Secreted frizzled-related proteins are required for Wnt/β-catenin signalling activation in the vertebrate optic cup

Pilar Esteve¹,²,³,*, Africa Sandonis¹,²,³, Carmen Ibañez³, Akihiko Shimono⁴, Isabel Guerrero³ and Paola Bovolenta¹,²,³,³,*

Summary
Secreted frizzled-related proteins (Sfrps) are considered Wnt signalling antagonists but recent studies have shown that specific family members enhance Wnt diffusion and thus positively modulate Wnt signalling. Whether this is a general and physiological property of all Sfrps remains unexplored. It is equally unclear whether disruption of Sfrp expression interferes with developmental events mediated by Wnt signalling activation. Here, we have addressed these questions by investigating the functional consequences of Sfrp disruption in the canonical Wnt signalling-dependent specification of the mouse optic cup periphery. We show that compound genetic inactivation of Sfrp1 and Sfrp2 prevents Wnt/β-catenin signalling activation in this structure, which fails to be specified and acquires neural retina characteristics. Consistent with a positive role of Sfrps in signalling activation, Wnt spreading is impaired in the retina of Sfrp1−/−;Sfrp2−/− mice. Conversely, forced expression of Sfrp1 in the wing imaginal disc of Drosophila, the only species in which the endogenous Wnt distribution can be detected, flattens the Wg gradient, suppresses the expression of high-Wg target genes but expands those typically activated by low Wg concentrations. Collectively, these data demonstrate that, in vivo, the levels of Wnt signalling activation strongly depend on the tissue distribution of Sfrps, which should be viewed as multifunctional regulators of Wnt signalling.

Key words: Ciliary margin, Wnt diffusion, Patterning, Mouse, Drosophila

Introduction
Secreted frizzled-related proteins (Sfrps) are a family of secreted factors involved in embryonic development and tissue homeostasis. Owing to their homology to the extracellular portion of the Wnt receptor Frizzled (Fzd), Sfrps have been described as scavengers of Wnt signal activation. Indeed, gain of Sfrp function has proven especially useful to antagonise both canonical and non-canonical Wnt signalling in a variety of contexts (Bovolenta et al., 2008). However, single or compound genetic inactivation or knockdown of individual Sfrps in different vertebrates has provided only partial support for the idea that, in the absence of Sfrps, Wnt signalling is overactivated (Esteve et al., 2004; Joesting et al., 2008; Misra and Matise, 2010; Satoh et al., 2006; Satoh et al., 2008; Trevant et al., 2008).

This discrepancy might be explained by the demonstration that Sfrps can interact with different proteins, thereby interfering with molecular cascades other than those activated by Wnts (Esteve et al., 2011; He et al., 2010; Kobayashi et al., 2009; Lee et al., 2006; Muraoka et al., 2006). Furthermore, Sfrps can modulate Fzd-mediated signalling independently of Wnts, forming complexes with both Fzd (Bafico et al., 1999; Dufourcq et al., 2005) and Wnts (Uren et al., 2000), possibly through differential domain binding (Lopez-Rios et al., 2008). This raised the hypothesis that Sfrps could promote Wnt-Fzd interaction, either by bringing ligand and receptor into proximity or by favouring ligand dispersion (Lopez-Rios et al., 2008). This raised the hypothesis that Sfrps could promote Wnt-Fzd interaction, either by bringing ligand and receptor into proximity or by favouring ligand dispersion (Lopez-Rios et al., 2008). According to the latter possibility, FrzB/Sfrp3 and Crescent, two Sfrp family members, appear to promote the diffusion and expand the signalling range of otherwise non-diffusible, exogenously added Wnts in gastrulating Xenopus embryos (Mii and Taira, 2009). These results provide a novel view of Sfp function and raise the question of whether this is a common Sfrp property. Indeed, Crescent has no apparent homologue in mammals and FrzB structurally diverges from Sfrp1/2/5 (Bovolenta et al., 2008). Furthermore, genetic inactivation of FrzB causes articular cartilage loss and increases Wnt signalling (Lories et al., 2007), leaving open the question of whether Sfrp knockout has physiological consequences compatible with a positive function in Wnt signalling activation.

Here, we have addressed these questions by demonstrating that Sfrp1 promotes the spreading of endogenous and exogenously added Wnts and provide genetic evidence that Sfrp1 and Sfrp2 are required for the activation of canonical Wnt signalling implicated in the specification of the mouse eye periphery.

Materials and methods
Animals
Sfrp1−/−;Sfrp2+/− mice were generated and intercrossed to generate Sfrp1−/−;Sfrp2−/− double-mutant embryos as described (Satoh et al., 2006; Esteve et al., 2011).

Antibodies
Antibodies used: mouse monoclonal BrdU (Boehringer Mannheim); Islet1, Wg, Cut, Myc; active β-catenin (ABC; Millipore); N-cadherin (Sigma); Myc (clone 9E10, DSHB); rat monoclonal Ci (Motzny and Holmgren, 1995); rabbit polyclonal Sfrp1, Otx2 (Abcam); P-Smad1,5,8, P-cJun, (Cell Signalling); cJun (Santa Cruz); calbindin (Swant); Pax6 (Covance); Par3 (Abcam); cJun (Santa Cruz); calbindin (Swant); Pax6 (Covance); Par3 (Upstate); Pax2 (Invitrogen); Chx10 (a kind gift from R. McMahon, The Sick Kids Hospital, Toronto, Canada); Distal-less (Panganiban et al., 1994); PH-H3 (Roche Diagnostics); Hh (a kind gift from S. Eaton, MPI, Dresden, Germany); guinea-pig polyclonal Vestigial (Kim et al., 1996); and Sens (a kind gift from H. Bellen, Jan and Dan Duncan Neurological Research Institute, Houston, TX, USA). Rabbit Alexa 488 or 594 and mouse Alexa 488 (Molecular Probes) were used as secondary antibodies.

¹Centro de Biología Molecular ‘Severo Ochoa’, CSIC-UAM, 28049 Madrid, Spain.
²CIBER de Enfermedades Raras (CIBERER), c/Nicolás Cabrera 1, 28049 Madrid, Spain. ³Instituto Cajal, CSIC, Avda. Doctor Arce 37, 28002 Madrid, Spain. 4Cancer Science Institute of Singapore, National University of Singapore, Singapore 117456.
*Authors for correspondence (pestreve@cbm.uam.es; pbovolenta@cbm.uam.es)

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In situ hybridisation (ISH) and immunohistochemistry (IHC)

E9-16.5 mouse embryos were processed for ISH and IHC as described (Esteve et al., 2003). In some cases, tissues were processed for antigen retrieval with an uncoating chamber (Biocare Medical) at 115°C for 2 minutes in 10 mM citrate buffer (pH 6). The employed digoxigenin-labelled antisense riboprobes were: *Otx1*, *Mxsl*, *Axin2*, *Lef1*, *Wnt2b*, *Wnt5a*, *Wnt3a*, *Wnt7a* and *Wnt7b*, *Bmp4*, *Bmp7*, cyclin D1 (*Cnd1*), *Cdx10* and *Crx*. For BrdU analysis, pregnant dams were injected intraperitoneally with BrdU (50 μg/g), sacrificed 1 hour later and processed for IHC. IHC of *Drosophila* imaginal discs was performed as described (Torroja et al., 2004). Tissues were examined with a confocal laser-scanning microscope (LSM510 Vertical, Zeiss) or a DM500 microscope (Leica).

Electroporation and detection of Wnts

Expression plasmids (pCAG) encoding Venus-tagged Wnt8 and Wnt11 [kindly provided by Dr M. Taira (Mii and Taira, 2009)] were electroporated into isolated retinas from E12.5 wild-type, *Sfrp1*–/– and *Sfrp1*–/–;*Sfrp2*–/– mouse embryos. Tissue was then cultured for 24 hours in DMEM/F12 medium supplemented with N2 (Gibco) with or without the addition of purified recombinant Sfrp1. Retinas were fixed in 4% PFA for 2 hours and flat mounted. The extent of fluorescent signal diffusion was analysed by confocal microscopy with the aid of ImageJ software.

Overexpression of Sfrp1 in *Drosophila*

*Sfrp* cDNA was fused in frame to a C-terminal Myc tag and cloned into the pUAST vector to generate transgenic fly lines expressing Sfrp1 under the UAS promoter. The Hh-Gal4 (Tanimoto et al., 2000) and MD638-Gal4 (scalloped-Gal4) (Mullor et al., 1997) drivers were used to express Sfrp1 in the posterior compartment or the entire wing disc, respectively. As a positive control for planar cell polarity defects, *dachsous* function (Matakatsu and Blair, 2004) was knocked down by double-stranded (ds) RNAi expression using the same Gal4 driver.

RESULTS AND DISCUSSION

The optic cup can be molecularly subdivided in a central region, which will differentiate into central neural retina (cNR) and retinal pigment epithelium (RPE), and a peripheral portion, from which the ciliary body and the iris are derived. The specification of the dorsal and peripheral optic cup (OCP) requires the activation of canonical Wnt signalling (Fuhrmann et al., 2009; Veien et al., 2008). Indeed, ectopic canonical signal activation in the cNR suffices to induce peripheral marker expression (Trimarchi et al., 2009). Conversely, OCP identity is lost after eye-specific conditional inactivation of β-catenin (Liu et al., 2007) or expression of a dominant-negative form of Lef1 (Cho and Cepko, 2006). However, the source of the Wnt ligand responsible for signalling activation remains undefined. The locally expressed *Wnt2b* is an unlikely possibility because its expression seems to be regulated by Wnt-mediated activation of Bmp signalling (Muller et al., 2007) and its ectopic expression does not induce peripheral characteristics (Liu et al., 2007). Lens-derived Wnts are also unlikely because genetic ablation of lens precursors does not affect initial OCP specification (Zhang et al., 2007).

Wnt3, Wnt5a, Wnt7a and Wnt7b are transiently expressed in the cNR (Liu et al., 2003) and would require long-range distribution to qualify as candidates. In this scenario, we asked whether *Sfrp1* and *Sfrp2*, which are abundantly expressed during murine eye development (Liu et al., 2003), are required for Wnt signalling activation. *Sfrp2* transcripts are predominant in the cNR, whereas *Sfrp1* localises to the OCP and RPE but its protein is also detected in the cNR (Esteve et al., 2011), probably because Sfrps efficiently diffuse in the extracellular space (Mii and Taira, 2009). According to this
overlapping distribution and the proposed Sfrp functional redundancy (Misra and Matise, 2010; Satoh et al., 2006; Satoh et al., 2008), the eye of Sfrp1 and Sfrp2 single-null mouse embryos appeared histologically normal (data not shown). By contrast, at E16.5, the latest viable stage, the eyes of Sfrp1−/−;Sfrp2−/− compound mutants were smaller and grossly altered (see Fig. S1 in the supplementary material) (Esteve et al., 2011), with preponderant defects in the dorsal OCP (Fig. 1A,B). This phenotype was fully penetrant. According to morphological and molecular analyses, optic cup formation was initiated normally in mutant embryos and defects were first observed at ~E10.5 (see Figs S1 and S2 in the supplementary material), when the OCP begins to be specified.

The OCP is characterised by the expression of Otx1 and Msx1 (Trimarchi et al., 2009) (Fig. 1C,E,G,I), a low proliferation rate [few BrdU-positive or phospho-histone H3 (PH-H3)-positive cells] and by the absence of expression of the cell-cycle regulator Ccnd1 (Fig. 1K,M,O), of cNR markers such as Chx10 (Vsx2 – Mouse Genome Informatics) or of retinal cell type-specific determinants, including Islet1, Otx2 and calbindin (Fig. 1Q,S,U,W). If Sfrps were to directly antagonise Wnt/β-catenin signalling according to the proposed antagonist function (Leyns et al., 1997), the OCP, and thus the expression of its specific markers, would be expected to be expanded in Sfrp1−/−;Sfrp2−/− embryos. By contrast, from E10.5, the expression of Otx1 and Msx1 was nearly absent in the dorsal (Fig. 1D,F,H,J) and, occasionally, ventral OCP of the mutants, and was replaced by Ccnd1/BrdU/PH-H3/Chx10-positive proliferating cells (Fig. 1L,N,P,R). Thus, the OCP of Sfrp1/2 compound mutants had lost its defining characteristics and acquired those of the cNR. Accordingly, at E16.5, when neurogenesis is ongoing, ectopic retina ganglion (RGC; Islet1+), amacrine (calbindin+), bipolar (Otx2+) and photoreceptor (Crx+/Otx2+) cells were ectopically observed in the mutant OCP (Fig. 1T,V,X; data not shown).

Thus, Sfrp1 and Sfrp2 cooperate to establish the border between the peripheral and central neural retina. In their absence, the dorsal and, to a minor extent, the ventral OCP fail to be specified, as observed after inactivation of canonical Wnt signalling (Cho and Cepko, 2006; Liu et al., 2007). Accordingly, and in contrast to what was observed in controls, expression of Lef1 and Axin2, which are targets and readout of the Wnt/β-catenin pathway, was nearly absent in the dorsal OCP and in the periconical mesenchyme of Sfrp1−/−;Sfrp2−/− embryos (Fig. 2A-F). Similarly, the non-phosphorylated active form of nuclear β-catenin was almost absent (Fig. 2G,H). This lack of Wnt/β-catenin signalling activation was unlikely to be an aftermath of early changes in ligand expression because the mRNA distribution of Wnt3, Wnt5a, Wnt7a and Wnt7b, which are expressed at early stages of eye development (Liu et al., 2003), was unchanged in the mutant optic cup (see Fig. S3 in the supplementary material).

Bmp signalling acts downstream of the canonical Wnt pathway in the dorsal OCP (Veien et al., 2008) and its inhibition prevents the differentiation of the ciliary body, which is substituted by retinal ganglion-like cells (Zhao et al., 2002), thereby resembling a milder form of the Sfrp1−/−;Sfrp2−/− phenotype. Consistently, the levels of P-Smad1/5/8, which are Bmp signalling effectors, and the expression of Msn1, which is a direct Bmp target, and of Bmp4 were strongly diminished in the dorsal OCP of E16.5 Sfrp1/2 compound mutants (Fig. 1G-J; Fig. 2I-L), although Bmp4 expression was initiated normally (see Fig. S1 in the supplementary material). Furthermore, Wnt2b expression, which is normally controlled by Bmp activity (Müller et al., 2007), was diminished in the mutant OCP (Fig. 2M,N).

Altogether, these data demonstrate that Sfrp1 and Sfrp2 cooperate to positively modulate Wnt/β-catenin signalling in the dorsal OCP. There are several possible scenarios to explain this finding. In a classical view, Sfrp1/2 could antagonise non-canonical Wnt signalling, which might normally prevent the activation of canonical activity in the OCP. This regulatory interaction has been proposed to explain the phenotypes obtained by altering the expression of Wnt/Fzd/Sfrp family members during eye field specification in fish (Cavodeassi et al., 2005; Esteve et al., 2004). To address this possibility we asked whether the levels of phospho-cJun, a readout of non-canonical signalling (Sokol, 2000), were different in wild-type and mutant retinas. Immunohistochemical and western blot analyses revealed no differences (see Fig. S2G-I in the supplementary material). Similarly, the distribution of Par3
and aPKC, two cell polarity markers linked to non-canonical signalling (Sokol, 2000), was also unchanged (see Fig. S2C-F in the supplementary material).

As a second possibility, Sfrp1/2 could directly activate Fzd, as shown in other contexts (DuFourcq et al., 2008; Rodriguez et al., 2005). Co-immunoprecipitation studies confirmed that Sfrp1 interacts with Fzd7 and Fzd4 (DuFourcq et al., 2008; Rodriguez et al., 2005) (data not shown), which are strongly expressed in the mouse OPC (Liu et al., 2003). However, Sfrp1 addition to Fzd-expressing cells has no effect on luciferase reporter expression coupled to β-catenin-responsive Tcf binding sites (Esteve et al., 2003; Lopez-Rios et al., 2008), suggesting that the Sfrp1-Fzd interaction is likely to influence only non-canonical components (DuFourcq et al., 2008; Rodriguez et al., 2005), unless additional, as yet unidentified, molecules participate in the interaction.

As a third possibility, Sfrp1 and Sfrp2 could favour Wnt spreading across the OCP, behaving similarly to Crescent and FrzB in Xenopus embryos (Mii and Taira, 2009). If this were the case, Wnt spreading should be impaired in the retina of Sfrp1/2 compound mutants. In vertebrates, detection of endogenously expressed Wnts is severely hampered by the lack of appropriate antibodies. Therefore, we took advantage of the Venus-tagged versions of Wnt8 and Wnt11 (Mii and Taira, 2009). The choice of these ligands seemed appropriate because Wnt11 is transiently expressed in the retina, whereas Wnt8, to our knowledge, is not. The Venus-Wnt11 and Venus-Wnt8 expression constructs contain a signal peptide and are normally secreted into the culture medium (Mii and Taira, 2009). Consistently, when the constructs were electroporated into E13 wild-type retinas, Venus-Wnt11, but not Venus-Wnt8 (not shown) or a control cytoplasmic eGFP (Fig. 3A), was released into the parenchyma and detected as a weak but measurable signal under confocal microscopy (Fig. 3B, C). This punctuate signal was intense up to 30 μm from the electroporation front and slowly decreased to background values beyond 80 μm (Fig. 3D), indicating diffusion of the fluorescent protein. By contrast, the Venus-Wnt11 signal was detected only in close proximity to the electroporation front in Sfrp1/2 null retinas, despite similarity in the areas targeted (Fig. 3E, F). Notably, increased fluorescence levels distant from the electroporation front were restored when Sfrp1/2 null retinas electroporated with Venus-Wnt11 were cultured for 22 hours in the presence of Sfrp1 protein (Fig. 3G, H).

These observations indicate that Sfrp1 (and likely Sfrp2) favours the spreading of Wnt11, supporting the proposal that, in vivo, Sfrp1/2 might promote Wnt-Fzd interaction by bringing ligand and receptor into proximity (Lopez-Rios et al., 2008). Alternatively, Sfrp1/2 might contribute to regulating the arrangement of the extracellular matrix, which, in turn, is fundamental for morphogen diffusion (Guerrero and Chiang, 2007). This last hypothesis would be consistent with the observation that Sfrps act as inhibitors of Wnt spreading should be impaired in the retina of Sfrp1/2 compound mutants. In vertebrates, detection of endogenously expressed Wnts is severely hampered by the lack of appropriate antibodies. Therefore, we took advantage of the Venus-tagged versions of Wnt8 and Wnt11 (Mii and Taira, 2009). Consistently, when the constructs were electroporated into E13 wild-type retinas, Venus-Wnt11, but not Venus-Wnt8 (not shown) or a control cytoplasmic eGFP (Fig. 3A), was released into the parenchyma and detected as a weak but measurable signal under confocal microscopy (Fig. 3B, C). This punctuate signal was intense up to 30 μm from the electroporation front and slowly decreased to background values beyond 80 μm (Fig. 3D), indicating diffusion of the fluorescent protein. By contrast, the Venus-Wnt11 signal was detected only in close proximity to the electroporation front in Sfrp1/2 null retinas, despite similarity in the areas targeted (Fig. 3E, F). Notably, increased fluorescence levels distant from the electroporation front were restored when Sfrp1/2 null retinas electroporated with Venus-Wnt11 were cultured for 22 hours in the presence of Sfrp1 protein (Fig. 3G, H).

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To distinguish between these two possibilities and to further test whether Sfrp1/2 can modify Wnt diffusion and hence signalling activation, we turned to the Drosophila wing imaginal disc, which has considerable advantages. First, in contrast to vertebrates, Drosophila is the only species in which the endogenous Wingless (Wg; a Wnt homologue) gradient can be easily detected with specific antibodies. Second, Wg activity has been very well characterised and can be unequivocally followed (Neumann and Cohen, 1997; Zecca et al., 1996). Third, no apparent SFRPs homologues have been identified in the Drosophila genome (Bovolenta et al., 2008), effectively offering an Sfrp null background. Despite this absence, Wg efficiently binds to Sfrp1 (Uren et al., 2000), mimicking vertebrate Wnt1 or Wnt8 interaction with Sfrp1 (Lopez-Rios et al., 2008).

Wg is expressed in a stripe of cells at the dorsoventral (DV) boundary of the wing imaginal disc and forms a symmetrical gradient in dorsal and ventral compartments (Neumann and Cohen, 1997; Zecca et al., 1996). UAS-mediated expression of Sfrp1 in the posterior compartment of the wing disc using the Hh-Gal4 driver (HhGal4–UAS-Sfrp1-Myc) affected extracellular Wg protein localisation (Fig. 4A). In contrast to the anteriormost compartment, which served as control, high levels of extracellular Wg were no longer observed at the posterior DV boundary. Rather, the Wg signal was widely distributed across the entire posterior compartment, colocalising with Sfrp1 (Fig. 4B). Notably, a similar colocalisation was observed in an 8- to 10-cell body-wide fringe of the anterior compartment (Fig. 4B, B’), indicating that Sfrp1 diffuses from the producing cells to influence Wg distribution non-cell-autonomously.

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**Fig. 3. Sfrps favour the long-range distribution of Wnt11.**

(A-C, E, G) Confocal images of E12.5 wild-type (A-C) and Sfrp1+/−, Sfrp2+/− (E, G) mouse retinas electroporated with expression plasmids for eGFP or Venus-Wnt11 as indicated. Explants in G were cultured in the presence of SFRP1, which restores diffusion of Wnt11. (D, F, H) Quantification of fluorescence pixel intensity within the six boxed regions shown in the corresponding confocal image. Results of a typical experiment are shown; the analysis was repeated four times in duplicate, with similar results. Scale bar: 20 μm.
Sfrp in Wnt signalling activation

Confirming a flattening of the gradient, the expression of Senseless (Sens), a canonical target normally activated by high Wg levels in two discrete stripes at the DV boundary (Nolo et al., 2000), was no longer observed in the posterior compartment or at the anterior edge of the anterior-posterior border (Fig. 4C,C’). By contrast, the expression of Distal-less (Dll) and Vestigial (Vg), two Wg targets that are activated in response to low ligand concentrations (Neumann and Cohen, 1997; Zecca et al., 1996), was expanded at the anterior boundary and in the posterior compartment, but not in the anteriormost compartment (Fig. 4C,D), indicating that Sfrp1 flattens the Wg gradient without blocking pathway activation. Furthermore, the expression of Cut, a marker of the wing DV boundary that is directly activated by Notch signalling (Micchelli et al., 1997), was completely absent in the posterior compartment (Fig. 4D,D’), consistent with the notion that Sfrp1 modulates Notch signalling through Adam10 regulation (Esteve et al., 2011). We have shown that forced co-expression of Sfrp1 and Kuzbanian, the Drosophila Adam10 homologue (Rooke et al., 1996), completely rescues Cut but not Sens expression (Esteve et al., 2011), indicating that Sfrp1-mediated regulation of Adam10 and Wnt signalling are independent events. This last observation, together with Sfrp1/Wg colocalisation, favours the hypothesis that Sfrp1/2 positively modulate canonical Wnt signalling by bringing Wnts into proximity with their receptors (Lopez-Rios et al., 2008).

Collectively, our data suggest that the presence of Sfrp proteins might be crucial to determine the levels and distribution of Wnts and that they act as fine regulators of canonical signalling activation and target gene expression. Whether Sfrps have a similar regulatory function in non-canonical signalling remains unclear. In Sfrp1/2 mutant eyes, developmental events linked to non-canonical signalling appeared normal. Similarly, scalloped-Gal4-mediated expression of Sfrp1 throughout the entire Drosophila wing primordium had no major effect on bristle or trichome orientation, which are planar cell polarity-mediated events (Strutt and Strutt, 2007). Instead, scalloped-Gal4-Ds-RNAi-mediated knockdown of Dachsous, a cadherin-like protein required for the establishment of planar cell polarity (Matakatsu and Blair, 2004), induced a characteristic phenotype (data not shown). Nevertheless, these data do not rule out the possibility that, in other contexts, Sfrp proteins might control the diffusion of Wnts responsible for the activation of non-canonical signalling.

Nonetheless, our findings, together with those of Mii and Taira (Mii and Taira, 2009), contrast with the general designation of Sfrp1/2 as Wnt inhibitors and provide an alternative mechanism by which these proteins modulate Wnt signalling. This alternative view offers a possible explanation for previous results showing that Sfrp1 is required for positive regulation of β-catenin in, for example, hematopoietic progenitors (Renstrom et al., 2009; Yokota et al., 2008).

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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