

RESEARCH ARTICLE

SPECIAL ISSUE

CILIA AND FLAGELLA: FROM BASIC BIOLOGY TO DISEASE

Skeletal ciliopathy variants of the dynein-2 DYNC2LI1 subunit impair osteogenic differentiation of mesenchymal stem cells

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ABSTRACT

Skeletal ciliopathies result from defects in primary cilia, which are crucial for embryonic development because they transduce extracellular signals, including Hedgehog. Selective transport of ciliary proteins is mediated by the intraflagellar transport (IFT) machinery, containing the IFT-A and IFT-B complexes and the kinesin-2 and dynein-2 motors. Biallelic loss-of-function variants in genes encoding dynein-2-specific subunits, including DYNC2LI1, cause skeletal ciliopathies. As mesenchymal stem cells (MSCs) differentiate into osteoblasts, we investigated the effects of pathogenic variants of DYNC2LI1 on osteogenic differentiation of the MSC-like line C3H10T1/2. *Dync2li1*-knockout cells expressing disease-causing DYNC2LI1 variants demonstrated defects in the retrograde ciliary protein trafficking, including Hedgehog pathway GPCRs, Smoothed and GPR161. Furthermore, *Dync2li1*-knockout cells expressing the pathogenic variants demonstrated impaired Hedgehog signaling, in particular, a reduced ratio of the GLI3 repressor form to total GLI3, resulting in impaired osteogenic differentiation of MSCs. By contrast, osteogenic differentiation via BMP signaling was derepressed in *Dync2li1*-knockout cells. This suggests that skeletal ciliopathies caused by DYNC2LI1 variants could be attributable in part to impaired osteogenic differentiation due to defects in Hedgehog signaling, resulting from defects in retrograde ciliary protein trafficking.

KEY WORDS: Cilia, Dynein-2, GLI2, GLI3, Hedgehog signaling, Osteoblast

INTRODUCTION

Primary cilia are crucial for embryonic development and adult homeostasis because they receive and transduce extracellular signals, such as Sonic Hedgehog (SHH) and Indian Hedgehog (IHH) belonging to the Hedgehog (Hh) family. To fulfill these functions, cilia rely on specific membrane proteins, including G protein-coupled receptors (GPCRs) involved in Hh signaling (Anvarian et al., 2019; Nachury and Mick, 2019). The specific protein composition of the ciliary membrane and in the ciloplasm is

maintained by the presence of the transition zone (TZ) as a diffusion and permeability barrier at the ciliary base (Garcia-Gonzalo and Reiter, 2017; Park and Leroux, 2022). The intraflagellar transport (IFT) machinery containing IFT-A and IFT-B complexes powered by the kinesin-2 and dynein-2 motors mediates the selective transport of ciliary proteins (Nakayama and Katoh, 2020). Heterotrimeric kinesin-2 drives anterograde trafficking of the IFT machinery from the base to the tip of cilia, whereas the dynein-2 complex drives retrograde trafficking after its transport to the ciliary tip as an anterograde IFT cargo. Dynein-2 is also required for passage of the IFT machinery through the TZ (De-Castro et al., 2022; Hiyamizu et al., 2023). The dynein-2 or IFT dynein complex is composed of dynein-2-specific subunits [DYNC2H1, DYNC2I1/WDR60, DYNC2I2/WDR34, DYNC2LI1 and DYNLT2B (also known as TCTEX1D2)] and light chain subunits shared with the cytoplasmic dynein-1 complex (Nakayama and Katoh, 2020; Vuolo et al., 2020; Webb et al., 2020). An architectural model of the dynein-2 complex has been proposed based on biochemical studies together with interactome analyses (Asante et al., 2014; Hamada et al., 2018; Mikami et al., 2002; Tsurumi et al., 2019; Vuolo et al., 2018). In the human dynein-2 complex structure revealed by cryoelectron microscopy (cryo-EM), two molecules of the motor subunit DYNC2H1 form an asymmetric dimer, with the light intermediate chain DYNC2LI1 (also known as LIC3) bound to each DYNC2H1 molecule (Toropova et al., 2019). The DYNC2H1–DYNC2LI1 heterodimers are connected by DYNC2I1 and DYNC2I2 (Toropova et al., 2019); DYNC2I1 and DYNC2I2 use different interactions to engage equivalent sites on the two DYNC2H1 molecules and stabilize the asymmetric conformation of the DYNC2H1–DYNC2LI1 dimers (Mukhopadhyay et al., 2024).

Mutations in IFT machinery components result in congenital disorders known as ciliopathies, with a broad spectrum of clinical features (Mill et al., 2023; Reiter and Leroux, 2017). Prominent features of several of the ciliopathies are skeletal and craniofacial dysplasia, often accompanied by polydactyly, indicating the importance of primary cilia in bone formation and skeletal patterning (Chinipardaz et al., 2022; Mill et al., 2023; Quadri and Upadhyai, 2023). Among the IFT machinery components, mutations in dynein-2-specific subunits and all of the IFT-A subunits often cause skeletal ciliopathies with a narrow thorax and polydactyly, such as short rib-polydactyly syndrome (SRPS) (McInerney-Leo et al., 2015; Schmidts, 2014; Zhang et al., 2018).

Bone formation occurs through two distinct processes during skeletal development (Ohba, 2020; Salhotra et al., 2020). Flat bones of the skull are produced via intramembranous ossification, during which mesenchymal stem cells (MSCs) are directly differentiated into osteoblasts. By contrast, long bones develop via endochondral ossification. MSCs are initially differentiated into chondrocytes to form the cartilage mold, which produces various extracellular matrix proteins and IHH. Cells in the perichondrium that surrounds

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the cartilage are then differentiated into osteoblasts under the control of chondrocytes.

Hh signaling is crucial for osteogenesis (Salhotra et al., 2020; Zhou and Jiang, 2022). Three ciliary transmembrane proteins and three glioma-associated oncogene (GLI) transcription factors play crucial roles in Hh signaling in vertebrates, namely, Patched 1 (PTCH1), a 12-pass transmembrane receptor for Hh ligands, Smoothened (SMO), a class-F GPCR, and GPR161, a class-A GPCR, and the GLI1, GLI2 and GLI3 transcription factors (Anvarian et al., 2019; Kopinke et al., 2021; Niewiadomski et al., 2019). In the off-state of Hh signaling, PTCH1 inhibits the entry of SMO into cilia, and GPR161 on the ciliary membrane activates protein kinase A (PKA) to phosphorylate GLI2 and GLI3. Phosphorylated GLI3 undergoes partial proteolytic processing to yield its repressor form (GLI3R), which suppresses the expression of *Glil* (see Fig. 8A). In the on-state, binding of the Hh ligand to PTCH1 induces its exit from cilia and reverses SMO inhibition to enable its entry into cilia. Concomitantly, GPR161 exits cilia, resulting in the derepression of PKA-mediated negative regulation, and enhancement of *Glil* expression by the activator form of GLI2 (GLI2A) to promote differentiation. A recent study has indicated that binding of SMO to the PKA catalytic subunit to inactivate it is the event crucial for cancellation of the negative regulation of Hh signaling (Walker et al., 2024).

Regarding DYNC2LI1, several pathogenic biallelic variants have been identified in individuals with skeletal ciliopathies (Kessler et al., 2015; Niceta et al., 2018; Taylor et al., 2015; Zhang et al., 2020). In an attempt to clarify the molecular basis of the skeletal ciliopathies caused by pathogenic *DYNC2LI1* variants, we recently performed biochemical interaction experiments and established *DYNC2LI1*-knockout (KO) cells from human telomerase reverse transcriptase-immortalized retinal pigment epithelial 1 (hTERT-RPE1) cells (Qiu et al., 2022). We found that the analyzed SRPS variants of DYNC2LI1 were impaired in their interaction with the DYNC2H1 and DYNC2I1 subunits. We also found that the *DYNC2LI1*-KO cells are impaired in retrograde trafficking of the IFT machinery, and that the exit of GPR161 from cilia induced by treating cells with Smoothened agonist (SAG) is thereby impaired. Furthermore, combinatorial expression of two SRPS variants, i.e. DYNC2LI1(Δ302–332) and DYNC2LI1(L117V), or DYNC2LI1(1–317) and DYNC2LI1(P120S) (Taylor et al., 2015; Zhang et al., 2020) in *DYNC2LI1*-KO RPE1 cells, both of which mimic the cellular conditions of SRPS individuals, did not restore retrograde trafficking or GPR161 exit from cilia upon Hh signaling stimulation (Qiu et al., 2022). These results indicate that the biallelic pathogenic variants of *DYNC2LI1* cause defects in retrograde ciliary protein trafficking and in ciliary GPCR export in the cells of SRPS individuals. However, the study using *DYNC2LI1*-KO cells derived from RPE1 cells was unable to clarify the causal association between the ciliary defects and abnormal osteogenesis in SRPS individuals.

To address this remaining issue, we used the mouse MSC-like line C3H10T1/2, which has been used as a model for Hh-mediated osteogenic differentiation (Nakamura et al., 1997; Ruiz i Altaba, 1999; Shimoyama et al., 2007; Yang and Wang, 2012); C3H10T1/2 cells can be differentiated into alkaline phosphatase (ALP)-positive osteoblasts when stimulated with a Hh ligand or SAG. We here show that *Dync2li1*-KO C3H10T1/2 cells with the exogenous expression of any of the pathogenic DYNC2LI1 variants alone or in combination to mimic the cellular conditions of SRPS individuals demonstrate not only ciliary retrograde trafficking defects and aberrant accumulation of SMO and GPR161 within cilia, but also defects in differentiation into ALP-positive cells upon stimulation

with SAG. Furthermore, *Dync2li1*-KO C3H10T1/2 cells expressing any of the pathogenic DYNC2LI1 variants demonstrated a decreased ratio of GLI3R to full-length GLI3 upon SAG treatment. Thus, our data suggest that osteogenic differentiation in SRPS individuals could be compromised in part owing to defective Hh signaling.

RESULTS

***Dync2li1*-KO C3H10T1/2 cells have shortened cilia and are impaired in their osteogenic differentiation**

In a previous study, we showed that when combinations of DYNC2LI1 compound heterozygous variants found in SRPS individuals are expressed in *DYNC2LI1*-KO cells derived from hTERT-RPE1 cells, they could not rescue defects in retrograde trafficking of the IFT machinery and the aberrant accumulation of GPR161 within shortened cilia (Qiu et al., 2022). However, the study did not address the association between the ciliary defects and the pathobiology of skeletal ciliopathies, because RPE1 cells were used. We therefore established *Dync2li1*-KO cells from the mouse MSC-like line C3H10T1/2, which undergoes osteogenic differentiation when the Hh pathway is stimulated (Nakamura et al., 1997; Ruiz i Altaba, 1999; Shimoyama et al., 2007; Yang and Wang, 2012).

The *Dync2li1*-KO cell line #LI1-2-2 (Fig. S1A,B), which was established using the CRISPR/Cas9 system by homology-independent DNA repair (Kato et al., 2017), has extremely shortened cilia compared with control C3H10T1/2 cells (see also Fig. 1W), although there was no reduction in the frequency of cilia formation as revealed by staining for ARL13B (a ciliary membrane protein) (Fig. S1C,D). These observations indicate that loss of DYNC2LI1 does not affect early stages of ciliogenesis, in line with a previous study using embryonic fibroblasts from *Dync2h1*^{−/−} mice (Quidwai et al., 2021). Furthermore, the *Dync2li1*-KO cells were impaired in their differentiation into ALP-positive cells upon SAG treatment to stimulate the Hh pathway compared with what was seen for control cells (Fig. S1E). The expression of mCherry-fused human DYNC2LI1(WT) in *Dync2li1*-KO cells restored ciliary length, which was slightly longer than in control cells (see Fig. 1C,W) and differentiation into ALP-positive cells upon Hh pathway stimulation (see Fig. 4A), excluding the possibility that the abnormal phenotypes were caused by off-target effects. Thus, the C3H10T1/2-derived *Dync2li1*-KO cells were confirmed to be useful for investigating the association between ciliary defects and impaired osteogenic differentiation.

SRPS variants of DYNC2LI1 are unable to rescue the ciliary length defects of *Dync2li1*-KO cells, and are compromised in their localization to the ciliary base

In a previous study (Qiu et al., 2022), we demonstrated that the expression in *DYNC2LI1*-KO RPE1 cells of a specific combination of DYNC2LI1 variants that were reported in compound heterozygous SRPS individuals, cannot rescue defects in ciliary retrograde trafficking of the IFT machinery and in export of ciliary GPCRs; namely, a combination of a deletion variant and a missense variant, DYNC2LI1(Δ302–332)+DYNC2LI1(L117V) or DYNC2LI1(1–309)+DYNC2LI1(P120S) (see Table S1) (Taylor et al., 2015; Zhang et al., 2020). Note that among the variants, in a previous study (Qiu et al., 2022) we used DYNC2LI1(1–317) instead of DYNC2LI1(1–309), as used here, because we previously were not able to successfully construct DYNC2LI1(1–309).

We here stably expressed these variants alone or in combination in *Dync2li1*-KO C3H10T1/2 cells. Stably expressed mCherry-DYNC2LI1(WT) was mainly found at the ciliary base, with a small amount within cilia (Fig. 1C; see also Fig. 1X) as previously

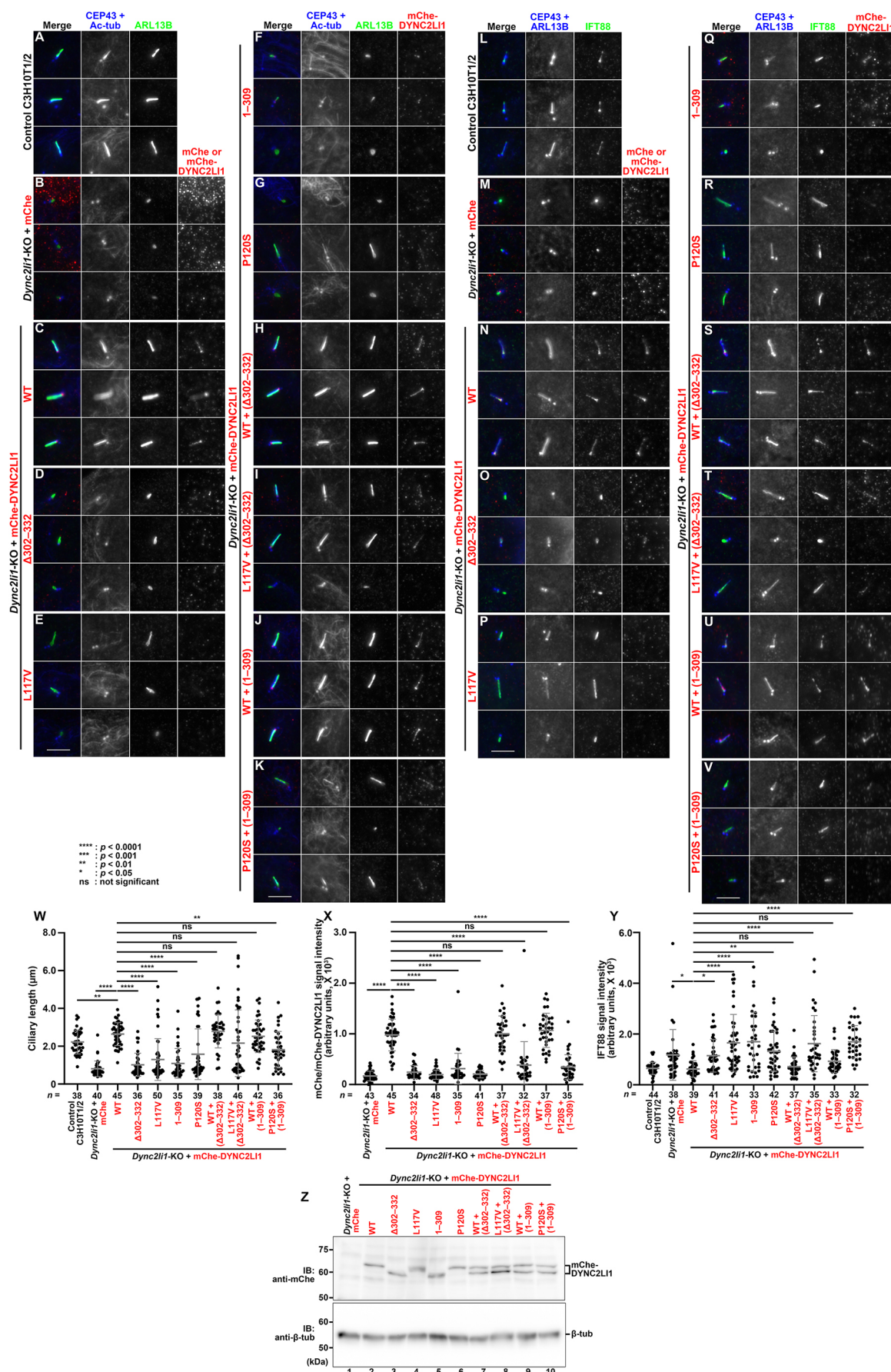


Fig. 1. See next page for legend.

Fig. 1. Abnormally short cilia in *Dync2li1*-KO cells expressing SRPS variants of DYNC2LI1. (A–V) Control C3H10T1/2 cells (A,L), *Dync2li1*-KO cells stably expressing mCherry (mChe) alone (B,M), mCherry (mChe)-fused DYNC2LI1(WT) (C,N), DYNC2LI1(Δ 302–332) (D,O), DYNC2LI1(L117V) (E,P), DYNC2LI1(1–309) (F,Q), DYNC2LI1(P120S) (G,R), DYNC2LI1(WT)+DYNC2LI1(Δ 302–332) (H,S), DYNC2LI1(L117V)+DYNC2LI1(Δ 302–332) (I,T), DYNC2LI1(WT)+DYNC2LI1(1–309) (J,U), or DYNC2LI1(P120S)+DYNC2LI1(1–309) (K,V) were serum-starved for 24 h to induce ciliogenesis, and then immunostained with antibodies against ARL13B (a ciliary membrane protein), RFP, CEP43 (previously referred to as FOP/FGFR1OP, a marker for the basal body/centrioles) and acetylated α -tubulin (Ac-tub, a marker of the ciliary axoneme) (A–K) or against IFT88, RFP, and ARL13B+CEP43 (L–V). Scale bars: 5 μ m. Images shown are representative of two experiments. (W) Ciliary lengths of individual ciliated cells in the experiments shown in A–K were measured and represented as scatter plots. (X,Y) Relative staining intensities of mChe or mChe-fused DYNC2LI1 (X) and those of IFT88 (Y) at the ciliary base and within cilia in the experiments shown in A–K and L–V, respectively, were estimated and represented as scatter plots. In W–Y, the numbers of analyzed cells (*n*) from a single experiment are indicated. Horizontal lines are means, and error bars are s.d. The immunostaining experiment was repeated twice to confirm the reproducibility. (Z) Expression of mChe-fused DYNC2LI1 proteins in *Dync2li1*-KO C3H10T1/2 cells. Lysates prepared from *Dync2li1*-KO C3H10T1/2 cells stably expressing the mChe-fused DYNC2LI1 constructs indicated were subjected to SDS-PAGE and immunoblotting analysis using antibodies against mChe or β -tubulin (β -tub). Images shown are representative of three experiments. **P*<0.05; ***P*<0.01; *****P*<0.0001; ns, not significant (one-way ANOVA followed by a Dunnett's multiple comparison test).

reported (Qiu et al., 2022; Vuolo et al., 2018; Weijman et al., 2024). The DYNC2LI1(WT) expression restored cilia length (compare Fig. 1C with Fig. 1A and B; see also Fig. 1W). By contrast, all of the analyzed SRPS variants were either marginally localized or not localized at the ciliary base (Fig. 1D–G; see also Fig. 1X), although expression of these mCherry-fused DYNC2LI1 proteins were confirmed by immunoblotting analysis of the cell lysates (Fig. 1Z). These observations suggest that these DYNC2LI1 variants are impaired in their incorporation into the dynein-2 complex as these variants are compromised in their interaction with the motor subunit DYNC2H1 (Qiu et al., 2022) and/or that the dynein-2 complex containing the pathogenic DYNC2LI1 variant was unstable as described previously (Taylor et al., 2015).

In support of this, we predicted whether there are differences between DYNC2LI1(WT) and the pathogenic variants regarding their interactions with DYNC2H1 using AlphaFold3 (Abramson et al., 2024). Compared with DYNC2LI1(WT), the C-terminal α -helix is displaced from the native position in DYNC2LI1(Δ 302–332) and lost in DYNC2LI1(1–309) (Fig. S2A). In agreement with the cryo-EM structure of the human dynein-2 complex (Mukhopadhyay et al., 2024; Toropova et al., 2019), DYNC2LI1(WT) was predicted to interact with bundles in DYNC2H1 via its N-terminal α -helix, central globular domain, and C-terminal α -helix (Fig. S2B). The C-terminal α -helix is displaced from the interface in DYNC2LI1(Δ 302–332), and lacking in DYNC2LI1(1–309), making it likely that these deletion variants have compromised interaction with DYNC2H1. By contrast, for DYNC2LI1(L117V) and DYNC2LI1(P120S), there was no clear difference from DYNC2LI1(WT) regarding the predicted interaction with DYNC2H1; the binding of these missense variants to DYNC2H1 might be destabilized by slight conformational variations and/or might be susceptible to interactions with other subunits.

In terms of ciliary length, there were differences between *Dync2li1*-KO cells expressing deletion and missense variants. The expression of mCherry-fused DYNC2LI1(Δ 302–332) or DYNC2LI1(1–309) did not significantly restore normal ciliary length (compare Fig. 1D and F with B; see also Fig. 1W). By contrast, in *Dync2li1*-KO cells

expressing DYNC2LI1(L117V) or DYNC2LI1(P120S), there were substantial variations in ciliary length among the cells (Fig. 1E,G).

When the mCherry-fused deletion variant DYNC2LI1(Δ 302–332) or DYNC2LI1(1–309) was coexpressed with mCherry–DYNC2LI1(WT) in *Dync2li1*-KO cells, which reflects the DYNC2LI1 proteins expressed from a healthy carrier, the localization of mCherry signals and the ciliary length were comparable to those of cells singly expressing mCherry–DYNC2LI1(WT) (compare Fig. 1H and J with C; see also Fig. 1W,X). In clear contrast, *Dync2li1*-KO cells coexpressing DYNC2LI1(Δ 302–332) with the missense variant DYNC2LI1(L117V) or DYNC2LI1(1–309) with the missense variant DYNC2LI1(P120S), which mimics the cellular conditions of individuals with SRPS (Taylor et al., 2015; Zhang et al., 2020), displayed phenotypes that were similar to *Dync2li1*-KO cells expressing the missense variant alone; namely, mCherry signals at the ciliary base and within cilia were significantly decreased compared with cells expressing mCherry–DYNC2LI1(WT), and the ciliary length tended to be variable among individual cells (compare Fig. 1I and K with H and J; see also Fig. 1W,X).

Accumulation of the IFT machinery within cilia in *Dync2li1*-KO cells expressing DYNC2LI1 variants

One of the prominent abnormal phenotypes of cells defective in dynein-2 subunits, including DYNC2LI1, is the aberrant accumulation of IFT machinery components, such as the IFT-B subunit IFT88 within cilia, indicative of defects in retrograde trafficking driven by dynein-2 (De-Castro et al., 2022; Hamada et al., 2018; Hiyaizumi et al., 2023; Mukhopadhyay et al., 2024; Qiu et al., 2022; Shak et al., 2023; Taylor et al., 2015; Tsurumi et al., 2019; Vuolo et al., 2018). This was also the case with *Dync2li1*-KO C3H10T1/2 cells. In control C3H10T1/2 cells and *Dync2li1*-KO cells expressing mCherry–DYNC2LI1(WT), IFT88 signals were found mainly at the ciliary base and weakly within cilia (Fig. 1L,N). By contrast, in *Dync2li1*-KO cells expressing mCherry alone, IFT88 signals were observed (Fig. 1M); however, cilia revealed by ARL13B staining (see Fig. S1D) were very short, making it difficult to distinguish between the base and the interior of cilia (see below). *Dync2li1*-KO cells expressing mCherry-fused DYNC2LI1(Δ 302–332) or DYNC2LI1(1–309) demonstrated intense IFT88 signals at the base or interior of very short cilia, like those expressing mCherry alone (compare Fig. 1O and Q with M; see also Fig. 1Y). As for *Dync2li1*-KO cells expressing mCherry-fused DYNC2LI1(L117V) or DYNC2LI1(P120S), IFT88 signals were observed inside cilia, which had recovered some length; this was in contrast to control C3H10T1/2 cells and DYNC2LI1(WT)-expressing *Dync2li1*-KO cells, in which IFT88 signals were observed mainly at the ciliary base and weakly within cilia (compare Fig. 1P and R with L and N; see also Fig. 1Y). Similar IFT88 staining patterns were observed in *Dync2li1*-KO cells expressing DYNC2LI1(L117V)+DYNC2LI1(Δ 302–332) or DYNC2LI1(P120S)+DYNC2LI1(1–309) (compare Fig. 1T and V with S and U; see also Fig. 1Y). Thus, *Dync2li1*-KO cells expressing any of the SRPS variants of DYNC2LI1 are impaired in retrograde IFT, although to varying degrees depending on the type of variant.

Next, we attempted to investigate how trafficking of the dynein-2 complex containing the DYNC2LI1 variant is impaired by observing the cells using total internal reflection fluorescence (TIRF) microscopy. However, the attempt was unsuccessful owing to a relatively low fluorescence intensity of the expressed mCherry-fused DYNC2LI1 construct. Another attempt to express DYNC2H1 and observe its movement was also unsuccessful due to the enormous size



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Fig. 2. Aberrant accumulation of SMO within cilia under basal conditions in *Dync2li1*-KO cells expressing SRPS variants of DYNC2LI1. (A–V) Cells as described in the legend for Fig. 1A–K were serum-starved for 24 h, and then left untreated (–SAG; A–K) or treated with 200 nM SAG (+SAG; L–V) for a further 24 h, and immunostained with antibodies against SMO, RFP and CEP43+ARL13B (markers for the basal body/centrioles and ciliary membrane, respectively). Scale bars: 5 μ m. Images shown are representative of two experiments. (W) Relative staining intensities of ciliary SMO were estimated, represented as scatter plots, and analyzed as described in the legend for Fig. 1Y. The numbers of analyzed cells (*n*) from a single experiment are indicated. The immunostaining experiment was repeated twice to confirm the reproducibility. Statistical significances among multiple groups and those between –SAG and +SAG were calculated using one-way ANOVA followed by a Dunnett's multiple comparison test and an unpaired two-tailed Student *t*-test, respectively. Horizontal lines are means, and error bars are s.d. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns, not significant.

of the DYNC2H1 coding sequence. Therefore, we turned to investigating how trafficking mediated by the IFT machinery is affected in cells defective in DYNC2LI1. To this end, we observed cells expressing EGFP–IFT88 (Fig. S3) by TIRF microscopy. In control C3H10T1/2 cells and *Dync2li1*-KO cells expressing mCherry–DYNC2LI1(WT) (Movie 1), bidirectional movement of EGFP–IFT88-positive particles within cilia was clearly observed; in these cells, the accumulation of EGFP–IFT88 at the ciliary tip to some extent might be due to its transient expression. Compared with what was seen in mCherry–DYNC2LI1(WT)-expressing *Dync2li1*-KO cells, EGFP–IFT88 was substantially accumulated at the tip in *Dync2li1*-KO cells expressing any of the pathogenic DYNC2LI1 variants, in particular, in those expressing the deletion variant at the bulged tip (Fig. S3D–G, Movie 1), and particle movement within cilia was not clearly observed in either direction (Movie 1). These observations are in line with a previous study on a temperature-sensitive *che-3* (DYNC2H1) mutant of *Caenorhabditis elegans* showing that anterograde as well as retrograde IFT within cilia was impaired after a prolonged period of time at restrictive temperature (Jensen et al., 2018). As a previous imaging study on *Chlamydomonas* IFT trains has suggested that IFT-B proteins from retrograde trains reenter the basal body pool of IFT components (IFT-A, IFT-B and motor proteins) from the cell body and are reused for assembly of anterograde trains (Wingfield et al., 2017), the persistent failure of retrograde trains due to dynein-2 defects might lead to depletion of building blocks required for anterograde train assembly, resulting in shortened cilia.

Impaired exit of ciliary GPCRs in *Dync2li1*-KO cells expressing DYNC2LI1 SRPS variants

In the basal state, GPR161 is on the ciliary membrane and negatively regulates Hh signaling via activating PKA, and SMO is excluded from cilia. By contrast, when cells are treated with Hh ligands or SAG, SMO is induced to enter cilia, while GPR161 exits cilia, resulting in the derepression of Hh signaling (Kopinke et al., 2021; Patel and Smith, 2024). Under basal conditions (without SAG treatment), SMO was not detectable within cilia in control C3H10T1/2 cells (Fig. 2A), probably because SMO is removed from the ciliary membrane once it has entered cilia (Hamada et al., 2018; Tsurumi et al., 2019). By contrast, SMO was aberrantly accumulated within very short cilia in *Dync2li1*-KO cells expressing mCherry alone (Fig. 2B; see also Fig. 2W); the SMO accumulation within short cilia under basal conditions in *Dync2li1*-KO cells is in line with previous observations in embryonic fibroblasts from *Dync2h1*^{−/−} mice (Quidwai et al., 2021). When mCherry–DYNC2LI1(WT) was expressed alone or together

with an SRPS variant of DYNC2LI1 in *Dync2li1*-KO cells, SMO accumulation within cilia as well as shortened cilia was rescued (Fig. 2C,H,I,W). By contrast, expression of any of the DYNC2LI1 SRPS variants alone in *Dync2li1*-KO cells did not eliminate the abnormal SMO accumulation in the basal state (Fig. 2D–G,W). Essentially the same results were obtained with *Dync2li1*-KO cells expressing compound heterozygous combinations of the DYNC2LI1 variants (Fig. 2I,K,W). When control cells and DYNC2LI1(WT)-expressing *Dync2li1*-KO cells were stimulated with SAG, SMO entered cilia (Fig. 2L,N; see also Fig. 2W). In *Dync2li1*-KO cells expressing any of the DYNC2LI1 variants, the ciliary SMO levels upon SAG treatment were not substantially different from the level in DYNC2LI1(WT)-expressing cells (Fig. 2N–W). Thus, these observations indicate that the exit of SMO from cilia under basal conditions is impaired in *Dync2li1*-KO cells and those expressing SRPS variants of DYNC2LI1.

We also investigated the localization of GPR161 under basal and SAG-stimulated conditions; however, in this case, we expressed GPR161–EGFP in cells, as commercially available anti-GPR161 antibodies can react with the human but not the mouse GPR161 protein for unknown reasons, as previously described by others (Gómez et al., 2022). In control C3H10T1/2 cells, GPR161–EGFP was found within cilia under basal conditions (Fig. 3A) but exited cilia upon treatment of cells with SAG (Fig. 3L; see also Fig. 3W). By contrast, the ciliary GPR161–EGFP level was not decreased upon SAG treatment in *Dync2li1*-KO cells expressing mCherry alone (Fig. 3B,M), but was significantly decreased in *Dync2li1*-KO cells expressing mCherry–DYNC2LI1(WT) (Fig. 3C,N; see also Fig. 3W). Similarly, *Dync2li1*-KO cells singly expressing mCherry-fused SRPS variants of DYNC2LI1 or expressing their combination did not show SAG-induced GPR161–EGFP exit (Fig. 3D–G,I,K,O–R,T,V; see also Fig. 3W). Thus, the induced exit of ciliary GPR161 upon SAG treatment was impaired in *Dync2li1*-KO cells expressing SRPS variants of DYNC2LI1.

Aberrant accumulation of the BBSome within cilia in *Dync2li1*-KO cells expressing SRPS variants of DYNC2LI1

The export of GPCRs from cilia is known to be mediated by the IFT machinery, which is powered by the dynein-2 complex. The BBSome, which is composed of eight subunits encoded by the causative genes of Bardet–Biedl syndrome (BBS), has been proposed to act as an adaptor between the IFT machinery and ciliary GPCRs for their export (Eguether et al., 2014; Liew et al., 2014; Nozaki et al., 2019; Nozaki et al., 2018; Ye et al., 2018; Zhou et al., 2022b). Based on the fact that ciliary GPCRs are aberrantly accumulated within cilia in the absence of DYNC2LI1 (Figs 2 and 3), we next investigated whether the BBSome is also accumulated within cilia.

The BBSome subunit BBS9 was not detectable within cilia in control C3H10T1/2 cells (Fig. S4A), whereas it was found within shortened cilia in *Dync2li1*-KO cells (Fig. S4B). The aberrant BBS9 accumulation as well as the shortening of cilia was reversed by the exogenous expression of mCherry–DYNC2LI1(WT) in *Dync2li1*-KO cells (Fig. S4C; see also Fig. S4L). In clear contrast, none of the SRPS variants of DYNC2LI1 could rescue the aberrant BBS9 accumulation within cilia (Fig. S4D–G,L). Furthermore, the combinatorial expression of DYNC2LI1(Δ 302–332) or DYNC2LI1(1–309) with DYNC2LI1(WT) eliminated BBS9 accumulation within cilia in *Dync2li1*-KO cells, whereas the combinatorial expression of DYNC2LI1(Δ 302–332) with DYNC2LI1(L117V) or DYNC2LI1(1–309) with DYNC2LI1(P120S) did not (Fig. S4H–L). Thus, in *Dync2li1*-KO cells and those expressing any of the DYNC2LI1 SRPS variants, it is likely that the BBSome as well as ciliary GPCRs cannot

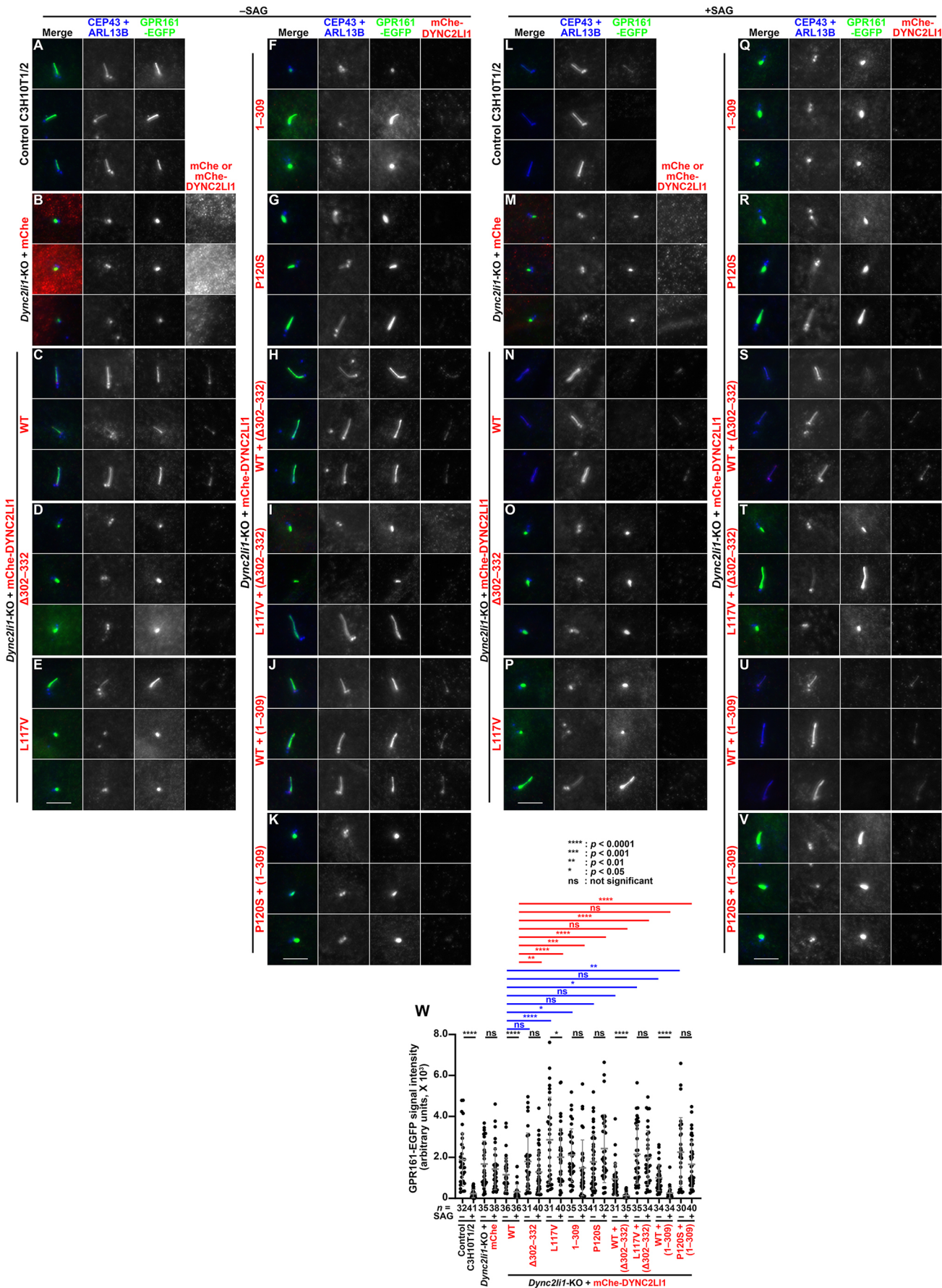


Fig. 3. See next page for legend.

Fig. 3. Impaired exit of ciliary GPR161 upon Hh signaling activation in *Dync2li1*-KO cells expressing SRPS variants of DYNC2LI1. (A–V) Cells as described in the legend for Fig. 1A–K were infected with a lentiviral vector for GPR161–EGFP. The cells were serum-starved for 24 h to induce ciliogenesis, and then left untreated (–SAG; A–K) or treated with 200 nM SAG (+SAG; L–V) for a further 24 h and immunostained with antibodies against GFP, RFP and CEP43+ARL13B. Scale bars: 5 μ m. Images shown are representative of three experiments. (W) Relative staining intensities of ciliary GPR161–EGFP in the experiments shown in A–V were estimated, represented as scatter plots, and analyzed as described in the legend for Fig. 1Y. For the quantification of GPR161–EGFP fluorescence intensity, only GFP-positive cells were analyzed. The numbers of analyzed cells (*n*) from a single experiment are indicated. The immunostaining experiment was repeated three times to confirm the reproducibility. Statistical significances among multiple groups and those between –SAG and +SAG were calculated using one-way ANOVA followed by a Dunnett's multiple comparison test and an unpaired two-tailed Student *t*-test, respectively. Horizontal lines are means, and error bars are s.d. ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns, not significant.

exit cilia owing to impairment in retrograde trafficking and passage through the TZ of the IFT machinery.

Osteogenic differentiation induced by Hh signaling activation is impaired in the absence of DYNC2LI1 or the presence of its SRPS variants

We then investigated whether *Dync2li1*-KO cells stably expressing any of the DYNC2LI1 SRPS variants can undergo osteogenic differentiation. As described above, control C3H10T1/2 cells were differentiated into ALP-positive cells by SAG treatment (Fig. 4A, column 1). *Dync2li1*-KO cells expressing mCherry alone did not undergo SAG-induced differentiation (column 2). Expression of mCherry–DYNC2LI1(WT) in *Dync2li1*-KO cells restored SAG-induced differentiation (column 3), although not as much as differentiation was as observed as with control C3H10T1/2 cells (column 1); although we do not know the exact reason for the incomplete rescue, it might be because not all cells expressed exogenous mCherry–DYNC2LI1(WT) or because the mCherry tag hampered efficient incorporation of DYNC2LI1(WT) into the dynein-2 complex. In clear contrast, expression of any of the pathogenic DYNC2LI1 variants or their combination did not result in SAG-induced differentiation (columns 4–7, 9 and 11).

The data on ALP staining were confirmed by reverse transcription-quantitative PCR (RT-qPCR) analysis of the transcript level of *Alpl* (bone mineralization-associated ALP). In control C3H10T1/2 cells, the *Alpl* transcript level was ~1000-fold increased by the treatment of cells with SAG (Fig. 4B,C). By contrast, in *Dync2li1*-KO cells expressing mCherry alone, the basal *Alpl* level was significantly higher than that in control cells (Fig. 4B). However, it is interesting to note that the *Alpl* level showed virtually no increase upon SAG treatment in *Dync2li1*-KO cells expressing mCherry alone (Fig. 4B,C). The expression of mCherry–DYNC2LI1(WT) restored the SAG-induced increase in *Alpl* level in *Dync2li1*-KO cells, although not as much as the increase in control C3H10T1/2 cells. As in *Dync2li1*-KO cells expressing mCherry, *Dync2li1*-KO cells expressing any of the SRPS variants of DYNC2LI1 or their pathogenic combination tended to show a moderate increase in basal *Alpl* level, which was not further increased by SAG treatment (Fig. 4B,C). By contrast, *Dync2li1*-KO cells expressing DYNC2LI1(Δ 302–332) or DYNC2LI1(1–309) with DYNC2LI1(WT), which reflects the DYNC2LI1 proteins expressed from the alleles of a healthy parent of an SRPS individual, demonstrated a low *Alpl* level in the basal state, and a significant increase in *Alpl* level under SAG-stimulated conditions, as in *Dync2li1*-KO cells expressing DYNC2LI1(WT)

(Fig. 4B,C). These results indicate that *Dync2li1*-KO cells or those expressing pathogenic DYNC2LI1 variants cannot undergo osteogenic differentiation under the control of Hh signaling, although the *Alpl* level under basal conditions is moderately increased in these cells compared with control cells.

We then analyzed the level of the *Gli1* transcript, as previous studies using *Gli1* reporter mice have shown that *Gli1*-positive cells are characterized as skeletal stem cells with osteogenic potential (Jin et al., 2024; Zhao et al., 2015). The tendency of *Gli1* expression levels was essentially the same as that of *Alpl* levels. Namely, as in control C3H10T1/2 cells, the *Gli1* level was relatively low under basal conditions, and increased considerably upon stimulation with SAG in *Dync2li1*-KO cells expressing DYNC2LI1(WT), whereas the *Gli1* level was relatively high in the basal state but was not further increased by SAG treatment in *Dync2li1*-KO cells expressing any of the pathogenic DYNC2LI1 variants (Fig. 4D,E). We also analyzed the expression level of *Ptch1*, which is under the control of the GLI transcription factors, and found that its expression level in *Dync2li1*-KO cells expressing DYNC2LI1 constructs did not vary substantially upon Hh pathway stimulation, similar to what was seen for *Alpl* and *Gli1* levels (Fig. 4F,G). Thus, these results indicate that SRPS variants of DYNC2LI1 impair the osteogenic differentiation of MSCs upon Hh signaling activation, at least in part via uncontrolled *Gli1* expression, whereas they slightly increase differentiation in the basal state.

SRPS variants of DYNC2LI1 decrease the relative level of GLI3R but block the SAG-induced decrease in total GLI3 protein level

We then analyzed the localization of GLI2 and GLI3. GLI2 and GLI3 have been reported to be enriched at the ciliary tip under Hh pathway-stimulated conditions (Endoh-Yamagami et al., 2009; Kim et al., 2009; Tukachinsky et al., 2010; Wen et al., 2010). Indeed, in control C3H10T1/2 cells, GLI2 and GLI3 became localized at the ciliary tip when the cells were treated with SAG (Figs 5A,L, and 6A,L; see also Figs 5W and 6W). In contrast to control cells, GLI2 or GLI3 was not detected in shortened cilia in *Dync2li1*-KO cells expressing mCherry, even under SAG-stimulated conditions (Figs 5B,M, and 6B,M; see also Figs 5W and 6W); note that for very short cilia, as we cannot distinguish the tip from the base, we refer to them simply as 'cilia' without distinguishing between them. The stable expression of mCherry–DYNC2LI1(WT) in *Dync2li1*-KO cells restored the ciliary tip localization of GLI2 and GLI3 upon SAG treatment (Figs 5C,N, and 6C,N; see also Figs 5W and 6W). Similarly, the SAG-induced localization of GLI2 and GLI3 to the ciliary tip was restored when pathogenic DYNC2LI1 variants were coexpressed with DYNC2LI1(WT) (Figs 5H,J,S,U, and 6H,J,S,U; see also Figs 5W and 6W). In clear contrast, all of the DYNC2LI1 variants, expressed singly or in combination (Figs 5D–G,I,K,O–R, T,V, and 6D–G,I,K,O–R,T,V) were impaired in their ability to restore the ciliary localization of GLI2 and GLI3 (see also Figs 5W and 6W). Thus, these results show that the ciliary localization of GLI2 and GLI3 is impaired in cells with defects in DYNC2LI1. More importantly, whether *Dync2li1*-KO cells expressing DYNC2LI1(WT) or its variants are able to undergo SAG-induced osteogenic differentiation and to induce the expression of *Alpl*, *Gli1* and *Ptch1* (Fig. 4) correlated very well with the ability of these cells to localize GLI2 and GLI3 to the ciliary tip upon SAG treatment (Figs 5A–W and 6A–W). The exact reason why GLI2 and GLI3 are not found at the ciliary tip in cells defective in DYNC2LI1 is unclear, but transport of GLI2 and GLI3 to the tip or their retention at the tip may be impaired as observed in *Dync2h1* mutant mice

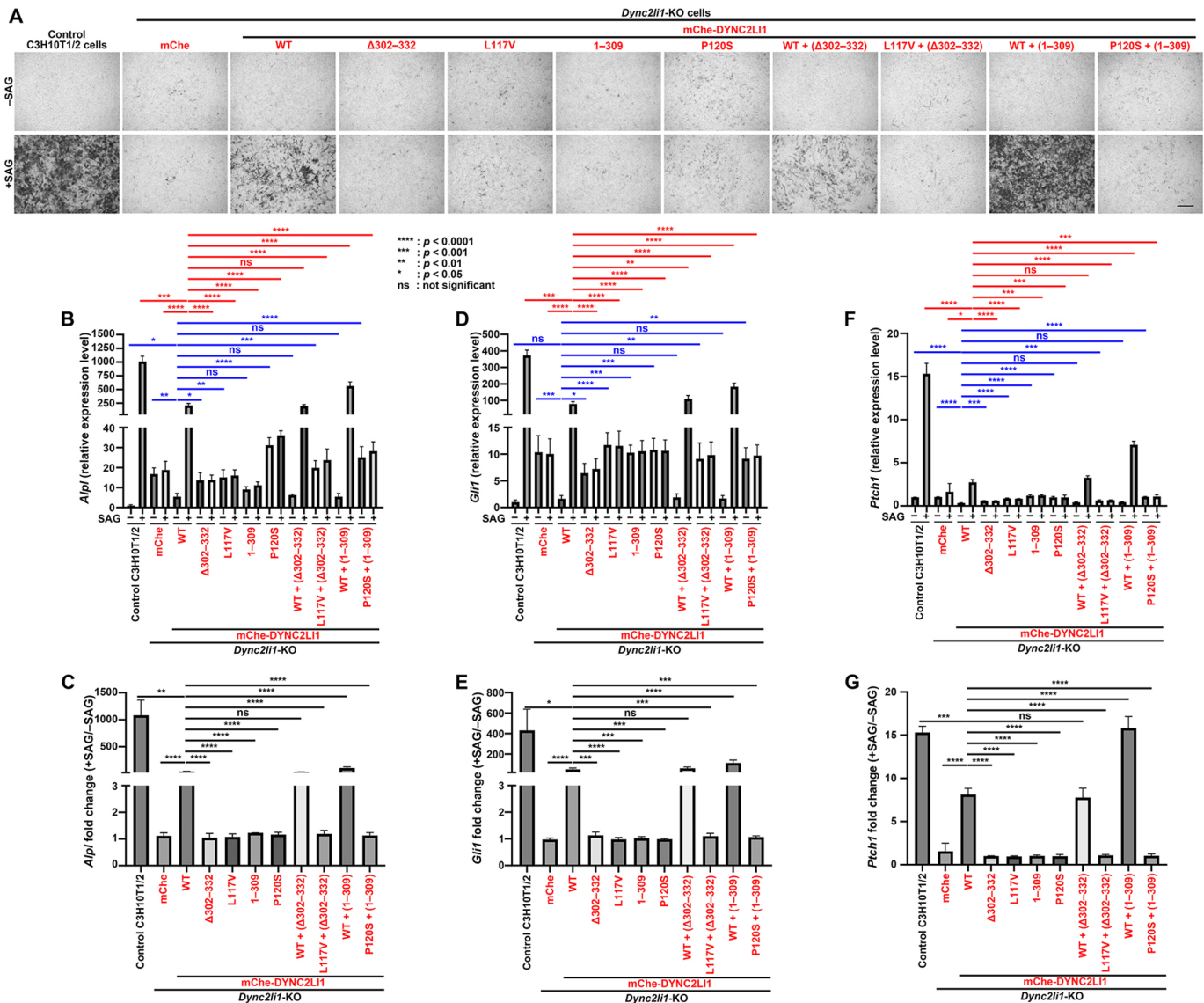


Fig. 4. Defects in osteogenic differentiation and Hh signaling of *Dync2li1*-KO cells expressing SRPS variants of DYNC2LI1 upon SAG treatment.

(A) Cells as described in the legend for Fig. 1A–K were cultured in the presence (+SAG) or absence (–SAG) of 200 nM SAG for 7 days, and then stained for ALP activity. Scale bar: 500 μm. Images shown are representative of four experiments. (B–G) RNAs extracted from the cells were subjected to RT-qPCR for the *Alpl* (B,C), *Gli1* (D,E) or *Ptc1* (F,G) transcript. In B, D and F, relative expression levels are shown as mean±s.d. of technical triplicates from a single experiment. In C, E and G, fold changes in the expression levels by SAG treatment are shown as mean±s.d. of technical triplicates from a single experiment. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns, not significant (one-way ANOVA followed by the Dunnett's multiple comparison test).

(Ocbina et al., 2011), because trafficking of anterograde as well as retrograde IFT particles is impaired (Movie 1).

We also investigated the GLI3 protein level, and the relative ratio of GLI3R to full-length GLI3 (GLI3FL), as GLI3 is required for normal skeletal development (Mo et al., 1997; Rice et al., 2010) and the balance between GLI3R and the activator form of GLI2 has been proposed to determine the expression of Hh target genes (Kopinke et al., 2021). The treatment of cells with SAG resulted in a significant decrease in total GLI3 protein level (GLI3FL+GLI3R) in control C3H10T1/2 cells but not in *Dync2li1*-KO cells expressing mCherry (Fig. 6X, lanes 1–4; see also Fig. 6Y). The stable expression of mCherry–DYNC2LI1(WT) alone (Fig. 6X, lanes 5 and 6) or in combination with an SRPS variant (lanes 15, 16, 19 and 20) in *Dync2li1*-KO cells restored the SAG-induced decrease in GLI3 protein level (see also Fig. 6Y). By contrast, expression of any of the DYNC2LI1 variants singly or in combination did not cause a

decrease in GLI3 protein level in response to SAG treatment (Fig. 6X, lanes 7–14, 17, 18, 21 and 22; see also Fig. 6Y). Thus, the SAG-induced decrease in GLI3 protein level appears to correlate well with the SAG-induced increase in GLI3 signals at the ciliary tip (Fig. 6A–W), and to be inversely associated with the SAG-induced increase in osteogenic differentiation (Fig. 4).

It is also interesting to note that there is common variation in the ratio of GLI3R to total GLI3 in *Dync2li1*-KO cells expressing different DYNC2LI1 constructs (Fig. 6Z). Namely, the ratios of GLI3R to total GLI3 in *Dync2li1*-KO cells expressing mCherry–DYNC2LI1(WT) alone (Fig. 6X, lanes 5 and 6) or in combination with its SRPS variant (lanes 15, 16, 19 and 20) were comparable to the ratio in control C3H10T1/2 cells (lanes 1 and 2) under both basal and SAG-stimulated conditions (see also Fig. 6Z). In clear contrast, in *Dync2li1*-KO cells expressing one of the SRPS variants (Fig. 6X, lanes 7–14) or their combination (lanes 17, 18, 21, and 22), the ratio

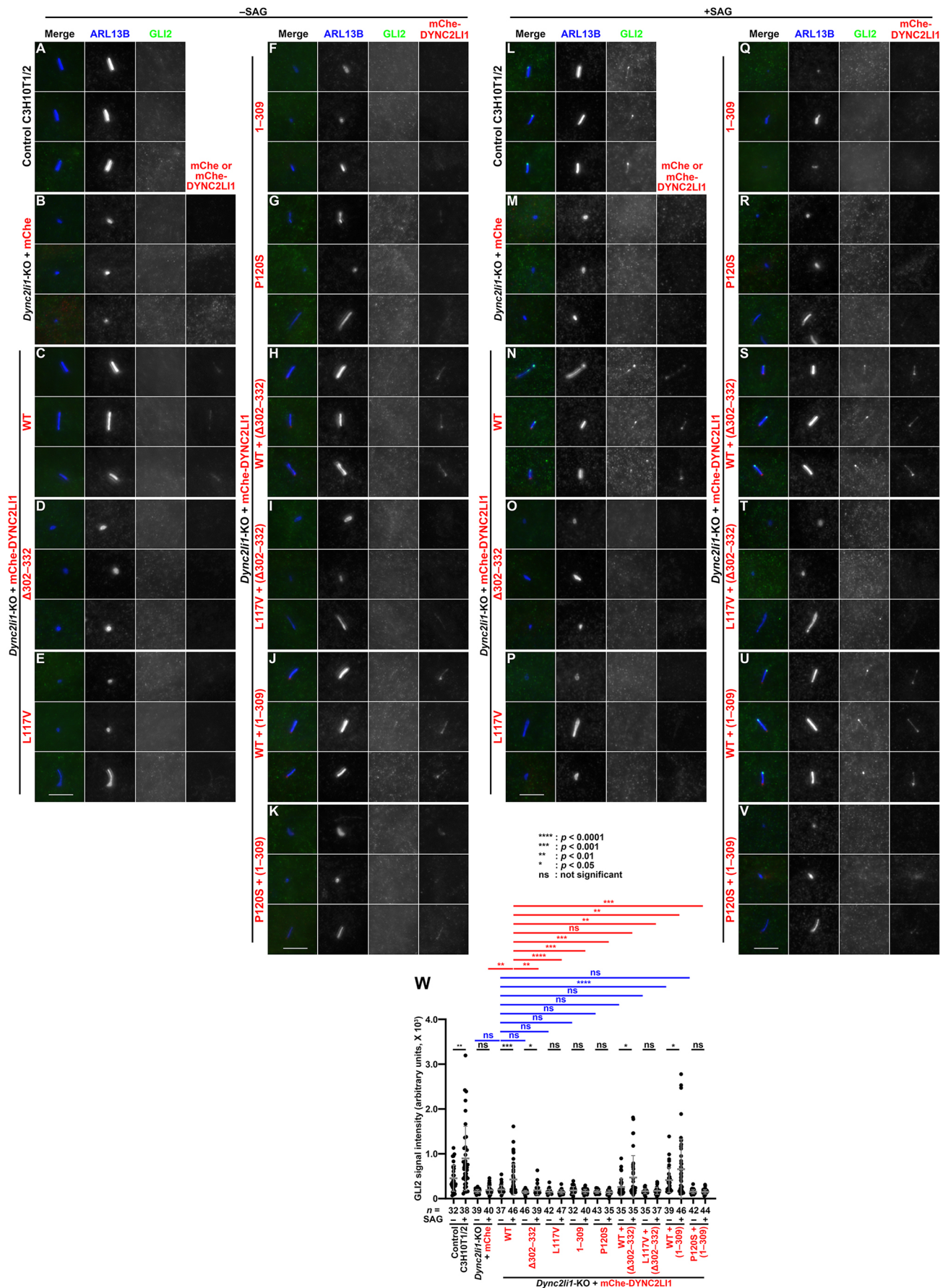


Fig. 5. See next page for legend.

Fig. 5. Impaired ciliary localization of GLI2 in *Dync2li1*-KO cells expressing SRPS variants of DYNC2LI1. (A–V) Cells as described in the legend for Fig. 2A–V were immunostained with antibodies against RFP, GLI2 and ARL13B. Scale bars: 5 μ m. Images shown are representative of a single experiment. (W) Relative staining intensities of ciliary GLI2 in the experiments shown in A–V were estimated, represented as scatter plots, and analyzed as described in the legend for Fig. 1Y. Data are shown as mean $\pm$ s.d. of a single experiment. The numbers of analyzed cells (*n*) from the single experiment are indicated. Statistical significances among multiple groups and those between –SAG and +SAG were calculated using one-way ANOVA followed by a Dunnett's multiple comparison test and an unpaired two-tailed Student *t*-test, respectively. Horizontal lines are means, and error bars are s.d. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns, not significant.

was significantly lower than that in DYNC2LI1(WT)-expressing *Dync2li1*-KO cells (Fig. 6X, lanes 5 and 6; see also Fig. 6Z). The data on the reduced ratio of GLI3R to total GLI3 in *Dync2li1*-KO cells expressing the pathogenic DYNC2LI1 variants are in good agreement with the ratio in fibroblasts derived from SRPS individuals (Taylor et al., 2015) and with the ratio in embryo extracts of *Dync2h1* mutant mice (Huangfu and Anderson, 2005).

Comparing the data shown in Fig. 6Z with the data shown in Fig. 4 demonstrates the following correlation: *Gli1* expression and osteogenic differentiation of cells are suppressed under basal conditions, and are promoted by SAG treatment of cells with a relative high ratio of GLI3R to total GLI3, such as control C3H10T1/2 cells and DYNC2LI1(WT)-expressing *Dync2li1*-KO cells; by contrast, *Alpl* and *Gli1* levels are slightly high in the basal state but are not further increased by SAG treatment in *Dync2li1*-KO cells expressing SRPS variant(s) of DYNC2LI1, in which the ratio of GLI3R to total GLI3 is relatively low. In addition, comparing the data shown in Fig. 6Y with that in Fig. 4 suggests that in response to SAG treatment, a reduction in the total level of GLI3 (GLI3FL+GLI3R) correlates with the increase in the expression of *Gli1* and osteogenic differentiation of the cells.

BMP signaling regulates osteogenic differentiation independently and compensatory of Hh signaling

We have so far investigated the effect of DYNC2LI1 deficiency on osteogenic differentiation by activating SMO with SAG. Next, we activated Hh signaling using SHH, a ligand for PTCH1. As shown in Fig. 7A (compare middle row with top row) and Fig. 7B, C (compare blue bars with black bars), the tendency of the examined cells to differentiate into ALP-positive cells upon SHH treatment was similar to what was seen with SAG treatment (Fig. 4A–C). Namely, the increase in ALP staining intensity and *Alpl* transcript levels observed in control C3H10T1/2 cells upon SHH treatment was significantly suppressed in *Dync2li1*-KO cells expressing mCherry alone and was restored by expression of mCherry–DYNC2LI1(WT) alone or in combination with a pathogenic variant, albeit to various degrees. By contrast, expression of any of the pathogenic DYNC2LI1 variants alone or in combination in *Dync2li1*-KO cells did not restore the ALP staining or *Alpl* levels. A similar trend was observed for *Gli1* expression, which was restored by DYNC2LI1(WT) expression and not by expression of any of the SRPS variants in *Dync2li1*-KO cells (Fig. 7D,E).

We also treated *Dync2li1*-KO cells expressing DYNC2LI1 variants with BMP2, as previous studies have suggested cooperation or interplay of BMP signaling with Hh signaling in osteogenic differentiation of MSCs (Guo et al., 2018; Hojo et al., 2015; Nakamura et al., 1997; Reichert et al., 2013; Yuasa et al., 2002; Zhou et al., 2022a). As shown in Fig. 7A (compare bottom

row with top and middle rows) and Fig. 7B,C (compare red bars with black and blue bars), we obtained somewhat unexpected, but interesting results. In control cells, BMP2 treatment increased the *Alpl* level by ~10-fold, although not as much as SHH treatment (>400-fold) (Fig. 7C). In striking contrast, in *Dync2li1*-KO cells expressing mCherry alone, the *Alpl* level was increased by ~60-fold by BMP2 treatment, whereas the SHH-induced increase was significantly suppressed (Fig. 7B,C). Expression of mCherry–DYNC2LI1(WT) in *Dync2li1*-KO cells reversed to some extent the BMP2-induced increase and the suppression of the SHH-induced increase in the *Alpl* level. By contrast, in *Dync2li1*-KO cells expressing any pathogenic DYNC2LI1 variant, the *Alpl* level elevated by BMP2 treatment remained high (Fig. 7B,C, red bars), and the SHH-induced increase in the *Alpl* level remained suppressed (Fig. 7B,C, blue bars). The BMP2-induced increase in the *Gli1* level in cells defective in DYNC2LI1 was less pronounced than the BMP2-induced increase in the *Alpl* level (Fig. 7D,E). These data make it likely that Hh signaling and BMP signaling are under coordinated, albeit at least partially separate, regulation from each other; such regulation might involve changes in the ratio of GLI3R to total GLI3.

We then examined the levels of osteogenic transcriptional regulators, *Runx2* and *Osterix* (*Osx*; also known as *Sp7*), as a previous study has revealed that RUNX2 regulates chromatin accessibility to the *Osx* enhancer to direct osteogenic differentiation and that RUNX2 binds to a region upstream of the *Alpl* gene in *Osx*-positive osteoblasts (Hojo et al., 2022). In control C3H10T1/2 cells, the *Runx2* level was not significantly changed by SHH treatment and slightly reduced by BMP2 treatment (Fig. 7F,G); this is compatible with previous studies showing that BMP2 increases the protein level and activity of RUNX2 via post-transcriptional regulation (Jeon et al., 2006; Ohba et al., 2007). The trend in the *Runx2* expression level upon treatment with SHH and BMP2 was not significantly altered in *Dync2li1*-KO cells or in those expressing any DYNC2LI1 variant, although in *Dync2li1*-KO cells expressing mCherry-fused DYNC2LI1(WT)+DYNC2LI1(Δ 302–332) or DYNC2LI1(L117V)+DYNC2LI1(Δ 302–332), the basal *Runx2* level was slightly increased compared to control cells (Fig. 7F,G).

In contrast to *Runx2*, the *Osx* level showed significant changes upon treatment with SHH and BMP2 (Fig. 7H,I). In control C3H10T1/2 cells, the *Osx* level was increased by >10-fold by SHH treatment and approximately doubled by BMP2 treatment. In *Dync2li1*-KO cells expressing mCherry or any of the DYNC2LI1 variants, BMP2 treatment increased the *Osx* level by >20-fold, whereas the SHH-induced increase in the *Osx* level was significantly reversed. mCherry–DYNC2LI1(WT) expression in *Dync2li1*-KO cells slightly increased the SHH-induced *Osx* level and significantly attenuated the BMP2-induced increase in the *Osx* level. Thus, the trend of SHH-induced and BMP2-induced changes in the *Osx* level in control cells and *Dync2li1*-KO cells expressing any of the DYNC2LI1 variants was similar to the trend of changes in the *Alpl* level (Fig. 7, compare H and I with B and C).

BMP2 induces chondrogenic differentiation independently of DYNC2LI1 loss

Next, we examined whether loss of DYNC2LI1 affects chondrogenic differentiation, as BMP2 but not SAG has been reported to induce chondrogenic differentiation of C3H10T1/2 cells (Date et al., 2004; Hojo et al., 2013). When control C3H10T1/2 and *Dync2li1*-KO cells were subjected to micromass culture, Alcian Blue was able to stain both control and *Dync2li1*-KO cells upon treatment with BMP2 but not with SAG (Fig. S5A). RT-qPCR

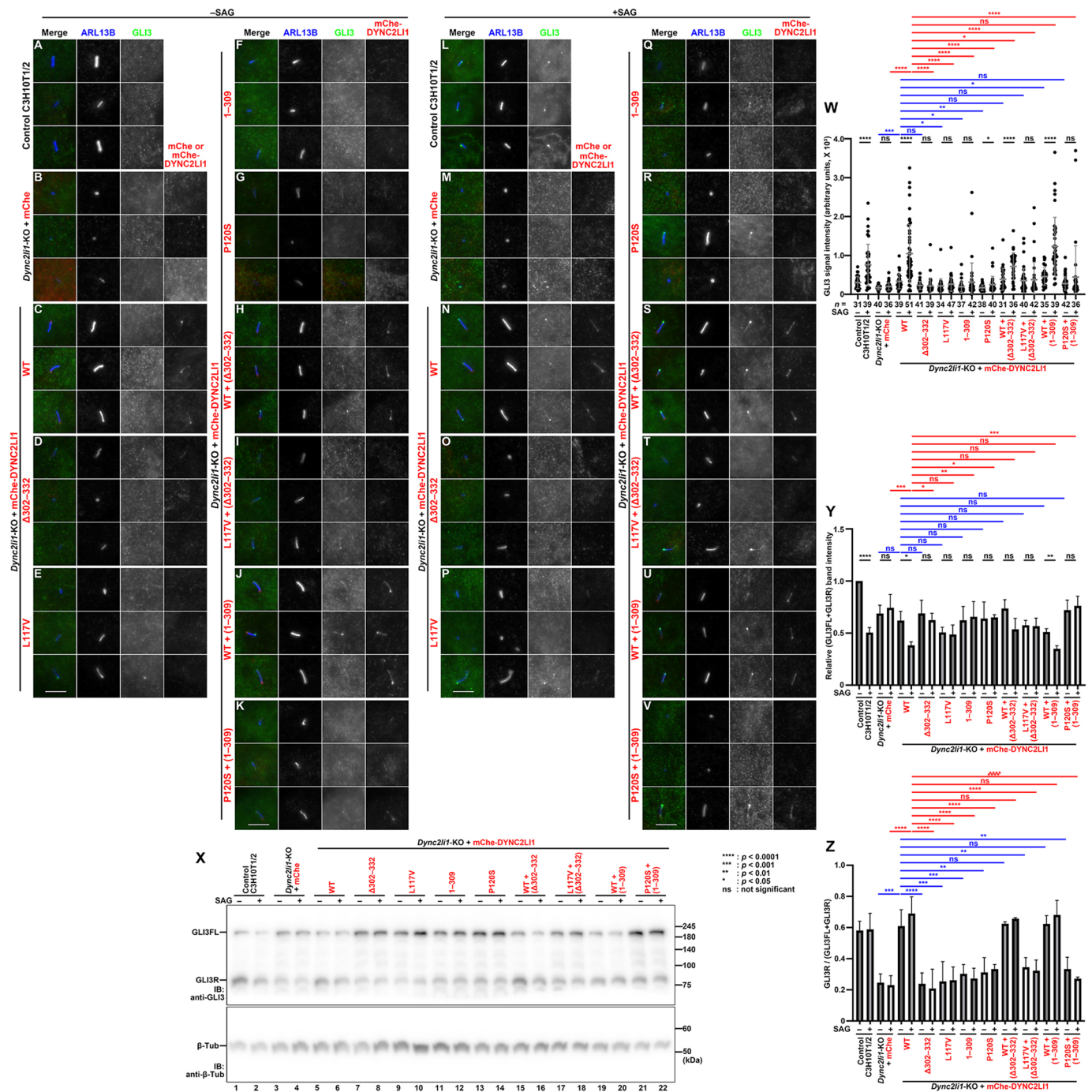


Fig. 6. Reduced ratio of GLI3R to total GLI3 in *Dync2l1*-KO cells and those expressing *DYNC2L1* variants. (A–V) Cells as described in the legend for Fig. 2A–V were immunostained with antibodies against RFP, GLI3 and ARL13B. Scale bars: 5 μm. Images shown are representative of a single experiment. (W) Relative staining intensities of ciliary GLI3 in the experiments shown in A–V were estimated, represented as scatter plots, and analyzed as described in the legend for Fig. 1Y. The numbers of analyzed cells (*n*) from the single experiment are indicated. Horizontal lines are means, and error bars are s.d. (X) Lysates prepared from cells in A–V were processed for SDS-PAGE and immunoblotting analysis using antibodies against GLI3 or β-tub. Images shown are representative of three experiments. (Y,Z) Band intensities of GLI3FL and GLI3R in X were measured, and band intensities of total GLI3 (GLI3FL+GLI3R) (Y) and the ratio of GLI3R to total GLI3 (Z) were compared. Quantification was performed three times using separate protein lysates. Data are shown as the mean±s.d. of three independent experiments. Statistical significances among multiple groups and those between –SAG and +SAG were calculated using one-way ANOVA followed by a Dunnett's multiple comparison test and an unpaired two-tailed Student *t*-test, respectively. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns, not significant.

analysis indicated that the *Col2a1* level was significantly elevated when both control and *Dync2l1*-KO cells were treated with BMP2 (Fig. S5B). Thus, BMP2-induced chondrogenic differentiation of C3H10T1/2 cells is not affected by loss of *DYNC2L1*.

DISCUSSION

In this study, we investigated the association between defects in ciliary protein trafficking and localization and defects in osteogenic differentiation of MSCs caused by skeletal ciliopathy variants of the



dynein-2 subunit DYNC2LI1. In agreement with a previous study using RPE1 cells (Qiu et al., 2022), retrograde trafficking of the IFT machinery was considerably impaired (Fig. 1) in *Dync2li1*-KO C3H10T1/2 cells and those expressing SRPs variants of DYNC2LI1. In these DYNC2LI1-defective cells, GPR161 was

aberrantly retained within cilia under SAG-stimulated conditions (Fig. 3), indicating continued negative regulation of Hh signaling even upon Hh pathway stimulation. By contrast, probably owing to impairment in its constant cycling in and out of cilia, SMO substantially accumulated within cilia under basal conditions in

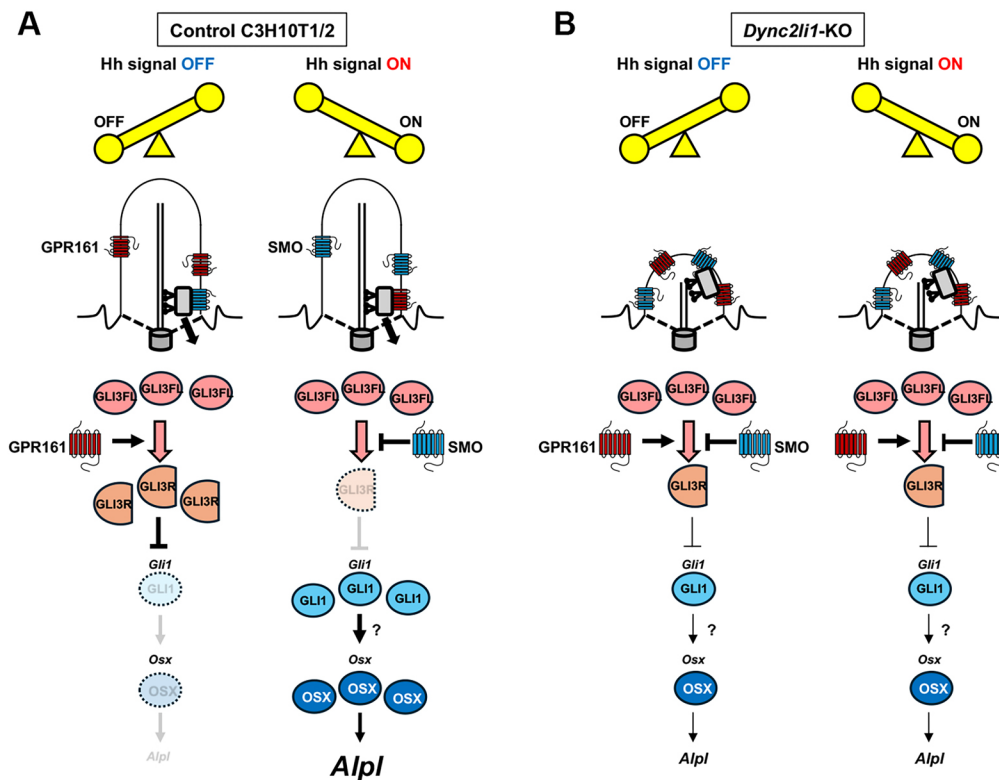


Fig. 8. Model diagrams of osteogenic differentiation via Hh signaling. (A) Control C3H10T1/2 cells. In the off-state of Hh signaling (left), GPR161 on the ciliary membrane promotes limited proteolytic processing of GLI3FL to GLI3R via activating protein kinase A-mediated GLI3 phosphorylation. GLI3R then represses expression of *Gli1*. In the on-state of Hh signaling (right), GPR161 exits cilia as SMO enters cilia, resulting in cancellation of the GLI3FL-to-GLI3R processing and thereby derepression of the *Gli1* expression. Whether GLI1 can induce *Alpl* expression directly or through induction of expression of transcription factors such as *Osx* is an issue for future study. (B) *Dync2li1*-KO cells and those expressing pathogenic DYNC2LI1 variants. Both GPR161 and SMO are present on the ciliary membrane, whether in the OFF state (left) or ON state (right), resulting in a low level of *Gli1* expression. Note that the upper half parts of these models are based on data from our previous study (Takahashi et al., 2025) as well as new results presented here.

Dync2li1-KO C3H10T1/2 cells and those expressing SRPS variants of DYNC2LI1 (Fig. 2), suggesting the possibility that Hh signaling is, at least in part, activated even in the basal state. Concomitant with GPR161 and SMO accumulation within cilia, the BBSome, which link the IFT machinery to ciliary GPCRs, are also accumulated within cilia (Fig. S4). Thus, exit of the IFT machinery, the BBSome and GPCRs from cilia are all impaired in cells with defects in DYNC2LI1. Presumably, BBSome-mediated export of other GPCRs, such as SSTR3, is also impaired in *Dync2li1*-KO cells and those expressing pathogenic DYNC2LI1 variants.

The above-mentioned data indicate that, in cells defective in DYNC2LI1, a situation is created in which GPR161 and SMO, which are negative and positive regulators of Hh signaling, respectively, always coexist on the ciliary membrane owing to impaired retrograde ciliary protein trafficking driven by the dynein-2 complex (Fig. 8B). Consistent with the constant coexistence of negative and positive regulators, the *Gli1* transcript level was not increased upon SAG treatment, whereas it was slightly increased under basal conditions in cells with defective DYNC2LI1, compared with control C3H10T1/2 cells (Fig. 4D–G). Moreover, in agreement with the *Gli1* level, osteogenic differentiation confirmed by increases in ALP staining and in the *Alpl* level in *Dync2li1*-KO cells and those expressing SRPS variants of DYNC2LI1 was slightly increased in the basal state compared with control cells, but no further increase was observed upon SAG treatment in these cells (Fig. 4A–C). In addition, the suppression of *Gli1* and *Alpl* levels under basal conditions correlated well with the ratio of GLI3R to total GLI3 (Fig. 6Z), and their SAG-induced increase tended to be inversely correlated with the level of total GLI3 (GLI3FL+GLI3R) (Fig. 6Y). Thus, the relatively high GLI3R-to-total GLI3 ratio in control cells likely contributes to the suppression of *Gli1* and *Alpl* levels in the basal state. By contrast, cells defective in DYNC2LI1 are unable to undergo basal suppression and SAG-induced increase in *Gli1* and *Alpl* levels,

owing to the relatively low GLI3R-to-total GLI3 ratio and lack of the SAG-induced decrease in total GLI3 level (probably the GLI3R level), respectively.

Altogether, the data presented in this study suggest the following scenario of skeletal ciliopathies caused by DYNC2LI1 variants, although there are other possibilities: (1) DYNC2LI1 variants cause defects in retrograde ciliary protein trafficking, probably owing to inefficient incorporation of the DYNC2LI1 variants into the dynein-2 complex and/or instability of the dynein-2 complex containing a DYNC2LI1 variant; (2) export of GPR161 and SMO from cilia is inhibited owing to impaired retrograde trafficking and impaired passage through the TZ; (3) constant coexistence of the negative regulator GPR161 and the positive regulator SMO on the ciliary membrane causes impaired or uncontrolled expression of *Gli1*, which is caused at least in part to the insufficient regulation of the ratio of GLI3R to total GLI3, namely, processing of GLI3FL to yield GLI3R, and of total GLI3 level (in particular, GLI3R level) (Fig. 8B); (4) impaired or uncontrolled expression of *Gli1* leads to impaired or uncontrolled expression of *Alpl* and in turn might lead to impaired or uncontrolled osteogenic differentiation; and (5) impaired or uncontrolled osteogenic differentiation at the cellular level could be responsible at least partly for the abnormal phenotypes of skeletal ciliopathy individuals with DYNC2LI1 variants.

Another important finding in this study is that BMP2 might induce osteogenic differentiation of C3H10T1/2 cells in a coordinated but at least partially separate manner from Hh signaling; in particular, in cells with defective DYNC2LI1, where Hh signaling is suppressed, BMP2-induced *Alpl* and *Osx* levels were significantly higher than those in control cells (Fig. 7B,C and H,I, respectively), suggesting a compensatory role of BMP signaling in osteogenic differentiation under conditions where Hh signaling is impaired. By contrast, chondrogenic differentiation of C3H10T1/2 cells was induced by BMP2 but not by SAG, and the BMP2-induced chondrogenic differentiation was unaffected in *Dync2li1*-KO cells (Fig. S5).

These results suggest that Hh signaling contributes to osteogenic differentiation, whereas BMP signaling contributes to both osteogenic and chondrogenic differentiation independently of *DYNC2LI1* deficiency. The compensatory role of BMP signaling might be involved in the pathological phenotypes caused by defects of Hh signaling in patients and mouse models of skeletal ciliopathies (Ashe et al., 2012; Rix et al., 2011; Taylor et al., 2015). Further studies are needed to elucidate the molecular mechanism underlying the crosstalk between Hh and BMP signaling in skeletal development, although participation of other signaling pathways is also possible (Hojo et al., 2015; Zhou et al., 2022a).

A limitation of this study is that although the suppression of *Gli1* and *Alpl* levels under basal conditions was shown to correlate well with the ratio of GLI3R to total GLI3, the reason why the SAG-induced increase in these levels does not occur in cells defective in *DYNC2LI1* was not fully clarified. The balance between GLI3R and a putative activator form of GLI2, GLI2A, is believed to control Hh signaling (Hwang et al., 2023; Kopinke et al., 2021; Niewiadomski et al., 2019; Takahashi et al., 2025). GLI2 and GLI3 were shown to be transported to the ciliary tip by SAG treatment in *Dync2li1*-KO cells expressing *DYNC2LI1*(WT), but not in cells expressing its SRPS variants (Figs 5A–V and 6A–V). Although the characteristics of GLI2A are not well understood, probably owing to its labile nature (Kopinke et al., 2021), GLI2 transport to the tip might contribute to formation of GLI2A via post-translational modifications, such as phosphorylation at sites not involving PKA or dephosphorylation (Baran et al., 2024, 2023; Yoshida et al., 2024; Zhou and Jiang, 2022). In addition, although the characteristics of putative GLI3A remain entirely unknown, the decrease in the total GLI3 protein level upon SAG treatment in control cells and *Dync2li1*-KO cells expressing *DYNC2LI1*(WT) (Fig. 6X,Y) might be attributable to the labile nature of GLI3A, whereas the absence of SAG-induced reduction of the total GLI3 level in *Dync2li1*-KO cells expressing an SRPS variant (Fig. 6X,Y) might reflect suppression of GLI3A production owing to impaired GLI3 transport to the ciliary tip.

Consistent with cooperativity of the IFT-A complex with the dynein-2 complex in retrograde ciliary protein trafficking, variants of not only all of the dynein-2-specific subunits but also all subunits of the IFT-A complex are known to cause skeletal ciliopathies (Corés et al., 2015; Lin et al., 2013; McInerney-Leo et al., 2015; Reiter and Leroux, 2017; Schmidts, 2014; Zhang et al., 2018). However, there are subtle differences in the phenotypes of ciliopathies caused by different protein variants. The degree of abnormality at the cellular level might lead to phenotypic differences at the individual level. Using C3H10T1/2 cells as an MSC-like model system, it might be possible to link abnormalities in ciliary protein trafficking and in osteogenic differentiation at the cellular level caused by different protein variants to differences in phenotypic severity of individuals with skeletal ciliopathies.

MATERIALS AND METHODS

Plasmids, antibodies, reagents and cell lines

Expression vectors for *DYNC2LI1* and the other dynein-2 subunits used in this study are listed in Table S2; some of them were constructed in our previous study (Qiu et al., 2022). The antibodies used in this study are listed in Table S2. SAG was purchased from Enzo Life Sciences. C3H10T1/2 clone 8 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB9080). For normal growth, C3H10T1/2 cells were cultured in basal medium Eagle (BME; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 2 mM L-glutamine at 37°C in 5% CO₂. For osteogenic differentiation, the cells were cultured in α -modified MEM (Nacalai Tesque) containing

10% FBS supplemented with 200 nM SAG. HEK293T cells were obtained from RIKEN BioResource Research Center (RBC2202) and cultured in DMEM with high glucose (Nacalai Tesque) supplemented with 5% FBS.

Establishment of *Dync2li1*-KO cells from C3H10T1/2 cells

Disruption of the *Dync2li1* alleles in C3H10T1/2 cells by the CRISPR/Cas9 system using homology-independent DNA repair was performed as described previously (Kato et al., 2017), with minor modifications (Okazaki et al., 2020; Qiu et al., 2021). Single-guide RNA (sgRNA) sequences targeting the mouse *Dync2li1* gene (see Table S3) were designed using CRISPOR (Haeussler et al., 2016). Double-stranded oligonucleotides for the target sequence were inserted into the all-in-one sgRNA expression vector pHFiCas9-2×sgRNA (Fujisawa et al., 2021). Approximately 2.0×10^4 C3H10T1/2 cells were plated onto a 12-well plate. On the next day, the cells were transfected with the all-in-one vector and the donor knock-in vector pDonor-tBFP-NLS-Neo(universal) (Addgene #80767) using X-tremeGENE9 Reagent (Roche Applied Science). After selection of the transfected cells in medium containing G418 (600 μ g/ml), cells were sorted using an SH800S cell sorter (SONY) at the Medical Research Support Center of Kyoto University. Genomic DNA extracted from the isolated cells were analyzed by PCR using three sets of primers (Table S3) and GoTaq Master Mixes (Promega) to distinguish the following three states of integration of the donor knock-in vector: forward integration, reverse integration, and no integration with a small indel. Direct sequencing of the genomic PCR products was performed to confirm the disruption of alleles of the *Dync2li1* gene.

Preparation of *Dync2li1*-KO cells expressing mCherry-fused *DYNC2LI1* constructs and GPR161-EGFP

pRRsinPPT-based vectors for the expression of *DYNC2LI1* variants fused to mCherry and GPR161-EGFP were constructed by a previously described method (Takahashi et al., 2012). The preparation of lentiviral vectors was performed as described previously (Kato et al., 2017; Takahashi et al., 2012). Briefly, the pRRsinPPT-based vectors for various *DYNC2LI1* constructs were transfected into HEK293T cells together with the packaging plasmids [pRSV-REV, pMD2.g, and pMDLg/pRRE; kind gifts from Peter McPherson, McGill University, Canada (Thomas et al., 2009)]. The culture medium was replaced 8 h after transfection, and collected at 24, 36 and 48 h after transfection. The medium containing viral particles was passed through a 0.45- μ m filter and centrifuged at 32,000 g at 4°C for 4 h. Precipitated lentiviral particles were resuspended in BME supplemented with 2 mM L-glutamine, and stored at –80°C until use. *Dync2li1*-KO cells expressing the mCherry-fused *DYNC2LI1* constructs were prepared by adding the lentiviral suspension to the culture medium, followed by selection in the presence of blasticidin (50 μ g/ml) or Zeocin (25 μ g/ml). *Dync2li1*-KO cells stably expressing the *DYNC2LI1* constructs were then infected with the lentiviral vector for GPR161-EGFP, and used for subsequent analyses.

ALP staining

Staining of cells for ALP activity was performed as described previously (Takahashi et al., 2025). Cells were cultured in the absence (–SAG) or presence (+SAG) of 200 nM SAG for 7 days, or in the presence of 100 ng/ml BMP2 or 100 ng/ml SHH for 5 days. The cells were fixed in 3% paraformaldehyde for 15 min at room temperature, washed with phosphate-buffered saline, and stained with Black Substrate Kit Alkaline Phosphatase (Vector Laboratories) for 30 min at room temperature. The stained cells were washed with 100 mM Tris-HCl (pH 9.5) for 5 min, and observed using an all-in-one-type fluorescence microscope EVOS M5000 (Thermo Fisher Scientific) using a 4×/0.13 NA objective lens.

Micromass culture and Alcian Blue staining

Cells were suspended in BME supplemented with 10% FBS and 2 mM L-glutamine to a final concentration of 10^7 cells/ml. A 10- μ l drop of the cell suspension was placed at the center of a well in a 24-well tissue culture plate. The cells were allowed to adhere for 1 h at 37°C in 5% CO₂. Following adhesion, 500 μ l of Ham's F-12 medium (Nacalai Tesque) supplemented with 10% FBS containing either 200 nM SAG or 100 ng/ml BMP2 was

added to the culture. The medium was replaced every 3 days, and cells were cultured for 5 days. For Alcian Blue staining, cells were fixed with methanol at -20°C for 20 min. After equilibration in 0.1 M HCl, cells were stained at room temperature for 2 h with a working solution prepared by mixing Alcian Blue solution (pH 2.5, Wako) and 0.2 M HCl at a 1:1 ratio. The cells were then washed three times with distilled water and observed under a microscope (BZ-8000, Keyence) with a 4 $\times$ /0.20 NA objective lens under constant imaging conditions.

RT-qPCR

Total RNAs were isolated from cultured cells using an RNeasy mini kit (QIAGEN). Reverse transcription was performed using ReverTra Ace qPCR RT Master Mix (TOYOBO) followed by RT-qPCR using the StepOnePlus™ system (Thermo Fisher Scientific) at Graduate School of Pharmaceutical Sciences, Kyoto University. THUNDERBIRD SYBR qPCR Mix (TOYOBO) containing 0.3 μM of a specific primer pair for each gene (Table S3) was used in the reactions. Data were quantified using the comparative CT method (ΔCT method) to estimate the mRNA copy number relative to that of mouse 18S rRNA, which was used as internal standard.

Immunofluorescence analysis

Parental C3H10T1/2 cells and *DYNC2LI1*-KO cells were cultured in BME supplemented with 10% FBS and 2 mM L-glutamine. To induce ciliogenesis, cells were grown to 100% confluence on coverslips, and starved for 24 h in BME supplemented with 0.5% FBS and 2 mM L-glutamine. Subsequent immunofluorescence analysis was performed as described previously (Hirano et al., 2017; Qiu et al., 2022; Takahashi et al., 2012). The cells were fixed and permeabilized with 3% paraformaldehyde at 37°C for 5 min, and subsequently in methanol at -20°C for 5 min, and washed three times with phosphate-buffered saline. The fixed and permeabilized cells were blocked with 10% FBS, stained with antibodies diluted in Can Get Signal immunostain solution A (TOYOBO) (for the detection of SMO) or 5% FBS (for the detection of the other proteins), and observed using an Axio Observer microscope (Carl Zeiss). All images acquired under the same setting and saved in CZI file format were processed and analyzed using ZEISS ZEN microscope software (version 3.1; Carl Zeiss). A region of interest (ROI) was constructed along the signal of ARL13B within cilia using a drawing tool, and the fluorescence intensity in the ROI was quantified. To correct for local background intensity, a ROI was set to a nearby region. Statistical analyses were performed using GraphPad Prism 10 software (version 10.1.2; GraphPad Software, Inc.). Statistical significances among multiple groups and those between $-SAG$ and $+SAG$ were calculated using one-way ANOVA followed by a Dunnett's multiple comparison test and an unpaired two-tailed Student's *t*-test, respectively.

TIRF microscopy

TIRF microscopy was performed as described previously (Hirano et al., 2017; Kobayashi et al., 2021). *Dync2li1*-KO cells stably expressing mCherry-fused *DYNC2LI1* constructs were infected with the lentiviral vector for EGFP-IFT88. The cells were then serum-starved for 24 h on a glass-bottom culture dish (Greiner Bio-One) and observed using a TIRFM ECLIPSE Ti microscope (Nikon) at a video rate using NIS-Elements imaging software.

SDS-PAGE and immunoblotting analysis

To induce ciliogenesis, cells were grown on six-well plates to 100% confluence, and starved for 24 h in BME supplemented with 0.5% FBS and 2 mM L-glutamine. Then, the culture medium was changed to the above medium containing 200 nM SAG, and the cells were incubated for a further 6 h. The cells were lysed in 100 μl of cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA) containing EDTA-free protease inhibitor cocktail (Nacalai Tesque). After 20 min on ice, the cell lysates were centrifuged at 16,100 *g* for 15 min at 4°C in a microcentrifuge. The supernatants were boiled in SDS-PAGE sample buffer, and separated by SDS-PAGE using gels prepared with TGX FastCast Acrylamide Solution Kit 10% (Bio-Rad). The

separated proteins were electroblotted onto an Immobilon-P membrane (Merck-Millipore), and the membrane was then subjected to immunoblotting analysis using an anti-GLI2, anti-GLI3, anti-mCherry or anti- β -tubulin antibody, followed by a peroxidase-conjugated secondary antibody. Protein bands were detected using a Chemi-Lumi One L kit (Nacalai Tesque), and images were captured using ImageQuant 800 (Cytiva). The intensity of the bands was measured using ImageJ software. Full uncropped images of blots from this paper are available in Fig. S6.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.I., H.-W.S., Y.K., K.N.; Formal analysis: Y.I., H.H., K.K.; Investigation: Y.I., H.H., K.K.; Project administration: Y.K., K.N.; Supervision: H.-W.S., Y.K., K.N.; Validation: Y.I.; Writing – original draft: K.N.; Writing – review & editing: Y.I., H.-W.S., Y.K., K.N.

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Data and resource availability

All relevant data can be found within the article and its supplementary information.

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Special Issue

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References

- Abramson, J., Adler, J., Dunger, J., Evans, R., Green, T., Pritzel, A., Ronneberger, O., Willmore, L., Ballard, A. J., Bambrick, J. et al. (2024). Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **630**, 493–500. doi:10.1038/s41586-024-07487-w
- Anvarian, Z., Mykityn, K., Mukhopadhyay, S., Pedersen, L. B. and Christensen, S. T. (2019). Cellular signaling by primary cilia in development, organ function and disease. *Nat. Rev. Nephrol.* **15**, 199–219. doi:10.1038/s41581-019-0116-9
- Asante, D., Stevenson, N. L. and Stephens, D. J. (2014). Subunit composition of the human cytoplasmic dynein-2 complex. *J. Cell Sci.* **127**, 4774–4787. doi:10.1242/jcs.159038
- Ashe, A., Butterfield, N. C., Town, L., Courtney, A. D., Cooper, A. N., Ferguson, C., Barry, R., Olsson, F., Liem, K. F., Jr, Parton, R. G. et al. (2012). Mutations in mouse *Ift144* model the craniofacial, limb and rib defects in skeletal ciliopathies. *Hum. Mol. Genet.* **21**, 1808–1823. doi:10.1093/hmg/ddr613
- Baran, B., Derua, R., Janssens, V. and Niewiadomski, P. (2024). PP2A phosphatase regulatory subunit PPP2R3C is a new positive regulator of the hedgehog signaling pathway. *Cell. Signal.* **123**, 111352. doi:10.1016/j.cellsig.2024.111352
- Baran, B., Kosieradzka, K., Skarzynska, W. and Niewiadomski, P. (2023). MRKCa β positively regulates Gli protein activity. *Cell. Signal.* **107**, 110666. doi:10.1016/j.cellsig.2023.110666
- Chinipardaz, Z., Liu, M., Graves, D. T. and Yang, S. (2022). Role of primary cilia in bone and cartilage. *J. Dental Res.* **101**, 253–260. doi:10.1177/00220345211046606
- Corés, C. R., Metzis, V. and Wicking, C. (2015). Unmasking the ciliopathies: craniofacial defects and the primary cilium. *Wiley Interdiscip. Rev. Dev. Biol.* **4**, 637–653. doi:10.1002/wdev.199
- Date, T., Doiguchi, Y., Nobuta, M. and Shindo, H. (2004). Bone morphogenetic protein-2 induces differentiation of multipotent C3H10T1/2 cells into osteoblasts,

- chondrocytes, and adipocytes in vivo and in vitro. *J. Orthop. Sci.* **9**, 503-508. doi:10.1007/s00776-004-0815-2
- De-Castro, A. R. G., Rodrigues, D. R. M., De-Castro, M. J. G., Vieira, N., Vieira, C., Carvalho, A. X., Gassmann, R., Abreu, C. M. C. and Dantas, T. J. (2022). WDR60-mediated dynein-2 loading into cilia powers retrograde IFT and transition zone crossing. *J. Cell Biol.* **221**, e202010178. doi:10.1083/jcb.202010178
- Eguether, T., San Agustín, J. T., Keady, B. T., Jonassen, J. A., Liang, Y., Francis, R., Tobita, K., Johnson, C. A., Abdelhamed, Z. A., Lo, C. W. et al. (2014). IFT27 links the BBSome to IFT for maintenance of the ciliary signaling compartment. *Dev. Cell* **21**, 279-290. doi:10.1016/j.devcel.2014.09.011
- Endoh-Yamagami, S., Evangelista, M., Wilson, D., Wen, X., Theunissen, J.-W., Phamluong, K., Davis, M., Scales, S. J., Solloway, M. J., De Sauvage, F. J. et al. (2009). The mammalian Cos2 homolog Kif7 plays an essential role in modulating Hh signal transduction during development. *Curr. Biol.* **19**, 1320-1326. doi:10.1016/j.cub.2009.06.046
- Fujisawa, S., Qiu, H., Nozaki, S., Chiba, S., Katoh, Y. and Nakayama, K. (2021). ARL3 and ARL13B GTPases participate in distinct steps of INPP5E targeting to the ciliary membrane. *Biol. Open* **10**, bio058843. doi:10.1242/bio.058843
- García-González, F. R. and Reiter, J. F. P. (2017). Open sesame: how transition fibers and the transition zone control ciliary composition. *Cold Spring Harb. Perspect. Biol.* **9**, a028134. doi:10.1101/cshperspect.a028134
- Gómez, A. E., Christman, A. K., Van De Weghe, J. C., Finn, M. and Doherty, D. (2022). Systematic analysis of cilia characteristics and Hedgehog signaling in five immortal cell lines. *PLoS ONE* **17**, e0266433. doi:10.1371/journal.pone.0266433
- Guo, Y., Yuan, Y., Wu, L., Ho, T. V., Jing, J., Sugii, H., Li, J., Han, X., Feng, J., Guo, C. et al. (2018). BMP-IHH-mediated interplay between mesenchymal stem cells and osteoclasts supports calvarial bone homeostasis and repair. *Bone Res.* **6**, 30. doi:10.1038/s41413-018-0031-x
- Haeussler, M., Schöning, K., Eckert, H., Eschstruth, A., Mianné, J., Renaud, J. B., Schneider-Maunoury, S., Shkumatava, A., Teboul, L., Kent, J. et al. (2016). Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biol.* **17**, 148. doi:10.1186/s13059-016-1012-2
- Hamada, Y., Tsurumi, Y., Nozaki, S., Katoh, Y. and Nakayama, K. (2018). Interaction of WDR60 intermediate chain with TCTEX1D2 light chain of the dynein-2 complex is crucial for ciliary protein trafficking. *Mol. Biol. Cell* **29**, 1628-1639. doi:10.1091/mbc.E18-03-0173
- Hirano, T., Katoh, Y. and Nakayama, K. (2017). Intraflagellar transport-A complex mediates ciliary entry and retrograde trafficking of ciliary G protein-coupled receptors. *Mol. Biol. Cell* **28**, 429-439. doi:10.1091/mbc.e16-11-0813
- Hiyamizu, S., Qiu, H., Vuolo, L., Stevenson, N., Shak, C., Heesom, K. J., Hamada, Y., Tsurumi, Y., Chiba, S., Katoh, Y. et al. (2023). Multiple interactions of the dynein-2 complex with the IFT-B complex are required for effective intraflagellar transport. *J. Cell Sci.* **136**, jcs260462. doi:10.1242/jcs.260462
- Hojo, H., Ohba, S. and Chung, U.-I. (2015). Signaling pathways regulating the specification and differentiation of the osteoblast lineage. *Regen. Ther.* **1**, 57-62. doi:10.1016/j.reth.2014.10.002
- Hojo, H., Ohba, S., Taniguchi, K., Shirai, M., Yano, F., Saito, T., Ikeda, T., Nakajima, K., Komiyama, Y., Nakagata, N. et al. (2013). Hedgehog-Gli activators direct osteo-chondrogenic function of bone morphogenetic protein toward osteogenesis in the perichondrium. *J. Biol. Chem.* **288**, 9924-9932. doi:10.1074/jbc.M112.409342
- Hojo, H., Saito, T., He, X., Guo, Q., Onodera, S., Azuma, T., Koebis, M., Nakao, K., Aiba, A., Seki, M. et al. (2022). Runx2 regulates chromatin accessibility to direct the osteoblast program at neonatal stages. *Cell Rep.* **40**, 111315. doi:10.1016/j.celrep.2022.111315
- Huangfu, D. and Anderson, K. V. (2005). Cilia and Hedgehog responsiveness in the mouse. *Proc. Natl. Acad. Sci. USA* **102**, 11325-11330. doi:10.1073/pnas.0505328102
- Hwang, S.-H., White, K. A., Somatilaka, B. N., Wang, B. and Mukhopadhyay, S. (2023). Context-dependent ciliary regulation of hedgehog pathway repression in tissue morphogenesis. *PLoS Genet.* **19**, e1011028. doi:10.1371/journal.pgen.1011028
- Jensen, V. L., Lambacher, N. J., Li, C., Mohan, S., Williams, C. L., Inglis, P. N., Yoder, B. K., Blacque, O. E. and Leroux, M. R. (2018). Role for intraflagellar transport in building a functional transition zone. *EMBO Rep.* **19**, e45862. doi:10.15252/embr.201845862
- Jeon, E.-J., Lee, K.-Y., Choi, N.-S., Lee, M.-H., Kim, H.-N., Jin, Y.-H., Ryoo, H.-M., Choi, J.-Y., Yoshida, M., Nishino, N. et al. (2006). Bone morphogenetic protein-2 stimulates Runx2 acetylation. *J. Biol. Chem.* **281**, 16502-16511. doi:10.1074/jbc.M512494200
- Jin, M., An, Y., Wang, Z., Wang, G., Liu, Z., Ding, P., Lu, D., Zhao, Z. and Bi, H. (2024). Distraction force promotes the osteogenic differentiation of Gli1⁺ cells in facial sutures via primary cilia-mediated Hedgehog signaling pathway. *Stem Cell Res. Ther.* **15**, 198. doi:10.1186/s13287-024-03811-3
- Katoh, Y., Michisaka, S., Nozaki, S., Funabashi, T., Hirano, T., Takei, R. and Nakayama, K. (2017). Practical method for targeted disruption of cilia-related genes by using CRISPR/Cas9-mediated homology-independent knock-in system. *Mol. Biol. Cell* **28**, 898-906. doi:10.1091/mbc.e17-01-0051
- Kessler, K., Wunderlich, I., Uebe, S., Falk, N. S., Gießl, A., Brandstätter, J. H., Popp, B., Klinger, P., Ekici, A. B., Sticht, H. et al. (2015). *DYNC2L1* mutations broaden the clinical spectrum of dynein-2 defects. *Sci. Rep.* **5**, 11649. doi:10.1038/srep11649
- Kim, J., Kato, M. and Beachy, P. A. (2009). Gli2 trafficking links Hedgehog-dependent activation of Smoothened in the primary cilium to transcriptional activation in the nucleus. *Proc. Natl. Acad. Sci. USA* **106**, 21666-21671. doi:10.1073/pnas.0912180106
- Kobayashi, T., Ishida, Y., Hirano, T., Katoh, Y. and Nakayama, K. (2021). Cooperation of the IFT-A complex with the IFT-B complex is required for ciliary retrograde protein trafficking and GPCR import. *Mol. Biol. Cell* **32**, 45-56. doi:10.1091/mbc.E20-08-0556
- Kopinke, D., Norris, A. M. and Mukhopadhyay, S. (2021). Developmental and regenerative paradigms of cilia regulated hedgehog signaling. *Sem. Cell Dev. Biol.* **110**, 89-103. doi:10.1016/j.semcdb.2020.05.029
- Liew, G. M., Ye, F., Nager, A. R., Murphy, J. P., Lee, J. S. H., Aguiar, M., Breslow, D. K., Gygi, S. P. and Nachury, M. V. (2014). The intraflagellar transport protein IFT27 promotes BBSome exit from cilia through the GTPase ARL6/BBS3. *Dev. Cell* **31**, 265-278. doi:10.1016/j.devcel.2014.09.004
- Lin, A. E., Traum, A. Z., Sahai, I., Keppler-Noreuil, K., Kukulich, M. K., Adam, M. P., Westra, S. J. and Arts, H. H. (2013). Sensenbrenner syndrome (Cranioectodermal dysplasia): clinical and molecular analyses of 39 patients including two new patients. *Am. J. Med. Genet.* **161A**, 2762-2776. doi:10.1002/ajmg.a.36265
- McInerney-Leo, A. M., Harris, J. E., Marshall, M. S., Gardiner, B., Kinning, E., Leong, H. Y., McKenzie, F., Ong, W. P., Vodopietz, J., Wicking, C. et al. (2015). Whole exome sequencing is an efficient, sensitive and specific method for determining the genetic cause of short-rib thoracic dystrophies. *Clin. Genet.* **88**, 550-557. doi:10.1111/cge.12550
- Mikami, A., Tynan, S. H., Hama, T., Luby-Phelps, K., Saito, T., Crandall, J. E., Besharse, J. C. and Vallee, R. B. (2002). Molecular structure of cytoplasmic dynein 2 and its distribution in neuronal and ciliated cells. *J. Cell Sci.* **115**, 4801-4808. doi:10.1242/jcs.00168
- Mill, P., Christensen, S. T. and Pedersen, L. B. (2023). Primary cilia as dynamic and diverse signaling hubs in development and disease. *Nat. Rev. Genet.* **24**, 421-441. doi:10.1038/s41576-023-00587-9
- Mo, R., Freer, A. M., Zinyk, D. L., Crackower, M. A., Michaud, J., Heng, H. H.-Q., Chik, K. W., Shi, X.-M., Tsui, L.-C., Cheng, S.-H. et al. (1997). Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* **124**, 113-123. doi:10.1242/dev.124.1.113
- Mukhopadhyay, A. G., Toropova, K., Daly, L., Wells, J. N., Vuolo, L., Mladenov, M., Seda, M., Jenkins, D., Stephens, D. J. and Roberts, A. J. (2024). Structure and tethering mechanism of dynein-2 intermediate chains in intraflagellar transport. *EMBO J.* **43**, 1257-1272. doi:10.1038/s44318-024-00060-1
- Nachury, M. V. and Mick, D. U. (2019). Establishing and regulating the composition of cilia for signal transduction. *Nat. Rev. Mol. Cell Biol.* **20**, 389-405. doi:10.1038/s41580-019-0116-4
- Nakamura, T., Aikawa, T., Iwamoto-Enomoto, M., Iwamoto, M., Higuchi, Y., Maurizio, P., Kinto, N., Yamaguchi, A., Noji, S., Kurisu, K. et al. (1997). Induction of osteogenic differentiation by Hedgehog proteins. *Biochem. Biophys. Res. Commun.* **237**, 465-469. doi:10.1006/bbrc.1997.7156
- Nakayama, K. and Katoh, Y. (2020). Architecture of the IFT ciliary trafficking machinery and interplay between its components. *Crit. Rev. Biochem. Mol. Biol.* **55**, 179-196. doi:10.1080/10409238.2020.1768206
- Niceta, M., Margiotti, K., Digilio, M. C., Guida, V., Bruselles, A., Pizzi, S., Ferraris, A., Memo, L., Laforgia, N., Dentici, M. L. et al. (2018). Biallelic mutations in *DYNC2L1* are a rare cause of Ellis-van Creveld syndrome. *Clin. Genet.* **93**, 632-639. doi:10.1111/cge.13128
- Niewiadomski, P., Niedziółka, S. M., Markiewicz, L., Uspienski, T., Baran, B. and Chojnowska, K. (2019). Gli proteins: regulation in development and cancer. *Cells* **8**, 147. doi:10.3390/cells8020147
- Nozaki, S., Katoh, Y., Kobayashi, T. and Nakayama, K. (2018). BBS1 is involved in retrograde trafficking of ciliary GPCRs in the context of the BBSome complex. *PLoS ONE* **13**, e0195005. doi:10.1371/journal.pone.0195005
- Nozaki, S., Castro Araya, R. F., Katoh, Y. and Nakayama, K. (2019). Requirement of IFT-B-BBSome complex interaction in export of GPR161 from cilia. *Biol. Open* **8**, bio043786. doi:10.1242/bio.043786
- Ocbina, P. J. R., Eggenschwiler, J. T., Moskowitz, I. and Anderson, K. V. (2011). Complex interactions between genes controlling trafficking in primary cilia. *Nat. Genet.* **43**, 547-553. doi:10.1038/ng.832
- Ohba, S. (2020). Hedgehog signaling in skeletal development: roles of Indian Hedgehog and the mode of its action. *Int. J. Mol. Sci.* **21**, 6665. doi:10.3390/ijms21186665
- Ohba, S., Ikeda, T., Kugimiya, F., Yano, F., Lichtler, A. C., Nakamura, K., Takato, T., Kawaguchi, H. and Chung, U.-I. (2007). Identification of a potent combination of osteogenic genes for bone regeneration using embryonic stem (ES) cell-based sensor. *FASEB J.* **21**, 1777-1787. doi:10.1096/fj.06-7571com
- Okazaki, M., Kobayashi, T., Chiba, S., Takei, R., Liang, L., Nakayama, K. and Katoh, Y. (2020). Formation of the B9-domain protein complex MKS1-B9D2-

- B9D1 is essential as a diffusion barrier for ciliary membrane proteins. *Mol. Biol. Cell* **31**, 2259–2268. doi:10.1091/mbc.E20-03-0208
- Park, K. and Leroux, M. R. (2022). Composition, organization and mechanisms of the transition zone, a gate for the cilium. *EMBO Rep.* **23**, e55420. doi:10.15252/embr.202255420
- Patel, K. and Smith, N. J. (2024). Primary cilia, A-kinase anchoring proteins and constitutive activity at the orphan G protein-coupled receptor GPR161: a tale about a tail. *Br. J. Pharmacol.* **181**, 2182–2196. doi:10.1111/bph.16053
- Qiu, H., Fujisawa, S., Nozaki, S., Katoh, Y. and Nakayama, K. (2021). Interaction of INPP5E with ARL13B is essential for its ciliary membrane retention but dispensable for its ciliary entry. *Biol. Open* **10**, bio057653. doi:10.1242/bio.057653
- Qiu, H., Tsurumi, Y., Katoh, Y. and Nakayama, K. (2022). Combinations of deletion and missense variations of the dynein-2 DYNC2L1 subunit found in skeletal ciliopathies cause ciliary defects. *Sci. Rep.* **12**, 31. doi:10.1038/s41598-021-03950-0
- Quadri, N. and Upadhyay, P. (2023). Primary cilia in skeletal development and disease. *Exp. Cell Res.* **431**, 113751. doi:10.1016/j.yexcr.2023.113751
- Quidwai, T., Wang, J., Hall, E. A., Petriman, N. A., Leng, W., Kiesel, P., Wells, J. N., Murphy, L. C., Keighren, M. A., Marsh, J. A. et al. (2021). A WDR35-dependent coat protein complex transports ciliary membrane cargo vesicles to cilia. *eLife* **10**, e69786. doi:10.7554/eLife.69786
- Reichert, J. C., Schmalz, J., Prager, P., Gilbert, F., Quent, V. M., Steinert, A. F., Rudert, M. and Nöth, U. (2013). Synergistic effect of Indian hedgehog and bone morphogenetic protein-2 gene transfer to increase the osteogenic potential of human mesenchymal stem cells. *Stem Cell Res. Ther.* **4**, 105. doi:10.1186/scrt316
- Reiter, J. F. and Leroux, M. R. (2017). Genes and molecular pathways underpinning ciliopathies. *Nat. Rev. Mol. Cell Biol.* **18**, 533–547. doi:10.1038/nrm.2017.60
- Rice, D. P. C., Connor, E. C., Veltmaat, J. M., Lana-Elola, E., Veistinen, L., Tanimoto, Y., Bellusci, S. and Rice, R. (2010). *GLI3/JIK1-J* mice exhibit lambdoid suture craniosynostosis which results from altered osteoprogenitor proliferation and differentiation. *Hum. Mol. Genet.* **19**, 3457–3467. doi:10.1093/hmg/ddq258
- Rix, S., Calmont, A., Scambler, P. J. and Beales, P. L. (2011). An *Ift80* mouse model of short rib polydactyly syndromes shows defects in hedgehog signaling without loss or malformation of cilia. *Hum. Mol. Genet.* **20**, 1306–1314. doi:10.1093/hmg/ddr013
- Ruiz I Altaba, A. (1999). Gli proteins encode context-dependent positive and negative functions: implications for development and disease. *Development* **126**, 3205–3216. doi:10.1242/dev.126.14.3205
- Salhotra, A., Shah, H. N., Levi, B. and Longaker, M. T. (2020). Mechanisms of bone development and repair. *Nat. Rev. Mol. Cell Biol.* **21**, 696–711. doi:10.1038/s41580-020-00279-w
- Schmidts, M. (2014). Clinical genetics and pathobiology of ciliary chondrodysplasias. *J. Pediatr. Genet.* **3**, 49–64. doi:10.3233/PGE-14089
- Shak, C., Vuolo, L., Uddin, B., Katoh, Y., Brown, T., Mukhopadhyay, A. G., Heesom, K., Roberts, A. J., Stevenson, N., Nakayama, K. et al. (2023). Disease-associated mutations in WDR34 lead to diverse impacts on the assembly and function of dynein-2. *J. Cell Sci.* **136**, jcs260073. doi:10.1242/jcs.260073
- Shimoyama, A., Wada, M., Ikeda, F., Hata, K., Matsubara, T., Nifuji, A., Noda, M., Amano, K., Yamaguchi, A., Nishimura, R. et al. (2007). Ihh/Gli2 signaling promotes osteoblast differentiation by regulating Runx2 expression and function. *Mol. Biol. Cell* **18**, 2411–2418. doi:10.1091/mbc.e06-08-0743
- Takahashi, S., Kubo, K., Waguri, S., Yabashi, A., Shin, H.-W., Katoh, Y. and Nakayama, K. (2012). Rab11 regulates exocytosis of recycling vesicles at the plasma membrane. *J. Cell Sci.* **125**, 4049–4057. doi:10.1242/jcs.102913
- Takahashi, Y., Ishida, Y., Yoshida, S., Shin, H.-W., Katoh, Y. and Nakayama, K. (2025). Counterregulatory roles of GLI2 and GLI3 in osteogenic differentiation via Gli1 expression. *J. Cell Sci.* **138**, jcs263556. doi:10.1242/jcs.263556
- Taylor, S. P., Dantas, T. J., Duran, I., Wu, S., Lachman, R. S., University of Washington Center for Mendelian Genomics Consortium, Nelson, S. F., Cohn, D. H., Vallee, R. B. and Krakow, D., Cohn, D. H. et al. (2015). Mutations in *DYNC2L1* disrupt cilia function and cause short rib polydactyly syndrome. *Nat. Commun.* **6**, 7092. doi:10.1038/ncomms8092
- Thomas, S., Ritter, B., Verbich, D., Sanson, C., Bourbonniere, L., McKinney, R. A. and McPherson, P. S. (2009). Intersectin regulates dendritic spine development and somatodendritic endocytosis but not synaptic vesicle recycling in hippocampal neurons. *J. Biol. Chem.* **284**, 12410–12419. doi:10.1074/jbc.M809746200
- Toropova, K., Zalyte, R., Mukhopadhyay, A. G., Mladenov, M., Carter, A. P. and Roberts, A. J. (2019). Structure of the dynein-2 complex and its assembly with intraflagellar transport trains. *Nat. Struct. Mol. Biol.* **26**, 823–829. doi:10.1038/s41594-019-0286-y
- Tsurumi, Y., Hamada, Y., Katoh, Y. and Nakayama, K. (2019). Interactions of the dynein-2 intermediate chain WDR34 with the light chains are required for ciliary retrograde protein trafficking. *Mol. Biol. Cell* **30**, 658–670. doi:10.1091/mbc.E18-10-0678
- Tukachinsky, H., Lopez, L. V. and Salic, A. (2010). A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu–Gli protein complexes. *J. Cell Biol.* **191**, 415–428. doi:10.1083/jcb.201004108
- Vuolo, L., Stevenson, N. L., Heesom, K. J. and Stephens, D. J. (2018). Dynein-2 intermediate chains play crucial but distinct roles in primary cilia formation and function. *eLife* **7**, e39655. doi:10.7554/eLife.39655
- Vuolo, L., Stevenson, N. L., Mukhopadhyay, A. G., Roberts, A. J. and Stephens, D. J. (2020). Cytoplasmic dynein-2 at a glance. *J. Cell Sci.* **133**, jcs240614. doi:10.1242/jcs.240614
- Walker, M. F., Zhang, J., Steiner, W., Ku, P.-I., Zhu, J.-F., Michaelson, Z., Yen, Y.-C., Lee, A., Long, A. B., Casey, M. J. et al. (2024). GRK2 kinases in the primary cilium initiate SMOOTHENED-PAK signaling in the Hedgehog cascade. *PLoS Biol.* **22**, e3002685. doi:10.1371/journal.pbio.3002685
- Webb, S., Mukhopadhyay, A. G. and Roberts, A. J. (2020). Intraflagellar transport trains and motors: insights from structure. *Sem. Cell Dev. Biol.* **107**, 82–90. doi:10.1016/j.semcdb.2020.05.021
- Weijman, J. F., Vuolo, L., Shak, C., Pugnetti, A., Mukhopadhyay, A. G., Hodgson, L. R., Heesom, K. J., Roberts, A. J. and Stephens, D. J. (2024). Roles for CEP170 in cilia function and dynein-2 assembly. *J. Cell Sci.* **137**, jcs261816. doi:10.1242/jcs.261816
- Wen, X., Lai, C. K., Evangelista, M., Hongo, J.-A., De Sauvage, F. J. and Scales, S. J. (2010). Kinetics of Hedgehog-dependent full-length Gli3 accumulation in primary cilia and subsequent degradation. *Mol. Cell Biol.* **30**, 1910–1922. doi:10.1128/MCB.01089-09
- Wingfield, J. L., Mengoni, I., Bomberger, H., Jiang, Y. Y., Walsh, J. D., Brown, J. M., Picariello, T., Cochran, D. A., Zhu, B., Pan, J. et al. (2017). IFT trains in different stages of assembly queue at the ciliary base for consecutive release into the cilium. *eLife* **6**, e26609. doi:10.7554/eLife.26609
- Yang, S. and Wang, C. (2012). The intraflagellar transport protein IFT80 is required for cilia formation and osteogenesis. *Bone* **51**, 407–417. doi:10.1016/j.bone.2012.06.021
- Ye, F., Nager, A. R. and Nachury, M. V. (2018). BBSome trains remove activated GPCRs from cilia by enabling passage through the transition zone. *J. Cell Biol.* **217**, 1847–1868. doi:10.1083/jcb.201709041
- Yoshida, S., Kawamura, A., Aoki, K., Wiriyaermkul, P., Sugimoto, S., Tomiyoshi, J., Tajima, A., Ishida, Y., Katoh, Y., Tsukada, T. et al. (2024). Positive regulation of Hedgehog signaling via phosphorylation of GLI2/GLI3 by DYRK2 kinase. *Proc. Natl. Acad. Sci. USA* **121**, e3230070121. doi:10.1073/pnas.2320070121
- Yuasa, T., Kataoka, H., Kinto, N., Iwamoto, M., Enomoto-Iwamoto, M., Iemura, S., Ueno, N., Shibata, Y., Kurosawa, H. and Yamaguchi, A. (2002). Sonic hedgehog is involved in osteoblast differentiation by cooperating with BMP-2. *J. Cell. Physiol.* **193**, 225–232. doi:10.1002/jcp.10166
- Zhang, W., Taylor, S. P., Ennis, H. A., Forlenza, K. N., Duran, I., Li, B., Ortiz Sanchez, J. A., Nevarez, L., Nickerson, D. A., Bamshad, M. et al. (2018). Expanding the genetic architecture and phenotypic spectrum in the skeletal ciliopathy. *Hum. Mut.* **39**, 152–166. doi:10.1002/humu.23362
- Zhang, X., You, Y., Xie, X., Xu, H., Zhou, H., Lei, Y., Sun, P., Meng, Y., Wang, L. and Lu, Y. (2020). Whole-exome sequencing identified two novel mutations of *DYNC2L1* in fetal skeletal ciliopathy. *Mol. Genet. Genomic Med.* **8**, e1524. doi:10.1002/mgg3.1524
- Zhao, H., Feng, J., Ho, T.-V., Grimes, W., Urata, M. and Chai, Y. (2015). The suture provides a niche for mesenchymal stem cells of craniofacial bones. *Nat. Cell Biol.* **17**, 386–396. doi:10.1038/ncb3139
- Zhou, M. and Jiang, J. (2022). Gli phosphorylation code in Hedgehog signal transduction. *Front. Cell Dev. Biol.* **10**, 846927. doi:10.3389/fcell.2022.846927
- Zhou, H., Zhang, L., Chen, Y., Zhu, C.-H., Chen, F.-M. and Li, A. (2022a). Research progress on the hedgehog signaling pathway in regulating bone formation and homeostasis. *Cell Prolif.* **55**, e13162. doi:10.1111/cpr.13162
- Zhou, Z., Qiu, H., Castro-Araya, R.-F., Takei, R., Nakayama, K. and Katoh, Y. (2022b). Impaired cooperation between IFT74/BBS22–IFT81 and IFT25–IFT27/BBS19 causes Bardet-Biedl syndrome. *Hum. Mol. Genet.* **31**, 1681–1693. doi:10.1093/hmg/ddab354