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Interaction of smoothened with integrin-linked kinase in primary cilia mediates Hedgehog signalling

Badia Barakat¹, Liang Yu¹, Camden Lo², Duc Vu¹, Elisabetta De Luca¹, Jason E. Cain¹, Luciano G. Martellotto¹, Shoukat Dedhar³, Anthony J. Sadler¹, Die Wang¹, D. Neil Watkins¹ & Gregory E. Hannigan¹⁺

¹Centre for Cancer Research, ²Monash Micro Imaging, Monash University and Monash Institute of Medical Research, Clayton, Victoria, Australia, and ³BC Cancer Agency and Department of Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada

Here we report that ILK localizes in the mouse primary cilium, a sensory organelle required for signalling by the Hedgehog (Hh) pathway. Genetic or pharmacological inhibition of ILK blocks ciliary accumulation of the Hh pathway effector smoothened (Smo) and suppresses the induction of Gli transcription factor mRNAs by SHh. Conditional deletion of ILK or Smo also inhibits SHh-driven activation of Gli2 in the embryonic mouse cerebellum. ILK regulation of Hh signalling probably requires the physical interaction of ILK and Smo in the cilium, and we also show selective cilia-associated interaction of ILK with β -arrestin, a known mediator of Smo-dependent signalling.

Keywords: cerebellar development; Hedgehog signalling; integrin-linked kinase; primary cilium

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INTRODUCTION

The primary cilium is a microtubule-based sensory organelle projecting singly from the surface of most vertebrate cells. The cilium coordinates protein interactions governing signal transduction by the Hedgehog (Hh) developmental pathway [1–5]. Signalling is initiated by Hh ligand interacting with and inducing movement of the Patched (Ptc1) receptor out of the cilium, thereby derepressing the essential pathway effector smoothened (Smo), a G-protein coupled receptor (GPCR)-like molecule. Activated Smo translocates from endomembranes into the cilium where it activates Gli transcription factors, which then transport to the

nucleus to regulate gene expression. In the absence of Hh ligand, full-length Gli factors are processed in the cilium to produce truncated repressors of Hh signalling. Hh-induced ciliary translocation and activation of Smo inhibits processing, producing active Glis that are thought to undergo retrograde transport to the nucleus [6]. Smo activation is incompletely understood [7], however it requires phosphorylation in the cilium by GRK2 and CK1 α [8]. The cilium forms at the location where the mother centriole (centrosome) docks to the plasma membrane after mitosis, forming the basal body, a microtubule-organizing centre that gives rise to the primary cilium in a process requiring bidirectional intraflagellar transport (IFT) proteins, which regulate ciliary protein transport and assembly [9]. Accordingly, IFT proteins mediate developmental Hh signalling [2], and *ift* mutations can deregulate signalling through altered localization of Smo and Gli within the cilium [10].

Sonic Hedgehog (SHh) is critical for development of the cerebellum, a hindbrain structure that governs fine motor coordination in mammals, and regulates proliferation of granule cell precursors (GCPs), which mature to comprise the major neuronal population in the adult cerebellum [11,12]. The integrin-linked kinase (ILK) is an essential effector of β 1 integrin signalling, and ablation of either ILK or β 1 integrin in the CNS causes defects in cerebellar development suggestive of interactions in the Hh pathway [13,14]. Inhibition of ILK [14], but not β 1 integrin [13], suppresses GCP proliferation *ex vivo*, indicating ILK has an integrin-independent role in SHh-induced GCP proliferation. Using embryonic cerebellar development as a model of Hh signalling, we present *in vivo* evidence for ILK-mediated Hh activation in GCPs. The identification of ILK in ciliary proteomic screens [15,16] prompted us to investigate a potential role in ciliary Hh signalling. We find that ILK functions to regulate Hh-induced Smo ciliary translocation, associated with close physical interaction with Smo in the cilium. ILK also interacts at the ciliary base with β -arrestins, which have an essential role in Smo ciliary translocation, thus indicating a novel interaction governing Smo-dependent signalling.

¹Centre for Cancer Research

²Monash Micro Imaging, Monash University and Monash Institute of Medical Research, 27–31 Wright St, Clayton, Victoria 3168, Australia

³BC Cancer Agency and Department of Biochemistry, University of British Columbia, Vancouver, British Columbia, Canada

⁺Corresponding author. Tel: +61 3 9902 4824; Fax: +61 3 9594 7114;

E-mail: greg.hannigan@monash.edu

- ◀ **Fig 1** | Integrin-linked kinase (ILK) is expressed in granule cell precursors (GCPs) and mediates Hedgehog (Hh) signalling *in vivo* and *in vitro*. (A) GCPs isolated from post-partum day (dpp) 7 mice were positive for HNK1, Math1 and ILK and have primary cilia (Arl13b, red). Scale bar, 5 μ M. (B, upper) Representative sagittal sections of H&E-stained E18.5 cerebellae from E18.5 *ilk*^{loxP/+} (control), *Math1-Cre;ilk*^{loxP/loxP} and *Math1-Cre;Smo*^{loxP/loxP} mice. Asterisks indicate shallow invaginations corresponding to fissure locations in control cerebellum. (B, lower) Sagittal sections as indicated, stained with antibody specifically recognizing activated Gli2^A. Arrows indicate GCPs in the EGL, bounded by a dashed line. Scale bar, 50 μ m. (C) Induction of alkaline phosphatase activity in ILK-depleted 10T1/2 cells by SHh-CM, recombinant Sonic Hedgehog (rhSHh) and SAG (smoothened agonist). Controls included conditioned medium (CM) from HEK293 cell cultures (293-CM) and SHh-CM pre-incubated with 5E1 SHh-neutralizing monoclonal antibody. IB: western blotting for ILK protein levels in control and ILK siRNA-treated cells. Data are presented as mean $\pm$ s.e.m., * P < 0.05, ** P < 0.01 (Student's *t*-test), n = 4 independent experiments with triplicate determinations in each. (D) Primary cultures of *ilk*^{loxP/loxP} MEFs were infected with Ad-GFP control or Ad-Cre^{GFP} for 24 h, serum starved for an additional 24 h before stimulation with rhSHh for 0, 12 or 24 h. Cells were harvested and RNA subjected to qRT-PCR to quantify ILK, Gli1 and Gli2 expression as described in Methods. (E) Primary MEF cultures were serum starved for 24 h, then pretreated with the indicated concentrations of QLT-0267 for 1 h before stimulation for 24 h with rhSHh. Cells were harvested and RNA subjected to quantitative RT-PCR (qRT-PCR) to quantify Gli1 and Gli2 induction. Results are inclusive of two independent experiments. Columns indicate the mean of two independent experiments with duplicate determinations in each experiment.

RESULTS AND DISCUSSION

ILK mediates Hh signalling *in vitro* and *in vivo*

We characterized GCPs from embryonic mouse cerebellum using the neuronal cell surface marker HNK1 [17], Math1 [18], ILK and ciliary marker Arl13b [19]. Sorting indicated that more than 90% of this population of cells co-expressed HNK1, Math1 and ILK. Cilia were also readily detected on ~50% of isolated GCPs, indicating competence for Hh signalling (Fig 1A).

Immunohistochemistry (IHC) demonstrated strong ILK staining in the external granule layer (EGL) (supplementary Fig S1 online). We asked if targeted *ilk* deletion in GCPs affects Hh signalling during cerebellar development, by crossing *ilk*^{loxP/loxP} mice to a strain carrying Cre recombinase under the control of the GCP-specific Math1 promoter [20]. ILK was not detected in the EGL of embryonic *Math1-Cre;ilk*^{loxP/loxP} cerebellae, whereas EGL of *ilk*^{loxP/+} control EGL stained strongly for ILK (supplementary Fig S1 online). As a control, we crossed *Smo*^{loxP/loxP} mice to the *Math1-Cre* strain for *Smo* knockout in GCPs. Cerebellae from *Math1-Cre;ilk*^{loxP/loxP}, *Math1-Cre;Smo*^{loxP/loxP} embryos were examined for SHh signalling by evaluating Gli2 activation using IHC [10,21]. Delayed foliation in *Gli2* mutant cerebellae is tightly linked to impaired SHh signalling [22,23], thus lack of foliation at E18.5 in *Math1-Cre;ilk*^{loxP/loxP} and *Math1-Cre;Smo*^{loxP/loxP} cerebellae is suggestive of impaired SHh signalling. Nuclear Gli2^A protein was detected in control cerebellum, with marked reduction in Gli2^A staining observed in *Math1-Cre;ilk*^{loxP/loxP} and *Math1-Cre;Smo*^{loxP/loxP} EGL (Fig 1B). These results indicate that ILK regulates SHh processing of Gli protein, or induction of Gli mRNA.

SHh signalling induces osteoblastic differentiation of C3H10T1/2 fibroblasts, measured by induction of alkaline phosphatase (AP) [24]. We inhibited ILK in 10T1/2 cells using ILK siRNA, before induction of AP with SHh. Cells were treated with conditioned medium (CM), SHh-CM, recombinant SHh (rSHh) or smoothened agonist (SAG) [25], a lipid agonist that binds to and activates Smo (Fig 1C). Both the Smo antagonist cyclopamine and SHh-neutralizing antibody 5E1 [26] suppressed AP induction by > 90%. SHh-CM induced AP equally in untransfected and control small interfering RNA (siRNA)-transfected cells, however, AP induction was inhibited by ~60% in ILK siRNA-transfected cells, consistent with the observed 70–75% knockdown of ILK in these cells. AP induction by rSHh and SAG was inhibited, respectively,

Fig. 2 | Integrin-linked kinase (ILK) localizes in primary cilia of epithelial and fibroblast cells. Stacked confocal images of IMCD3 (A) and C3H10T1/2 (B) cells transfected with mCherry-tagged ILK. Primary cilia were labelled with anti- α -tub^{acet} antibody (green). Channels are shown as a single overlay (merge), with signal in the primary cilium shown as shifted-overlay (inset). (C) Confocal images of C3H10T1/2 cells labelled for ILK with an anti-ILK mouse monoclonal antibody (mILK, green) and centrosomes using γ -tubulin antibody (γ -tub, red). (D) Confocal images of IMCD3 cells labelled with an anti-ILK rabbit monoclonal antibody (rILK, red) and α -tub^{acet} antibody (green). Lower panel represents the corresponding confocal image in the *x*-*z* plane. Arrows indicate primary cilia. Scale bar, 10 μ m.

Fig 3 | Integrin-linked kinase (ILK) and smoothened (Smo) associate in the primary cilium. Protein–protein interactions were determined using a Venus-based BiFC assay. (A,B) Z-stack confocal images of (A) β -arrestin 2 (arrB2) and Smo and (B) ILK and Smo protein–protein interactions (green). Primary cilia and basal bodies were identified by immunofluorescence using anti- α -tub^{acet} (magenta) and anti- γ -tub (blue) antibodies, respectively. Transfected cells were identified by immunofluorescence with anti-GFP antibody (red) specifically detecting the V2 tag. (C) Representative images of Venus signal in cilia and its quantification using 3D isosurface rendering and interaction density maps as detailed in Methods. (D) Quantification of the mean Venus interaction intensity relative to the amount of split Venus components present. The signal ratio of reconstituted Venus (green) to total GFP (red) in cilia of transfected cells is indicated below graph. Results from one representative experiment are graphed. Scale bar, 5 μ M for panels A and B.

by 90% and 60% in ILK siRNA-transfected 10T1/2 cells (Fig 1C). To examine Hh signalling directly, isolated MEFs from *ilk^{loxP/loxP}* mice were infected with adenovirus expressing Cre recombinase to delete *ilk*, and GFP to mark infected cells (Ad-Cre^{GFP}). After 24 h infection, cells were treated with SHh-CM for 12 and 24 h, then harvested and analysed by quantitative RT–PCR (qPCR) for levels of *ilk*, and induction of *Gli1* and *Gli2* mRNA expression. Ad-Cre^{GFP} infection reduced *ilk* mRNA by ~70%. SHh induction of *Gli1* and *Gli2* mRNAs at 24 h was suppressed by ~50% relative to controls (Fig 1D). We next analysed the effects of the selective small-molecule ILK inhibitor, QLT-0267 [27], on SHh induction of *Gli1/2* mRNA expression in primary MEF cultures. Wild-type MEFs were serum starved overnight, followed by pretreatment with vehicle, 5 μ M or 10 μ M QLT-0267 before stimulation with SHh-CM for 24 h. Quantitative RT–PCR analyses indicated that induction of both genes was inhibited in a dose-dependent manner, with 10 μ M QLT-0267 effectively blocking induction

(Fig 1E). Inhibition of *Gli1/2* mRNA inductions by QLT-0267 suggests that ILK kinase activity mediates Hh signalling downstream of Ptch1. Interestingly, QLT-0267 treatment results in more complete inhibition of Gli mRNA induction than seen with Ad-Cre^{GFP} infections. Residual ILK kinase activity is probably sufficient to mediate significant, albeit reduced levels of SHh signalling in Ad-Cre^{GFP} infected *ilk^{loxP/loxP}* MEFs.

ILK is not required for ciliogenesis

Transfected mCherry-tagged ILK co-localizes with the ciliary marker, acetylated α -tubulin (α -tub^{acet}), in 10T1/2 cells and renal intermedullary collecting duct cells, IMCD3 (Fig 2A,B). Apico-basal orientation of cilia was established using the basal body marker γ -tubulin (γ -tub). Using mouse and rabbit monoclonal anti-ILK, anti- α -tub^{acet} and anti- γ -tub antibodies we observed co-localization of endogenous ILK with α -tub^{acet} in cilia of both cell types (Fig 2C,D).

Fig 4 | β -arrestin 2 interacts with integrin-linked kinase (ILK) and Kif3a at base of the cilium. Z-stack confocal images of V2- β -arrestin 2 (arrB2) co-transfected with (A) V1-ILK or (B) V1-Kif3a. Interaction of V1- and V2-tagged proteins (green) is localized relative to the primary cilium (magenta, α -tub^{acet}) and basal bodies (blue, γ -tub), as in Fig 3. 3D isosurface renderings for A and B were generated using IMARIS software. Scale bar, 5 μ M (A), with the same magnification shown in B.

The kinesin II motor subunit, Kif3a, is an IFT required for assembly of the cilium [28]. ILK- and Kif3a-specific siRNA suppressed respective protein levels by 71–75% in 10T1/2 and IMCD3 cells, and siKif3a inhibited cilia formation by 65–75% relative to control and ILK siRNA (supplementary Fig S2A–C online). To confirm this, MEFs were isolated from *ilk*^{loxP/+} and *ilk*^{loxP/loxP} mice and cultures infected with Ad-Cre^{GFP} to delete *ilk*. Western blotting demonstrated that >75% reduction in cellular ILK protein levels did not reduce cilia numbers in *ilk*-depleted MEFs (supplementary Fig S2D–F online). As another parameter of ciliogenesis, we measured cilia length in cultures of control and ILK siRNA-transfected IMCD3 cells. ILK knockdown did not affect ciliary length relative to controls (supplementary Fig S2G online), consistent with a recent proteomic screen which included, but did not identify ILK as a regulator of cilium length [29].

Cilium-associated ILK interacts with Smo and β -arrestins

We used bimolecular fluorescence complementation (BiFC) to detect and localize potential interactions between ILK and Hh proteins. Proteins were tagged with either the NH₂- (V1) or COOH-terminal (V2) fragment of Venus, a YFP variant. Close physical association of co-expressed V1- and V2-tagged proteins reconstitutes the mature fluorophore [30,31]. V1-ILK interacted with tagged partners V2- α -Parvin or V2-PINCH at focal adhesions in both C3H10T1/2 and IMCD3 cells (supplementary Figs S3A,B and S4A,B online). BiFC assays were performed using characterized binding-deficient mutants of ILK, ILK^{M402A/K403A} and ILK Δ ANK, which did not interact with Parvin

and PINCH, respectively, as expected (supplementary Figs S3C,D and S4C–E online).

β -arrestin adaptors regulate GPCR signalling [32]. β -arrestins 1 and 2 (arrB1, arrB2) localize at basal bodies, and to the primary cilium in response to Hh ligand [8]. ArrB mediates Smo translocation [33], thus we asked whether these two proteins form ciliary complexes. Using BiFC, we observed interaction of Smo with either arrB2 (Fig 3A) or arrB1 in cilia (supplementary Fig S4F online), in which we also observed Smo interaction with ILK (Fig 3B,C). As reported by others, we find that ectopic expression localizes Smo to the cilium independently of Hh ligand, thus we cannot examine SHh-induced ciliary interactions using BiFC. ILK-Smo, and arrB1/2-Smo complexes are present in 90–100% of cilia of transfected cells, and quantitation of ciliary BiFC signals indicated similar levels of Smo/ILK and Smo/arrB complexes (Fig 3D).

While ILK and Smo interact in the cilium, ILK-arrB2 (Fig 4A), ILK-arrB1 (supplementary Fig S4G online) and Kif3a-arrB2 (Fig 4B) complexes were observed at the ciliary base in close association with the basal body. Three-dimensional reconstruction of high-resolution Z-stack images and interaction density maps indicated that ILK-arrB2 and ILK-arrB1 complexes assemble at the transition zone, situated between the basal body and primary cilium (Fig 4A; supplementary Fig S4G online). Interestingly, ILK and arrB1/2 do not interact in cilia, although ectopic ILK and arrB1/2 proteins localize here (Fig 3A,B). Thus, ILK interacts selectively and possibly directly with arrB1/2 at the ciliary base, and in the cilium with Smo.

ILK localizes to integrin-dependent, basal focal adhesion sites and assembles signalling complexes via direct interactions

Fig 5 | Integrin-linked kinase (ILK) mediates ciliary translocation of smoothened (Smo). (A) Single-plane confocal images of IMCD3 cells untreated or transfected with either scrambled siRNA (siScr) or ILK siRNA (siILK) and treated with either SHh-CM, 1 μ g/ml recombinant Sonic Hedgehog (rhSHh) or 200 nM smoothened agonist (SAG) for 6 h. Smo protein (red) is detected by immunofluorescence. Primary cilia are labelled with anti- α -tub^{acet} antibody (green), the centrosome with γ -tubulin (magenta) and nuclei with DAPI (blue). Scale bar, 10 μ M. (B,C) Number of cilia positive for Smo as detected by immunofluorescence was quantitated in IMCD3 (B) and C3H10T1/2 (C) cells and expressed as a percentage of cilia count. Data are shown as mean $\pm$ s.e.m., asterisk indicates significance, $P < 0.01$ (Student's *t*-test), $n = 3$ independent experiments.

with PINCH and Parvin adaptor proteins [34,35]. To test whether ILK-PINCH-Parvin complexes mediate ciliary ILK localization, mCherry-tagged ILK^{M402A/K403A}, mutated in the Parvin-binding site [36], was transfected into IMCD3 cells. After 24 h cultures were serum starved for an additional 24 h to induce cilia, into which mCherry-ILK^{M402A/K403A} localized efficiently (supplementary Fig S5A online). Next we tested for localization and interaction of the PINCH non-binding ILK mutant, ILK Δ ANK [37], containing an intact kinase domain but lacks the ankyrin repeat domain. V1-ILK Δ ANK interacts with V2-Smo and V2-arrB, thus regionalizing Smo and arrB interactions to the catalytic domain of ILK (supplementary Fig S5B,C online). We conclude

that interactions with its canonical focal adhesion partners do not mediate ILK ciliary localization.

ILK mediates SHh-stimulated ciliary transport of Smo

Our observation of ILK-arrB2 complexes at the base of the cilia suggested that ILK regulates Smo ciliary translocation. We tested SHh-induced Smo translocation in cilia of ILK-depleted IMCD3 and 10T1/2 cells (Fig 5A). SHh-CM or rSHh treatment for 6 h induced Smo ciliary translocation in 85–90% of cells transfected with a scrambled control siRNA (Fig 5B,C). In marked contrast, the number of Smo-positive cilia was reduced to only 17–20% in ILK siRNA-transfected IMCD3, and $\sim$ 11% ILK-depleted 10T1/2 cells.

Thus, ILK mediates SHh-induced Smo translocation in fibroblasts and epithelial cells.

To determine whether ILK regulation occurs at the level of Ptc1-mediated Smo derepression, the lipid agonist SAG [25] was used to directly activate Smo in ILK-inhibited and non-inhibited cells. SAG induced Smo translocation in 82% of IMCD3, and 92% of 10T1/2 cells transfected with scrambled siRNA. This was reduced to 27% and 26% in ILK-inhibited IMCD3 and C3H10T1/2 cells, respectively (Fig 5B,C). These data indicate ILK does not regulate the Hh pathway at the level of Ptc1.

Ciliary transport is thought to be a tightly regulated process, entailing accumulation of cargo proteins at the ciliary base before IFT-dependent movement across the selective transition zone [38]. The association of arrB with ILK and Kif3a at the basal body/transition zone indicates an IFT-mediated mechanism mediating constitutive ciliary ILK localization. Targeted *ilk* deletion in GCPs suppresses cerebellar activation of Gli2, indicating a direct role for ILK in mediating Smo-dependent signalling *in vivo*. A key challenge will be to identify specific ILK-mediated phosphorylations governing Hh signalling in the primary cilium, and it will be of interest to determine whether ILK has a broader role in signalling in the primary cilium.

METHODS

Mice. Math1-Cre;*ilk^{loxP/loxP}* mice were generated by mating Math1-Cre^{+/−} [20] with *ilk^{loxP/loxP}* and backcrossing the Math1-Cre;*ilk^{loxP/+}* progeny with *ilk^{loxP/loxP}* [39]. Math1-Cre;*Smo^{loxP/loxP}* mice were generated by mating Math1-Cre^{+/−} with *Smo^{loxP/loxP}* [40] and backcrossing the Math1-Cre;*Smo^{loxP/+}* progeny with *Smo^{loxP/loxP}*.

Immunohistochemistry. Whole heads from E18.5 mice were fixed in formalin for 2 days at room temperature. Paraffin-embedded sagittal sections (4 μm) of the vermis region were used for IHC.

GCP isolation and FACS analysis. The cerebellum was dissected from day 7 post-partum mice, minced into small pieces and digested with Dispase (BD) for 15 min, then DnaseI for 10 min and fixed in 4% PFA, followed by sorting using HNK1 antibody.

Plasmids. Split Venus plasmids (referred to as V1 and V2) were kindly provided, by Drs Stephen Michnick and Jean-François Paradis (University of Montreal) for BiFC assays.

Cell culture and transfections. IMCD3 and C3H10T1/2 cells were obtained from ATCC and maintained in DMEM containing 10% FCS. For siRNA experiments, cells were transfected with non- or gene-targeted siRNAs (0.1 μM, SmartPool, Dharmacon) using Lipofectamine 2000 (Invitrogen). Primary MEFs were isolated from E13.5 wild-type and *ILK^{loxP/loxP}* mice.

Quantitative RT-PCR. mRNA was extracted using innuPREP RNA mini kit (Analytik Jena AG, Germany) and cDNA synthesized using using Cells-to-Ct reverse transcription kit (Applied Biosystems), and transcript levels measured using Taqman probes and normalized to *GAPDH* levels.

Western blotting. Cells were lysed in RIPA buffer and western blots conducted using standard protocols for the Odyssey system (Licor). ILK, panActin and GFP (detecting V2 or V1 fluorophore, Roche) were detected with either AlexaFluor 680 (Invitrogen) or IRDye800 (Rockland) secondary antibodies.

Immunofluorescence. Cells were fixed in formalin for 10 min at RT, permeabilized and then re-fixed in formalin. Cells were

blocked in blocking buffer and incubated with the relevant primary antibodies for 1 h at room temperature. Antibodies were detected with AlexaFluor (Invitrogen) secondary antibodies before mounting with Prolong Gold Antifade (Invitrogen).

Immunofluorescence data acquisition. Immunofluorescence data for Smo translocation were collected using a Nikon C1 laser scanning confocal microscope, and data for Venus experiments were collected using a Deltavision confocal microscope.

Venus quantification and Primary Cilia analyses. Venus interactions and primary cilia identification and quantitation were performed using Imaris analysis software (Bitplane AG, v7.0), using the fluorescence signal of acetylated α-tubulin to trace out enclosed volumes. Cilia length was analysed using Imaris v7.6.1 (Bitplane AG).

AP induction by SHh and SAG in C3H10T1/2 cells. C3H10T1/2 AP assay was conducted as previously described [24].

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

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Author contributions: G.H. conceived the project and wrote the manuscript. G.H. and D.N.W. planned and designed experiments. B.B., J.E.C., E.D.L., D.V., L.G.M., L.Y. performed experiments and/or analyses. S.D. provided QLT-0267; A.J.S. and D.W. provided expertise in BiFC experiments; C.L. wrote algorithms and performed image analyses.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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