of spen in pupal eye is in agreement with previous reports showing that spen is ubiquitous during embryogenesis (Kuang et al., 2000; Wiellette et al., 1999). To determine the contribution of each lattice cell type in spen-mediated IOCs survival, we performed a statistical analysis of spen mosaic ommatidia at the border of spen mutant clones as previously described for other retinal mutations (Domingos et al., 2004b). In such mosaic ommatidia, the proportion of wild type and mutant lattice cells is random and it was possible to determine the contribution of each mutant lattice cell subtype in IOC death (Fig. 5). We thus analyzed a large number of spen mosaic ommatidia and scored for IOC loss. We first observed that spen is not required in cell autonomously in IOCs for their survival as the number of wild type and spen mutant IOCs was in average identical in mosaic ommatidia (Fig. 5c). To further examine the role of spen in IOC, we used the pigment cell specific 54C-GAL4 which is specifically expressed in 2’s and 3’s at the time of death and was able to potently suppress red color of the Drosophila eye by the expression of white RNAi (Fig. 5d, e). In agreement with spen mosaic analysis, specific silencing of spen in IOCs using 54C-GAL4 driver did not induce extra IOC loss (Fig. 5h and k). In addition, we found no requirement of spen in primary pigment cell or bristle cells for IOC survival (Fig. 5d and e). Thus spen is not required in cell autonomously in IOCs for their survival. In agreement, bristle hairs were largely present after silencing or in mutant spen whole eye clone, exhibiting an abnormal shape and sometimes in pairs likely due to the ectopic loss of secondary and tertiary pigment cells separating bristle cells (Fig. S5). This suggests that spen is not required in bristles cells for IOCs survival but rather for its terminal differentiation allowing bristle hair secretion and shaping. In contrast, we showed that the number of IOCs was clearly diminished when one or more cone cells were mutants (Fig. 5f). This indicates that spen is required in cone cells for IOC survival.

The requirement of spen in cone cells suggests that it positively regulates the expression of a secreted factor required for IOC survival. The EGFR ligand, Spitz, has been proposed to regulate EGFR and survival of IOCs (Monserrat and Brachmann, 2007); we thus examined Spitz expression in spen mutant clones (Fig. 6). While endogenous wild type Spitz was observed at low level in IOCs, we could detect a very clear accumulation of Spitz in cone cells and IOCs of spen mutant clones at 42 h APF but not in larval eye disk at third instar (Fig. 6a–d and data not shown). This effect is cell autonomous although occasional IOCs at the border of the clone were ambiguous due to the nuclear localization of GFP. To determine whether deregulated Spitz affects EGFR signaling, we used antibody specific for the phosphorylated form of MAPK/Erk (dpERK) in spen mutant clones (Helman and Paroush, 2010; Fig. 6e–g). We found a weak but consistent reduction in dpERK staining which indicates that EGFR signaling is downregulated in spen mutant clones. Together, these results indicate a requirement of spen for Spitz/EGFR signaling in the pupal retina.

Discussion

The elimination of superfluous IOCs during eye development allows the proper arrangement of ommatidial units to form the perfect hexagonal array. It requires cell–cell communication and precise coordination of death and life signals permitting the apoptotic death of pre-defined number of IOCs. We found that spen is required for survival of IOCs during pupal development (Fig. 1). The conditional silencing of spen during pupation led to extra IOC indicating that spen acts during pupation avoiding the extensive elimination of IOCs. We showed that the loss of spen induces ectopic loss of IOCs by apoptosis. This is based on the observation that loss of IOCs is associated with an increased cleaved Dcp1 staining in spen mutant clone and that IOC loss was inhibited by the expression of the caspase inhibitor p35 (Fig. 2). Interestingly, our analysis also demonstrated that spen is required non-autonomously in cone cells for the survival of IOCs (Figs. 4 and S3). This result clarifies the source of survival signal in the Drosophila pupal eye. Previous studies using laser ablation or live imaging have shown that primary pigment and/or cone cells are important for IOC survival but could not distinguish between these two retinal subtypes (Miller and Cagan, 1998; Monsserrat and Brachmann, 2007). Our study is the first one to perform systematic analysis of spen mosaic clones that unambiguously demonstrate a role of spen in cone cells for IOCs survival. This result suggests that cone cells are the primary source of survival signal for IOCs during retinal pupal development.
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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.03.021

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