The Golgi matrix protein giantin is required for normal cilia function in zebrafish

Dylan J. M. Bergen1,2, Nicola L. Stevenson1, Roderick E. H. Skinner2, David J. Stephens1,*,† and Christina L. Hammond2,*,‡

ABSTRACT
The Golgi is essential for glycosylation of newly synthesised proteins including almost all cell-surface and extracellular matrix proteoglycans. Giantin, encoded by the golgb1 gene, is a member of the golgin family of proteins that reside within the Golgi stack, but its function remains elusive. Loss of function of giantin in rats causes osteochondrodysplasia; knockout mice show milder defects, notably a cleft palate. In vitro, giantin has been implicated in Golgi organisation, biosynthetic trafficking, and ciliogenesis. Here we show that loss of function of giantin in zebrafish, using either morpholino or knockout techniques, causes defects in cilia function. Giantin morphants have fewer cilia in the neural tube and those remaining are longer. Mutants have the same number of cilia in the neural tube but these cilia are also elongated. Scanning electron microscopy shows that loss of giantin results in an accumulation of material at the ciliary tip, consistent with a loss of function of retrograde intraflagellar transport. Mutants show milder defects than morphants consistent with adaptation to loss of giantin.

KEY WORDS: Golgi, Golgi matrix, Cilia, Zebrafish

INTRODUCTION
The Golgi apparatus is the main hub of the secretory pathway, responsible for modifying proteins derived from the endoplasmic reticulum (ER) prior to transportation to the plasma membrane. It is estimated that one-third of the proteome traverses the ER-to-Golgi transport pathway (Zanetti et al., 2011). This includes delivery of key developmental signalling receptors to the plasma membrane and assembly of the extracellular matrix (ECM), which presents a major secretory load during development of the early embryo (Zhong, 2011). The organisation of Golgi membranes into flattened disc shapes is orchestrated by 11 members of the Golgi matrix (golgins). Giantin is present in all vertebrate genomes but is not found in invertebrates including Caenorhabditis elegans or Drosophila. In those organisms that express it, giantin is widely expressed and is found in almost all tissues. Giantin is required for the localisation of the dynein-2 intermediate chain WD-repeat protein 34 (WDR34) at the base of the cilium (Asante et al., 2013). Giantin is required for the localisation of the dynein-2 intermediate chain WD-repeat protein 34 (WDR34) at the base of the cilium. Since dynein-2 is the retrograde motor for IFT within cilia, we reasoned that loss of giantin might affect transport within the cilium and explain the defects in both ciliogenesis and length control.

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(Katayama et al., 2011). More recently, golgb1 knockout mice were generated using both N-ethyl-N-nitrosourea (ENU) and CRISPR/Cas9 mutagenesis (Lan et al., 2016). These animals show no gross abnormalities in skeletal development, including development of craniofacial structures, other than cleft palate and an abnormal deposition of glycosylated bone ECM as recognised by peanut agglutinin (PNA) lectin (Lan et al., 2016). The discrepancies in severity of the phenotypes of these rodent models could be due to species differences or genetic background. Nonetheless, both show extensive defects in ECM secretion and/or assembly.

Here, we describe that acute depletion of giantin in zebrafish leads to defects in cilia number and structure. These data show that acute depletion of giantin in vivo recapitulates ciliary defects seen in vitro. They also suggest that species-specific adaptive changes might underlie the phenotypic variability seen in these giantin knockout animals.

RESULTS

Acute knockdown and genetic knockout approach to assess giantin function in the zebrafish

Zebrafish giantin is encoded by 20 exons, with the translational start site (TSS), the human to zebrafish conserved coding sequence for the p115 binding site, and C-terminal TMD at exon 2, 3, and 20, respectively (Fig. 1A). The relative exon position of existing mutations identified or generated in rat and mouse models (Katayama et al., 2011; Lan et al., 2016) shows that all mutations affect evolutionarily conserved exons, ranging from exon 10 to 15 (Fig. 1A, yellow, blue, and brown coloured bars). The golgb1 gene was not duplicated in the teleost lineage. Aligning zebrafish giantin protein sequence [obtained from Ensembl (release 87, www.ensembl.org)] resulted in a 31.8% conservation to human, 29.5% to rat, and 32% to mouse, with a better conservation in the C-terminal half of the protein especially in the region of exon-11 and exon-14 (Fig. S1).

We chose to analyse the role of giantin in zebrafish using a combination of morpholino knockdown and genetic mutants. To knockdown giantin, two antisense morpholinos (MOs) were designed against the zebrafish golgb1 gene, the first targeting the TSS (ATG MO) located in exon 2 (ATG MO, Fig. 1A), and a second targeting a highly-conserved splice site located across intron 13 and the start of exon 14 (E14 MO, Fig. 1A). We validated the efficacy of the E14 MO using reverse transcription (RT)-PCR on cDNA generated from RNA extracted from controls and E14 morphants. Abnormal splicing against the zebrafish golgb1x3078 gene, that giantin plays in ciliogenesis in vivo, we visualised cilia in the ventral neural tube of morphants at 24 hpf by labelling for acetylated tubulin (Fig. 2A). These ventral neural tube ependymal cells project a mixture of primary cilia (Borovina et al., 2010) and motile 9+0 cilia, both of which are able to perform ciliary signal transduction (Bay and Caspary, 2012; Sedykh et al., 2016). Cilia observed in a two-nuclei window were counted showing a significant reduction in cilia abundance in both ATG and E14 morphants compared to controls (decreased by 22% and 33.5%, respectively, Fig. 2B). Measuring the length of individual cilia in the same area showed a significant increase in length of remaining cilia (Fig 2C-E). In contrast to morphants, no significant difference was seen in cilia number in the neural tube of giantin knockouts compared to controls (Fig. 3C). Quantifying cilia length in both golgb1Q294XX and
Fig. 1. See next page for legend.
**Fig. 1. Experimental knockdown and knockout of giantin in vivo.**
(A) Schematic representation of the zebrafish golgb1 transcript (ENSDBT00000131402.2) showing the binding sites of the designed morpholinos (ATG and E14), the relative exonic location of the human annotated p115 binding site, transmembrane domain (TMD), the various mouse mutants (black asterisks) and the ocd rat allele (white asterisk) as described in Lan et al. (2016), and Katayama et al. (2011), respectively. Coloured bars show the interspecies conserved sequence regions as assigned in Enssembl (release 87). Red asterisks indicate the location of zebrasfish giantin mutant alleles.
(B) Alternative spliced PCR products (blue arrows) from 32 hpf RT-PCR CDNA of two amplicons in E14 MO binding region. Black arrows indicate the expected size of PCR product. (C) SaT1389 line carrying a point mutation from C to T (yellow highlight, red letters) resulting in a premature stop codon at Q2948 (golgb1Q2984X) from the EZRC. (D) TALEN site directed mutagenesis resulted in an 8 bp deletion (spaccer sequence in yellow, red letters), resulting in golgb1X3078 mutant line. (E) Alignment of GolgI1 WT protein sequence (Ensembali release 87) with predicted GolgI1 X3078 protein sequence showing translated spacer sequence (red) and site of deletion T3028 and A3029 with a subsequent frameshift from E3027 (red asterisk) changing 51 amino acids to a predicted stop codon at position 3078. Part of cDNA exon 14 sequence.
(F) Stereoemicroscope images of 48 hfpc control, ATG, and E14 morphants exhibiting defects in the eye, heart, cranium, and various axis orientations (blue, green, black, red arrow, respectively). (G) Percentile quantification of scored phenotypes. (H) Stereoemicroscope images of heterozygote in-cross 5 dpf showing_E14_3078 and A3029 with a subsequent frameshift from E3027 (red asterisk) changing 51 amino acids to a predicted stop codon at position 3078. Part of cDNA exon 14 sequence.

\[\text{golgb1}^{X3078/X3078}\] mutants at 1 dpf showed a reproducible increase in ciliary length in the ventral neural tube (15.9% and 19.8%, respectively) compared to wild type (WT) (Fig 3D showing scatter dot plot, Fig 3E cumulative frequency plot of pooled data from multiple fish, and Fig 3F average cilia length per individual).

Since we saw an increase in cilia length in both morphants and mutants in vivo upon loss of giantin, we wanted to assess the appearance of the cilia. At 3 dpf, the olfactory placode is located at the anterior side of the head and projects 9–12 microtubule kinocilia into the lumen for sensory purposes (Hansen and Zeiske, 1998; Vina et al., 2015). Imaging of these olfactory pit neuropethelial cilia using scanning electron microscopy (SEM) showed that control morphants had a high density of olfactory cell cilia with a uniform appearance along their length, including at the tip. In contrast, both ATG and E14 morphants displayed a lower density of these cilia and an obvious bulbous appearance to the ciliary tip (Fig 4A). This phenotype could be reproduced in vitro, using the porcine kidney LLC-PK1 cell line transfected with short interfering RNAs (siRNA) targeting giantin, which led to bulbous ciliary tips compared to the smooth cilia in mock knockdown cells (Fig 4B).

Consistent with depletion of giantin causing defects in cilia function, MO knockdown of giantin using either ATG or E14 splice MOs caused heart laterality defects. The heart appeared morphologically normal but was situated either centrally or to the right rather than in its usual left position in a significant percentage of larvae at 32 hpf (Fig 5A). We then sought to rescue the effect of either the ATG or E14 giantin morpholino by co-injection with mRNA encoding FLAG-tagged giantin from Rattus norvegicus (Rn-GolgI1). Rat giantin was seen to localise to Golgi-like structures by immunofluorescence (Fig 5B). Randomisation of the heart position could be partially rescued (17% and 43%, respectively) by co-injecting the morpholino with mRNA encoding Rn-GolgI1 (Fig 5B,C). This ability to rescue the phenotype using mRNA from a mammalian species demonstrates that the function of giantin is conserved between rat and zebrafish.

The availability of a mutant null for giantin enabled us to test the specificity of our MO. We injected the E14 splice morpholino into the Q2984X mutant line. As described in Fig. 1, when the MO was injected into WT embryos 73% of larvae exhibited the ‘severe’ ciliopathy-like phenotype. In contrast, golgb1Q2948X/Q2948X embryos injected with the same concentration of E14 morpholino did not display the most severe phenotypes, instead a majority (77.5%) of the mutants displayed a mild phenotype (Fig 5D, quantified in 5E).

**DISCUSSION**

We show that the largest Golgi matrix protein, giantin, plays a role in ciliogenesis and control of ciliary length in vivo. Previous work showed that giantin is required for the ciliary localisation of the WDR34 intermediate chain subunit of the retrograde IFT motor dynein-2 (Asante et al., 2013). Knockdown of giantin in mammalian cells also led to a reduction in cilia number whilst the remaining cilia were longer (Asante et al., 2013). Mutations in human dynein-2 components lead to skeletal ciliopathies (McInerney-Leo et al., 2013; Schmidts et al., 2013a,b; Schmidts et al., 2013b), where defects in cilia formation and/or function result in pathologies associated with formation of the skeleton (Huber and Cornnier-Daire, 2012). These ciliogenesis defects seen in cells are phenocopied here in vivo where we show that golgb1 morpholino knockdown leads to a ciliopathy-like phenotype. Bulbous ciliary tips are seen upon acute depletion of giantin in zebrafish and in cultured pig kidney epithelial cells, consistent with a defect in retrograde IFT. This is also consistent with other work showing loss of the dynein-2 motor heavy chain subunit leads to shorter cilia and bulbous tips in the zebrafish (Krock et al., 2009). In agreement with morphant data, we found that cilia in the neural tube were significantly longer in homozygous mutant fish. This phenotype was not fully penetrant as cilia number was normal, potentially through tissue-specific compensation upon chronic loss of giantin.

Some phenotypic discrepancies between giantin morphants and homozygous mutants were observed, suggesting a compensation
mechanism following gene knockout. Overall, the effect of giantin knockout is less significant than depletion by morpholino injection. It has been estimated that 90-95% of genomic zebrafish knockouts do not resemble the acute morpholino knockdown phenotypes (Kok et al., 2015). This discrepancy between knockdown and genetic knockouts has also been shown in other model systems including mouse (Daude et al., 2012; De Souza et al., 2006) and Arabidopsis (Gao et al., 2015). Recently, Rossi et al. (2015) described that ‘long term’ ECM-dependent compensatory mechanisms are active to compensate for chronic loss of vascular ECM regulator egfl7. RNA sequencing screening led to the identification of Emilin2 and Emilin3 ECM components as compensatory factors in genetic egfl7 knockouts (Rossi et al., 2015). Our work suggests that genetic compensation in the mutant fish is unlikely to have occurred through changes in expression of other golgins, as mRNA transcript levels were equivalent in WT and mutant fish. Owing to a lack of effective reagents, we were not able to determine the effect on protein expression by immunoblot or...
immunofluorescence. Similarly, we were not able to rescue phenotypes on exogenous stimulation of hedgehog signalling and we could detect no defects in smoothened trafficking to cilia in siRNA duplex giantin-depleted cells in culture.

We also note considerable variation in phenotypes between the mouse (Lan et al., 2016), rat (Katayama et al., 2011), and zebrafish giantin knockout animal models. Knockout mice have non-syndromic cleft palate but show limited skeletal phenotypes; knockout rats show osteochondrodysplasia with oedema. Both rodent models die during late embryonic development. In contrast, our fish survive to adulthood, and this might reflect differences in developmental pathways, or in the mechanisms of compensation for loss of giantin function. Our previous *in vitro* work implicated giantin in the secretion of ECM (McCaughey et al., 2016) and we are now actively studying this in our knockout models. It is possible that the capacity to adapt the ECM during development varies

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**Fig. 3. Knockout of giantin results in defects in cilia length.** (A,B) Lateral view confocal images of 24 hpf cilia in ventral neural tube of Q2948X mutant line (A) and X3078 mutant line (B). Scale bars: 10 μm. White arrows indicate ciliary axonemes. (C) Cilia number per field of image (two per individual) in a two nuclei wide area adjacent to the notochord. (D,E) Ciliary length of all cilia measured depicted in a scatter dot plot (D) and a cumulative frequency plot (E) showing shift (blue arrow Q2948X and green arrow X3078) in percentile abundance of binned (0.25 μm bins) ciliary length between WT and homozygous 24 hpf embryos. (F) Average ciliary length per individual imaged. Data shown from at least three independent in-crosses. Bars show mean with standard deviation. Data was normally distributed and a subsequent unpaired *t*-test was performed between wild-type and homozygous groups. *P*<0.05, **P*<0.01, and ***P*<0.001.
Fig. 4. Scanning electron microscopy reveals structural defects in cilia following knockdown of giantin. (A) SEM images olfactory pit (3 dpf) indicating ciliary tips (red arrows), (B) SEM images of LLC-PK1 cells exhibit bulbous ciliary tips (red arrow) with visible ciliary extension (blue arrow) after Ss-Golgbl1 knockdown.

between both tissues and species and that this could compound the variation in phenotypes observed. This variety of phenotypes may also partially explain why acute knockdown and chronic knockout of golgb1 show differences, since acute knockdown may not trigger an adequate compensatory response due to giantin protein remnants. In summary, our data using in vivo models complement our previous in vitro work to support a role for giantin in controlling cilia formation and function.

MATERIALS AND METHODS
Zebrafish husbandry and transgenic lines
The London AB (AB), Tupfel long fin (TL), and AB/TL strains were used and maintained according to standard conditions (Westerfield, 2000) and staged accordingly (Kimmel et al., 1995). Transgenic lines: Arl13-GFP fusion protein [Tg(Arl13b::GFP)] (Borovina et al., 2010), Gli::mCherry [Tg(GLi2::mCherry)] (Schwend et al., 2010), and Cmcl2::GFP [Tg(myl7::eGFP)] were used. Experiments were approved by the University of Bristol Ethical Review Committee and the UK Home Office.

Morpholino knockdown
A translational block morpholino (Golgbl1-ATG; AACATGCTGACTGCAAGAAAATA) and a splice site blocker morpholino at the boundary between the preceding intron and exon 14 (Golgb1-E14; CTGTTCCAGCTACCTATTTGAAAAAATA) were obtained (Ensembl release 69) along with standard control morpholinos (GeneTools LLC, Philomath, OR). 3.8 ng morpholino with 0.1 M KCl and 0.1% phenol red was injected in one- to two-cell stage embryos. Lower dose morpholinos were injected at a calculated dose of 2.0 ng. N-capped Rn-flagGolgb1 mRNA was in vitro transcribed (Ambion™ T7 mMessage mMachine kit, Fischer Scientific) and both arm transcripts (170 pg each) were injected in one-cell stage embryo cytoplasm.

In vitro tissue culture, short-interference RNA knockdown
Pig kidney epithelial cells (LLC-PK1) cells were maintained under standard tissue culture, short-interference RNA knockdown conditions (37°C in 5% CO2) in DMEM (Medium-199, Sigma-Aldrich). Pig kidney epithelial cells (LLC-PK1) cells were maintained under standard conditions (37°C in 5% CO2) in DMEM (Medium-199, Sigma-Aldrich) plus 10% fetal calf serum (FCS). Calcium phosphate knockdown (Watson and Stephens, 2006) was performed with siRNA duplexes Sir/cSi/SsGolgbl1 (GUUCAGUGAUUGCAUAUCA) and Sir/dSiSsGolgbl1 (UCACAGUGUGUACCGAGGU) (MWG-Eurofins, Germany).

Human telomerase immortalised retinal pigment epithelial cells (hTERT-RPE1, Takara Bioscience, Kusatsu, Japan) were grown in DMEM...
F12 HAM supplemented with 10% FCS (Life Technologies, Paisley, UK). For giantin siRNA knockdown experiments, cells were first reverse transfected and then forward transfected 48 h later using Lipofectamine 2000 according to the manufacturer’s guidelines (Invitrogen, Carlsbad, CA, USA). In each round of transfection 200 pmol each of siRNA duplex 1 (ACUUCAUGCGAAGGCCAAATT) and siRNA duplex 2 (AGAGAGGCUCUUAUGAAUCAATT) targeting giantin were pooled together or for mock transfections 400 pmol siRNA targeting GL2 (GUACGCGGAAUCUUCGAUU) was used.

Immunofluorescence, microscopy, and ciliary length measurements
Whole-mount immunofluorescence and immunohistochemistry embryos or larvae (fixed 4% PFA for 1 h room temperature and post fixed in 100% methanol −20°C) were blocked in 10% FCS plus 1% bovine serum albumin blocking buffer. Primary antibodies: mouse α-acetylated tubulin (T6793, Sigma-Aldrich, UK), mouse M2 α-flag (F1804, Sigma-Aldrich). After primary antibody incubation samples were blocked again to reduce background staining, secondary antibodies were highly cross-adsorbed Alexa-dye labelled anti-IgG (Thermo Fisher Scientific). Images were acquired using confocal microscopy using either a Leica SP5-II or SP8 AOBS confocal laser scanning microscope attached to a Leica DM I6000 inverted epifluorescence microscope.

Lateral view 24 hpf agarose embedded embryos were imaged using a Leica SP5-II (405 nm diode and Argon lasers, 63× lens, oil, 1.4 numerical aperture). Neural tube around the cloaca was consistently imaged, with the same microscope settings per experiment, taking 0.25 µm thick z-slices. A two nuclei window directly dorsal of the notochord was drawn (white dotted line) from full projection images and cilia were quantified within that window using Fiji (Schindelin et al., 2012). Cilia crossing the white line were included in these measurements. Clusters of cilia without a clear start-to-end axoneme were excluded from length assessment.

Scanning electron microscopy
Both LLC-PK1 and hTERT-RPE1 cells were grown to confluence and serum-starved for 24 h prior to methanol fixation. Cells were washed in PBS, fixed in 100% MeOH at −20°C for 3 min, rinsed in PBS and blocked in 3% BSA-PBS for 30 min at room temperature. Antibodies were diluted in block and primary and secondary antibody labelling processed for 1 h each, washing 3× in between. Finally, cells were labelled with DAPI [4,6-diamidino-2-phenylindole (Life Technologies, Paisley, UK, D1306)] and mounted in Mowiol (Calbiochem, San Diego, CA, USA). Fixed cells were imaged using an Olympus IX70 microscope with 60×1.42 NA oil-immersion lens, Exfo 120 metal halide illumination with excitation, dichroic and emission filters [Semrock, Laser 2000 (Huntingdon, United Kingdom)], and a Photometrics Coolsnap HQ2 CCD, controlled by Volocity 5.4.1 (Perkin Elmer). Chromatic shifts in images were registration corrected using TetraSpek fluorescent beads (Thermo Fisher). Images were acquired as 0.2 µm z-stacks unless otherwise stated in the figure legend.
steps were: 25%, 50%, 70%, 80%, 90%, 96%, absolute ethanol, absolute ethanol over a course of 2 days. Critical point drying (Leica, EM CPD 300) was performed to remove excess of ethanol (12 cycles, 50% CO₂ infusion speed). Sputter coating with gold palladium supplied with Argon gas (Emitech K575X) was performed prior to imaging under high vacuum.

Reverse transcriptase PCR and quantitative Real-Time PCR

Larvae (5 dpf, n=30) were pooled and total RNA was isolated using RNeasy mini kit (cat# 74104, Qiagen) and reverse transcriptase reaction was performed by using Superscript III (cat# 1808093, Thermo Fisher Scientific) according to the manufacturers’ protocols. Quantitative Real-Time PCR (qPCR) reaction (primers: Table S1) was undertaken with DyNaMo HS SYBR green (F410L, Thermo Fisher Scientific) cycling (40 times) at 95°C 25 s, 57.5°C 30 s, and 70°C 45 s followed by a standard melt curve (QuantStudio3, Applied Biosystems).

Smoothened agonist, SHH and smoothened antagonist-1 treatment

Co-injected embryos with 3.8 ng of control, ATG, or E14 MO with either 1 ng control or p53TGG MO (Chen et al., 2005) were treated with final concentrations of 0.15%/v/v DMSO, 20 µM smoothened agonist (Sigma Aldrich), or 20 µM smoothened antagonist-1 (SANT-1) (Sigma, Dorset, UK, S4572) from 4 hpf until 48 hpf. Embryos were dechorionated and put in fresh treatment solution at 24 hpf. Images were taken with a fluorescence stereomicroscope (Leica Microsystems, Mannheim, Germany).

For cell experiments, kTERT-RPE1 cells were subjected to two rounds of transfection. Six hours after the second round, cells were serum starved in un-supplemented DMEM F12 HAM overnight, then treated with either 500 ng/ml recombinant Sonic Hedgehog (Shh) (C42II, N-terminus, R&D systems, Abingdon, UK, 1845-SH-025), 500 ng/ml SANT-1 (Sigma, Dorset, UK, S4572) or an equivalent volume of drug vehicle control for 24 h before methanol fixation and immunolabelling.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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mutations underlie Jeune asphyxiating thoracic dystrophy with impaired retrograde intraflagellar transport. Nat. Commun. 6, 7074.


Figure S1: Protein alignment showing conserved regions of zebrafish Golgb1 with human, mouse, and rat Golgb1. (A) Relatively conserved region corresponding to zebrafish exon-11 covering coiled-coiled domains number 23 and 24 counted from the N-terminus. (B) Evolutionarily conserved regions mapped to zebrafish exon-14 protein sequence, coding for coiled-coiled domains number 32 until 36 included, from a total of 40 annotated coiled-coiled domains in the zebrafish. Clustal OMEGA protein alignment tool (EMBL-EBI (Sievers et al., 2011)) was used to align Golgb1 protein sequences obtained from Ensembl (release #87).
Figure S2: Smoothened agonist treatment does not alter the ciliopathy-like phenotypes in giantin morphants. (A) Quantification of phenotypes observed (48 hpf). Note, no difference is seen with 1 ng p53 morpholino co-injection in all conditions. (B) Gli::mCherry expression was reduced in somites after SANT1 treatment, but not in SAG-treated individuals (48 hpf). SAG: smoothened agonist, SANT1: smoothened antagonist 1.
Figure S3: Depletion of giantin by RNAi in hTERT-RPE1 cells does not affect translocation of smoothened to the primary cilium. (A) Immunofluorescence of hTERT-RPE1 cells incubated with GL2 siRNA or two giantin siRNAs incubated for 96 hours, immunolabelled against acetylated Tubulin and smoothened. Smoothened translocation was detected in both conditions upon Shh stimulation (red arrow). (B) Quantification of Smo translocation to the cilium of three independent experiments (5 fields per images per condition). Scale bar = 5 µm. SANT1: smoothened antagonist 1, Shh: Sonic Hedgehog. Statistical test: 1-way ANOVA with Dunn’s multiple comparison test. * p<0.05, ** p<0.01, and *** p<0.001
Table S1: Primer sequences used for Quantitative RT-PCR of golgins.

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