Accelerated evolution of an *Lhx2* enhancer shapes mammalian social hierarchies

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Supplementary Information Text

Motor Coordination, Balance

Results of the rota-rod test[1] revealed that no significant difference in motor coordination and balance between $PAS1^{c/c}$ and $PAS1^{c/m}$, and between $PAS1^{-/m}$ and $PAS1^{-/-}$ male mice (Two-tailed permutation test, $P = 0.914$, and 0.394, respectively) (Supplementary information, Fig. S13). However, a significant difference in motor coordination and balance was observed between $PAS1^{w/m}$ and $PAS1^{w/w}$ male mice (Two-tailed permutation test, $P = 0.030$). Results of the pole test[2] showed that all 3 pairs of male mice had no significant difference in motor coordination and balance (Supplementary information, Fig. S13) (Two-tailed permutation test, $P = 0.972$, 0.076, and 0.996, respectively).

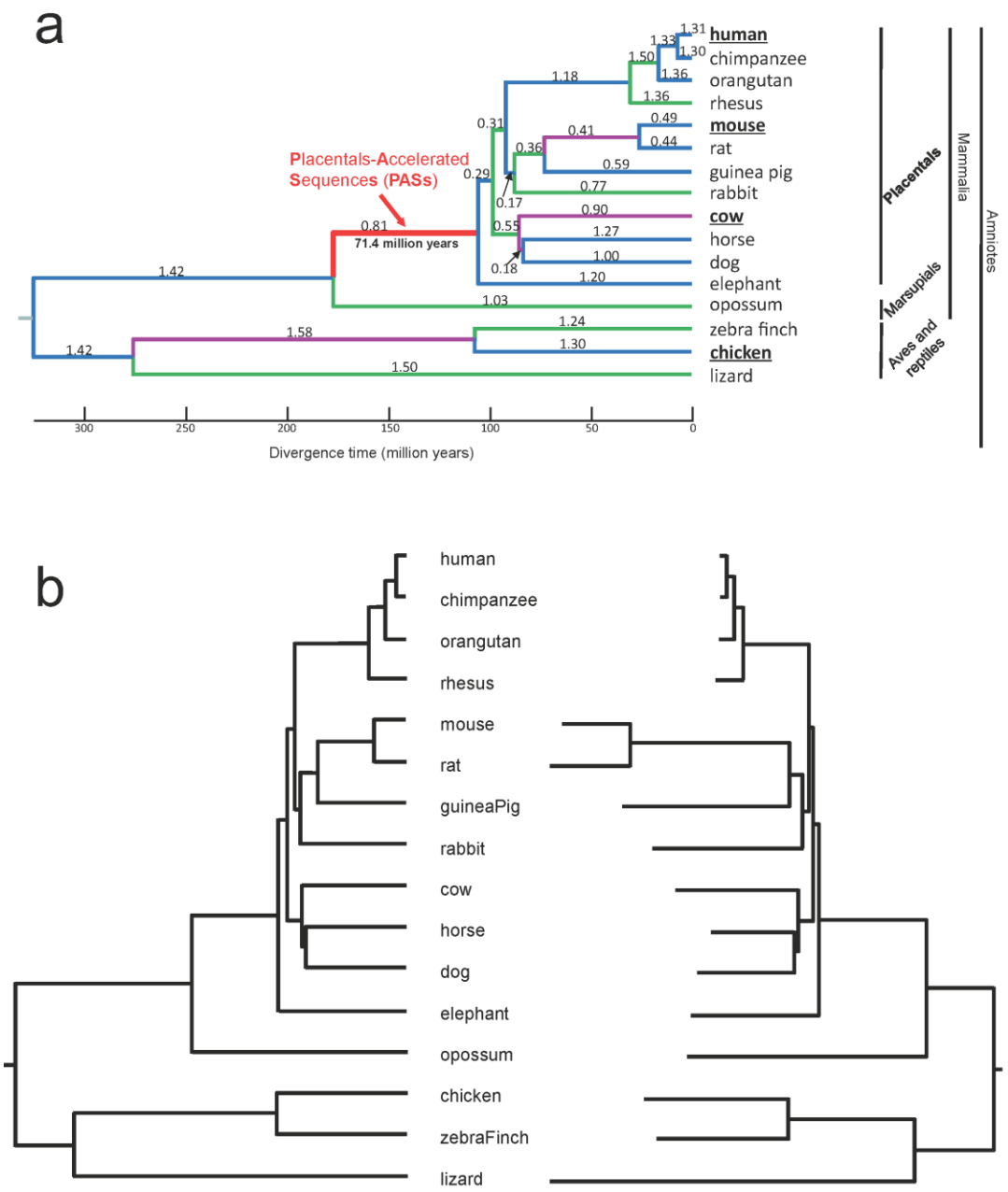


Fig. S1. Phylogenetic tree of 16 amniotes for studying placentals-accelerated sequences (PASs).

(a) The branch sets of tree and the branch-specific normalization factors. Connected branches with the same color (excluding the red branch) were considered as a branch set. There were 17 branch sets. The average evolutionary rate of each window in each branch set was calculated. The local evolutionary rate of the *i*-th branch was normalized by the branch-specific normalization factor α_i. α_i was shown above the branch. The normalization factors are larger than 1.0 for primates and smaller than 1.0 for rodents, consistent with the previous conclusions of slow-evolving primates and fast-evolving rodents.[3]

(b) Comparison between the original phylogenetic tree (left) and the reconstructed neighbor-joining tree[4] based on the genome-wide alignments using the eGPS software (right).[5]

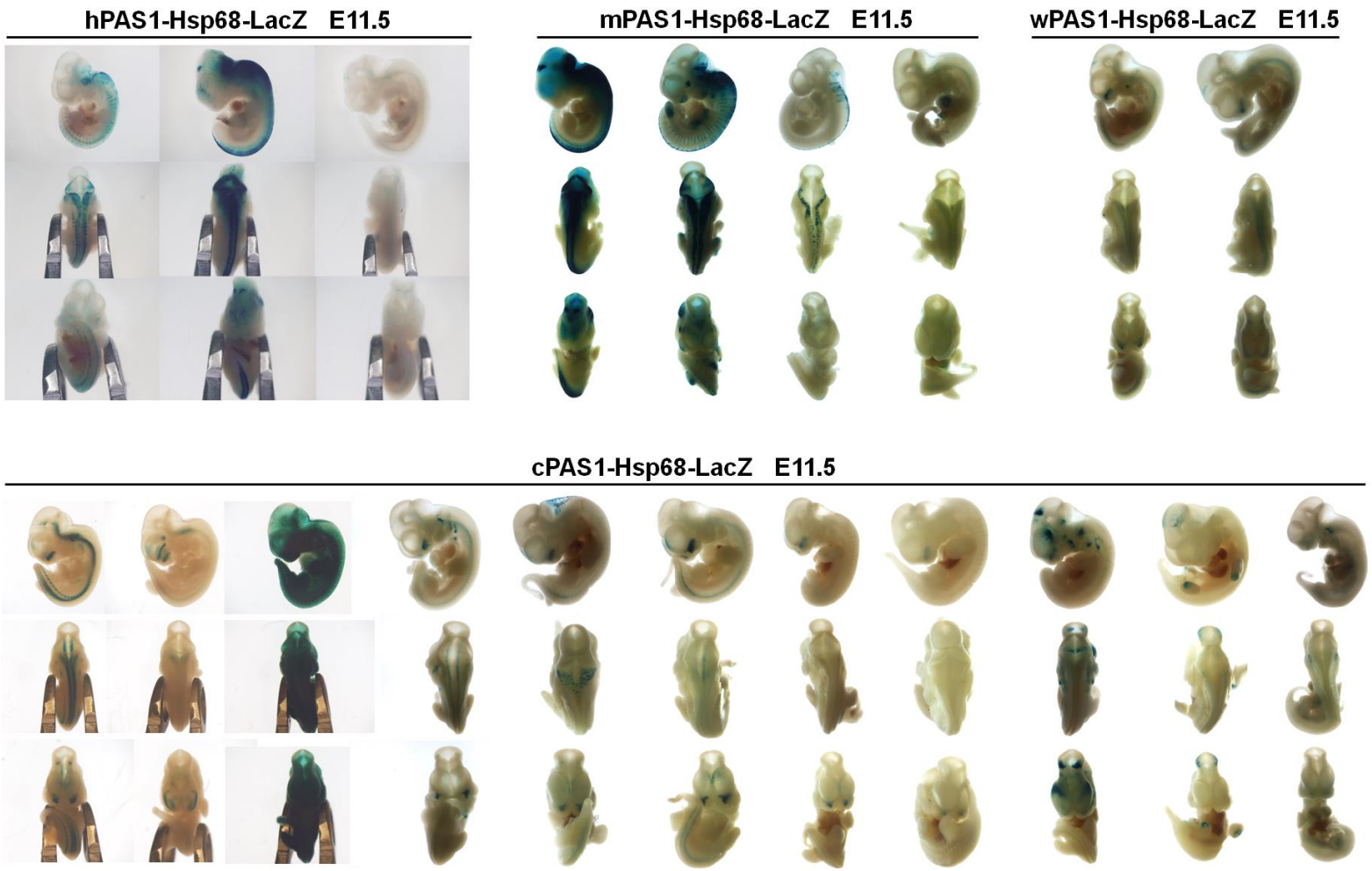


Fig. S2. LacZ-positive E11.5 embryos from hPAS1, mPAS1, wPAS1, and cPAS1 transgenic mouse enhancer assays. Lateral (upper), dorsal (middle), and ventral (bottom) views of all embryos are shown. The second mPAS1-Hsp68-LacZ embryo, the first wPAS1-Hsp68-LacZ embryo, and the sixth cPAS1-Hsp68-LacZ embryo were presented in Figure 2c.

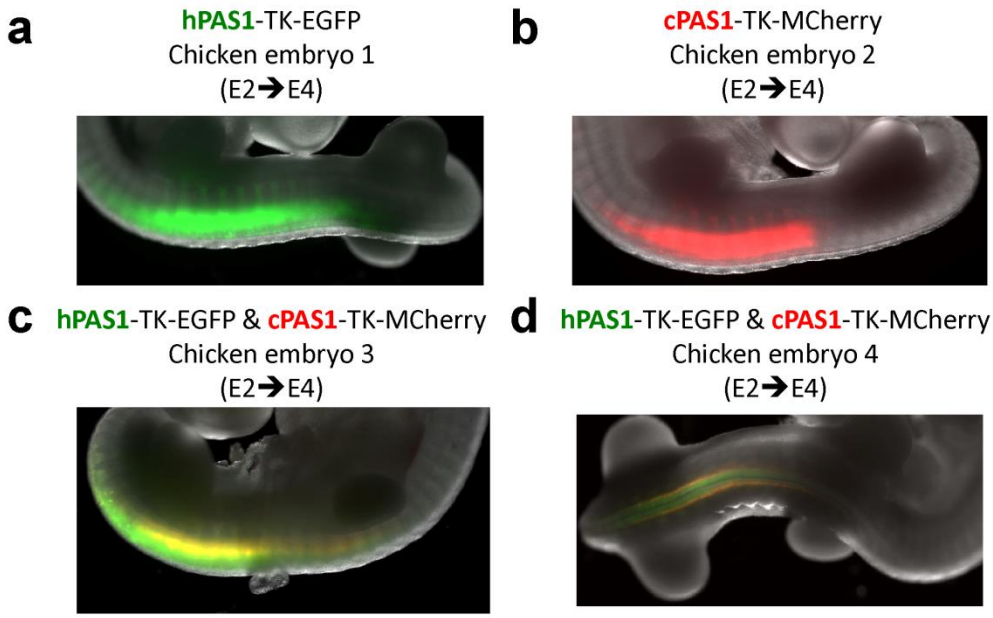


Fig. S3. Effect of hPAS1 and cPAS1 on the expression of the fluorescent protein gene in the spinal cord of chicken embryo.

(a) and (b): E4 chicken embryos after electroporation of hPAS1-TK-EGFP (green) or cPAS1-TK-mCherry (red) plasmid into the neural tube of E2 chicken embryos. Both EGFP and mCherry fluorescence signals were detected in the spinal cord of chicken embryos.

(c) and (d): Co-expression of EGFP (green) and mCherry (red) in the neural tube of chicken embryos after electroporation of hPAS1-TK-EGFP and cPAS1-TK-mCherry plasmids at 1:1 ratio.

(a, b, c) lateral view (d) dorsal view. The TK promoter is a minimal promoter and does not drive the expression of the reporter genes without an enhancer.

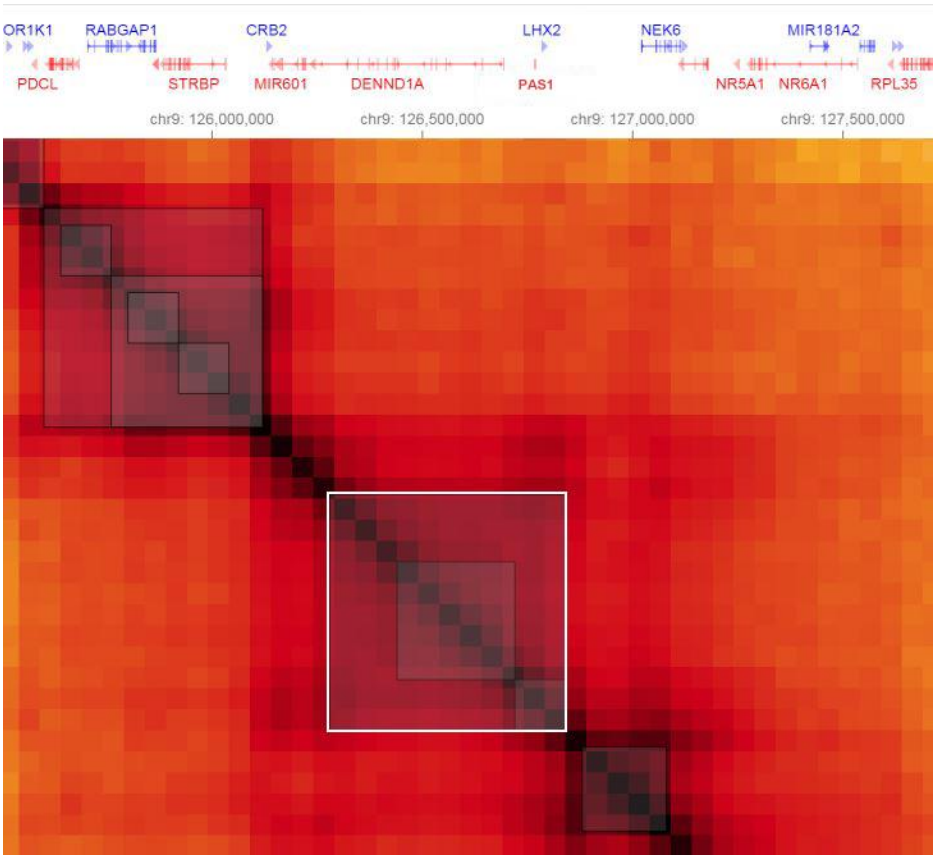


Fig. S4. Identification of topologically associating domain (TAD) around PAS1. The TAD was determined by TADTree,[6] and indicated with an empty square. The TAD was taken as the boundary of the region to which PAS1 may act. Data were obtained from.[7]

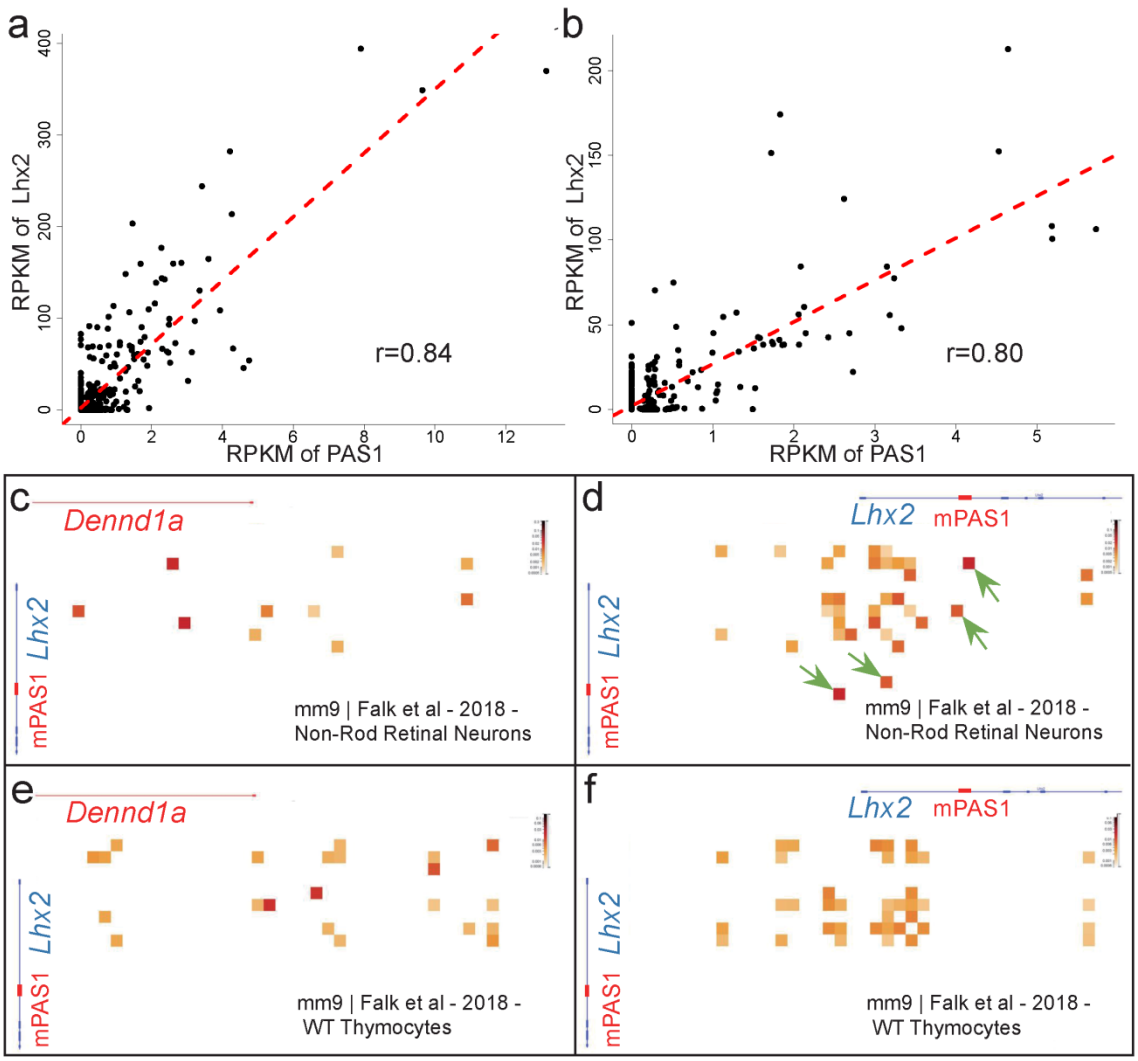


Fig. S5. Evidences for PAS1 regulating the expression of the *Lhx2* gene.

(a) Positive correlation between the CAGE signals of PAS1 and *Lhx2* expression in mouse samples. $P < 2.2 \times 10^{-16}$. The publicly available data were obtained from FANTOM. RPKM: Reads per kilobase per million mapped reads.

(b) Positive correlation between the CAGE signals of PAS1 and *Lhx2* expression in human samples. $P < 2.2 \times 10^{-16}$. The publicly available data were obtained from FANTOM. RPKM: Reads per kilobase per million mapped reads.

(c) – (f) Hi-C signals of PAS1 and *Lhx2* TSS in different mouse cell lines.[8] Hi-C signals (green arrow heads) were observed on PAS1 and *Lhx2*, but not on PAS1 and *Dennd1a* (C, and D); No Hi-C signal was detected in non-neural cells (E, and F)

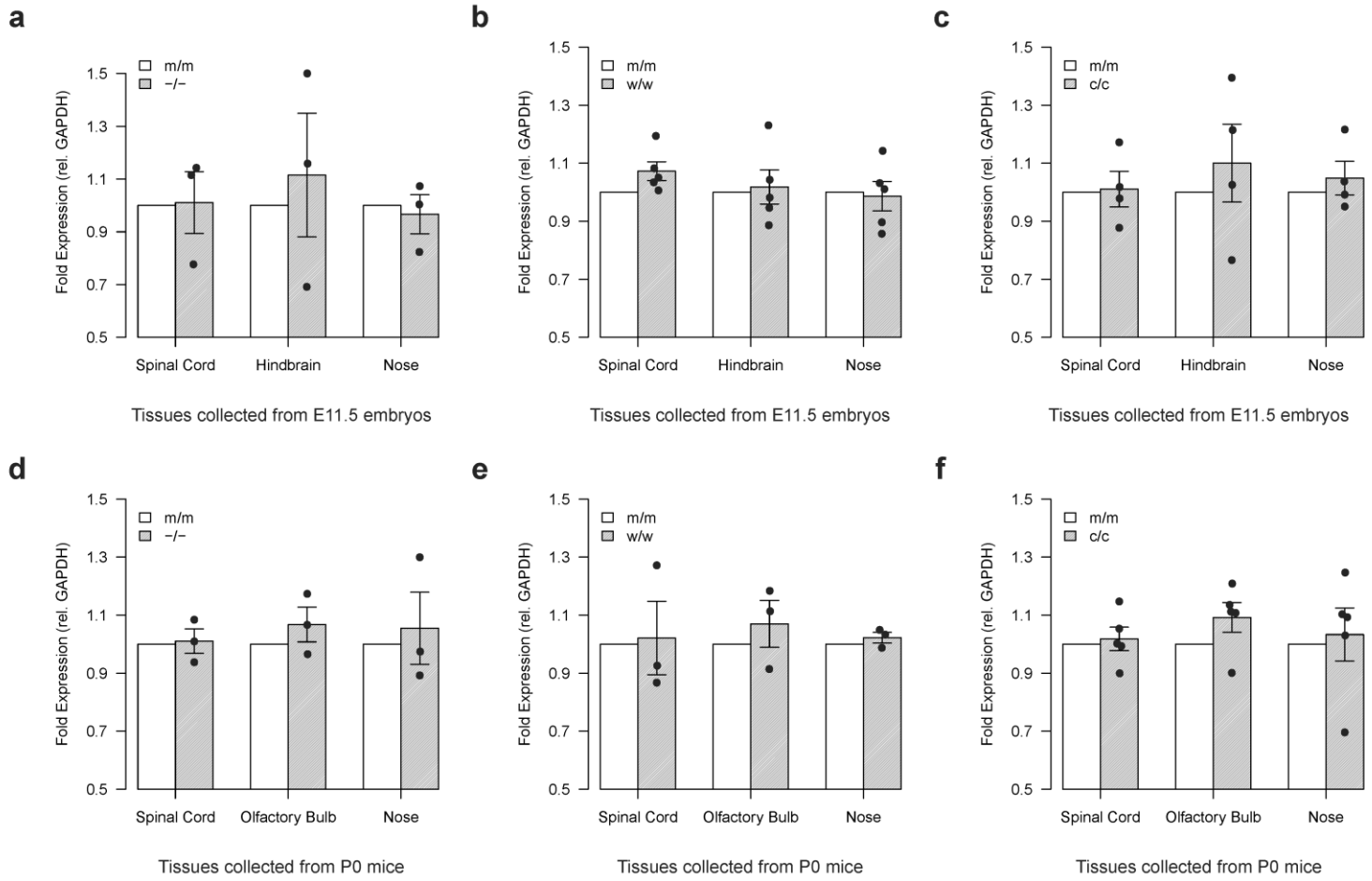


Fig. S6. mRNA levels of *Dennd1a* in E11.5 embryos (a–c) and P0 (d–f) of PAS1$^{-/-}$, PAS1$^{w/w}$ and PAS1$^{c/c}$ mice compared with those of wide-type littermates. mRNA levels of *Dennd1a* determined by RT-qPCR were normalized to those of *Gapdh*. Error bars represent the s.e.m of at least three biological replicates and three technical replicates for each experiment.

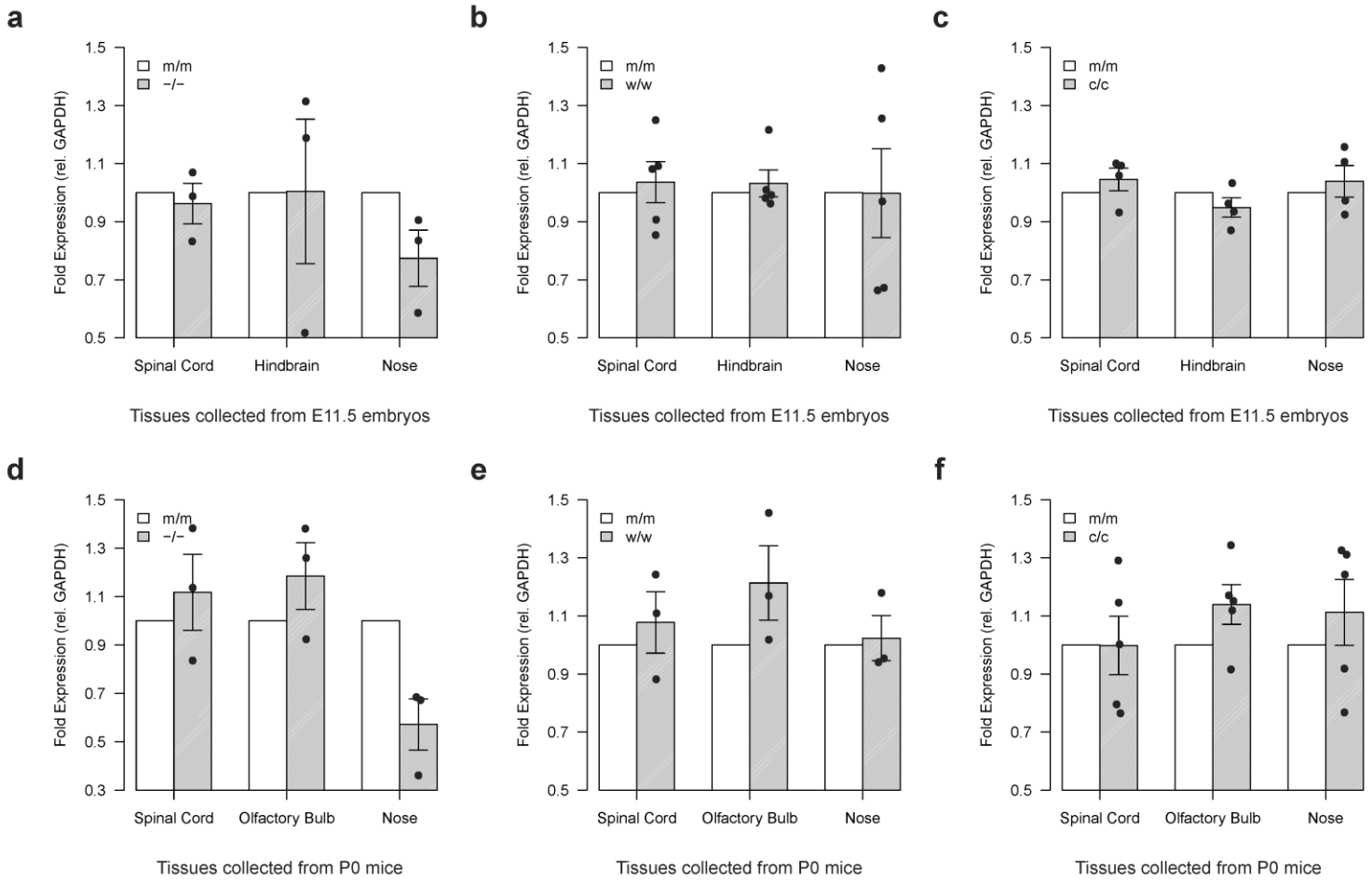


Fig. S7. mRNA levels of *Gm27197* in E11.5 embryos(a–c) and P0(d–f) of PAS1$^{-/-}$, PAS1$^{w/w}$, PAS1$^{c/c}$ mice compared with those of wide-type littermates. mRNA levels of *Gm27197* determined by RT-qPCR were normalized to those of *Gapdh* gene. Error bars represent the s.e.m from at least three biological replicates with three technical replicates for each experiment.

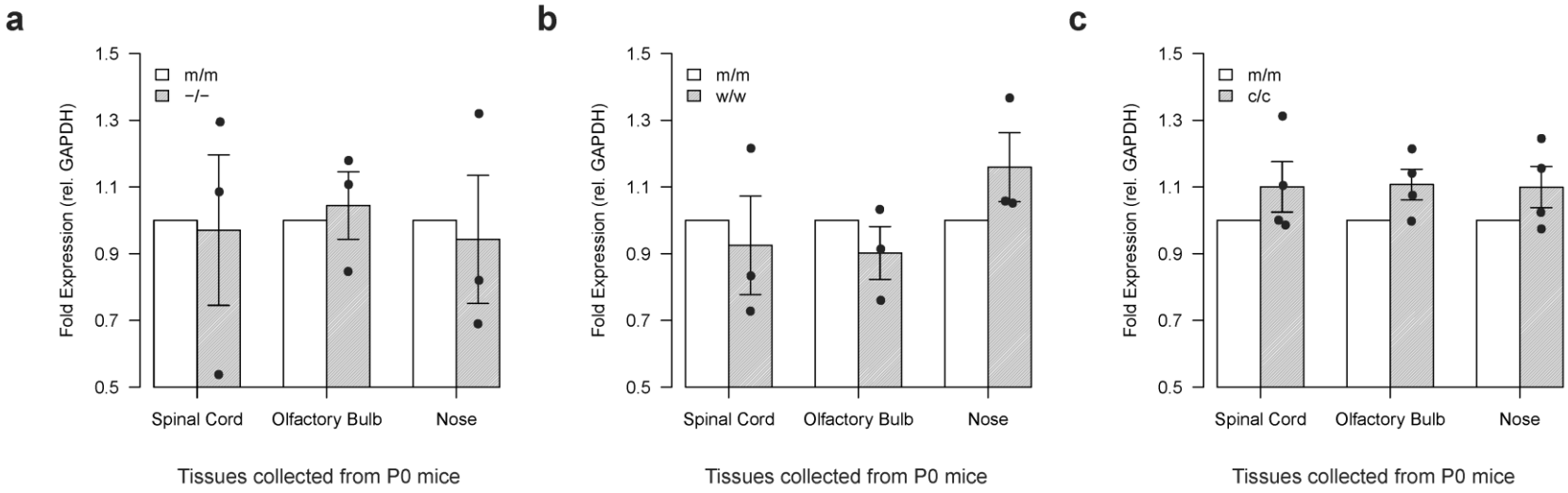


Fig. S8. *Lhx2* mRNA levels determined by RT-qPCR in P0 of PAS1$^{-/-}$, PAS1$^{w/w}$ and PAS1$^{c/c}$ mice compared with those of wide-type littermates. *Lhx2* mRNA levels were normalized to those of *Gapdh*. Error bars represent the s.e.m of at least three biological replicates (three for PAS1$^{-/-}$, three for PAS1$^{w/w}$, four for PAS1$^{c/c}$) and three technical replicates for each experiment.

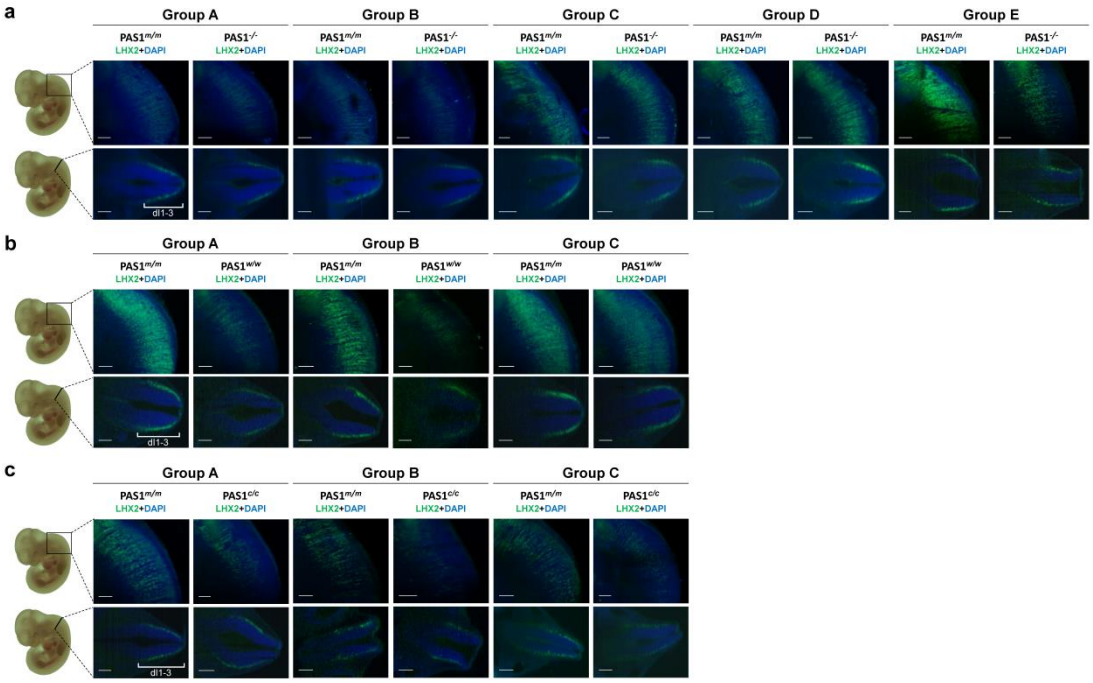


Fig. S9. Effect of PAS1s on the expression of *Lhx2* in the primitive spinal cord. E11.5 PAS1$^{-/-}$, PAS1$^{w/w}$, PAS1$^{c/c}$ and wild-type mouse embryos were immuno-stained for LHX2 (green) after CUBIC clearing. The region in which *Lhx2* was expressed in the wild-type (PAS1$^{m/m}$) embryo is marked (dI1-3, three neuronal cell types).[9] For each mouse strain, at least three groups of embryos were examined. Each group of embryos had two age-matched littermates of specific PAS1 genotypes. Scale bar, 200 μm.

(a) Five groups of PAS1$^{m/m}$ and PAS1$^{-/-}$ pair.

(b) Three groups of PAS1$^{m/m}$ and PAS1$^{w/w}$ pair. The PAS1$^{m/m}$ embryo in Group A was used in Figure 3H as the wild-type control.

(c) Three groups of PAS1$^{m/m}$ and PAS1$^{c/c}$ pair.

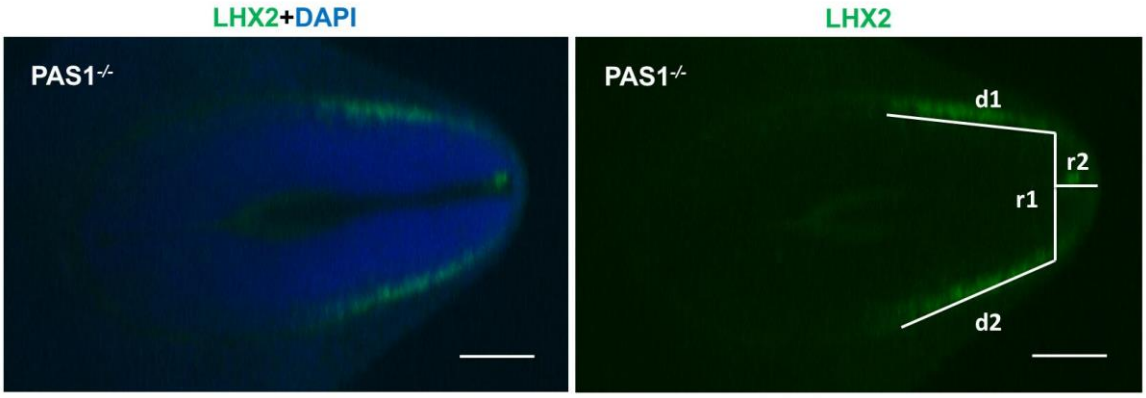


Fig. S10. Schematic of four summary statistics used for examination of LHX2 expression in spinal cord sections. d1 and d2 define the width of the areas in which the LHX2 protein was expressed, r1 and r2 denote the distances of the areas with LHX2 protein expression away from the dorsal vertex of the spinal cord. Scale bar, 200 μm.

Fig. S11. Stability of tube test ranks.

The social dominance tube test was performed daily for 7 to 10 consecutive days. The winner of each test is indicated with a colored dot, which represents a specific PAS1 genotype of the mice tested. Day1 indicates the first tube test trial after the three-day training. Please note that we applied the tube test only to cage mice living together for at least 2 weeks (Table S5-7).

(a) Tests of PAS1$^{w/m}$ *vs.* PAS1$^{w/w}$ male mice. All winners were of the PAS1$^{w/m}$ genotype.

(b) Tests of PAS1 $^{c/m}$ *vs.* PAS1$^{c/c}$ male mice. All winners were of the PAS1$^{c/c}$ genotype.

(c, d, e, f, g, h) Tests of PAS1$^{-/m}$ *vs.* PAS1$^{-/-}$ male mice. The genotype of the winner varied.

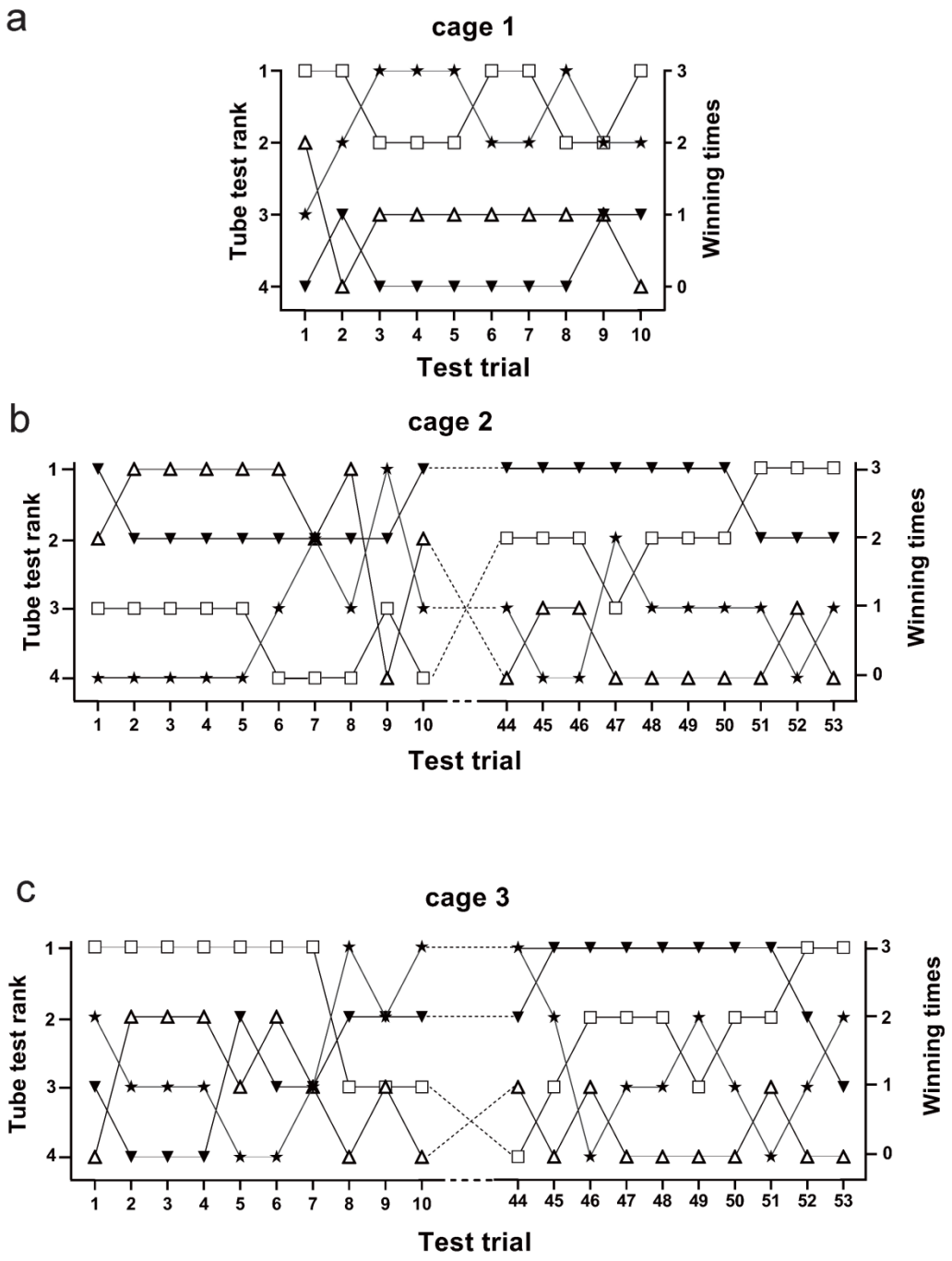


Fig. S12. PAS1$^{-/-}$ male mice lack social stratification.

The detailed information of PAS1$^{-/-}$ mice caged together was provided in Table S8.

(a) The social dominance tube test was performed daily for 10 consecutive days.

(b, c) The social dominance tube test was performed 20 times (once per day) within 53 consecutive days.

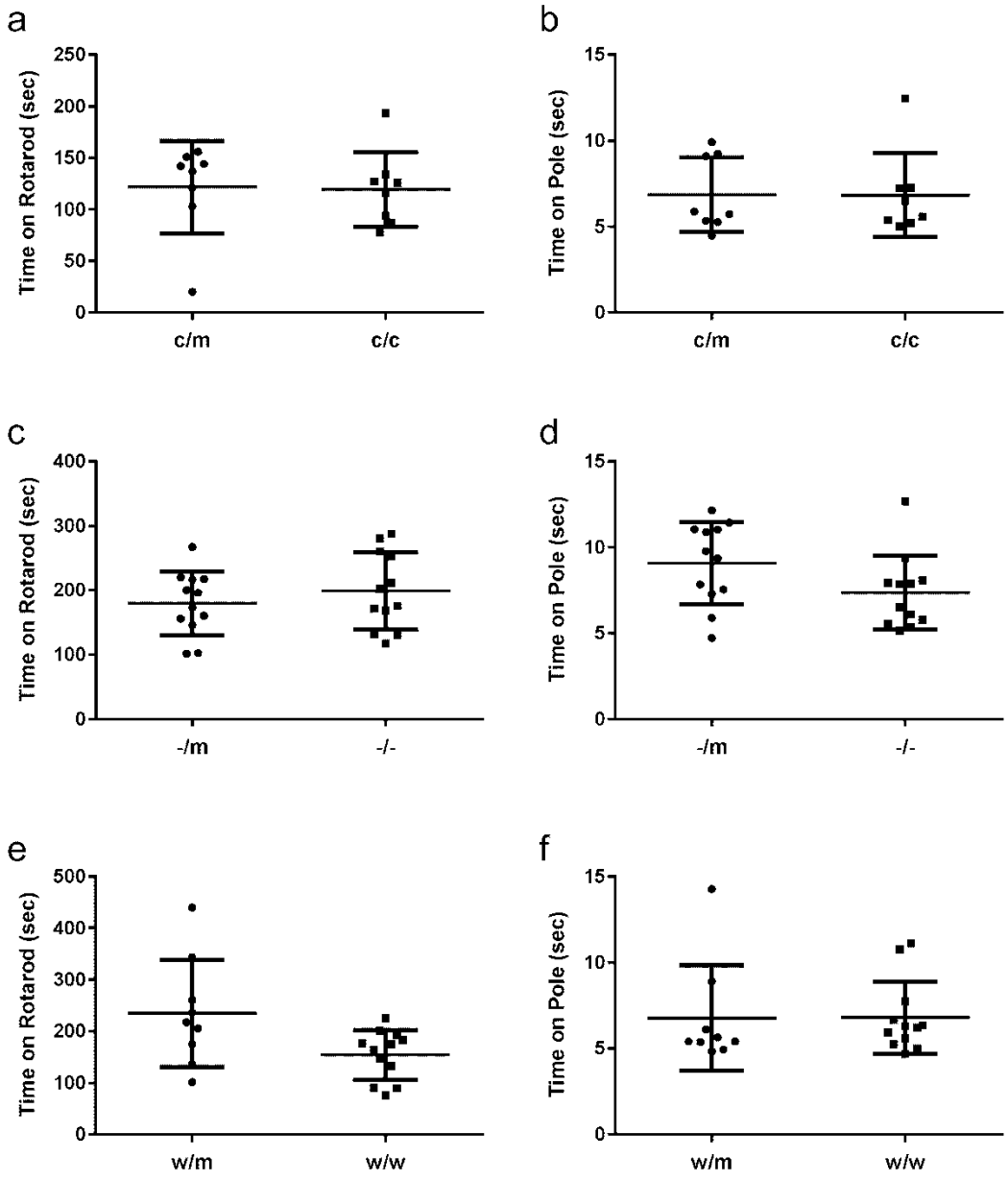


Fig. S13. Motor and activity tests. Data are presented as mean±SD.
(a) Rota-rod tests of PAS1$^{c/m}$ (n=8) and PAS1$^{c/c}$ (n=8) mice.
(b) Pole tests of PAS1$^{c/m}$ (n=8) and PAS1$^{c/c}$ (n=8) mice
(c) Rota-rod tests of PAS1$^{-/m}$ (n=12) and PAS1$^{-/-}$ (n=12) mice.
(d) Pole tests of PAS1$^{-/m}$ (n=12) and PAS1$^{-/-}$ (n=12) mice.
(e) Rota-rod tests of PAS1$^{w/m}$ (n=9) and PAS1$^{w/w}$ (n=12) mice.
(f) Pole tests of PAS1$^{w/m}$ (n=9) and PAS1$^{w/w}$ (n=12) mice.

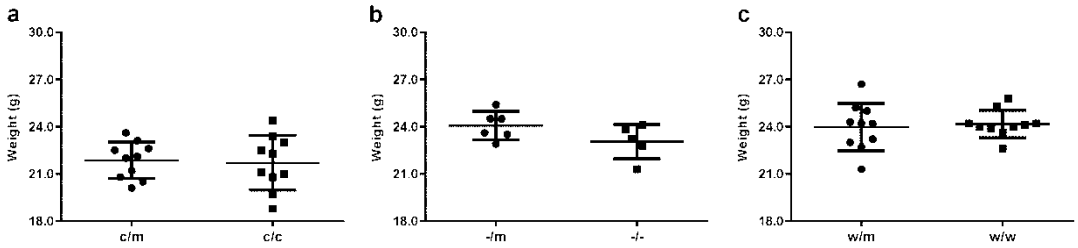
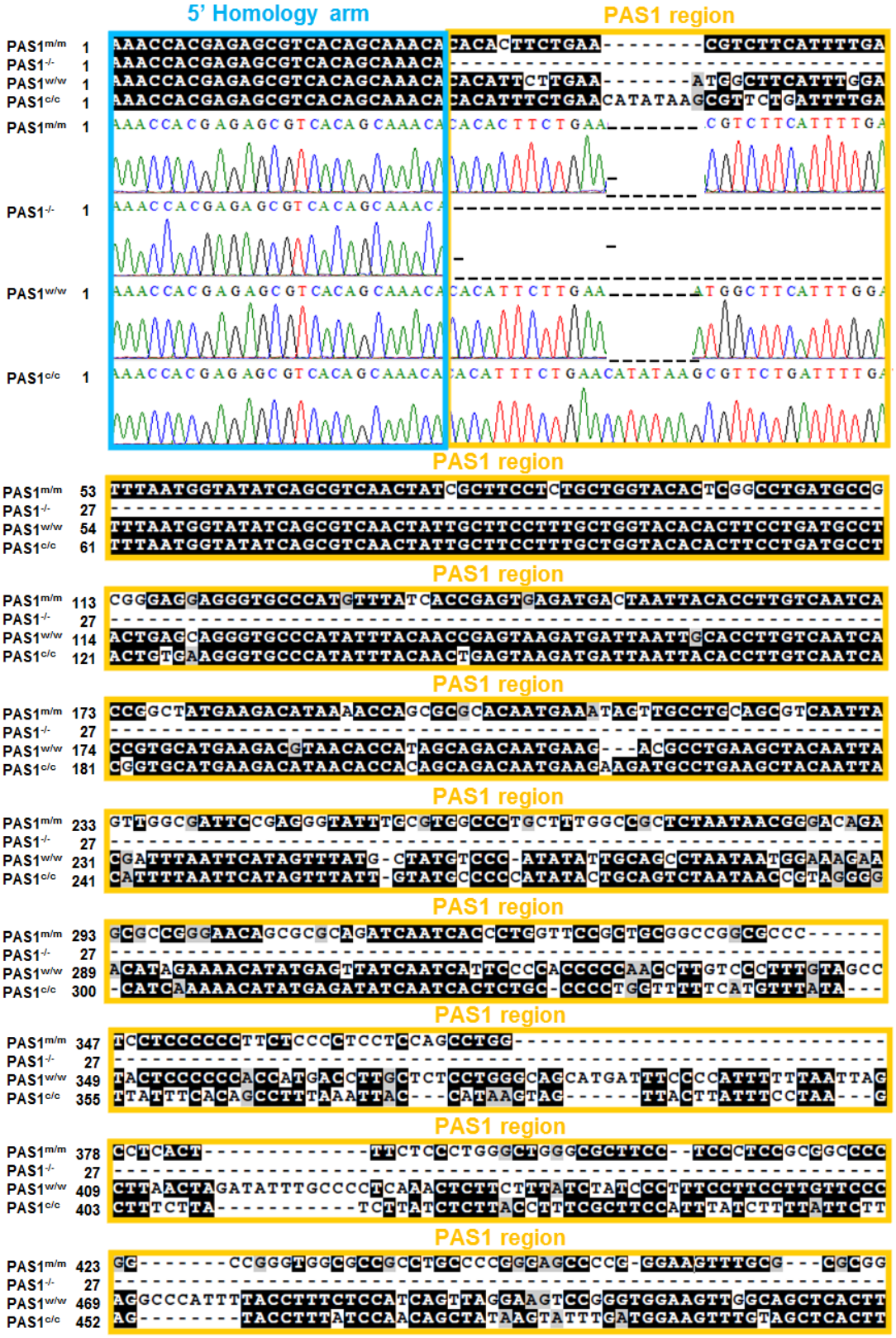


Fig. S14. Body weight of mice at two months (before group-housing). Data are presented as mean ±SD.

(a) Body weight of PAS1$^{c/m}$ (n=10) and PAS1$^{c/c}$ mice (n=10) at 9.0-10.0 weeks of age. One-way analysis of variance P = 0.822.

(b) Body weight of PAS1$^{-/m}$ (n=6) and PAS1$^{-/-}$ mice (n=5) at 9.4-10.4 weeks of age. One-way analysis of variance P = 0.122.

(c) Body weight of PAS1$^{w/m}$ (n=10) and PAS1$^{w/w}$ mice (n=10) at 9.5-10.5 weeks of age. One-way analysis of variance P = 0.734.


5' Homology arm
PAS1 region
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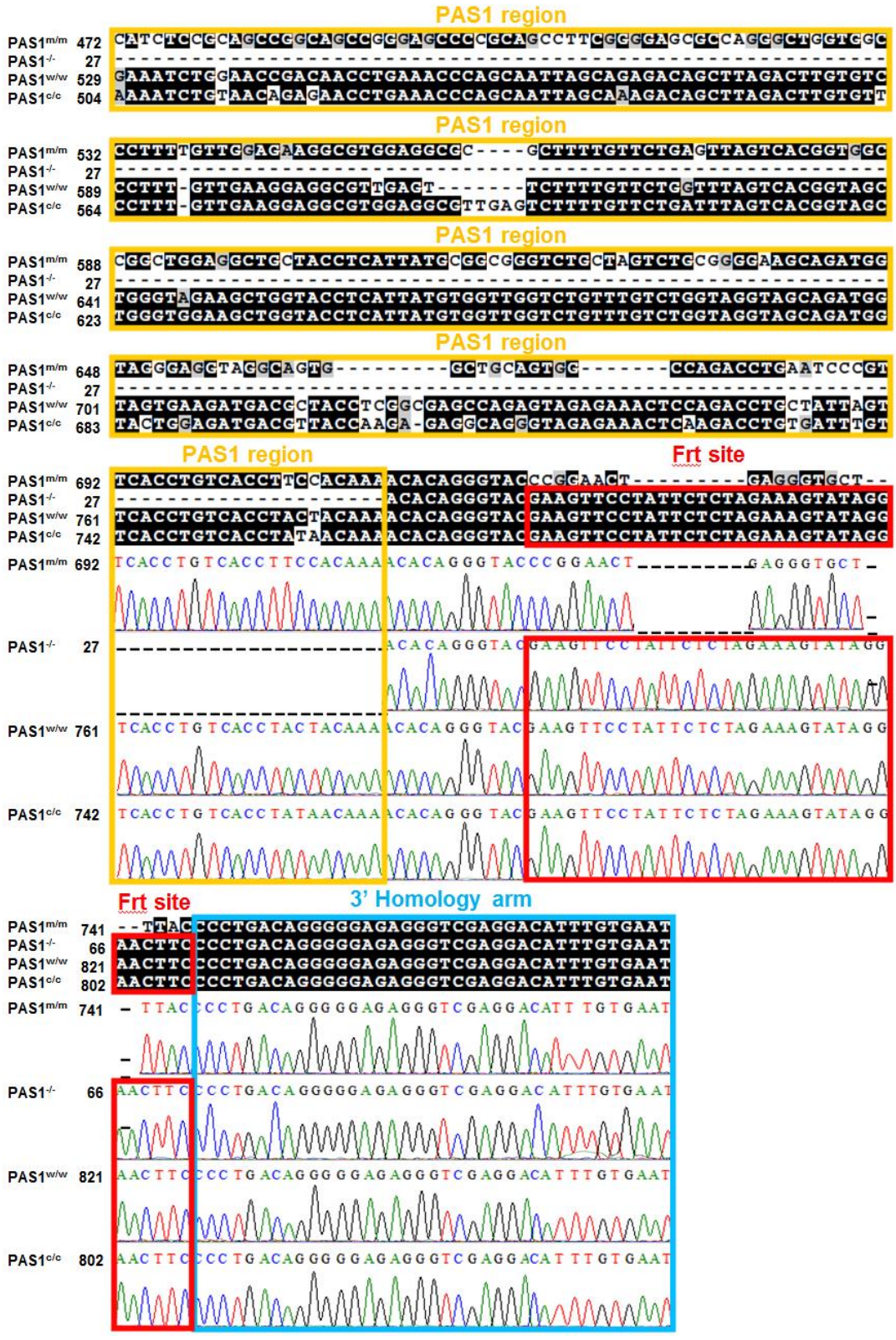
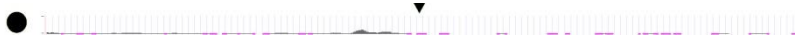
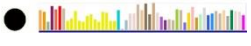
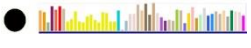
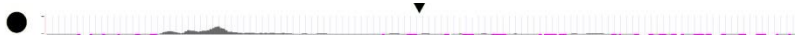


Fig. S15. Alignments of nucleotide sequences of PAS1$^{m/m}$ (wild-type), PAS1$^{-/-}$, PAS1$^{w/w}$ and PAS1$^{c/c}$ mice.

Table S1. List of the accelerated regions in the ancestral lineage of placental mammals. Genomic locations of accelerated regions and the most adjacent protein coding genes were determined by searching the Ensembl/Havana database (GRCh37). The threshold of *P*-values was 3.06×10^{-9} after Bonferroni correction. H3K27ac signals[10] within 30-kb regions were obtained from ENCODE/LICR on the UCSC genome browser with a vertical viewing range of [0.2, 10]. The accelerated regions are marked with inverted triangle, located in the middle of the 30-kb regions. Median gene expression levels in 51 tissues and 2 cell lines, based on RNA-seq data of 8,555 tissue samples from 570 adult post-mortem individuals from the NIH Genotype-Tissue Expression (GTEx) project (V6, October 2015),[11] were visualized with UCSC genome browser. GTEx gene expression levels were log-transformed. Green circle – Passed related filters; Black circle – Failed to pass related filters.

Rank	Coordinates	Genomic Location	Closest Coding Genes	*P*-value	Active enhancer signal (H3K27ac) in whole mouse brain E14.5	GTEx Gene Expression in 53 adult human tissues (Brain in yellow)
1	hg19 chr9:126770941-126771040 mm9 chr2:38203835-38203933	Non-coding	*Lhx2*	3.15×10^{-18}		
2	hg19 chr9:126770921-126771020 mm9 chr2:38203815-38203913	Non-coding	*Lhx2*	5.94×10^{-18}		
3	hg19 chr9:126770901-126771000 mm9 chr2:38203795-38203894	Non-coding	*Lhx2*	7.14×10^{-18}		
4	hg19 chr9:126770961-126771060 mm9 chr2:38203855-38203953	Non-coding	*Lhx2*	2.77×10^{-18}		
5	hg19 chr11:8310741-8310840 mm9 chr2:160352318-160352420	intergenic	*Lmo1*	9.63×10^{-13}		
6	hg19 chr9:126771021-126771120 mm9 chr2:38203914-38204013	Non-coding	*Lhx2*	1.92×10^{-12}		
7	hg19 chr20:39498201-39498300 mm9 chr2:160352318-160352420	intergenic	*Top1*	2.78×10^{-11}		
8	hg19 chr9:126770601-126770700 mm9 chr2:38203489-38203588	Non-coding	*Lhx2*	8.28×10^{-11}		
9	hg19 chr20:57485821-57485920 mm9 chr2:174171826-174171924	coding	*Gnas*	1.26×10^{-10}		
10	hg19 chr9:126770541-126770640 mm9 chr2:38203429-38203528	Non-coding	*Lhx2*	3.15×10^{-10}		
11	hg19 chr9:126770641-126770740 mm9 chr2:38203529-38203629	Non-coding	*Lhx2*	3.84×10^{-10}		
12	hg19 chr3:71113981-71114080 mm9 chr6:98978164-98978259	intronic	*Foxp1*	5.27×10^{-10}		
13	hg19 chr3:71114041-71114140 mm9 chr6:98978223-98978313	intronic	*Foxp1*	5.48×10^{-10}		
14	hg19 chr4:1804621-1804720	coding	*Fgfr3*	5.95×10^{-10}		

	mm9 chr5:34073910-34074009				
15	hg19 chr9:126770981-126771080 mm9 chr2:38203875-38203973	Non-coding	*Lhx2*	7.18×10^{-10}	
16	hg19 chr9:126770561-126770660 mm9 chr2:38203449-38203548	Non-coding	*Lhx2*	7.37×10^{-10}	
17	hg19 chr11:8358781-8358880 mm9 chr7:116382622-116382722	intergenic	*Stk33*	8.08×10^{-10}	
18	hg19 chr20:39498181-39498280 mm9 chr2:160352303-160352398	intergenic	*Top1*	1.10×10^{-9}	
19	hg19 chr9:126771041-126771140 mm9 chr2:38203934-38204033	Non-coding	*Lhx2*	1.10×10^{-9}	
20	hg19 chr2:80531301-80531400 mm9 chr6:77193092-77193192	5' UTR intronic	*Lrrtm1* *Ctnna2*	1.28×10^{-9}	
21	hg19 chr2:80531321-80531420 mm9 chr6:77193072-77193172	5' UTR intronic	*Lrrtm1* *Ctnna2*	1.28×10^{-9}	
22	hg19 chr11:8358761-8358860 mm9 chr7:116382602-116382702	intergenic	*Stk33*	1.29×10^{-10}	
23	hg19 chr14:94519381-94519480 mm9 chr12:104648175-104648275	coding	*Ddx24*	1.36×10^{-9}	
24	hg19 chr5:52776361-52776460 mm9 chr13:115248934-115249033	5' UTR	*Fst*	1.38×10^{-9}	
25	hg19 chr18:6882121-6882220 mm9 chr17:68213881-68213980	coding	*Arhgap28*	1.77×10^{-9}	
26	hg19 chr20:57478741-57478840 mm9 chr2:174167325-174167424	coding	*Gnas*	1.80×10^{-9}	
27	hg19 chr14:103355881-103355980 mm9 chr12:112489325-112489424	intronic	*Traf3*	2.51×10^{-9}	
28	hg19 chr20:57478721-57478820 mm9 chr2:174167305-174167404	coding	*Gnas*	2.98×10^{-9}	

Table S2. Summary statistics of *Lhx2* gene expression levels in spinal cord sections of PAS1$^{-/-}$ E11.5 mouse embryos. Images of spinal cord sections of E11.5 PAS1$^{m/m}$ (wild-type) and PAS1$^{-/-}$ embryos immuno-stained for LHX2 after CUBIC clearing were captured and analyzed with the Vision4D software (version 2.12.3, Arivis). Five groups of embryos were examined, and each group of embryos was from age-matched littermates. Three consecutive sections of each embryo were examined. d1 and d2 define the width of the areas with LHX2 protein expression. r1 and r2 denote the distances of the areas with LHX2 protein expression away from the dorsal vertex of the spinal cord. Measurements were conducted by two experts independently, and results are presented in the following two sub-tables. Two-tailed permutation test was used, **P* < 0.05, ***P* < 0.01.

		Group A		**Group B**		**Group C**		**Group D**		**Group E**		***P*-value**
		PAS1$^{m/m}$	**PAS1$^{-/-}$**	**PAS1$^{m/m}$**	**PAS1$^{-/-}$**	**PAS1$^{m/m}$**	**PAS1$^{-/-}$**	**PAS1$^{m/m}$**	**PAS1$^{-/-}$**	**PAS1$^{m/m}$**	**PAS1$^{-/-}$**	
Expert 1												
d1 (μm)	Section1	539.56	430.83	494.04	439.24	484.56	467.70	460.42	370.85	582.06	482.09	
	Section2	507.35	417.62	477.64	427.49	470.54	462.40	444.07	396.12	537.89	420.00	**0.00001** **
	Section3	559.73	410.76	531.60	416.65	481.26	464.10	448.82	270.45	532.01	407.67	
d2 (μm)	Section1	608.89	496.80	536.04	416.15	506.87	497.80	468.25	383.84	581.28	505.56	
	Section2	567.11	494.62	580.97	463.60	484.77	487.69	461.89	417.95	535.26	509.69	**0.00004** **
	Section3	574.08	434.10	564.94	416.18	541.19	434.00	425.55	377.96	510.70	444.90	
r1 (μm)	Section1	253.62	315.56	253.62	333.43	152.09	285.81	131.75	356.98	489.63	395.94	
	Section2	242.13	352.30	242.13	314.31	166.74	297.59	167.84	318.95	474.67	370.93	**0.00445** **
	Section3	260.09	353.08	260.09	351.72	159.37	277.10	126.43	360.40	433.02	377.27	
r2 (μm)	Section1	61.72	137.31	61.72	142.46	27.94	111.96	35.57	108.26	68.50	163.00	
	Section2	63.81	116.90	63.81	139.78	48.28	107.39	43.19	120.51	66.07	157.50	**0.00003** **
	Section3	66.06	116.73	66.06	172.94	53.35	101.81	43.19	118.32	64.65	170.30	
Expert 2												
d1 (μm)	Section1	*529.66*	*431.91*	*482.04*	*430.18*	*478.68*	*455.64*	*472.13*	*350.64*	*583.06*	*470.67*	
	Section2	*543.56*	*411.86*	*539.55*	*421.66*	*498.29*	*382.47*	*478.54*	*469.43*	*627.06*	*460.97*	***0.00004*** **
	Section3	*575.63*	*372.20*	*765.87*	*417.68*	*493.55*	*451.34*	*439.37*	*300.39*	*574.94*	*468.98*	
d2 (μm)	Section1	*656.01*	*442.12*	*545.45*	*406.20*	*552.24*	*502.86*	*518.37*	*459.56*	*604.80*	*510.91*	
	Section2	*578.58*	*511.04*	*614.04*	*520.80*	*509.50*	*478.95*	*425.55*	*412.17*	*571.81*	*526.14*	***0.00001*** **
	Section3	*613.61*	*373.40*	*604.75*	*470.22*	*564.01*	*424.54*	*441.14*	*414.47*	*475.52*	*414.73*	
r1 (μm)	Section1	*259.98*	*346.97*	*199.36*	*328.92*	*199.32*	*279.94*	*121.89*	*310.39*	*532.40*	*396.75*	
	Section2	*266.80*	*314.83*	*205.22*	*324.53*	*172.68*	*296.50*	*143.62*	*282.44*	*475.38*	*365.27*	***0.00401*** **
	Section3	*235.67*	*366.30*	*153.75*	*269.73*	*148.15*	*281.83*	*113.28*	*326.01*	*434.92*	*373.14*	
r2 (μm)	Section1	*63.51*	*140.30*	*67.27*	*158.72*	*47.56*	*106.71*	*43.19*	*135.19*	*90.75*	*183.27*	
	Section2	*66.34*	*108.27*	*73.68*	*151.16*	*56.86*	*101.81*	*63.79*	*115.79*	*97.32*	*168.74*	***0.00003*** **
	Section3	*55.89*	*146.72*	*58.45*	*106.74*	*50.01*	*94.20*	*45.73*	*139.38*	*89.12*	*186.26*	

Table S3. Summary statistics of *Lhx2* gene expression levels in spinal cord sections of PAS1$^{w/w}$ E11.5 mouse embryos. Images of spinal cord sections of E11.5 PAS1$^{m/m}$ (wild-type) and PAS1$^{w/w}$ embryos immuno-stained for LHX2 after CUBIC clearing were captured and analyzed with the Vision4D software (version 2.12.3, Arivis). Three groups of embryos were examined, and each group of embryos was from age-matched littermates. Three consecutive sections of each embryo were examined. d1 and d2 define the width of the areas with LHX2 protein expression. r1 and r2 denote the distances of the areas with LHX2 protein expression away from the dorsal vertex of the spinal cord. Measurements were conducted by two experts independently, and results are presented in the following two sub-tables. Two-tailed permutation test was used, **P* < 0.05, ***P* < 0.01.

		Group A		Group B		Group C		*P*-value
		PAS1$^{m/m}$	PAS1$^{w/w}$	PAS1$^{m/m}$	PAS1$^{w/w}$	PAS1$^{m/m}$	PAS1$^{w/w}$	
Expert 1								
d1 (μm)	Section1	512.05	446.78	645.23	403.00	532.79	548.83	**0.08092**
	Section2	558.82	417.99	647.42	397.57	600.04	638.70	
	Section3	556.00	391.57	602.79	380.79	579.55	561.50	
d2 (μm)	Section1	617.43	380.64	648.93	459.64	542.19	626.77	**0.08068**
	Section2	531.43	462.33	680.30	416.86	575.26	575.73	
	Section3	586.85	444.22	642.15	435.91	544.53	595.07	
r1 (μm)	Section1	539.57	630.17	322.17	342.87	292.01	384.59	**0.00465 ****
	Section2	487.55	612.02	290.03	305.63	264.44	385.46	
	Section3	447.78	534.52	291.35	359.43	253.72	367.33	
r2 (μm)	Section1	54.82	193.13	45.98	150.92	53.35	119.65	**0.00543 ****
	Section2	43.66	175.52	53.57	160.98	76.23	122.42	
	Section3	40.28	154.95	53.67	166.35	66.05	96.65	
Expert 2								
d1 (μm)	Section1	*629.95*	*368.60*	*708.30*	*446.72*	*521.27*	*508.43*	***0.00513*** ******
	Section2	*554.77*	*324.79*	*667.57*	*431.84*	*609.81*	*515.76*	
	Section3	*630.44*	*401.84*	*605.03*	*353.81*	*576.36*	*555.17*	
d2 (μm)	Section1	*743.56*	*520.68*	*680.43*	*474.58*	*454.84*	*652.03*	***0.00469*** ******
	Section2	*532.03*	*507.15*	*664.14*	*427.24*	*521.73*	*512.31*	
	Section3	*649.75*	*431.86*	*669.80*	*417.93*	*496.01*	*611.85*	
r1 (μm)	Section1	*521.03*	*630.17*	*291.93*	*309.32*	*297.49*	*337.34*	***0.00401*** ******
	Section2	*483.39*	*621.99*	*262.32*	*305.57*	*242.40*	*350.87*	
	Section3	*445.59*	*528.85*	*236.00*	*335.22*	*231.50*	*333.03*	
r2 (μm)	Section1	*76.11*	*155.01*	*73.56*	*180.61*	*92.12*	*121.37*	***0.00551*** ******
	Section2	*70.72*	*167.68*	*73.12*	*168.56*	*78.97*	*129.87*	
	Section3	*68.70*	*150.88*	*66.70*	*182.32*	*84.23*	*114.84*	

Table S4. Summary statistics of *Lhx2* gene expression levels in spinal cord sections of PAS1$^{c/c}$ E11.5 mice embryos. Images of spinal cord sections of E11.5 PAS1$^{m/m}$ (wild-type) and PAS1$^{c/c}$ embryos immuno-stained for LHX2 after CUBIC clearing were captured and analyzed with the Vision4D software (version 2.12.3, Arivis). Three groups of embryos were collected, and each group of embryos was from age-matched littermates. Three consecutive sections of each embryo were examined. d1 and d2 define the width of the areas with LHX2 protein expression. r1 and r2 denote the distances of the areas with LHX2 protein expression away from the dorsal vertex of the spinal cord. Measurements were conducted by two experts independently, and results are presented in the following two sub-tables. Two-tailed permutation test was used, **P* < 0.05, ***P* < 0.01.

		Group A		Group B		Group C		*P*-value
		PAS1$^{m/m}$	PAS1$^{c/c}$	PAS1$^{m/m}$	PAS1$^{c/c}$	PAS1$^{m/m}$	PAS1$^{c/c}$	
Expert 1								
d1 (μm)	Section1	447.39	329.85	613.81	340.18	548.26	454.95	**0.00561 ****
	Section2	360.18	300.99	552.89	425.39	578.09	402.69	
	Section3	395.50	279.46	570.85	382.50	554.51	434.37	
d2 (μm)	Section1	479.68	402.71	630.48	321.16	603.36	463.33	**0.00527 ****
	Section2	420.08	380.98	547.54	414.26	585.54	481.43	
	Section3	368.65	300.09	577.24	445.73	528.37	482.88	
r1 (μm)	Section1	97.26	258.24	336.25	458.48	156.00	320.50	**0.00492 ****
	Section2	83.00	256.39	341.98	465.72	180.02	339.66	
	Section3	98.74	268.99	301.19	482.01	162.02	292.31	
r2 (μm)	Section1	37.91	162.56	64.74	178.73	58.44	159.70	**0.00506 ****
	Section2	43.20	146.49	72.24	144.85	60.62	163.56	
	Section3	35.57	169.43	66.36	129.95	51.15	155.89	
Expert 2								
d1 (μm)	Section1	*461.63*	*368.90*	*600.44*	*357.35*	*578.65*	*436.46*	***0.00475 *****
	Section2	*411.37*	*356.28*	*510.31*	*350.69*	*592.05*	*391.90*	
	Section3	*408.59*	*429.95*	*583.41*	*339.36*	*552.68*	*423.07*	
d2 (μm)	Section1	*487.84*	*380.99*	*664.64*	*334.8*	*612.24*	*532.67*	***0.33588***
	Section2	*468.05*	*403.48*	*582.39*	*405.96*	*562.76*	*556.23*	
	Section3	*363.75*	*682.42*	*567.68*	*455.13*	*537.00*	*463.04*	
r1 (μm)	Section1	*56.76*	*276.06*	*347.75*	*462.42*	*150.19*	*331.35*	***0.00544 *****
	Section2	*40.03*	*284.21*	*345.64*	*478.24*	*162.18*	*326.84*	
	Section3	*35.75*	*273.09*	*307.92*	*478.54*	*162.02*	*314.14*	
r2 (μm)	Section1	*48.48*	*199.18*	*67.27*	*213.48*	*80.75*	*185.69*	***0.00504 *****
	Section2	*26.87*	*215.29*	*83.85*	*179.20*	*91.68*	*213.69*	
	Section3	*22.87*	*189.90*	*83.85*	*190.30*	*83.82*	*203.44*	

Table S5. Results of social dominance tube test of PAS1$^{w/m}$ *vs.* PAS1$^{w/w}$ mice.

Cage number[a]	No. of PAS1$^{w/m}$ *vs.* No. of PAS1$^{w/w}$	Winning rate of PAS1$^{w/m}$	Winning rate of PAS1$^{w/w}$	Age at group-housing (weeks)	Weight at group-housing (g)	Housed before tube test (weeks)[b]	Age at tube test (weeks)	Weight at tube test (g)
Cage 1	2:2	100%	0%	7.7	19.5-21.7	5.0	12.7	22.8-24.0
Cage 2	2:2	100%	0%	7.4	19.8-22.3	5.0	12.4	24.0-25.6
Cage 3	2:2	50%	50%	7.4-7.7	22.1-23.8	5.0	12.4-12.7	26.3-29.3
Cage 4	2:2	50%	50%	7.4-7.7	21.0-22.5	5.0	12.4-12.7	24.4-27.8
Cage 5	2:2	100%	0%	10.4	22.6-24.2	2.7	13.1	23.8-28.2
Cage 6	2:2	100%	0%	10.4	24.2-26.7	2.7	13.1	24.5-27.3
Cage 7	2:2	100%	0%	7.7-8.4	19.5-23.4	3.1	10.9-11.6	22.2-26.8
Cage 8	3:3	67%	33%	8.9-9.7	23.0-26.4	2.1	11.0-11.9	24.7-27.9
Cage 9	4:4	69%	31%	12.7-13.0	24.0-27.8	3.6	16.3-16.6	27.2-30.6
Cage 10	4:4	69%	31%	14.7-15.0	25.0-30.3	3.6	18.3-18.6	27.0-30.1
Cage 11	4:4	81%	19%	9.7-10.4	21.3-24.3	4.1	13.9-14.6	22.5-28.7
Summary		75.6%	24.4%					

[a]Male mice with the same genotype were not paired. There were no female mice in cages 1 to 4.
[b]The exact time to acquire a stable rank was not examined in this study, but it was known to be less than the duration of housing time before tube test.

Table S6. Results of social dominance tube test of PAS1$^{c/m}$ *vs.* PAS1$^{c/c}$ mice.

Cage number[a]	**No. of PAS1$^{c/m}$ *vs.* No. of PAS1$^{c/c}$**	**Winning rate of PAS1$^{c/m}$**	**Winning rate of PAS1$^{c/c}$**	**Age at group-housing (weeks)**	**Weight at group-housing (g)**	**Housed before tube test (weeks)** [b]	**Age at tube test (weeks)**	**Weight at tube test (g)**
Cage 1	2:2	0%	100%	11.6-11.7	24.8-27.3	3.4	15.0-15.1	27.4-29.6
Cage 2	2:2	0%	100%	11.6-11.7	23.2-24.6	3.4	15.0-15.1	22.8-26.5
Cage 3	2:2	0%	100%	9.6-9.9	23.0-24.4	3.3	12.9-13.1	25.8-26.9
Cage 4	2:2	0%	100%	9.6-9.9	19.7-22.6	3.3	12.9-13.1	23.1-25.5
Cage 5	2:2	0%	100%	9.1-9.6	18.8-22.1	3.1	12.3-12.7	22.5-24.6
Cage 6	2:2	0%	100%	9.1	21.2-23.4	3.9	13.0	23.0-26.3
Cage 7	2:2	0%	100%	9.1	20.1-21.1	3.9	13.0	22.5-23.6
Summary		0.0%	100.0%					

[a]Male mice with the same genotype were not paired.
[b]The exact time to acquire a stable rank was not examined in this study, but it was known to be less than the duration of housing time before tube test.

Table S7. Results of social dominance tube test of PAS1$^{-/m}$ *vs.* PAS1$^{-/-}$ mice.

Cage number	No. of PAS1$^{-/m}$ *vs.* No. of PAS1$^{-/-}$	Winning rate of PAS1$^{-/m}$	Winning rate of PAS1$^{-/-}$	Age at group-housing (weeks)	Weight at group-housing (g)	Housed before test (weeks)	Age at tube test (weeks)	Weight at tube test (g)	Time to achieve stable social hierarchy
Cage 1	2:2	73%	27%	9.4-11.1	23.8-25.4	4.3	13.7-15.4	27.1-29.7	
Cage 2	2:2	45%	55%	9.4-9.6	21.9-23.5	4.3	13.7-13.9	24.2-28.9	
Cage 3	2:2	58%	42%	9.4	22.8-24.5	2.7	12.1	24.5-26.1	Unstable during
Cage 4	2:2	68%	32%	7.9-8.7	21.1-22.5	4.0	11.9-12.7	23.7-25.2	the examined
Cage 5	2:2	38%	62%	7.9-8.1	22.6-24.7	4.0	11.9-12.1	25.7-28.8	period of time.
Cage 6	2:2	88%	12%	7.9-8.7	23.1-25.7	4.0	11.9-12.7	26.9-29.9	
Summary		58.8%	41.2%						

Male mice with the same genotype were not paired.
Average winning rates of PAS1$^{-/m}$ and PAS1$^{-/-}$ in 10 consecutive days are shown.

Table S8. Results of social dominance tube test of PAS1$^{-/-}$ mice.

Cage number	No. of PAS1$^{-/-}$	Age at group-housing (weeks)	Weight at group-housing (g)	Housed before tube test (weeks)	Age at tube test (weeks)	Weight at tube test (g)	Time to achieve stable social hierarchy
Cage 1	4	9.3-9.9	22.4-24.1	3.7	13.0-13.6	25.9-28.1	Unstable during the examined period of time.
Cage 2	4	9.3-9.9	24.0-25.4	3.7	13.0-13.6	27.7-29.5	
Cage 3	4	9.3-9.9	25.5-26.0	3.7	13.0-13.6	27.7-28.7	

Table S9. List of primers.

Experiments	Primers	Note
Cell transfection	hPAS1_Fx:CCGctcgagCTGCCTAGGAAGAGGAAGGA hPAS1_Rx:CCGctcgagAACCTGCCTTTTGTGGGAGG	XhoI site built in
	mPAS1_Fx:CCGctcgagCTGCCTAGGAAGAGGAAGGA mPAS1_Rx:CCGctcgagACCCTGTGTTTTGTGGAAGG	
	cowPAS1_Fx:CCGctcgagCTGCCTAGGAAAAGGAAAGA cowPAS1_Rx:CCGctcgagAACCTGCCTTCTGTGGGAGG	
	wPAS1_Fx:CCGctcgagTTGCCTATAAAAATGAAGGA wPAS1_Rx:CCGctcgagAACCTGTATTTTGTAGTAGG	
	cPAS1_Fx:CCGctcgagTTGCCTATAAAAAAGAAGAA cPAS1_Rx:CCGctcgagAACCTGTATTTTGTTATAGG	
	miniPro_F:agcttTAGAGGGTATATAATGGAAGCTCGACTTCCAGA miniPro_R:agcttCTGGAAGTCGAGCTTCCATTATATACCCTCTAA	HindIII site built in
	RVP3_F:CTAGCAAAATAGGCTGTCCC PGL411_R:CCATGGTGGCTTTACCAACA	For verification of uptake of the plasmid carrying the reporter gene
Chicken embryo electroporation	hPAS1_Fs:ACGCgtcgacCTGCCTAGGAAGAGGAAGGA hPAS1_Rs:ACGCgtcgacAACCTGCCTTTTGTGGGAGG	SalI site built in
	cPAS1_Fs:ACGCgtcgacTTGCCTATAAAAAAGAAGAA cPAS1_Rs:ACGCgtcgacAACCTGTATTTTGTTATAGG	
	CF:TGCGGGCCTCTTCGCTATTA CR:TGAGGAGCAGTTCTTTGATT	For verification of uptake of the plasmid carrying the reporter gene
Genotyping of ES cell targeting mouse	KO_P1:CATACTCCAAGAGTAACATCCCTGC KO_P2:GAATCGCCAACTAATTGACGCTGCA KO_P3:AGAGAATAGGAACTTCGTACCC	For PAS1^{-} model
	KI_P1:TTGTTCTGAGTTAGTCACGGTGGCC KI_P2:GGGTACGAAGTTCCTATTCTCT KI_P3:AAACGCAGCCTAGAGAAATGCA	For PAS1^{w} and PAS1^{c} models
	InternalControl_F:CAACCACTTACAAGAGACCCGTA InternalControl_R:GAGCCCTTAGAAATAACGTTCACC	
RT-qPCR	Lhx2_F:GCCGAGAAAGCGCAAGAGT Lhx2_R:TGTTCAGCATCGTTCTCGTTACA	
	Lhx2_203F: GCTGCCGAGGGCTCACGAAG Lhx2_203R: CTGCCAGCAGGTAGTAGCGG	
	Gapdh_F:CGTGTTCCTACCCCCAATGT	

Gapdh_R:TGTCATCATACTTGGCAGGTTTCT
Gm27197_F: AAGCGGGCGCCCAGATTTCT
Gm27197_R: CGCAGAGCCCTTCCAGGATG
Dennd1a_F:TTTGATGACCTCCAGAGCCT
Dennd1a_R:CATCACCTGTGGTTGTAGAG

Table S10. Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-LHX2 Antibody	Millipore	ABE1402 (RRID:AB_2722523)
ALEXA FLUOR 488 goat anti-rabbit IgG (H+L)	Thermo Scientific	A11034 (RRID:AB_2576217)
Chemicals		
N,N,N',N'-Tetrakis(2-Hydroxypropyl)ethylenediamine	ALDRICH	122262
Agarose	Biowest	111860
MEM, NEAA, powder	Gibco	41500034
Trypsin (0.25%), phenol red	Gibco	15050057
Opti-MEM® I Reduced Serum Medium	Gibco	51985042
PureLink® Quick Plasmid Miniprep Kit	Invitrogen	K210010
SlowFade® Diamond Antifade Mountant with DAPI	Molecular Probes	S36964
FuGENE® HD Transfection Reagent	Promega	E2311
Dual-Glo® Luciferase Assay System	Promega	E2920
Dual-Luciferase® Reporter Assay System	Promega	E1910
HiSpeed Plasmid Maxi Kit (25)	Qiagen	12663
QIAGEN Plasmid Mini Kit (25)	Qiagen	12123
Tissue-Tek® O.C.T. Compound	SAKURA	4583
Paraformaldehyde	Sangon	A500684
Fetal Bovine Serum	SIGMA	12007C
Fast Green FCF Dye	SIGMA	F7252
Triton™ X-100	SIGMA	T9284
4', 6-Diamidino-2-phenylindole dihydrochloride	SIGMA	D8417-1MG
Takara MiniBEST Agarose Gel DNA Ectraction Kit	TaKaRa	9762
MiniBEST Universal RNA Extraction Kit	TaKaRa	9767
PrimeScript RT-PCR Kit	TaKaRa	RR014A
Takara Ex Taq® Hot Start Version	TaKaRa	RR006A
FastDigest® HindIII	Thermo Scientific	FD0504
FastDigest® XhoI	Thermo Scientific	FD0694
FastAP Thermosensitive Alkaline Phosphatase	Thermo Scientific	EF0654
GeneGreen Nucleic Acid Dye	TIANGEN	RT210
DNA Marker I	TIANGEN	MD101-02
T4 DNA Ligase	TIANGEN	RT406
DH5α	TIANGEN	CB101-01
TIANquick Midi Purification Kit	TIANGEN	DP204
RNAstore Reagent	TIANGEN	DP408-02
pGM-T Cloning Kit	TIANGEN	VT202-01
TransStart® FastPfu DNA Polymerase	TransGen	AP221

Trans1-T1 Phage Chemically Competent Cell	TransGen	CD501
TransScript® II All-in-One First-Strand cDNA Synthesis SuperMix for qPCR	TransGen	AH341-01
TransStart® Top Green qPCR SuperMix	TransGen	AQ131-02
Bovine Serum Albumin，Lyophilized Powde	VETEC	V900933
Sucrose	VETEC	V900116
Urea	VETEC	V900119
Triethanolamine	VETEC	V900257
Consumables		
Thermo Scientific™ Nunc™	Thermo Scientific	142475
Assay Plate, 96 Well, No Lid	COSTAR	3362
LightCycler® 480 Multiwell Plate 96, white	Roche	04729692001
4IN THINWALL GL 1.0 OD/.75 ID	WPI	TW100F-4
Microloader 20μl	Eppendorf	5242856.003
Equipments		
Microplate reader	Bio-Tek	Synergy H1
Inverted fluorescence Microscope	Zeiss	Axio observer Z1
Micropipette Puller	SUTTER INSTRUMENT	MODEL P-2000
Microinjection pump	HARVARD APPARATUS	Pump 11 Elite
Stereoscopic microscope	Zeiss	Stemi 2000-C
3mm-diameter L-shaped electrode	BEX	LF613P3
In vivo electroporator	BEX	CUY21EDIT II
Stereo fluorescence microscope	Zeiss	AXIO Zoom.V16
Real-time quantitative PCR instrument	Roche	LightCycler® 480
Light sheet fluorescence microscopy	Zeiss	Lightsheet Z.1
Rota-rod	Shanghai Mobile Datum Information Technology Co.,LTD / Ugo basile	RD1123RS-G / Code: 47650
Experimental Models: Organisms/Strains		
Mouse: FVB (transgenic mouse enhancer assays)	Cyagen	http://www.cyagen.com/us/en/
Mouse: C57BL/6	Cyagen	http://www.cyagen.com/us/en/
	Shanghai Model Organisms Center,Inc	http://www.shmo.com.cn/English/

Mouse: C57BL/6 PAS1$^{-/-}$ (mouse PAS1 knock-out) Mouse: C57BL/6 PAS1$^{w/w}$ (wallaby PAS1 knock-in) Mouse: C57BL/6 PAS1$^{c/c}$ (chicken PAS1 knock-in)	This project (donated)	NM-KO-190421 NM-KI-190001 NM-KI-190002
Plasmids for cell and chicken embryos transfection		
pGL4.11 vector	Promega	E6661
pGL4.74 reporter vector	Promega	E6921
miniPro-pGL4.11 reporter vector (miniPro-luc2P) hPAS1-miniPro-pGL4.11 reporter vector mPAS1-minoPro-pGL4.11 reporter vector cowPAS1-miniPro-pGL4.11 reporter vector wPAS1-miniPro-pGL4.11 reporter vector cPAS1-miniPro-pGL4.11 reporter vector	This project	N/A
PUC19 reporter vector	TransGen	N/A
pCAG-mCherry reporter vector	Addgene	41583 (RRID:Addgene_41583)
pCAG-EGFP reporter vector	This project	N/A
TK-EGFP reporter vector	Provided by Dr. Naihe Jing	N/A
TK-mCherry reporter vector hPAS1-TK-EGFP reporter vector cPAS1-TK-mCherry reporter vector	This project	N/A
Oligonucleotides		
Primers for PAS1, see Table S9	This project	N/A
Primers for Genotyping of ES cell targeting mouse, see Table S9	This project	N/A
Primers for RT-qPCR, see Table S9	This project	N/A
Recombinant DNA		
Hsp68-LacZ vector hPAS1-Hsp68-LacZ reporter vector mPAS1-Hsp68-LacZ reporter vector wPAS1-Hsp68-LacZ reporter vector cPAS1-Hsp68-LacZ reporter vector ES cell targeting vector, mm9-KOS141218, PAS1^{-} ES cell targeting vector, mm9-KIS141215, PAS1^{w} ES cell targeting vector, mm9-galGal3, PAS1^{c}	This project	N/A
Cell Lines		
Mouse: Neuro2a	Cell Bank of CAS	TMC29
Human: HEK-293	Cell Bank of CAS	GNHu43
Software		
KungFuPanda	This project	http://www.picb.ac.cn/evo

		lgen/softwares/
eGPS[5]		http://www.egps-software.net/
Vision4D 2.12.3	Arivis	http://www.arivis.com/
BLASTN[12]		https://blast.ncbi.nlm.nih.gov/Blast.cgi
MULTIZ[13]		http://www.bx.psu.edu/miller_lab
UCSC Kent utilities		http://hgdownload.cse.ucsc.edu/admin/jksrc.zip

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