878

879

880 SUPPLEMENTARY FIGURE LEGENDS

881 **Supplementary Figure 1. Generation of *KIF3A*$^{-/-}$ and *KIF3B*$^{-/-}$ hPSC mutants using CRISPR**
882 **gene editing.** (**a**) Table of gene edited hPSC carrying loss of function mutations in *KIF3A* or *KIF3B*
883 generated using CRISPR gene editing. The genetic background, sgRNA used for targeting, and
884 resulting mutation is indicated for each cell line. (**b**) Chromatograms of homozygote mutants showing
885 the edited *KIF3A* or (**c**) *KIF3B* regions. The sgRNA sequence used for targeting is labelled in yellow
886 in the respective control chromatograms. (**d**) Sanger sequencing chromatogram of compound
887 heterozygote *KIF3A* or (**e**) *KIF3B* mutants and the separated allele sequences after TOPO cloning.
888 Deletions and insertions are indicated in the chromatograms.

889

890 **Supplementary Figure 2. CRISPR gene editing results in loss of KIF3A or KIF3B protein.** (**a**)
891 KIF3A immunoblot showing three distinct control and *KIF3A*$^{-/-}$ H9 hPSC lines at passages 4 and 17.
892 Cropped areas used in Figure 1 are shown in bracket box. β-actin was blotted as a loading control.
893 (**b**) KIF3A immunoblot for one control (C1) and two distinct *KIF3A*$^{-/-}$ WTC11 hPSC (M1 and M2).
894 GAPDH was blotted as a loading control. (**c**) KIF3B and β-actin immunoblot for one H9 control (C1)
895 and two *KIF3B*$^{-/-}$ mutant (M1, M2) hPSC. (**d**) KIF3B and β-actin immunoblot for three distinct control
896 and four *KIF3B*$^{-/-}$ WTC11 mutant hPSC. Cropped areas used in Figure 1 are shown in bracket box.
897 kDa sizes of the protein standards are indicated. (**e**) Band intensity quantification from immunoblots
898 of the indicated genotype (mean ± s.e.m., n ≥ 4 independent biological replicates).

Supplementary Figure 3. Kinesin-2 is dispensable for hPSC morphology and epiblast spheroid formation. (**a**) Confocal immunofluorescence images of acetylated α-tubulin (AcTub) and DNA in representative fields of undifferentiated control and *KIF3A*$^{-/-}$ hPSCs. Orthogonal (top) and volume (bottom) views are shown. (**b**) Phase contrast images of representative control and *KIF3A*$^{-/-}$ epiblast spheroids. (**c**) Quantification of lumen area as percentage of the spheroid area. (**d-e**) Confocal sections showing immunofluorescence for pluripotency, polarity, and ciliary markers in spheroids. A midbody is seen in a *KIF3A*$^{-/-}$ spheroid (arrow), but not cilia. Scale bars, 25 μm.

Supplementary Figure 4. Kinesin-2 knockout hPSCs establish a renewable source of diverse human cell types lacking cilia. (**a**) Neuroepithelial (AcTub), endodermal (AFP), and mesodermal (SMA) lineages in EBs. Zoom shows magnification of dashed boxed area with AcTub and DNA intensities increased for clarity. Scale bar, 50 μm. (**b**) Images and (**c**) quantification of pigmented EBs. Scale bar, 500 μm. Knockout (KO) represent pooled data from both *KIF3A*$^{-/-}$ and *KIF3B*$^{-/-}$ EBs (mean ± s.e.m., n ≥ 9 independent biological replicates per condition from a total of 8 distinct cell lines). (**d**) Representative images and (**e**) quantification of OCT4 and BRY immunofluorescence intensities in hPSCs after treatment with increasing doses of CHIR99021 in mTeSR1 for 48 hours. Each dot represents a single cell. Data are pooled from three separate experiments. bpp, bits per pixel (raw intensity). Scale bars, 50 μm.

Supplementary Figure 5. Kinesin-2 knockout tumors exhibit differentiation defects. (**a**) Representative photographs of whole, unfixed growths retrieved from immunodeficient animals at the same time point. Growths are sliced through their centers to reveal the internal surface. (**b**) A second set of tumors, photographed intact after retrieval from the animals. (**c-e**)

921 Quantification of tissue subtypes within teratomas as a fraction of total area (mean ± s.e.m., n
922 ≥ 5 independent biological replicates per condition from a total of 14 distinct cell lines). (**f**) Ratio
923 of area occupied by $SOX2^+$ cells in $TUJ1^+$ patches of teratoma sections (mean ± s.e.m., n ≥ 4
924 independent biological replicates per condition from a total of 6 distinct cell lines; *, $p < 0.05$).

925 **Supplementary Figure 6. Hedgehog switching is defective in kinesin-2 knockout**
926 **organoid cultures.** (**a**) Quantification of SOX2+ area per 96-well of kidney organoid cultures
927 (mean ± s.e.m., n ≥ 3 independent biological replicates per condition from a total of 11 distinct
928 cell lines. **, $p < 0.01$). (**b**) Wide-field immunofluorescence images of kidney organoid
929 differentiations in a representative 96-well plate. The three left wells contain control cell lines
930 and the three right wells contain *KIF3A*$^{-/-}$ cell lines. Zoom of boxed regions is shown below each
931 of the wells. Each kidney organoid contains distal tubular (ECAD), proximal tubular (LTL), and
932 podocyte (NPHS1) epithelial cells. Arrow indicates a cluster of $ECAD^+$ cells without proximal
933 tubule and podocyte segments. **(c)** Heatmap of the expression level of genes differentially
934 expressed as a combined function of *KIF3A* or *KIF3B* loss in hPSCs (FDR < 0.2). Gene names
935 and labels at right refer to genes associated with enriched gene ontology processes as a
936 function of *KIF3B*$^{-/-}$ loss in hPSCs in Fig. 5a. (**d**) Representative GLI3 immunoblot of control,
937 *KIF3A*$^{-/-}$, and *KIF3B*$^{-/-}$ undifferentiated hPSCs, with (**e**) band intensity quantification (mean ±
938 s.e.m., n ≥ 3 independent biological replicates per condition from a total of 11 distinct cell lines).
939 **(f)** Band intensity quantification of the blot shown in Fig. 5e, comparing cultures on day 0 to day
940 18. (**g**) Immunoblot of GLI3 in kidney organoid cultures on days 7, 11, and 18 of differentiation.
941 3A and 3B indicate *KIF3A*$^{-/-}$ and *KIF3B*$^{-/-}$ mutants, respectively. **(h)** Schematic of organoid
942 microdissection (left), with immunoblot of GLI3 on day 18 of differentiation in lysates of whole

wells (w), microdissected organoids (o), or remnant stroma (s). **(i)** GLI1 immunoblot of the samples shown in Fig. 5e, comparing cultures on day 0 to day 18.

Supplementary Figure 7. (a) Representative GLI3 immunoblot in organoid cultures on day 18. Blot is from independent biological replicates, compared to the blot shown in Fig. 6d. (**b**) Individual measurements of GLI3F and (**c**) GLI3R bands for the sample set quantified in ratiometric form in Fig. 6d. (**d**) Representative GLI1 blot of these conditions, with (**e**) quantification of band intensities (mean ± s.e.m., n ≥ 3 independent biological replicates per condition from a total of 6 distinct cell lines).

Supplementary Figure 8. Comparison between kinesin-2 and polycystin knockout mutants. (**a**) Quantification of organoid area in 12 month old suspension cultures. (**b**) Representative confocal optical sections showing tubular epithelial markers in cyst-lining epithelia of *PKD1* versus *KIF3B* mutant organoids. (**c-e**) Representative immunoblot showing PC1 and PC2 levels in *KIF3A*$^{-/-}$ hPSCs, compared to isogenic controls. β-actin is shown as a loading control. (**f**) Quantification of band intensities from whole cell lysates of kinesin-2 knockout or control hPSCs (n ≥ 5 independent biological replicates per condition). (**g**) Immunoblot showing PC2, KIF3A, and GAPDH loading control in kinesin-2 knockout hPSCs, isogenic controls, or PKD2 knockout. (**h**) Quantification of PC2 band intensities from immunoblots of kinesin-2 knockout hPSCs and isogenic controls (n ≥ 3 independent biological replicates per condition).

Supplementary Figure 9. hPSCs release ciliary proteins in EV via kinesin-2. (**a**) Quantification of protein fraction in EV (SN), compared to whole cell lysates (LY), as a percentage of total protein (SN + LY), using BCA assay (n = four distinct hPSC lines, plotted separately). (b) Representative immunoblot showing IFT88 levels in LY vs. SN. Total protein load is indicated in µg above each lane. (c) Quantification of IFT88 band intensities per lane at increasing total protein loads in SN and LY (n = 4 independent biological replicates from a total of 4 distinct cell lines). (**d-e**) Representative immunoblots and (**f**) band intensity quantifications of key ciliary proteins in EV, in *KIF3B*$^{-/-}$ hPSCs compared to isogenic controls (mean ± s.e.m., n ≥ 2 independent biological replicates from a total of 4 distinct cell lines).

Supplementary Figure 10. Kinesin-2 knockout hPSCs exhibit secretion defects with normal cytoplasmic expression. (**a**) Representative immunoblots of endogenous PC1 or (**b**) PC2 proteins in supernatants (SN) and lysates (LY) from control, *PKD1*$^{-/-}$, *PKD2*$^{-/-}$, and *KIF3A*$^{-/-}$ hPSCs, with FLOT1 loading control. $PC2_{tetra}$ refers to the tetrameric form of PC2. (**c**) Silver stain of whole supernatants from two control and three *KIF3A*$^{-/-}$ hPSC lines. No consistent differences in banding pattern are observed between the two groups. (**d**) Uncropped immunoblots for PTCH1 and IFT88 in EV, from Fig. 8b and 8c, respectively.

Supplementary Table 1. Lines and n used for statistical analysis.

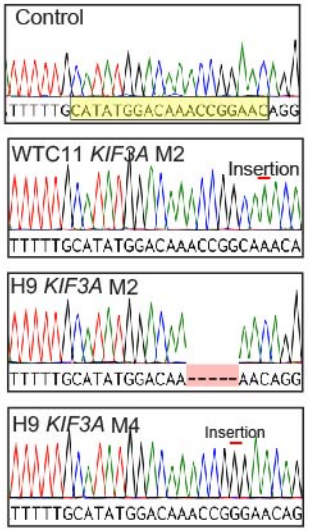
Supplementary Figure 1

a

Background	ID	gRNA	Mutation
H9 *KIF3A-/-*	Mutant 1	CATATGGACAAACCGGAAC	p.(Thr104LysfsTer16);(Thr102AsnfsTer11)
	Mutant 2	CATATGGACAAACCGGAAC	p.(Thr102AsnfsTer14)
	Mutant 3	CATATGGACAAACCGGAAC	p.(Tyr99LysfsTer16);(Gly103AsnfsTer14)
	Mutant 4	CATATGGACAAACCGGAAC	p.(Thr104AsnfsTer14)
WTC11 *KIF3A-/-*	Mutant 1	CATATGGACAAACCGGAAC	p.(Thr104AsnfsTer14);(Ile96_Thr107del)
	Mutant 2	CATATGGACAAACCGGAAC	p.(Thr104AsnfsTer14)
	Mutant 3	CATATGGACAAACCGGAAC	p.(Gly94AsnfsTer14); p.(Gly103del)
H9 *KIF3B-/-*	Mutant 1	TTCGCTGTCGGCCCATGAA	p.(Met19IlefsTer10);(Cys16_Met19del)
	Mutant 2	TTCGCTGTCGGCCCATGAA	p.(Met19IlefsTer10)
	Mutant 3	TACACCATGGAAGGAATCCG	p.(Arg110ProfsTer2)
WTC11 *KIF3B-/-*	Mutant 1	TTCGCTGTCGGCCCATGAA	p.(Met19IlefsTer10)
	Mutant 2	TTCGCTGTCGGCCCATGAA	p.(Pro18GlnTer7)
	Mutant 3	TACACCATGGAAGGAATCCG	p.(Tyr104ValfsTer35)
	Mutant 4	TACACCATGGAAGGAATCCG	p.(Glu107ValfsTer34)

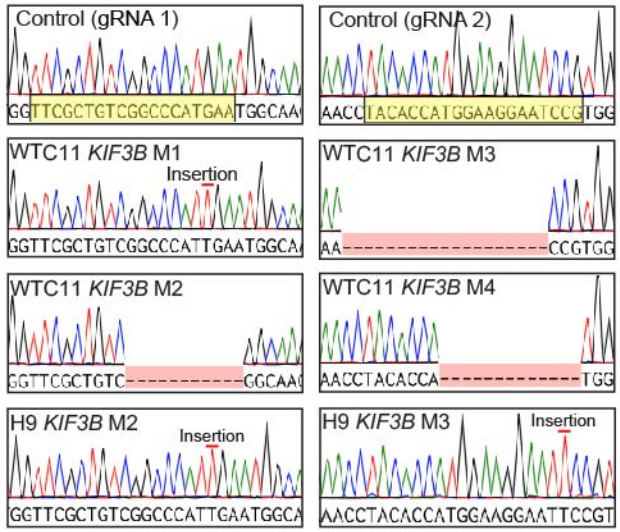
b

KIF3A homozygotes



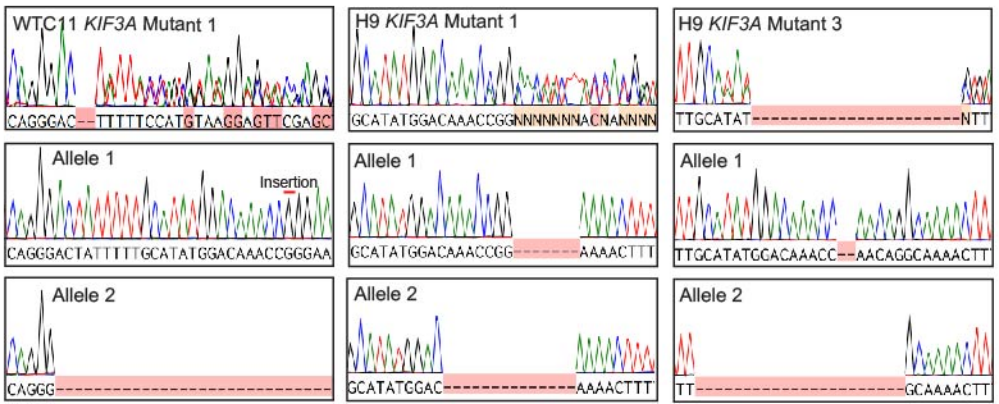
c

KIF3B homozygote mutants



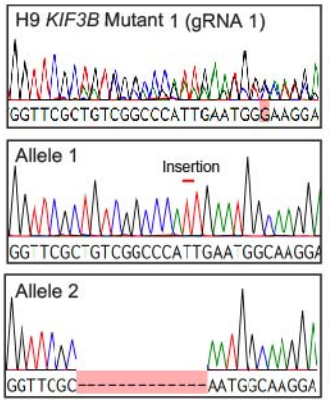
d

KIF3A compound heterozygote mutants

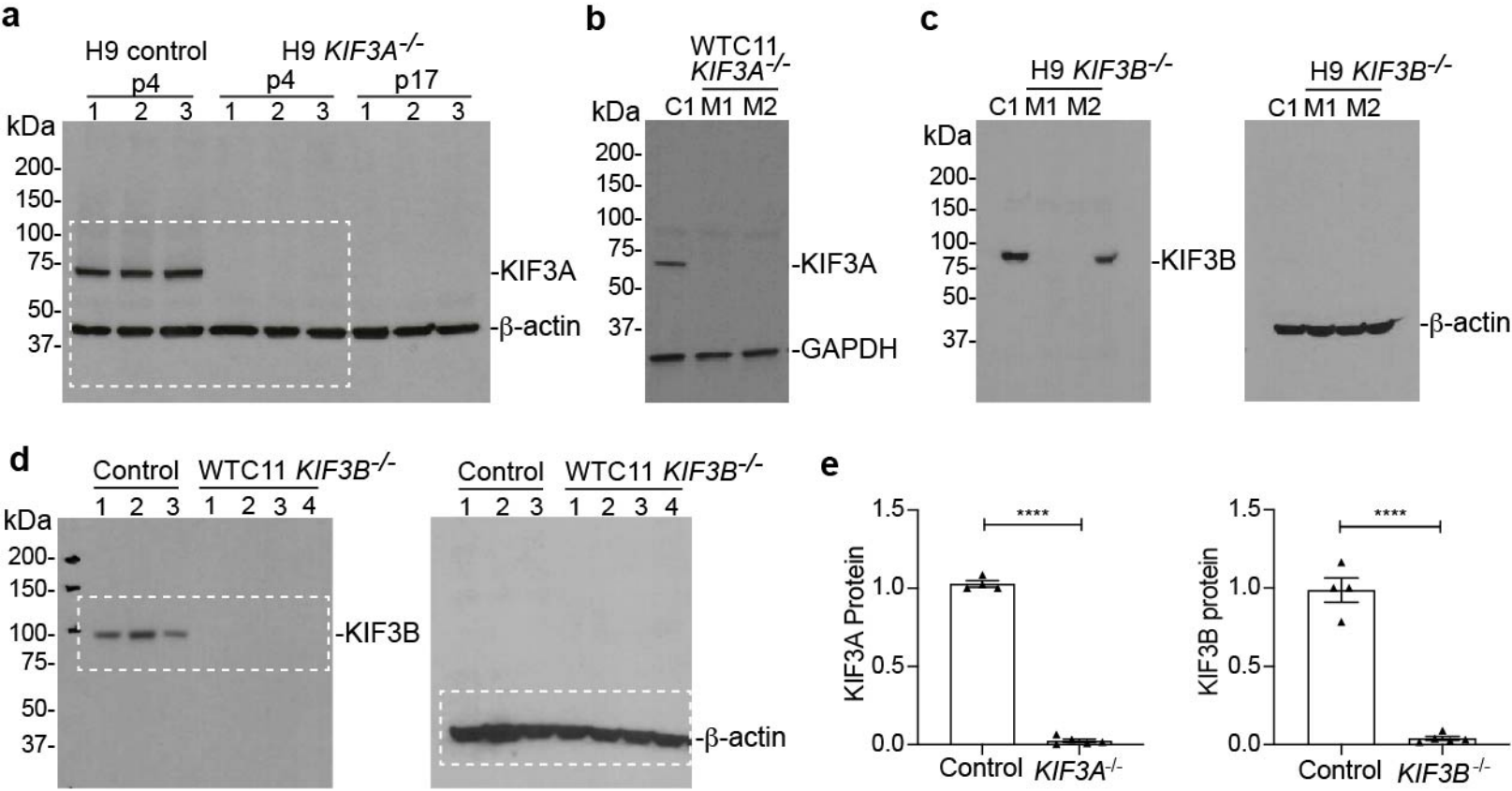


e

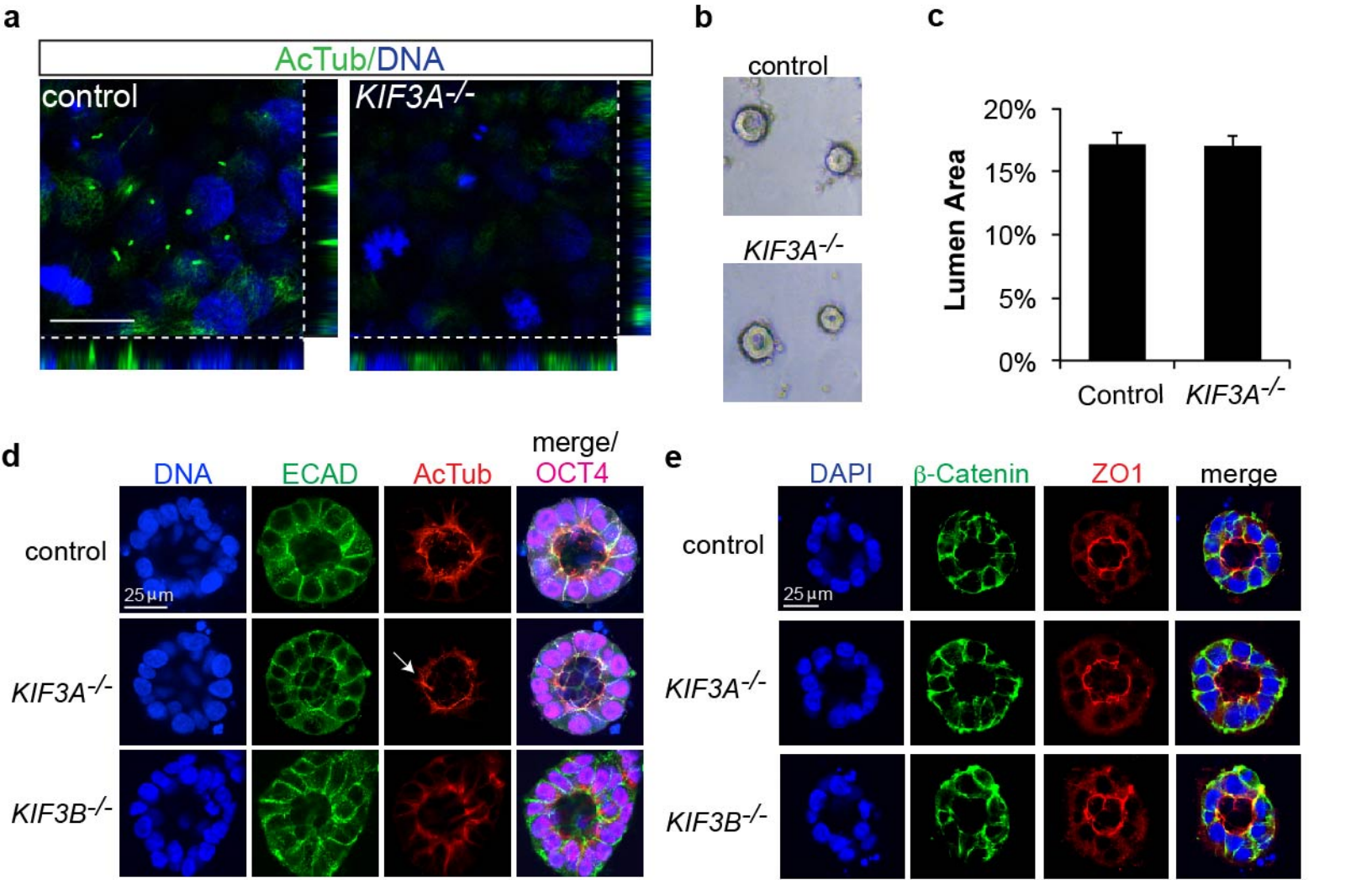
KIF3B compound heterozygote



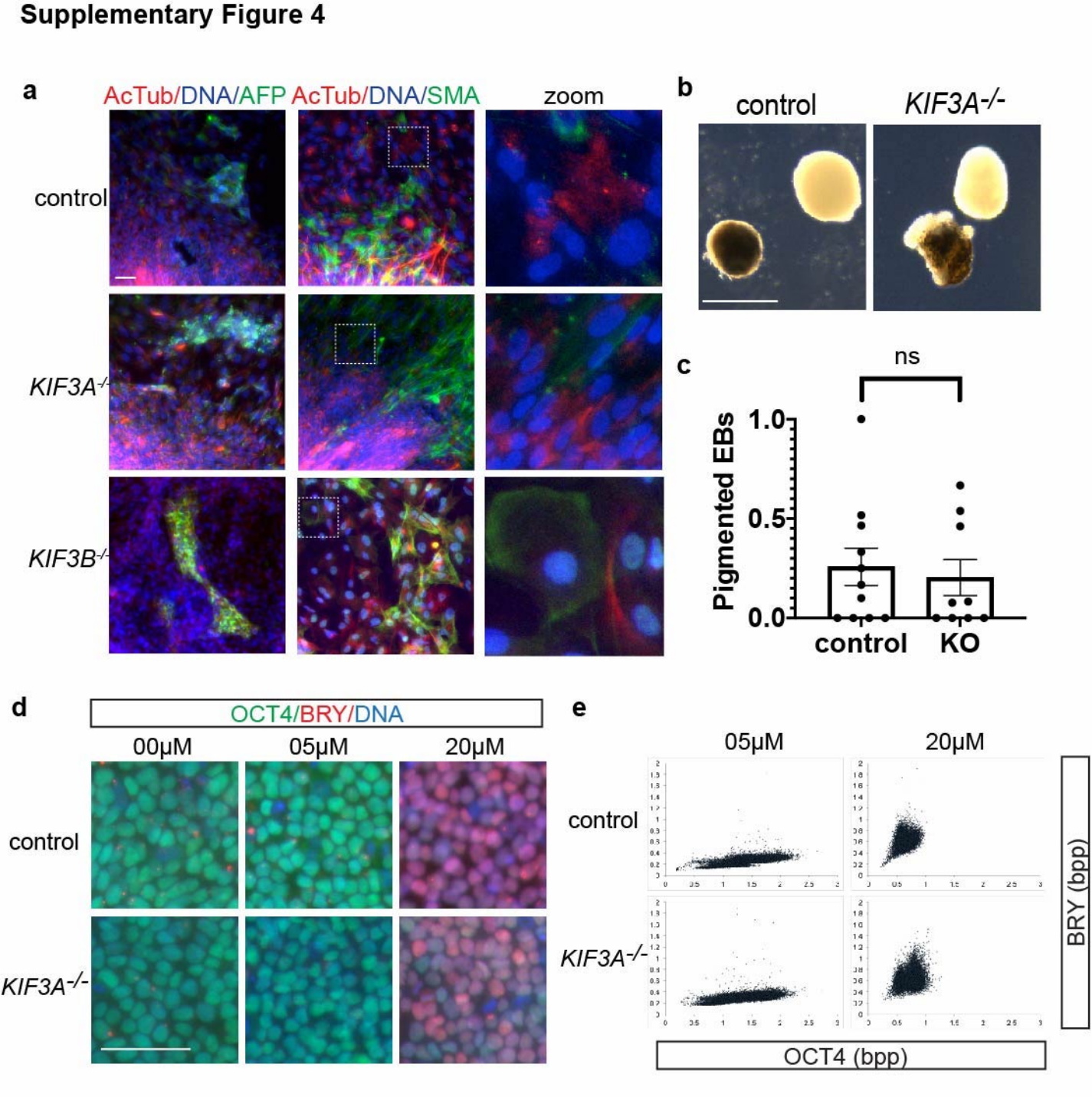
Supplementary Figure 2



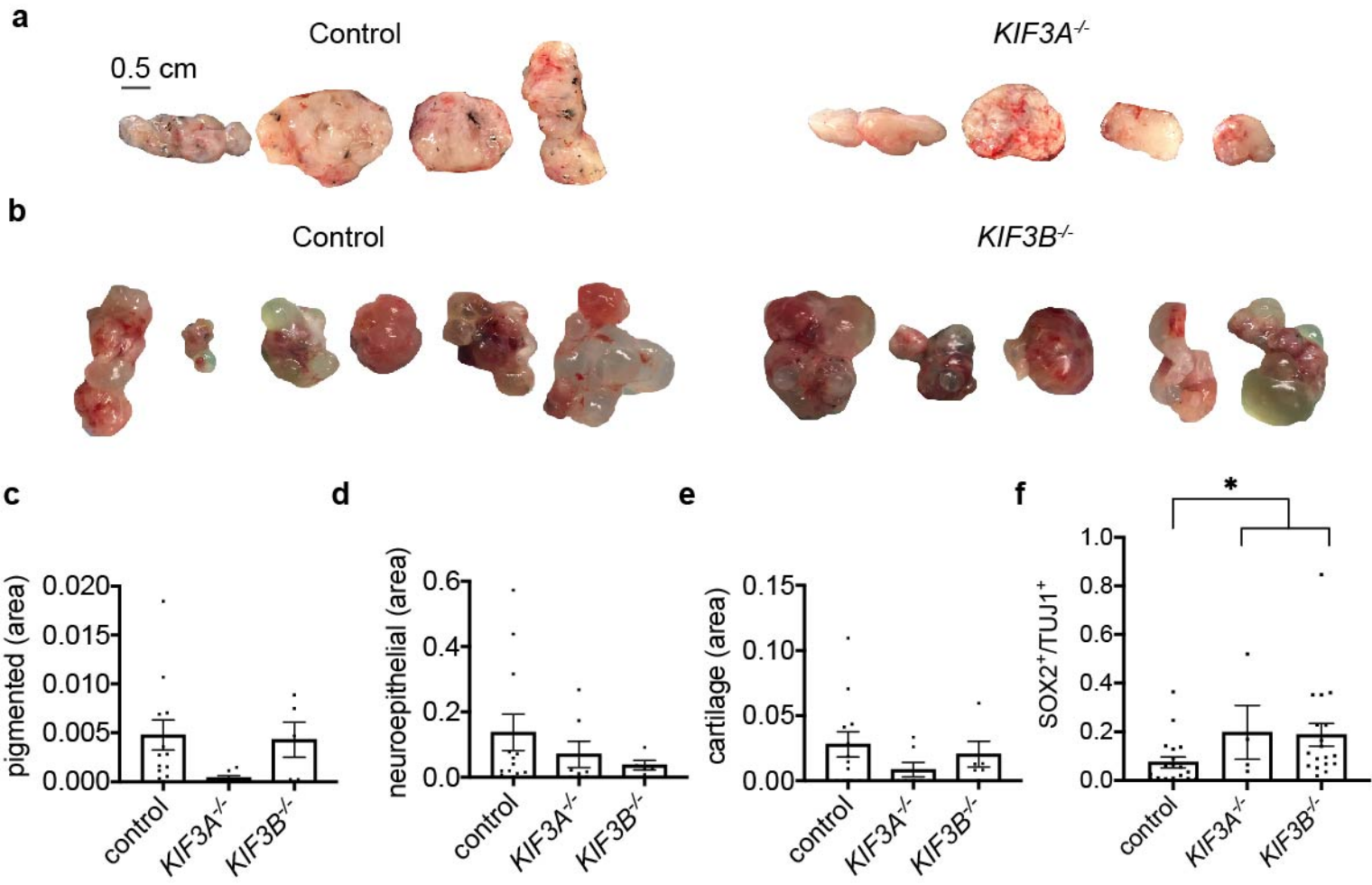
Supplementary Figure 3



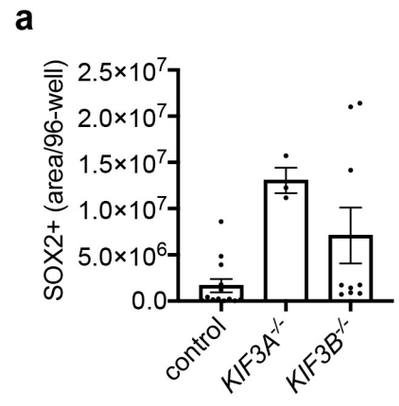
Supplementary Figure 4

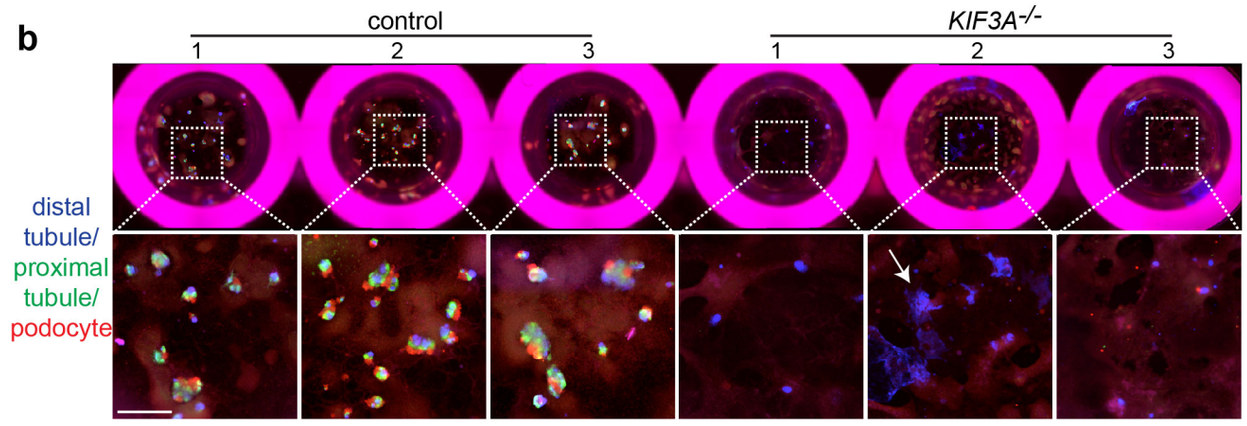


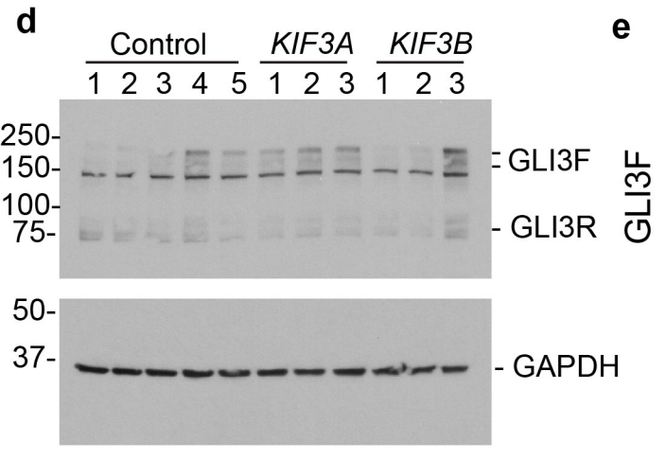
Supplementary Figure 5

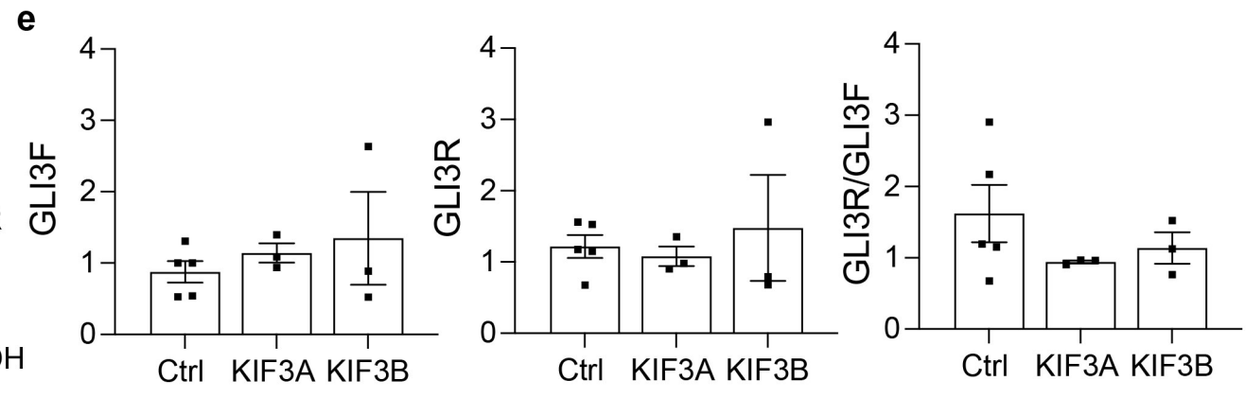


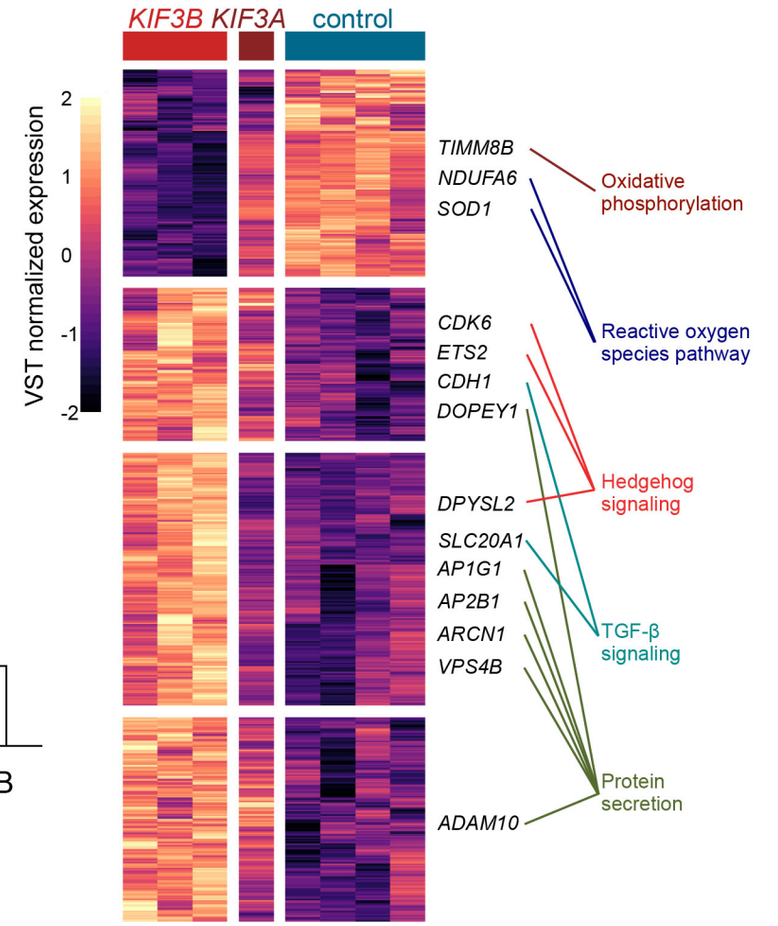
Supplementary Figure 6

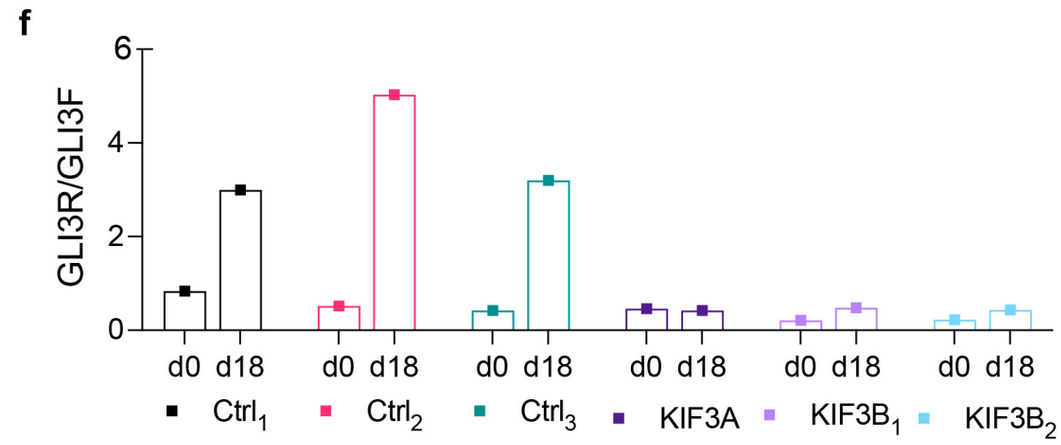


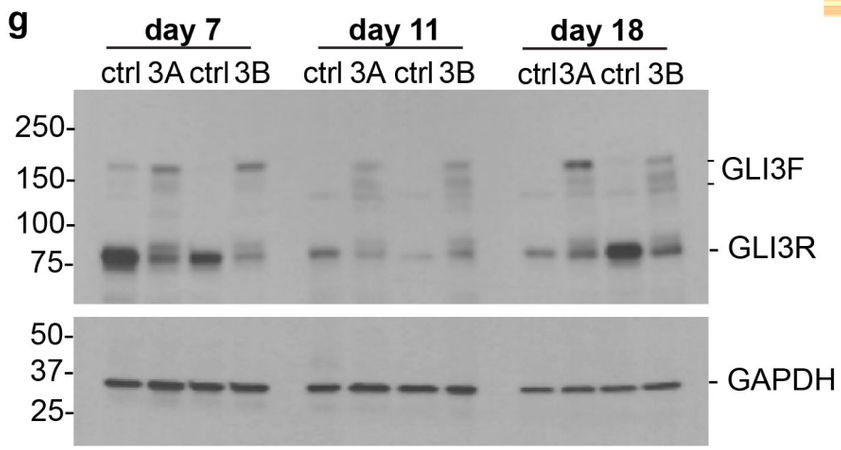


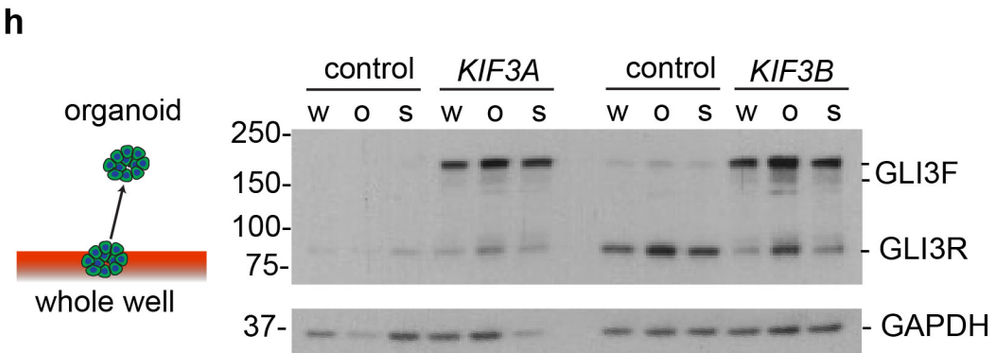


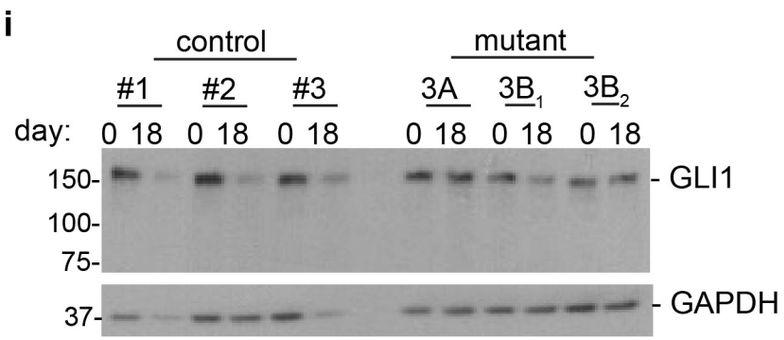




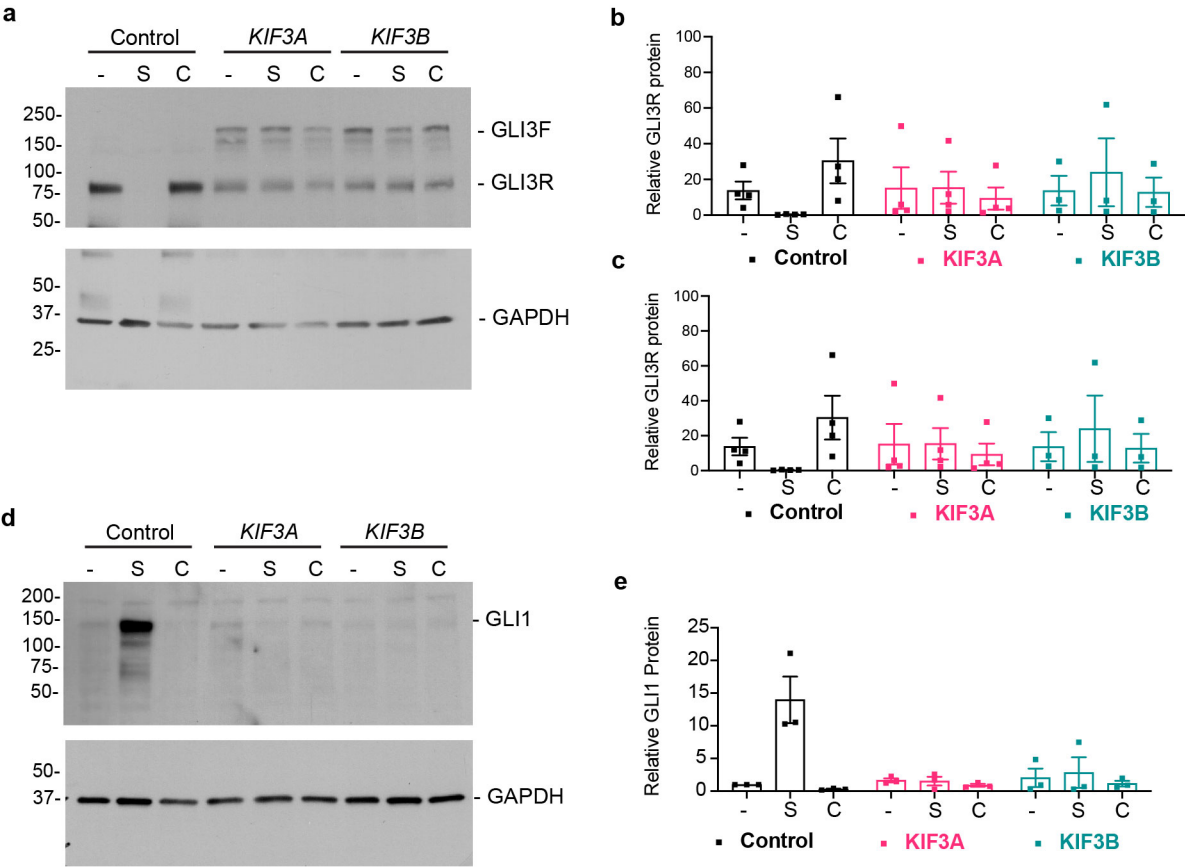




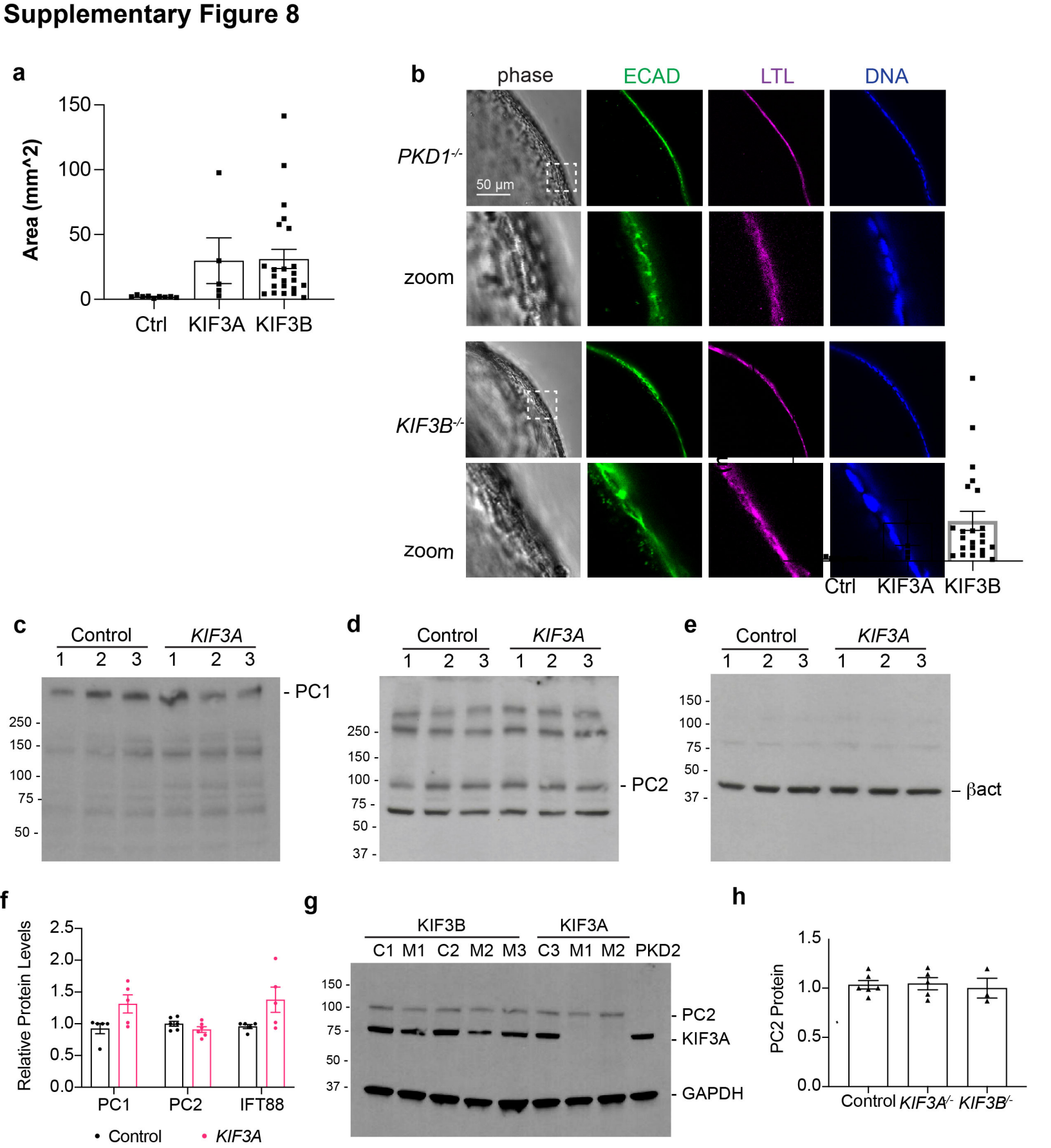




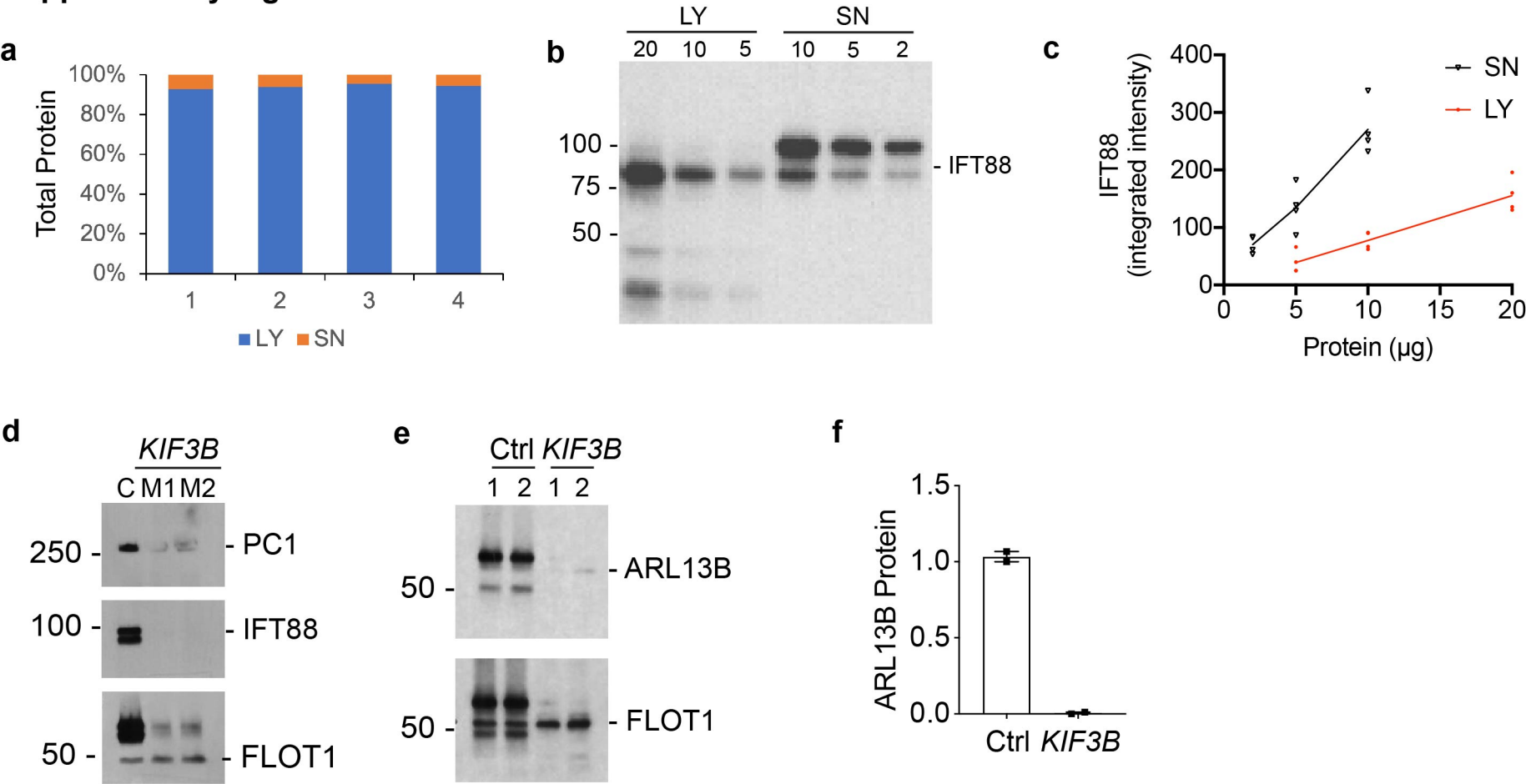
Supplementary Figure 7



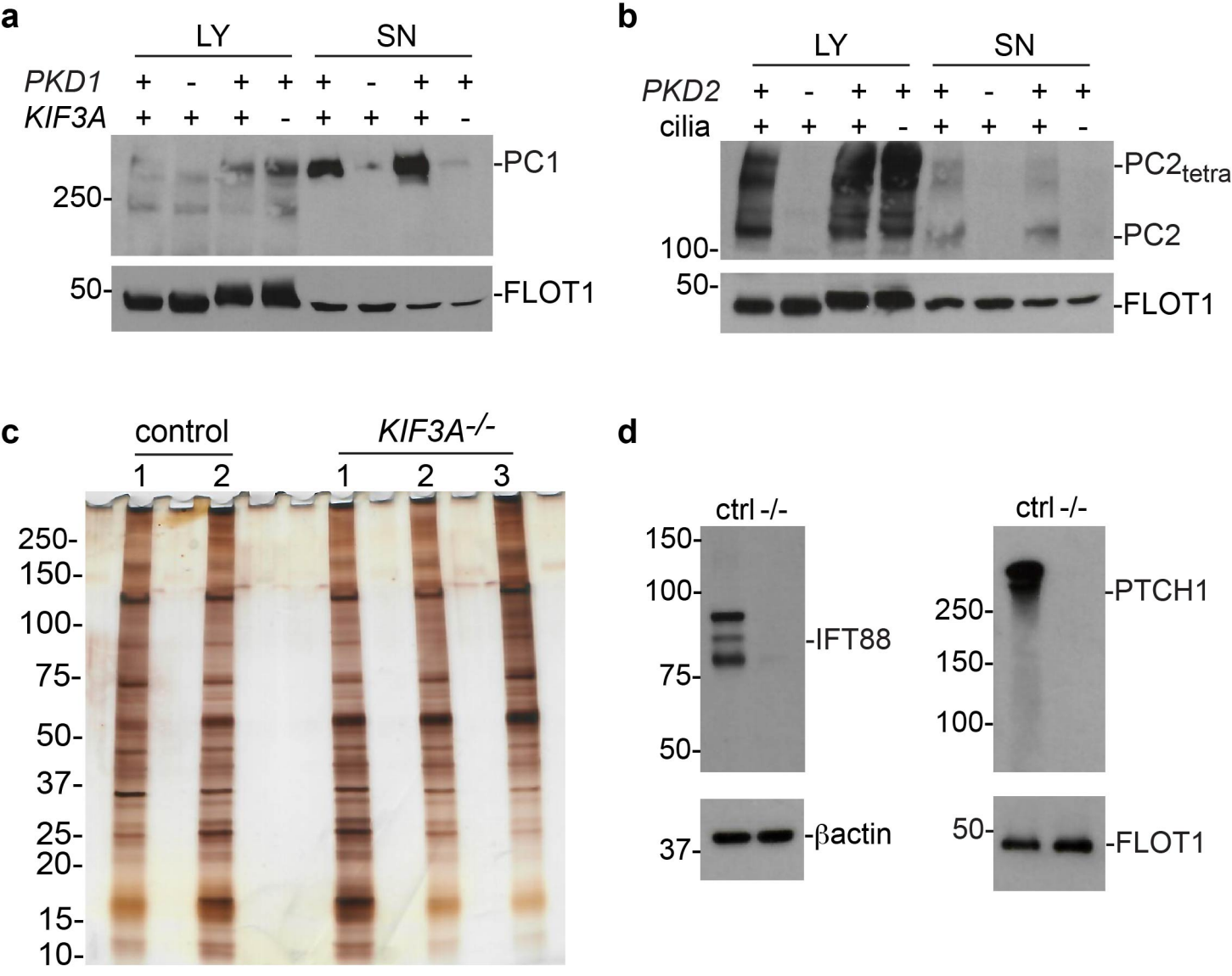
Supplementary Figure 8



Supplementary Figure 9



Supplementary Figure 10



Supplementary Table 1. Lines and n used for statistical analyses

Figure	Panel	Total n ≥	Total lines	KIF3A		KIF3B		control	
				n	Lines	n	Lines	n	Lines
1	e	7	24	7	7	7	7	10	10
2	b	3	9	3	3	3	3	3	3
2	c	6	11	10	4	7	1	11	6
2	g	3	8	3	3	3	2	4	3
2	i	2	9	5	3	2	2	7	4
3	a	6	14	7	4	6	4	13	6
3	d	5	14	7	4	5	4	12	6
3	g	9	8	9	2	11	2	18	4
4	c	3	11	3	3	9	3	13	5
4	f	7	13	10	4	7	4	12	5
6	d	3	8	4	3	3	2	4	3
6	f	4	4					4	4
6	g	10	4					10	4
6	g	10	4					10	4
7	c	4	10	5	3	4	2	10	5
8	f	6	14	6	4	6	4	8	6
8	g	3	6	3	3			3	3
8	i	3	6			3	3	3	3