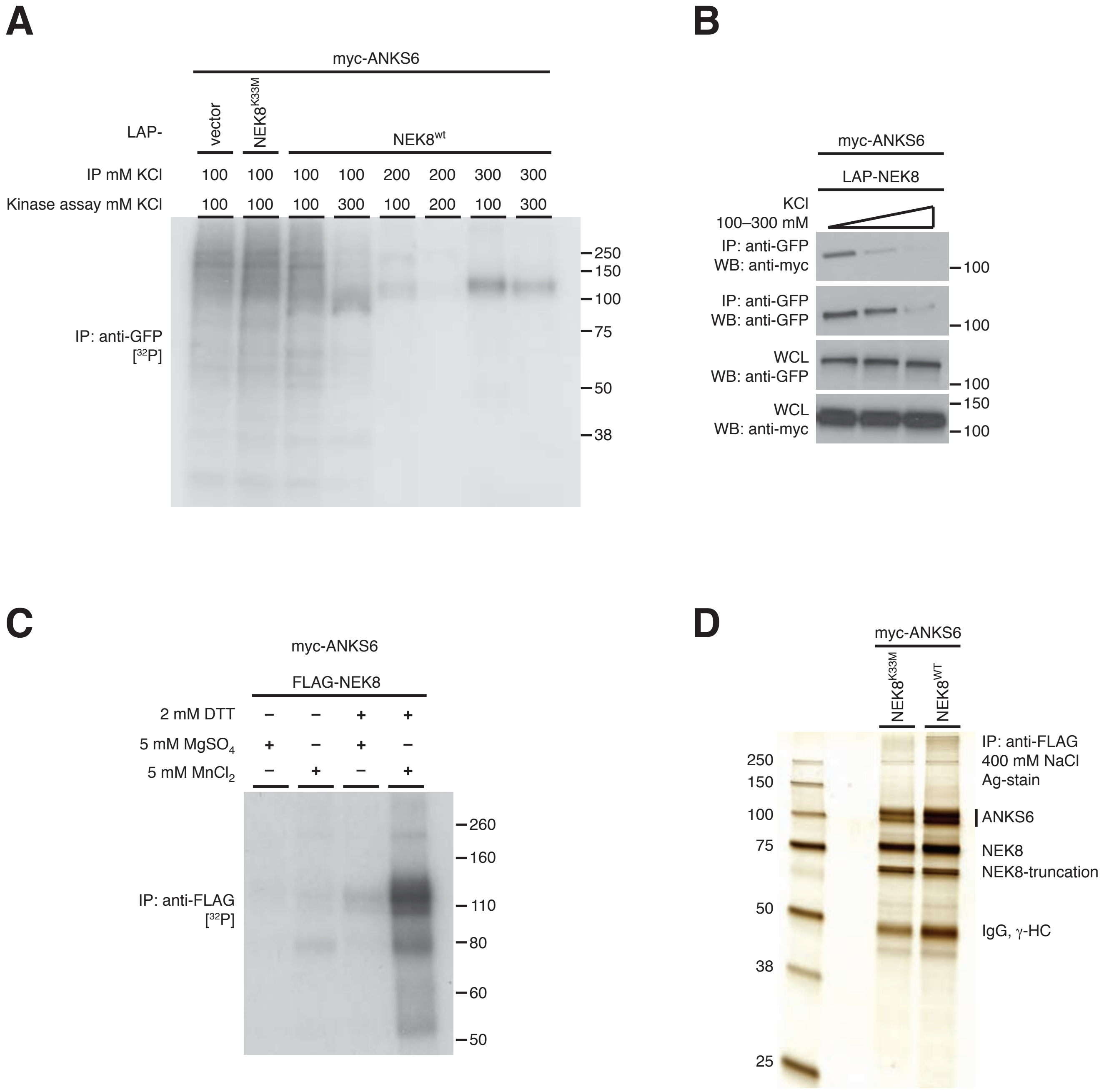
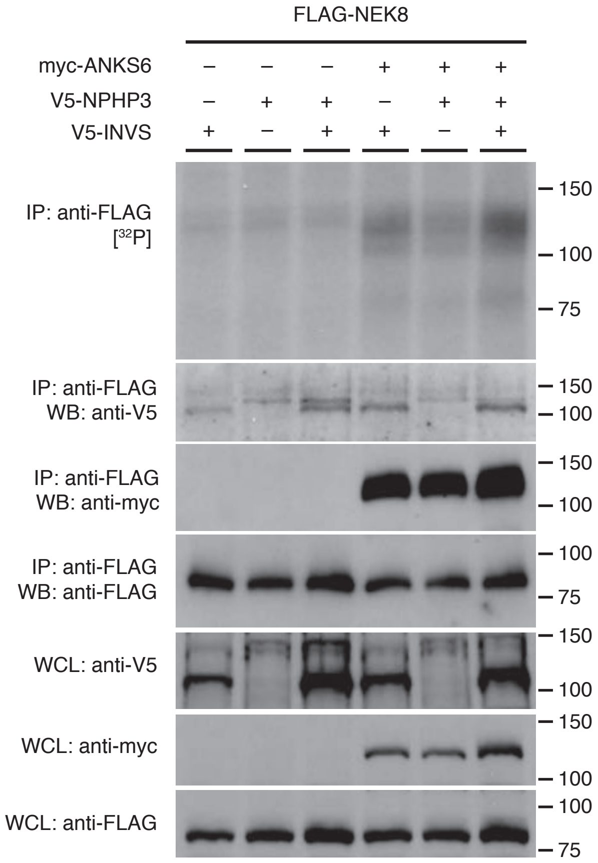
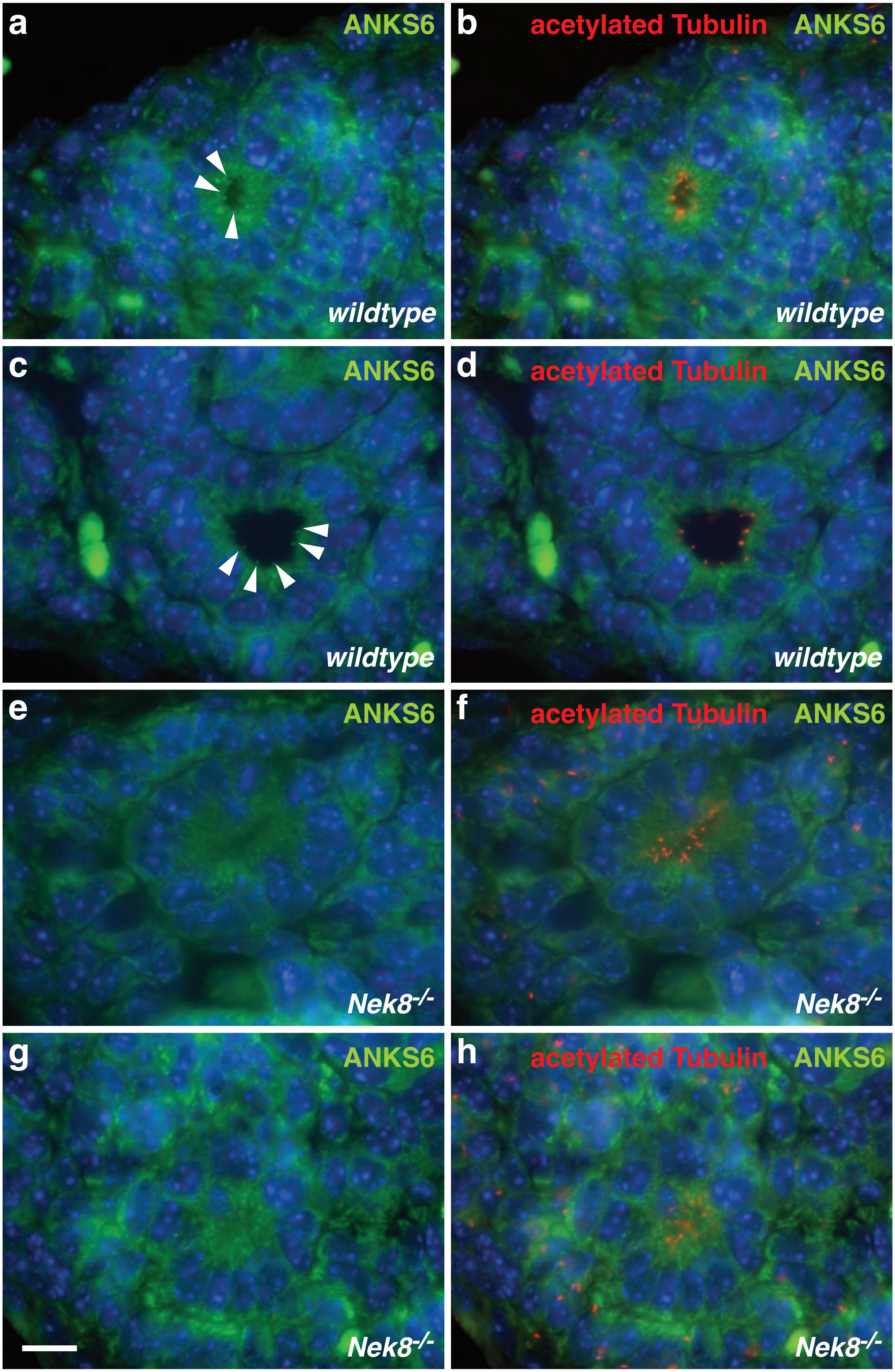


Supplementary Figure 1 | NEK8/ANKS6 kinase activity conditions

A, B – Higher ionic strength conditions interfere with efficient IP of LAP-NEK8 with anti-GFP antibody (B), but also permit higher specificity of phosphorylation reactions (A). Note that LAP-NEK8 and myc-ANKS6 appear at about the same molecular weight. Low-salt conditions are favorable for the kinase reaction itself, but result in much less specific phosphorylation. Importantly, nonspecific copurifying kinase activity produces an autoradiographic background in the case of empty vector or the “kinase-dead” NEK8^{K33M} allele (1st and 2nd lane) that is indistinguishable from wildtype kinase activity with IP and kinase assay salt concentrations of 100 mM KCl. C – NEK8/ANKS6 kinase activity is higher with Mn^{2+}, as compared to Mg^{2+} and strongly depends on the presence of reducing conditions. D – FLAG-NEK8 wildtype and K33M coprecipitate with myc-ANKS6. Cell lysis, IP and washing at 400 mM NaCl gives very clean results, as demonstrated by silver staining. The major bands, as analyzed by mass spectrometry, represent ANKS6, NEK8, a coprecipitating intracellular NEK8 truncation, and the immunoglobulin used for IP.

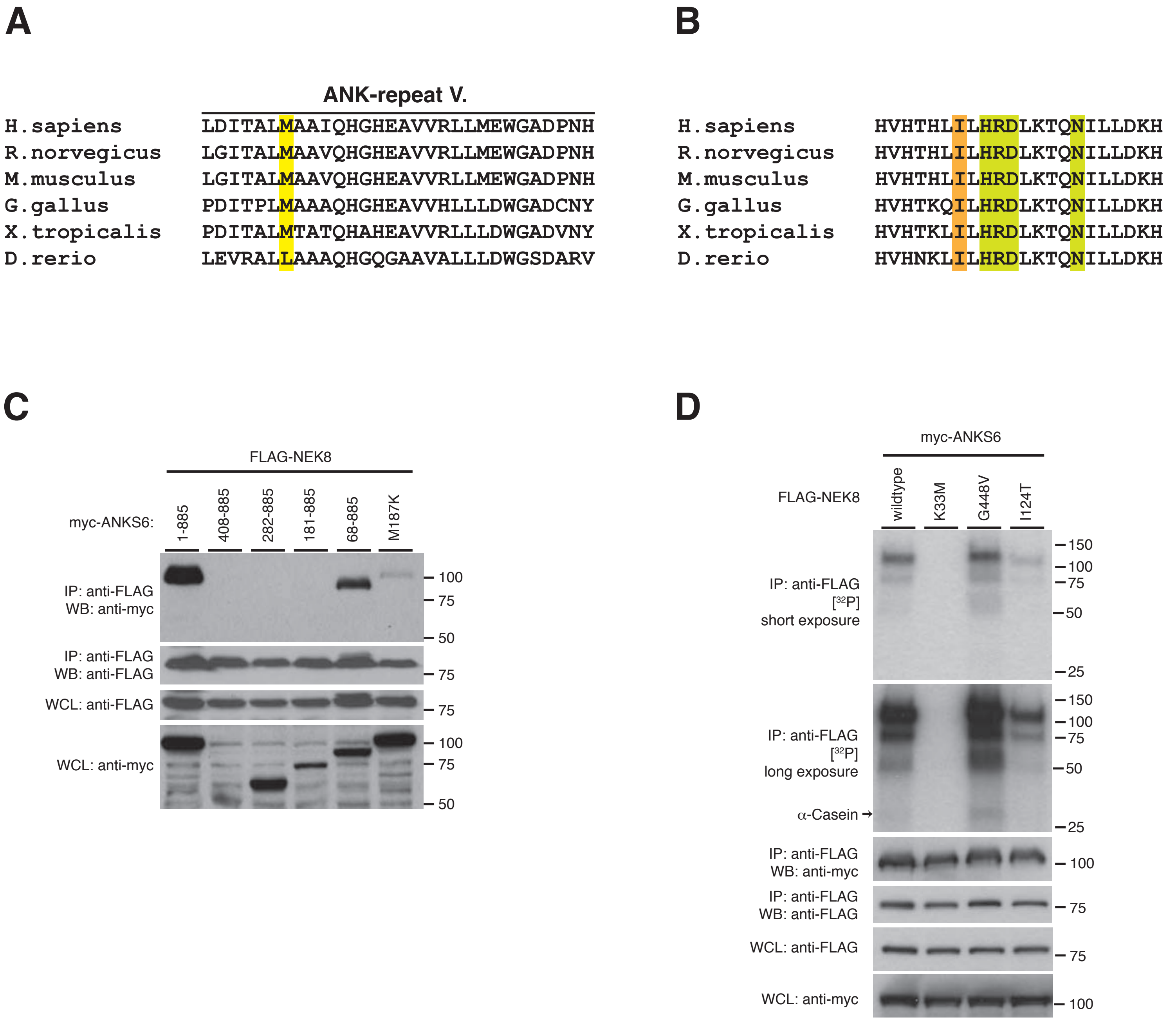


Supplementary Figure 2 | ANKS6 is unique within the NEK8/ANKS6/INVS/NPHP3 complex to stimulate NEK8 phosphorylation activity and serve as a kinase substrate
Coexpression, IP and kinase assays of NEK8 in combination with all other IC components reveals an exclusive dependency on ANKS6 for activation of phosphorylation activity. Significant phosphorylation of inversin or NPHP3 cannot be clearly identified. Note that cell lysis and IP conditions require an ionic strength of not higher than 100 mM NaCl in order to recover the entire complex, while most other lysis and IP protocols presented here employ 400 mM NaCl. The higher background in the autoradiogram is due to this adjustment (compare also Supplementary Fig. 1a).

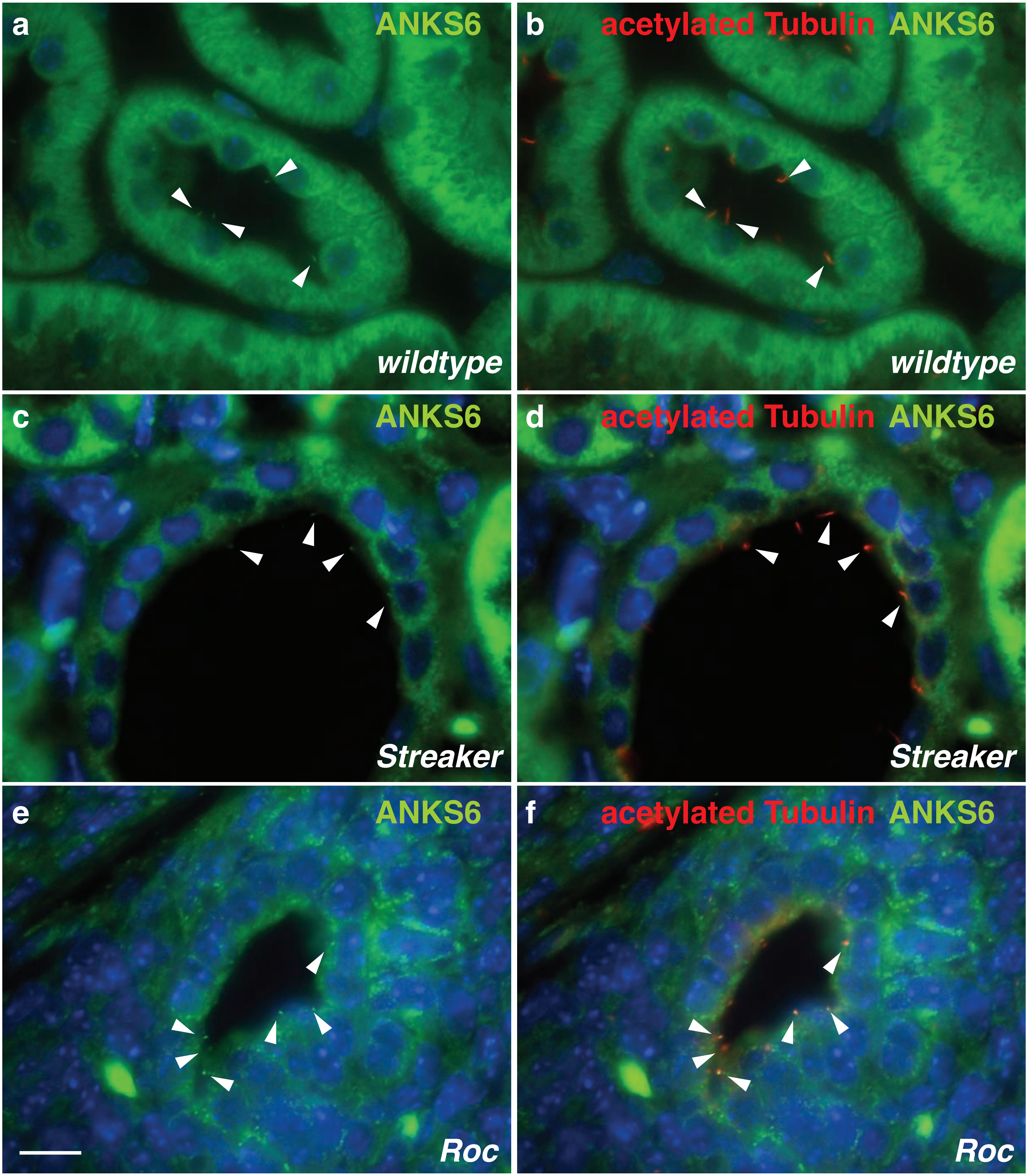

a
ANKS6
wildtype
b
acetylated Tubulin
ANKS6
wildtype
c
ANKS6
wildtype
d
acetylated Tubulin
ANKS6
wildtype
e
ANKS6
Nek8-/-
f
acetylated Tubulin
ANKS6
Nek8-/-
g
ANKS6
Nek8-/-
h
acetylated Tubulin
ANKS6
Nek8-/-

Supplementary Figure 3 | Fluorescence immunohistochemistry of ANKS6 in neonatal wildtype and Nek8$^{-/-}$ mutant kidneys

Native ANKS6 is only detected in a dot-like appearance in the proximal segment of renal tubular epithelial cilia, corresponding to the localization of the ciliary inversin compartment. This pattern is only observed in wildtype kidney sections (a, c; green labels ANKS6), and could not be detected in kidney sections of Nek8$^{-/-}$ mutants (e, g). Counterstaining against acetylated tubulin allows identification of cilia in the same sections (b, d, f, h: red labels cilia). Scale bar: 5 μm.



Supplementary Figure 4 | Alignment of mutant ANKS6 and NEK8 sequences
A – The M187K mutation (highlighted in yellow) is located within the ankyrin repeat V of ANKS6. B – The I124T mutation (highlighted in orange) is located within the kinase domain of NEK8. The invariant catalytic loop HRD-motif and asparagine are highlighted in green. C – Direct comparison of ANKS6 truncations and M187K mutation in co-IP with NEK8. The ANKS6(68-885) variant shows reduced binding to NEK8, as compared to the wildtype; the binding of ANKS6(M187K) is even more reduced. D – Direct comparison of kinase activities of wiltype, K33M, G448V^{jck} and I124T^{roc} mutants of NEK8, in presence of ANKS6. K33M serves as a negative control. There are no significant differences between wildtype and G448V, but again, a striking reduction in kinase activity of I124T. Mild background kinase activity of I124T can be visualized after prolonged autoradiography exposure.


a
ANKS6
wildtype
b
acetylated Tubulin ANKS6
wildtype
c
ANKS6
Streaker
d
acetylated Tubulin ANKS6
Streaker
e
ANKS6
Roc
f
acetylated Tubulin ANKS6
Roc

Supplementary Figure 5 | Fluorescence immunohistochemistry of ANKS6 in neonatal wildtype, Streaker and Roc mutant kidneys
Native ANKS6 is detected in a dot-like appearance in the proximal segment of renal tubular epithelial cilia, corresponding to the localization of the ciliary inversin compartment. This pattern is observed in all examined mutants (a, c, e; green labels ANKS6); counterstaining against acetylated tubulin allows identification of cilia in the same sections (b, d, f: red labels cilia). Scale bar: 10 μm.

Czarnecki PG et al., Supplementary Figure 6

Fig 1C

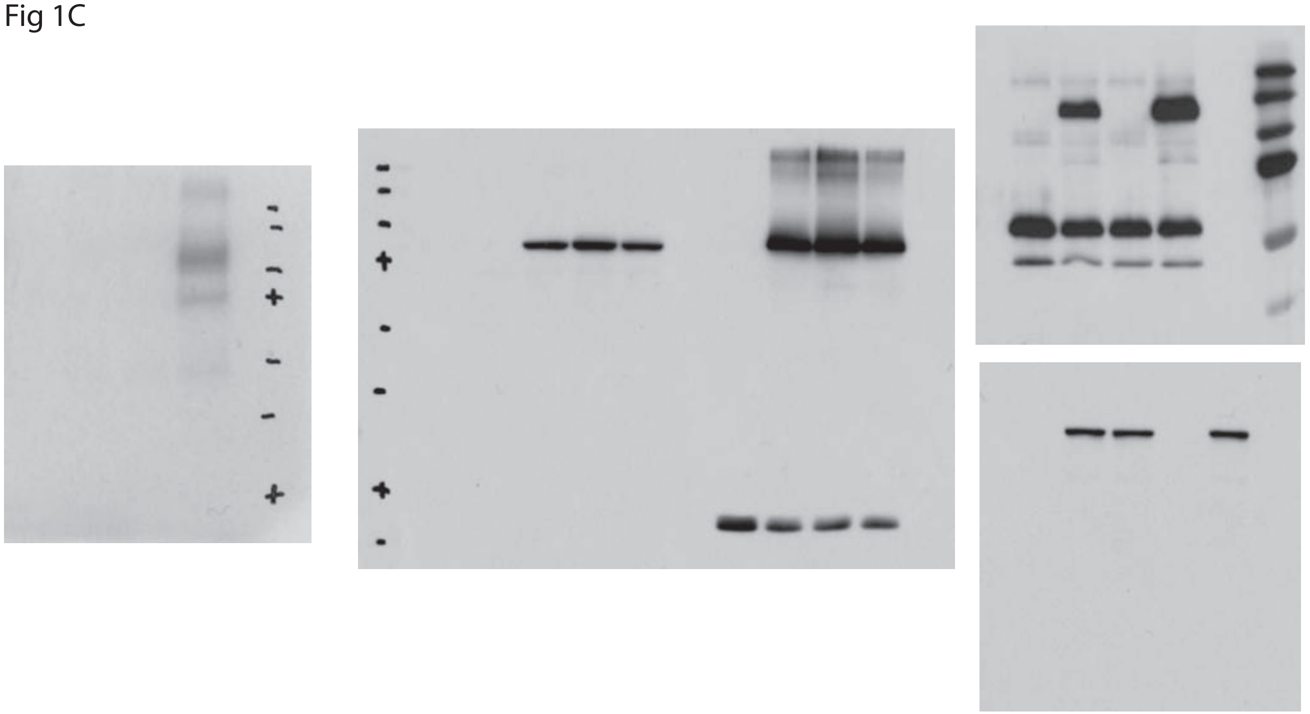


Fig 2B

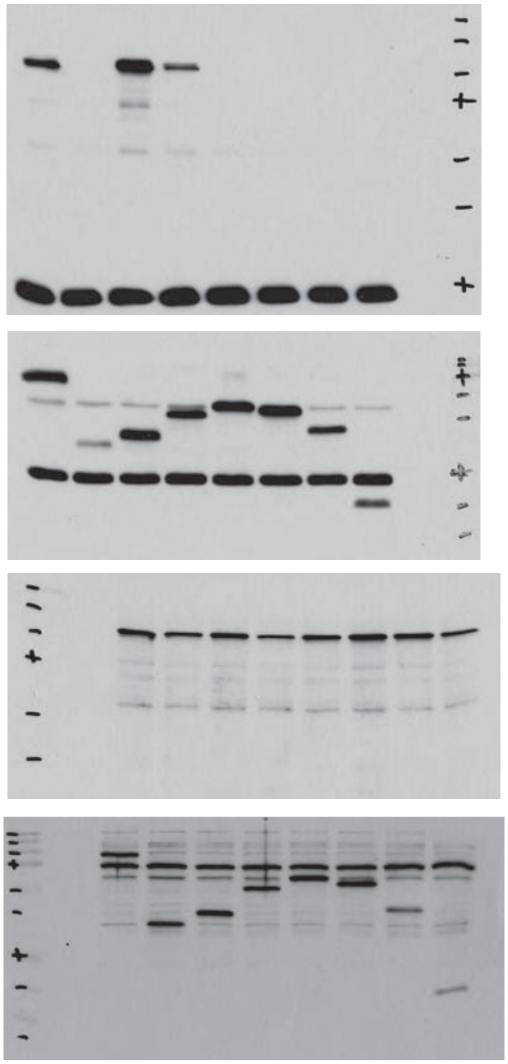


Fig 2C

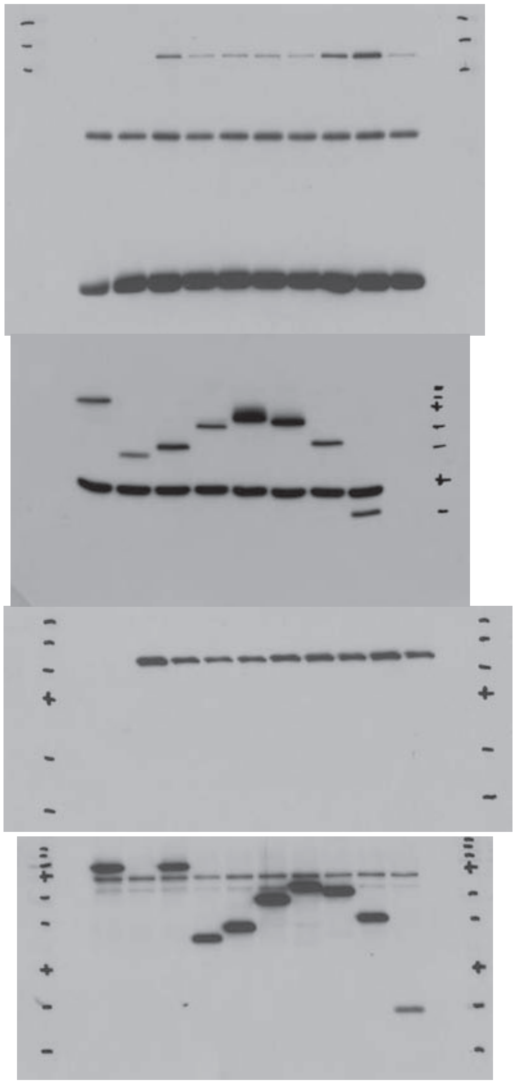
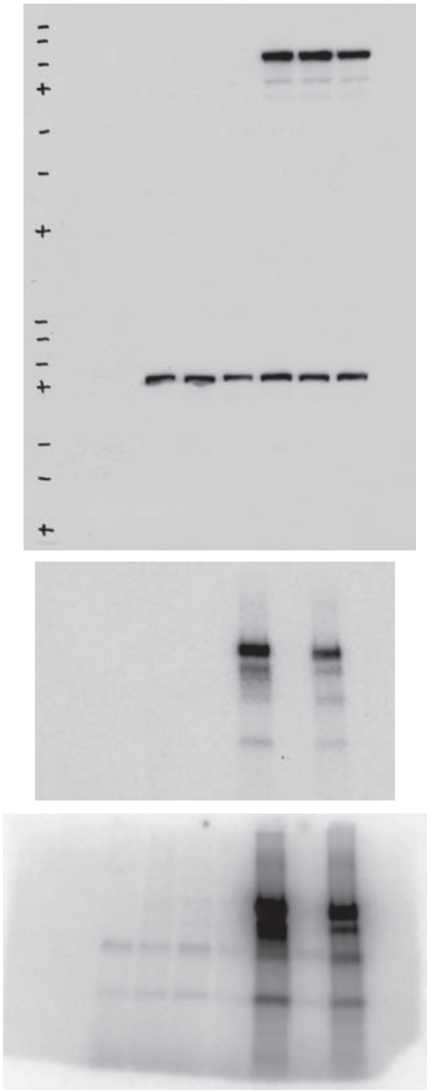


Fig 1D



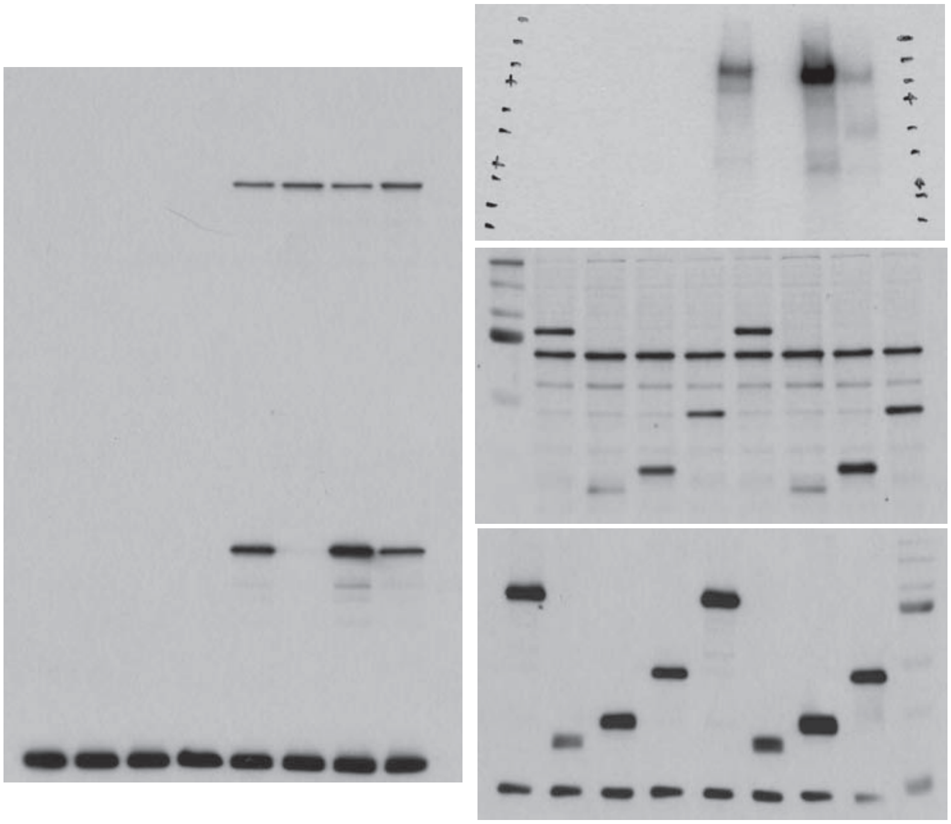


Fig. 2D

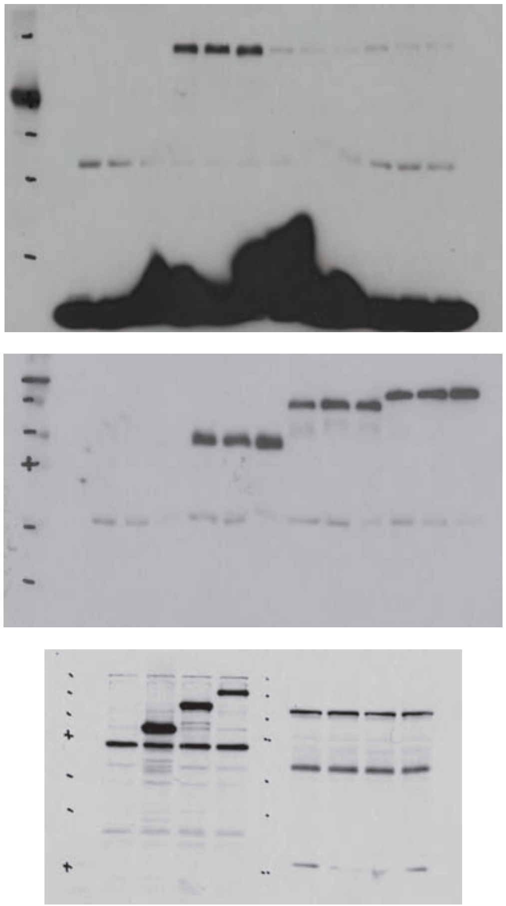


Fig 2E

Fig 6E

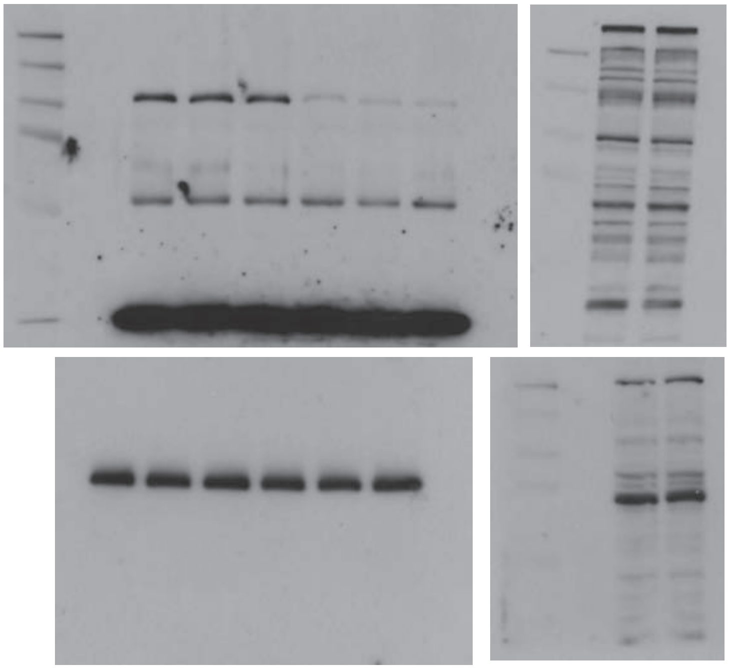
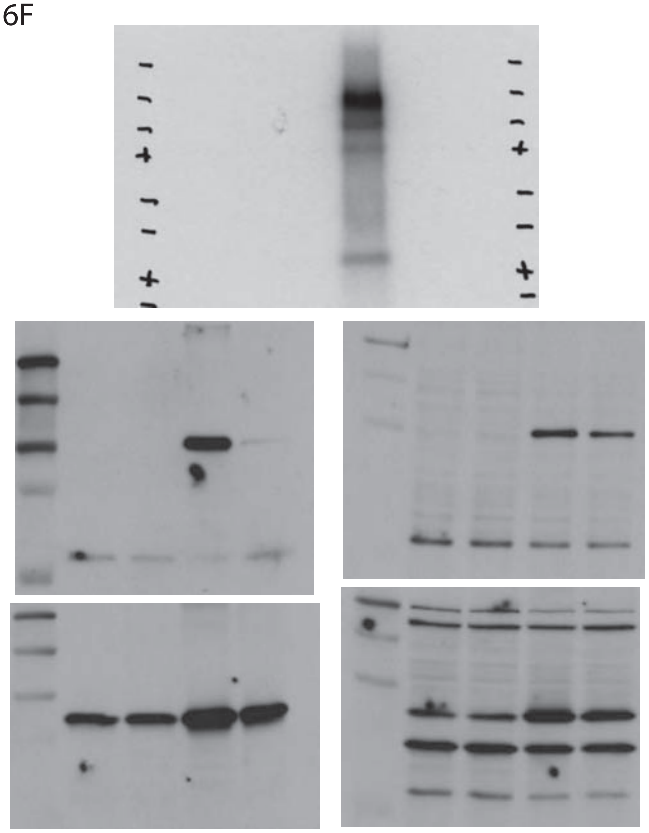


Fig 6F



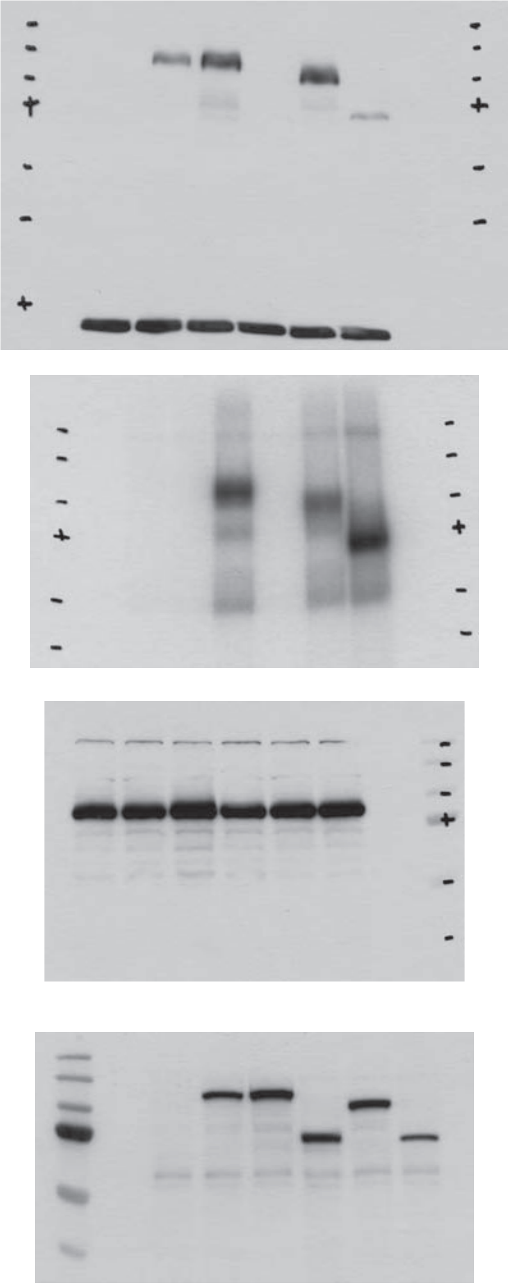


Fig 3B

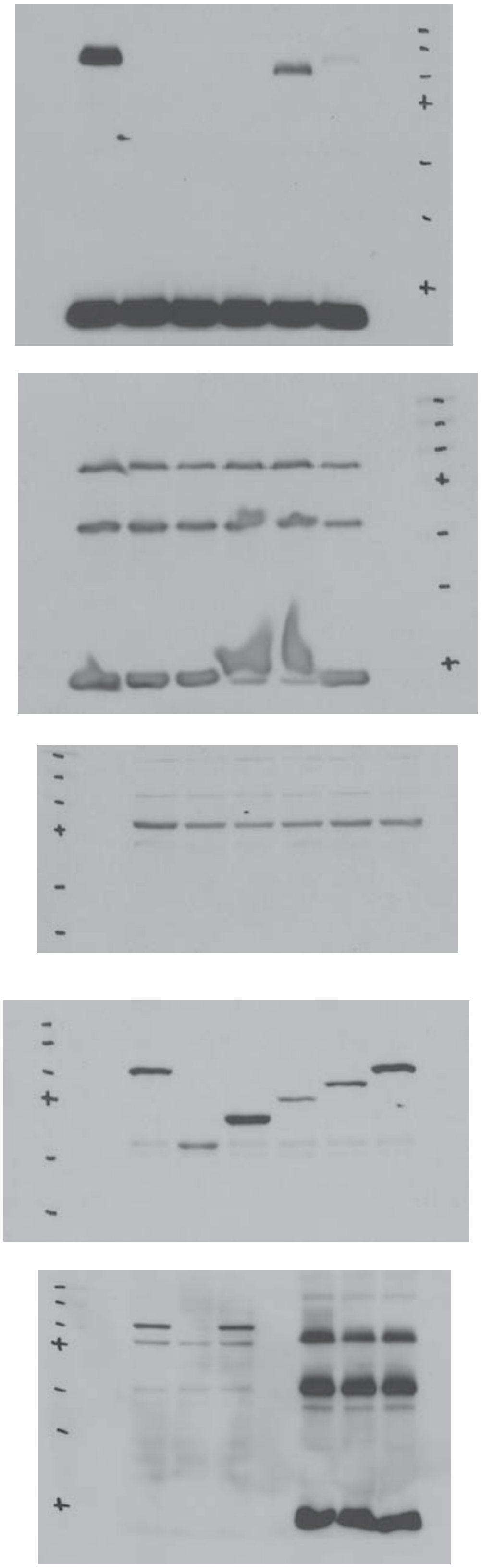


Fig 3C/6D

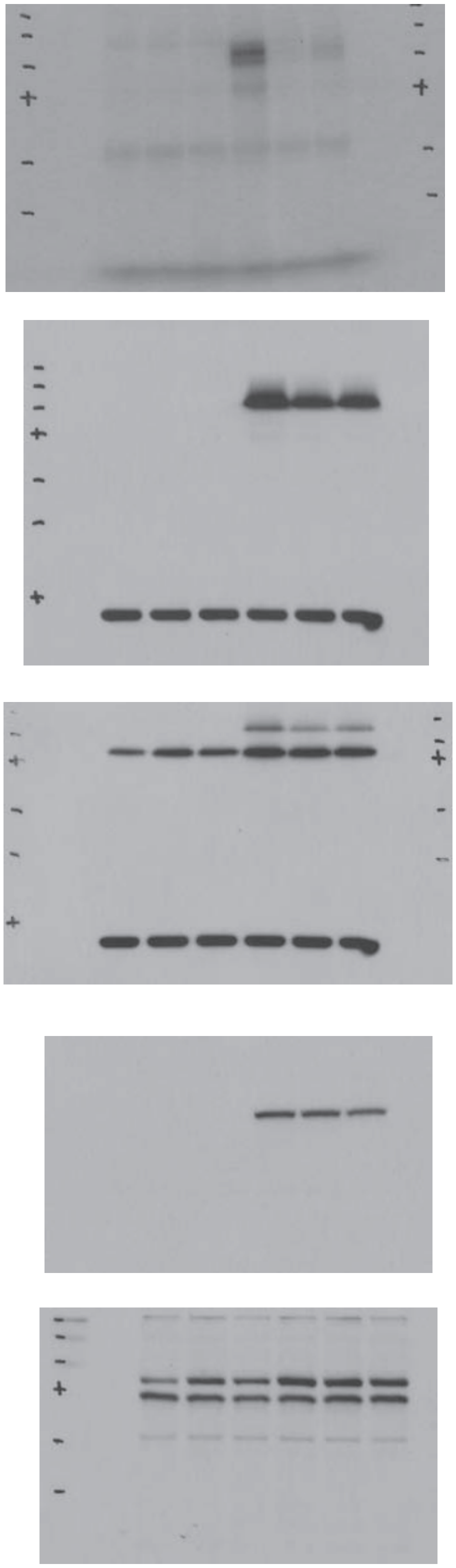


Fig 6H

	Left	Right	Midline	n	**% laterality defect**
Uninjected	34	3	0	37	**8**
ANKS6-MO1	34	17	1	52	**35**
Uninjected	41	3	4	48	**15**
NEK8 ATG MO	31	15	8	54	**43**

Supplementary Table 1 | Heart looping phenotypes in zebrafish ANKS6 and NEK8 morphants

Zebrafish ANKS6 and NEK8 morphants exhibit defective L/R-asymmetry determination. Heart looping laterality was observed after in situ hybridization (http://zfin.org/ZFIN/Methods/ThisseProtocol.html) for the cardiac marker *cmlc2* at 48 hours post fertilization, and after injection of 250 μM of the respective morpholino (MO; GeneTools, Philomath, OR) at the one-cell-stage. Zebrafish were maintained according to established IACUC protocols. Please refer to Supplementary Table 4 for morpholino sequences.

	Situs	Heart	Loop	Arch	Atria	OFT[2]	Septal Defects[3]	IVC[4]	Van[5] Praagh	Epart[6] Bronch	Lung	Abdm Situs[7]	Liver	Stom	Spleen	Pancr
1	HTX	Left	D	Left	RAI	D-TGA	AVSD	Dual	S,D,D	LI	RI (4R/3L)	Dsc	Nml	Right	Right	Right
2	HTX	Right	L	Right	Inv	D-TGA	None	Left	I,L,L	LI	RI (4R/3L)	Inv	Inv	Right	Right	Right
3	HTX	Right	D	Left	Inv	D-TGA	AVSD, mVSD	Left	I,D,D	Right	RI (4R/3L)	Dsc	Nml	Right	Right	Right
4	HTX	Right	L	Right	Inv	DOmRV	pmVSD	Left	I,L,L	Left	Inv (2R/3L)	Nml	Nml	Left	Left	Left
5	HTX	Right	L	Right	Inv	D-TGA	AVSD	Right	I,L,L	Left	RI (4R/3L)	Nml	Nml	Left	Left	Left
6	HTX	Right	L	Right	Inv	DOmRV	AVSD, pm/mVSD	Left	I,L,L	Left	Inv (2R/3L)	Dsc	Nml	Right	Right	Right
7	HTX	Right	L	Double	Inv	L-TGA	mVSD	Right	I,L,L	Left	Inv (2R/3L)	Inv	Inv	Right	Right	Right
8	HTX	Right	D	Right	RAI	D-TGA	AVSD o+mVSD	Dual	A,D,D	RI	Inv (2R/3L)	Nml	Nml	Left	Left	Left
9	HTX	Left	D	Left	Inv	D-TGA	AVSD	Left	A,D,D	Right	RI (4R/3L)	Inv	Inv	Right	Right	Right
10	HTX	Right	L	Right	Inv	oAo	mVSD	Left	I,L,I	Left	Inv (2R/3L)	Dsc	Nml	Right	Right	Right
11	HTX	Right	L	Right	Inv	DOmRV	pmVSD	Right	I,L,L	Left	RI (4R/3L)	Nml	Nml	Left	Left	Left
12	HTX	Right	L	Right	RAI	L-TGA	AVSD	Dual	A,L,L	LI	RI (4R/3L)	Dsc	Inv	Left	Left	Left
13	HTX	Left	D	Left	ND	Nml	ND	Right	ND	ND	Nml (3R/2L)	Dsc	Nml	left	Right	Right
14	HTX	Right	L	Right	RAI	Inv	AVSD, mVSD	Dual	A,L,I	Left	LI (1R/1L)	Nml	Nml	Left	Left	Left
15	HTX	Right	L	Right	RAI	L-TGA	pmVSD	Dual	A,L,L	Left	LI (1R/2L)	Inv	Inv	Right	Right	Right
16	HTX	Left	D	Left	RAI	D-TGA	ND	Right	A,D,D	Right	RI (4R/3L)	Dsc	Nml	Left	Left	Left
17	HTX	Left	D	Left	Inv	D-TGA	AVSD, pmVSD	Left	I,L,L	LI	RI (4R/3L)	Inv	Inv	Right	Right	Right
18	HTX	Right	D	Left	RAI	D-TGA	AVSD	Dual	A,D,D	LI	RI (4R/3L)	Nml	Nml	Left	Left	Left
19	HTX	Left	D	Left	Nml	Nml	None	Middle	S,D,S	Left	Am (3R/1L)	Dsc	Nml	Right	Right	Right
20	HTX	Left	D	Left	Nml	Nml	None	Right	S,D,S	ND	Nml (3R/2L)	Inv	Inv	Right	Right	Right
21	SIT	Right	L	Right	Inv	Inv	None	Left	I,L,L	Left	Inv (2R/3L)	Inv	Inv	Right	Right	Right
22	SIT	Right	L	Right	Inv	Inv	None	Left	I,L,I	Left	Inv (2R/3L)	Inv	Inv	Right	Right	Right
23	SIT	Right	L	Right	Inv	Inv	None	Right	I, L, I	Left	Inv (2R/3L)	Inv	Inv	Right	Right	Right
24	SIT	Right	L	Right	ND	Inv	ND	Left	ND	ND	Inv (2R/3L)	Inv	Inv	Right	Right	Right
25	SIT	Right	L	Right	Inv	Inv	None	Left	I,L,I	Left	Inv (2R/3L)	Inv	Inv	Right	Right	Right
26	SIT	Right	L	Right	Inv	Inv	None	left	I L,I	Left	Inv (2R/3L)	Inv	Inv	Right	Right	Right
27	SIT	Right	L	Right	Inv	Inv	None	ND	I,L,I	ND	Inv (2R/3L)	Inv	Inv	Right	Right	Right
28	SIT	Right	L	Right	Inv	Inv	None	Left	I,L,I	ND	Inv (2R/3L)	Inv	Inv	Right	Right	Right
29	SIT	Right	L	Right	Inv	Inv	None	Left	I,L,I	ND	Inv (2R/3L)	Inv	Inv	Right	Right	Right
30	SS	Left	D	Left	Nml	Nml	ND	Right	S,D,S	ND	Nml (3R/2L)	Nml	Nml	Left	Left	Left
31	SS	Left	D	Left	Nml	Nml	ND	Right	S,D,S	ND	Nml (3R/2L)	Nml	Nml	Left	Left	Left
32	SS	Left	D	Left	Nml	Nml	ND	Right	S,D,S	ND	Nml (3R/2L)	Nml	Nml	Left	Left	Left
33	SS	Left	D	Left	Nml	Nml	ND	Right	S,D,S	ND	Nml (3R/2L)	Nml	Nml	Left	Left	Left
34	SS	Left	D	Left	Nml	Nml	None	Dual	S,D,S	Right	Nml (3R/2L)	Nml	Nml	Left	Left	Left
35	SS	Left	D	Left	Nml	Nml	None	Right	S,D,S	ND	Nml (3R/2L)	Nml	Nml	Left	Left	Left
36	SS	Left	D	Left	Nml	Nml	None	Right	S,D,S	ND	Nml (3R/2L)	Nml	Nml	Left	Left	Left
37	SS	Left	D	Left	Nml	Nml	None	Right	S,D,S	ND	Nml(3R/2L)	Nml	Nml	Left	Left	Left
38	SS	Left	D	Left	Nml	Nml	None	Right	S,D,S	ND	Nml (3R/2L)	Nml	Nml	Left	Left	Left
39	SS	Left	D	Left	Nml	Nml	None	Right	S,D,S	ND	Nml (3R/2L)	Nml	Nml	Left	Left	Left

[1] HTX:heterotaxy, SIT: situs inversus totalis, SS: situs solitus; ND=not determined; None: no defect; Inv: inverted situs; Red fill=mirror symmetric, blue fill =normal, green fill=abnormal; [2]OFT: outflow tract; TGA: transposition of the great arteries, DOmRV: doublet outlet morphological right ventricle; [3]AVSD: atrioventricular septal defect, mVSD: muscular VSD, pmVSD: perimembranous VSD, oVSD: outlet VSD; [4]IVC: inferior vena cava; [5]Van Praagh classification of atrioventricular situs; [6]Epart Bronch: eparterial bronchus branch; [5]Abdm Situs: abdominal situs with combined assessment of stomach, spleen, liver situs. Dsc: discordant; [7]Abdm Situs: abdominal situs including position of stomach (Stom), spleen and pancreas (Pancr). Dsc: discordant.

Supplementary Table 2 | Cardiac and Visceral Organ Situs Phenotype in *Anks6*^{strker} Homozygous Mutants[1]

Cardiac and visceral organ situs phenotypes in *Anks6*^{Strkr} homozygous mutant mice.

	Situs	Heart	Atria	Loop	Aortic Arch	Heart	OFT[2]	Septal Defect[3]	IVC[4]	Van[5] Praagh	Epiart[6] Bronch	Lung	Abdm Situs[7]	Liver	Stom	Spleen	Pancr
1	HTX	Left	ND	D	Left	ND	Nml	ND	Right	S,D,I		Nml (3R/1L)	Dsc	LI (2R/2L)	Right	Right	Right
2	SIT	Right	Inv	L	Right	Inv	Inv	pmVSD, mVSD	Left	I,L I	Left	Inv (1R/4L)	Inv	Inv (2R/1L)	Right	Right	Right
3	HTX	Left	Nml	D	Left	Nml	Nml	pmVSD	Right	S,D,S	Right	Nml (3R/1L)	Dsc	LI (2R/2L)	Right	Right	Right
4	SIT	Right	Inv	L	Right	Inv	Inv	None	Left	I,L,I	Left	Inv (1R/4L)	Inv	Inv (2R/1L)	Right	Right	Right
5	HTX	Left	RAI	D	Left	RAI	DORV	AVSD, mVSD	Dual	A,D,D	Right	LI (1R/2L)	Dsc	Nml (1R/2L)	Right	Right	Right
6	HTX	Left	RAI	D	Left	RAI	Nml	mVSD	Dual	A,D,S	Right	RI (2R/1L)	Dsc	LI (2R/2L)	Right	Right	Right
7	SS	Left	ND	D	Left	ND	Nml	ND	Right	S,D,S	ND	Nml(3R/1L)	Nml	Nml (1R/2L)	left	left	left
8	SS	Left	ND	D	Left	ND	Nml	ND	Right	S,D,S	ND	Nml (3R/1L)	Nml	Nml (1R/2L)	left	left	left
9	HTX	Left	ND	D	Left	ND	Nml	ND	Right	S,D,S	ND	LI (1R/1L)	Nml	Nml (1R/2L)	left	left	middle

[1] HTX:heterotaxy, SIT: situs inversus totalis, SS: situs solitus; ND=not determined; None: no defect; Inv: inverted situs; Red fill=mirror symmetric, blue fill =normal, green fill=abnormal;
[2] OFT: outflow tract; TGA: transposition of the great arteries, DOmRV: doublet outlet morphological right ventricle
[3] AVSD: atrioventricular septal defect, mVSD: muscular VSD, pmVSD: perimembranous VSD, oVSD: outlet VSD
[4] IVC: inferior vena cava
[5] Van Praagh classification of atrioventricular situs.
[6] Epart Bronch: eparterial bronchus branch; [5]Abdm Situs: abdominal situs with combined assessment of stomach, spleen, liver situs. Dsc: discordant.
[7] Abdm Situs: abdominal situs including position of stomach (Stom), spleen and pancreas (Pancr). Dsc: discordant.

Supplementary Table 3 | Cardiac and Visceral Organ Situs Phenotype in *Nek8*^{Roc} Homozygous Mutants[1]
Cardiac and visceral organ situs phenotypes in *Nek8*^{Roc} homozygous mutant mice.

Primer	Sequence 5’ to 3’
mmNek8-BamHI-fwd	CGGGATCCATGGAGAAGTACGAGCGAATCCGAG
mmNek8-NotI-rev	ATAGTTTAGCGGCCGCTAGGGGGGAACTGGTTCAT
mmNek8-258-NotI-rev	ATAGTTTAGCGGCCGCTAGCAGAGGGGCTGCGC
mmNek8-295-NotI-rev	ATAGTTTAGCGGCCGCTACCTGCTCCCTGTGCTGCCAG
mmNek8-415-NotI-rev	ATAGTTTAGCGGCCGCTATCGGTCTGTCAAGCAAGC
mmNek8-258-BamHI-fwd	CGGGATCCATCCGGGCCCTACTCAAC
mmNek8-295-BamHI-fwd	CGGGATCCAGGGCCACCAGTGCCCGAT
mmNek8-415-BamHI-fwd	CGGGATCCGGCATTATCATGACGTTCGG
mmNek8-K33M-fwd	GGTGATCCTCATGCAGATCCCAGT
mmNek8-K33M-rev	ACTGGGATCTGCATGAGGATCACC
mmNek8-G448V-fwd	AAGCCTTGCTGGTCTATGAGATGGT
mmNek8-G448V-rev	ACCATCTCATAGACCAGCAAGGCTT
mmNek8-I124T-fwd	ACACACATCTCACCCTGCATCGGG
mmNek8-I124T-rev	CCCGATGCAGGGTGAGATGTGTGT
rnAnks6-BamHI-fwd	CGGGATCCATGGGCGAGGGCGCG
rnAnks6-XbaI-rev	TGCTCTAGACTACCTCCTGCTCGACACTGTTTCTTCTGGCCTTACC
rnAnks6-68-BamHI-fwd	CGGGATCCGCGGGCAACTCGGCGC
rnAnks6-181-BamHI-fwd	CGGGATCCCTGGGCATCACAGCCCTG
rnAnks6-282-BamHI-fwd	CGGGATCCAAGAGGCGACCTGATATTTTCC
rnAnks6-408-BamHI-fwd	CGGGATCCTGCATGCAGGTGAATAAGGACCG
rnAnks6-607-XbaI-rev	TGCTCTAGACTAGAATTTGACTGGCCTGCCAGCAGC
rnAnks6-758-XbaI-rev	TGCTCTAGACTAGTGGGACGAGGAGGAAGAC
rnAnks6-M187K-fwd	CACAGCCCTGAAGGCTGCCGTCC
rnAnks6-M187K-rev	GGACGGCAGCCTTCAGGGCTGTG
hsINVS-BamHI-fwd	CGGGATCCATGAACAAGTCAGAGAACCTG
hsINVS-NotI-rev	ATAGTTTAGCGGCCGCTAAGGTTTTGTTTTGTTTTTTGGC
hsNPHP3-BamHI-fwd	CGGGATCCATGGGGACCGCCTCGT
hsNPHP3-NotI-rev	ATAGTTTAGCGGCCGCTACCTTTGTCCTTGCTGAAG

Morpholino	Sequence 5’ to 3’
drANKS6-MO1	TCAGCGCCGTGTTTCCATCCTCATC
drNEK8-ATG-MO	CTTCTCATACTTCTCCATGTTTTCG

Supplementary Table 4 | Primer and morpholino sequences