Supplementary materials

Disruption of *Gen1* causes congenital anomalies of the kidney and urinary tract in mice

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Materials and Methods

VUR test

Adult mice were dissected using an anterior midline incision to expose kidneys and the urinary tract. The bladder was punctured with a 25-gauge needle to manually inject methylene blue (1 mg/ml in PBS) at a rate of 100 μl/min until dye exited through the urethra.

BUN and creatinine test

Serum BUN and creatinine tests were performed using VetScan VS2 (catalog No. 500-0038-12), according to the manufacturer's instructions.

Kidney primordial culture

Hoxb7/myr-Venus mice were generated as previously described[1] and bred with *Gen1* mutants. Kidney primordia were isolated from *Hoxb7/myr-Venus* positive embryos at E10.75 and cultured with 8 ng/μl recombinant GDNF (R&D systems) for 60 hours as previously described [2].

Plasmids

To generate N-terminal tagged Gen1 plasmids, mouse *Gen1* CDS was amplified from E12.5 embryos through RT-PCR, and cloned into the pGEM-T vector to generate pGEM-T-F1B3. A SalI-ApaI fragment containing the CDS was then cloned between the SalI and ApaI sites of pNTAP-2Flag to form 2Flag-Gen1, while the SalI-KpnI fragment of 2Flag-Gen1 was further cloned between the SalI and KpnI sites of pEGFP-C1 to get GFP-Gen1.

To generate the C-terminal tagged Gen1 plasmids, primers Gen1-CDS-F1 (5'-GCGTCGACATGGGAGTGAATGACTTATGGC-3') and Gen1-CDS-B8 (5'-CTCCCATGGCAGTATTATAAAATCCACTTTGAGT-3') were used to amplify a *Gen1* CDS without the stop codon. The PCR product was then ligated into pGEM-T to form pGEM-T-F1B8. A SalI-NcoI fragment containing the CDS was bluntly ligated into the EcoRV site of pCTAP-2Flag to get Gen1-2Flag.

To generate expression vectors for *Six1, Six2, Six4, Eya1, Foxc1*, and *Pax2*, we first performed RT-PCR to recover target CDS from E11.5 metanephric mesenchyme, then cloned individual CDS between the EcoRI and XhoI sites of pcDNA4-HA to add an HA tag at the N-terminal of the CDS. Resulted plasmids were named as HA-Six1, HA-Six2, HA-Six4, HA-Eya1, HA-Foxc1, and HA-Pax2, respectively. We also used pNTAP-2Flag as the vector for *Six1* to generate a 2x Flag tagged expression vector 2Flag-Six1.

Gdnf-luc2, the luciferase reporter of Six1/Eya1 complex was generated by inserting a 300 bp Xho1-Sma1 fragment composed of 6x consensus SIX1 binding motif (aldolase A MEF3) and a minimal CMV promoter between the Xho1 and EcoRV sites of pGL4.20[luc2-puro] [3].

Legends to Figures

Supplementary Figure 1

Vesicoureteral reflux (VUR) in one-month old *Gen1* mutants. Methylene blue injected in the bladder could be observed in the ureter of homozygotes with VUR (b), but never in wild type mice. Scale bar, 2 mm. BUN (c) and creatinine (d) concentration in the serum of six four-month old wild type and $Gen1^{PB/PB}$ mice was shown as mean ± SEM.

Supplementary Figure 2

Delayed neural tube closure in *Gen1* mutants. (a) Kinky tail defects in homozygous mice. (b) Micro CT images of hemi-vertebrae (arrow) and fused vertebrae (arrowhead) in a homozygous mutant. Scale bar, 10 mm. (c-d) In E9.5, the posterior neuropore (PNP) is closed in the heterozygotes (c), but not in the homozygotes (d). Scale bar, 0.5 mm. (e-f) H&E staining of the paraffin sections along the solid line in (c-d). nt, neural tube. Scale bar, 0.05 mm. (g-h) In E13.5, PNP is closed in both heterozygous (g) and homozygous (h) embryos. Scale bar, 0.2 mm.

Supplementary Figure 3

Generation of $Gen1^{PB}$ revertants. (a) Crossing strategy to induce precise excision of the PB insertion. (b) Number of mice with various kidney and tail morphologies observed in *Gen1* revertants (Rev) and their homozygous littermates.

Supplementary Figure 4

Expression pattern of *Gen1.* RNA *in situ* hybridization of E10.5 embryos revealed weaker *Gen1* expression in the homozygotes (b) than in the wild type littermates (a). Transverse sections showed the neural tube and nephric ducts (arrows). Scale bar, 0.2 mm.

Supplementary Figure 5

Examination of the interaction between GEN1 and different transcription factors. FLAG-tagged GEN1 could not pull down HA-tagged SIX2 (a), FOXC1 (b), EYA1 (c), PAX2 (d), or SIX4 (e) in co-IP.

Supplementary Figure 6
Full-length blots are presented for Figure 4A and B.

Supplementary Figure 7
Full-length blots are presented for Figure 5D.

Table s1
RNA-seq results of important markers for UB formation at E10.5 wild type and *Gen1*$^{PB/PB}$ embryos. Significance was defined as $P < 0.01$ and the absolute value of fold change ($|\log_2|$) > 0.5.

1 Chi, X., Hadjantonakis, A. K., Wu, Z., Hyink, D. & Costantini, F. A transgenic mouse that reveals cell shape and arrangement during ureteric bud branching. *Genesis* **47**, 61-66, doi:10.1002/dvg.20452 (2009).

2 Michos, O. *et al.* Reduction of BMP4 activity by gremlin 1 enables ureteric bud outgrowth and GDNF/WNT11 feedback signalling during kidney branching morphogenesis. *Development* **134**, 2397-2405, doi:10.1242/dev.02861 (2007).

3 Spitz, F. *et al.* Expression of myogenin during embryogenesis is controlled by Six/sine oculis homeoproteins through a conserved MEF3 binding site. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 14220-14225 (1998).

Fig. s1

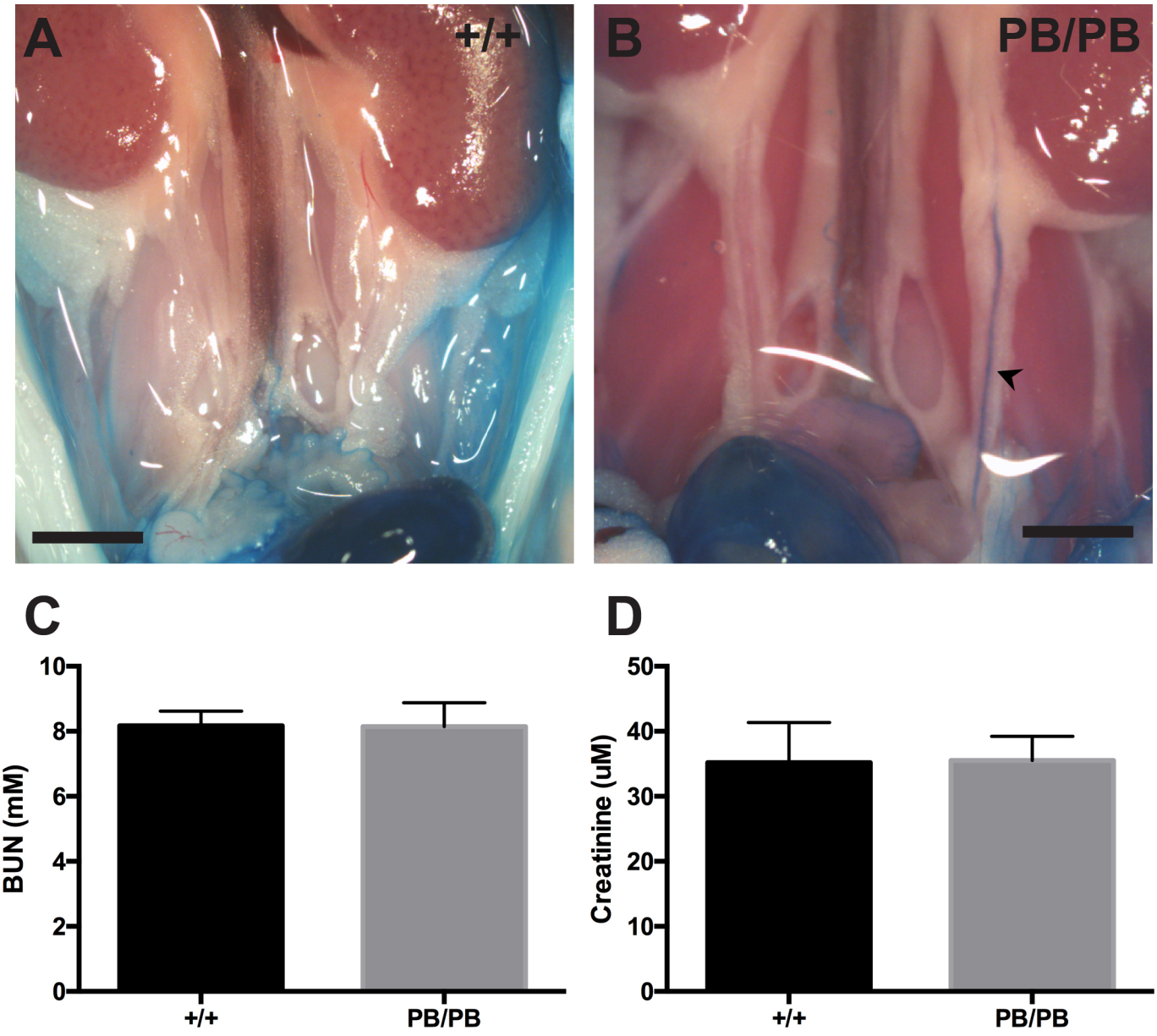

A
+/+
B
PB/PB
C
BUN (mM)
10
8
6
4
2
0
+/+
PB/PB
D
Creatinine (uM)
50
40
30
20
10
0
+/+
PB/PB

Fig. s2

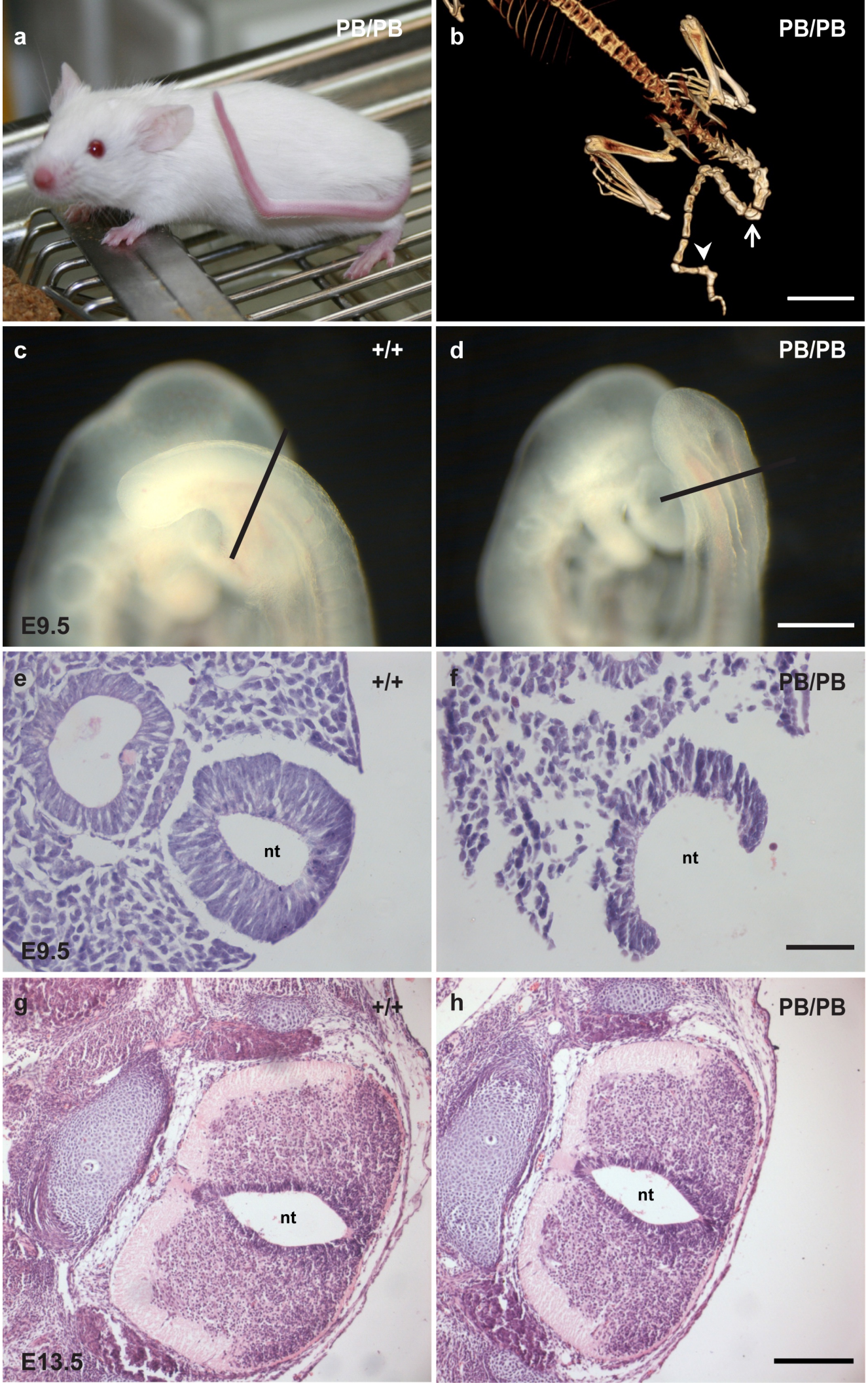
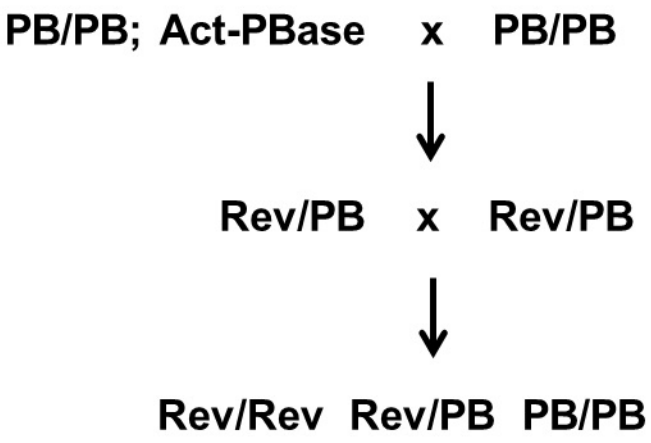


Fig. s3

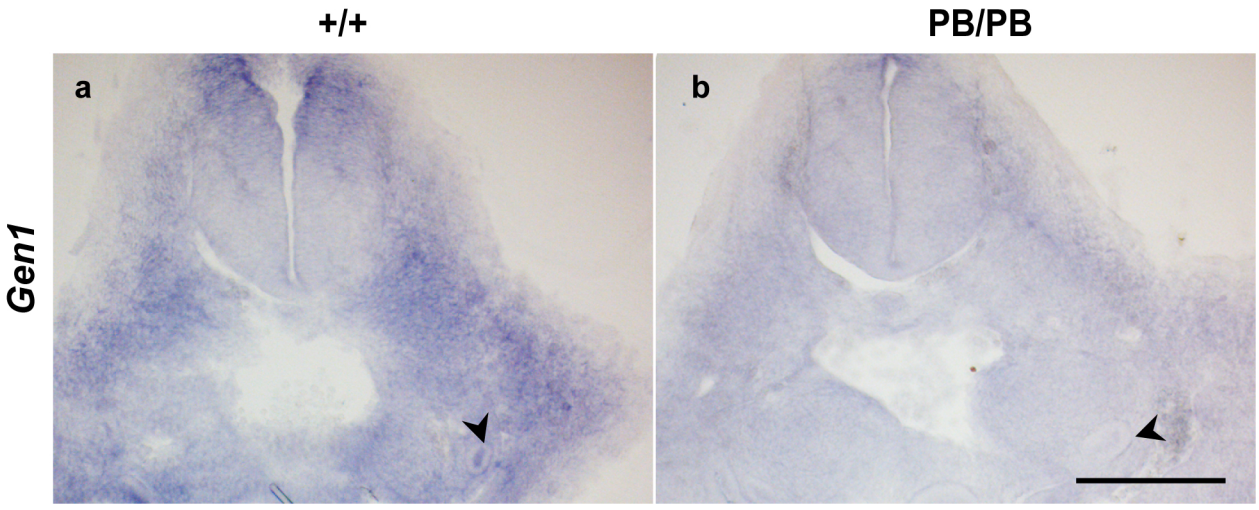
a



b

	Rev/Rev	PB/PB
solitary kidney	0	8
duplex kidney	0	4
hydronephrosis	0	0
kinky tail	0	21
total	25	21

Fig. s4



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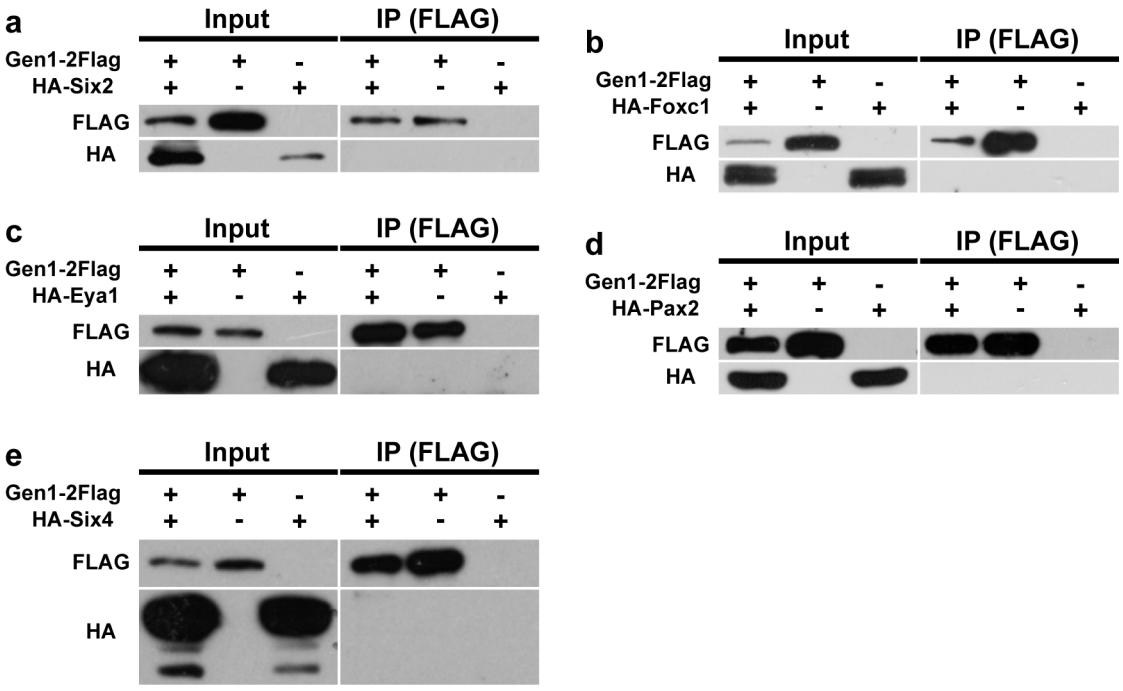
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IP (FLAG)
Gen1-2Flag + + - + + -
HA-Six2 + - + + - +
FLAG
HA
b
Input
IP (FLAG)
Gen1-2Flag + + - + + -
HA-Foxc1 + - + + - +
FLAG
HA
c
Input
IP (FLAG)
Gen1-2Flag + + - + + -
HA-Eya1 + - + + - +
FLAG
HA
d
Input
IP (FLAG)
Gen1-2Flag + + - + + -
HA-Pax2 + - + + - +
FLAG
HA
e
Input
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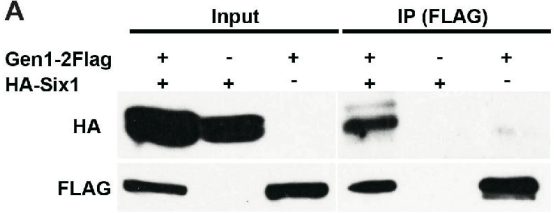
Gen1-2Flag + + - + + -
HA-Six4 + - + + - +
FLAG
HA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Input
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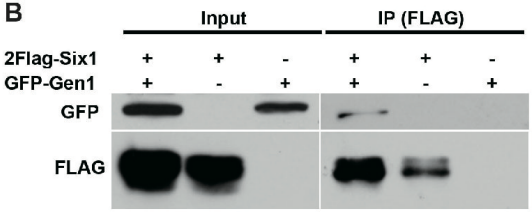
Gen1-2Flag + - + + - +
HA-Six1 + + - + + -
HA
FLAG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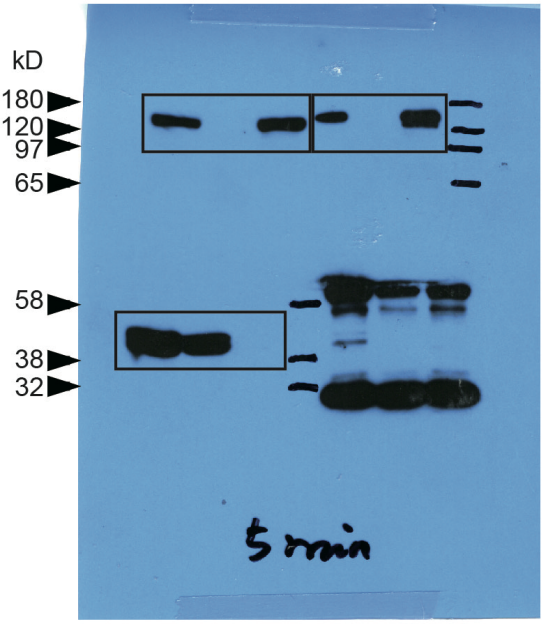
2Flag-Six1 + + - + + -
GFP-Gen1 + - + + - +
GFP
FLAG

Fig. s6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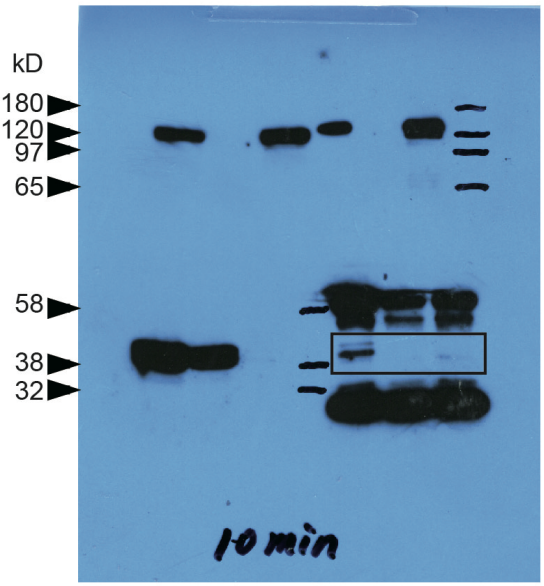
120
97
65
58
38
32
5 min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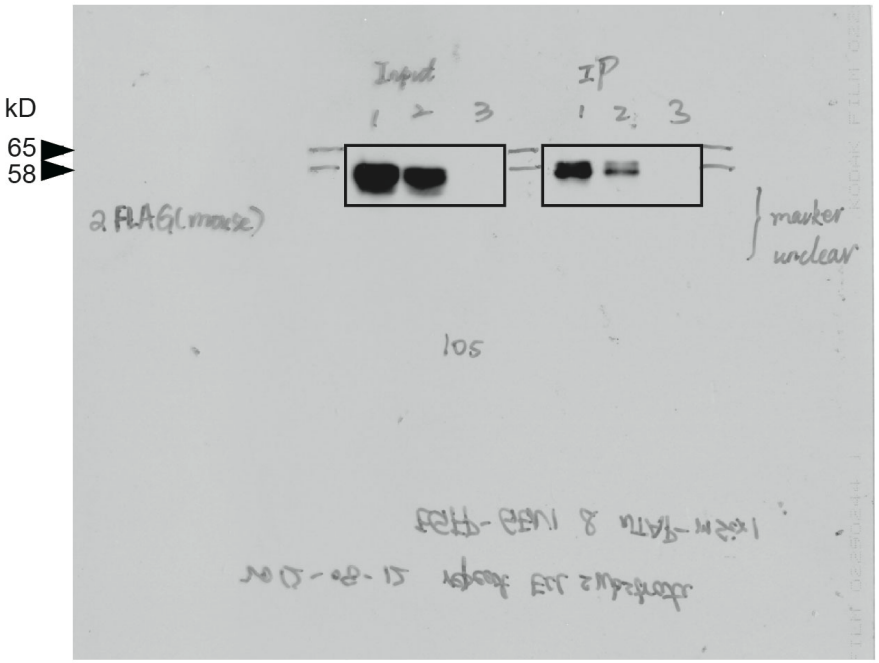
120
97
65
58
38
32
10 min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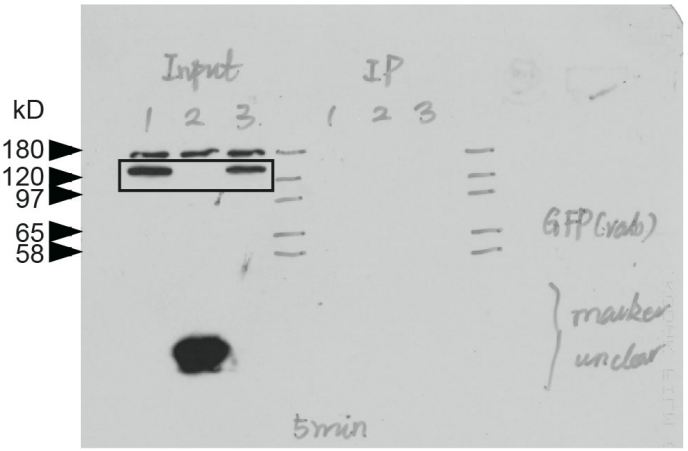
1 2 3
kD
65
58
2 FLAG(mouse)
marker
unclear
los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kD
180
120
97
65
58
GFP(rab)
marker
unclear
5min

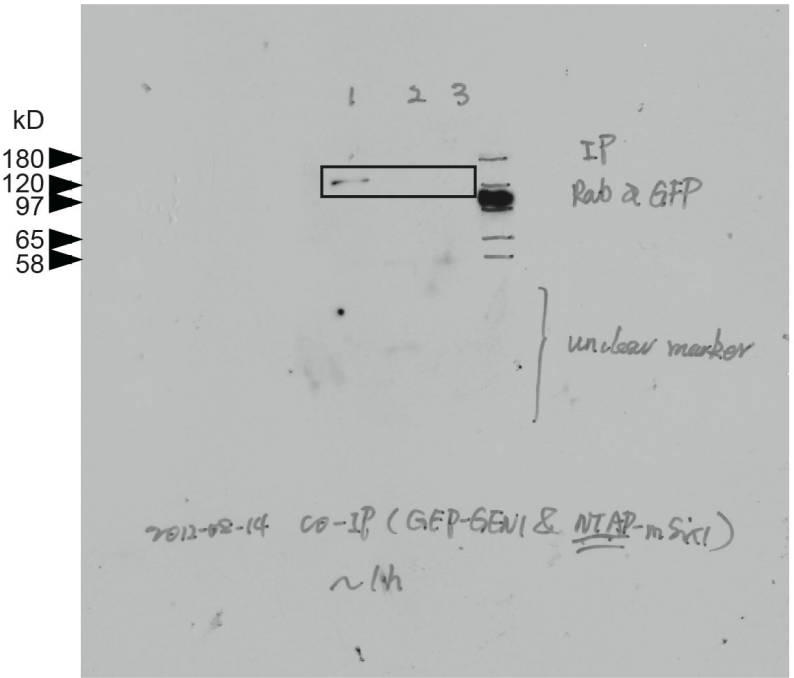
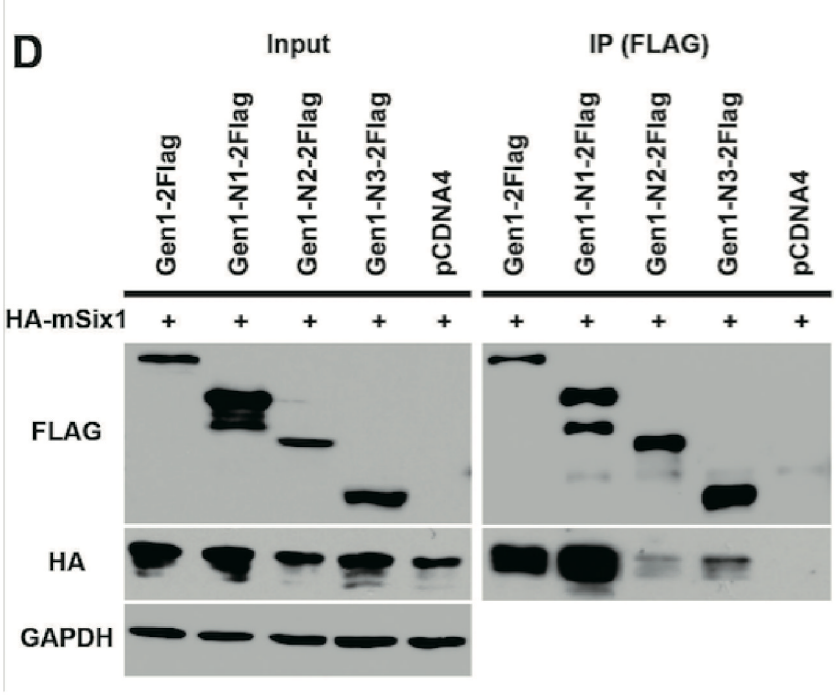
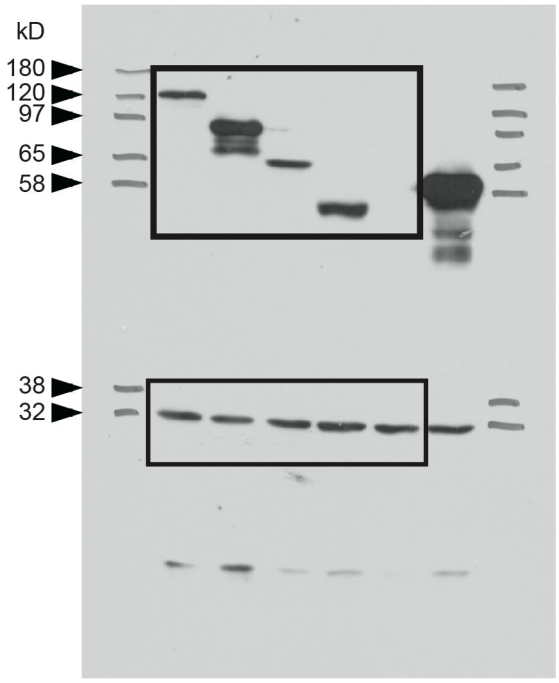

1 2 3
kD
180
120
97
65
58
IP
Rab a GFP
unclear marker
2012-02-14 Co-IP (GFP-GEN1 & NTAP-mSix1)
~1h

Fig. s7


D
Input
IP (FLAG)
Gen1-2Flag
Gen1-N1-2Flag
Gen1-N2-2Flag
Gen1-N3-2Flag
pCDNA4
HA-mSix1
FLAG
HA
GAPDH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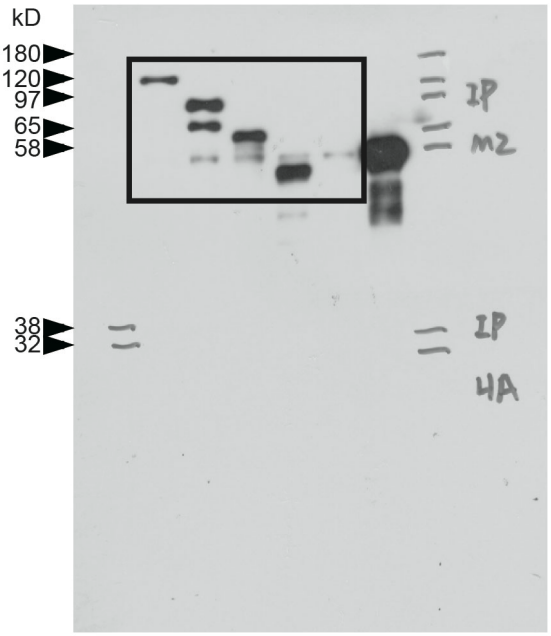
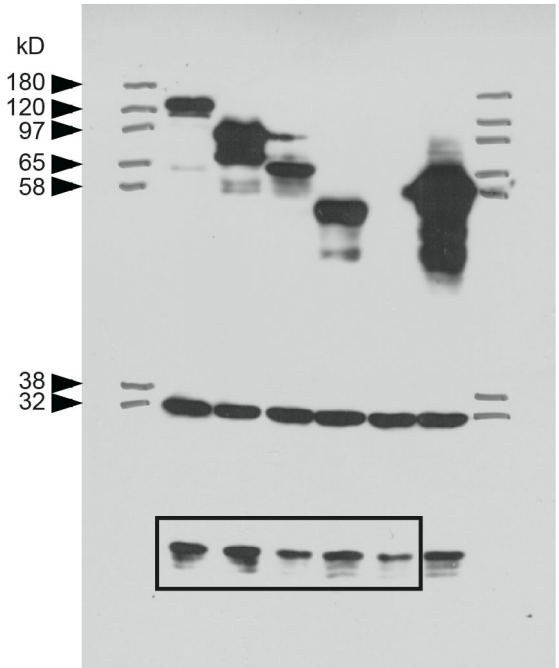
180
120
97
65
58
38
32


kD
180
120
97
65
58
38
32
IP
M2
IP
HA


kD
180
120
97
65
58
38
32

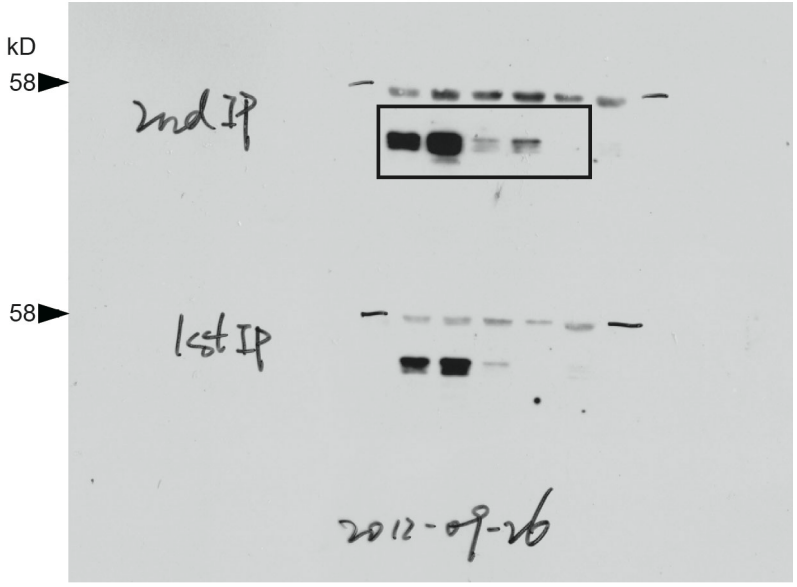

kD
58
2nd IP
58
1st IP
2012-09-26

Table s1. RNA-seq results of important markers for UB formation at E10.5 wild type and *Gen1*$^{PB/PB}$ embryos

Gene	WT	HO	$\log_2$ (fold change)	p value	significance
Eya1	33.441	22.9414	-0.54366	0.0055	yes
Fgf10	4.89641	3.61296	-0.43854	0.15305	no
Foxc1	48.2228	36.3601	-0.40736	0.0221	no
Gdnf	6.76248	5.72477	-0.24033	0.3093	no
Gen1	5.15105	0.943758	-2.44838	0.00005	yes
Grem1	1.94183	4.6153	1.24901	0.00025	yes
Osr1	41.1034	39.0901	-0.07245	0.75215	no
Pax2	26.3983	13.9442	-0.92078	0.00045	yes
Pax8	3.79289	2.05639	-0.88319	0.00755	yes
Ret	11.4155	8.08402	-0.49785	0.02065	no
Robo2	56.5725	43.2177	-0.38848	0.39255	no
Sall1	27.0615	17.2282	-0.65147	0.00035	yes
Six1	8.13363	13.7013	0.75235	0.0798	no
Six2	85.4764	48.8568	-0.80697	0.00005	yes
Six4	6.20722	7.80129	0.32977	0.1706	no
Slit2	12.6644	7.49956	-0.75590	0.04835	no
Spry1	11.2036	10.5149	-0.09152	0.6838	no
Wt1	74.1311	43.7538	-0.76067	0.00155	yes