

Supplementary information

Reciprocal regulation of Shh trafficking and H₂O₂ levels via a noncanonical BOC-Rac1 pathway

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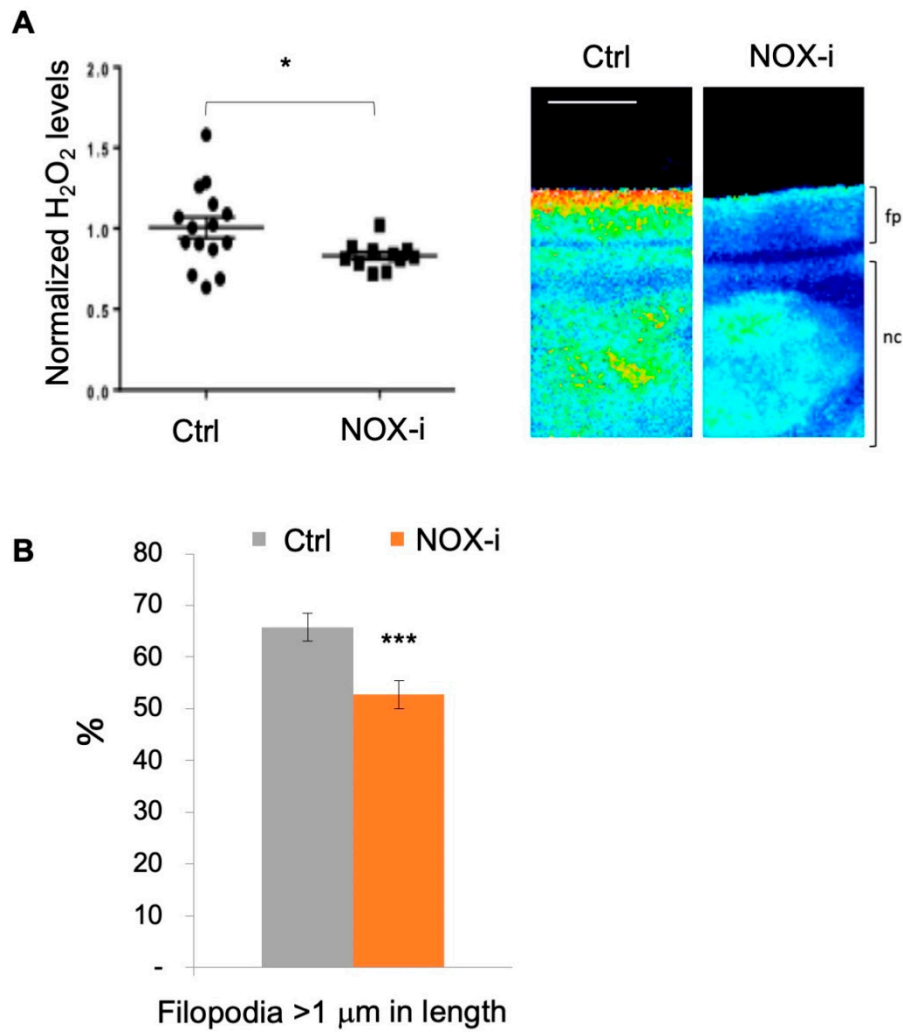


Figure S1. NOX-i treatment induces a reduction of H_2O_2 levels as well as a reduction of filopodia length in the MFP.

A, H_2O_2 levels decreased after NOX-i treatment. 2.4Shha-ABC:GalA-FF/UAS:HyPer7 embryos were incubated in NOX-i (10 μM), and H_2O_2 levels were quantified in the MFP at 46 hpf. Representative images are shown. fp: floor plate; nc: notochord. Scale Bar: 10 μm .

B, Quantification of filopodia length in MFP cells (see Methods) in Ctrl and NOX-I treated larvae. Details on statistics in Material and Methods

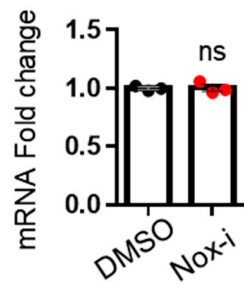


Figure S2. *Shha* expression is not affected by Nox-i treatment

Gene expression was analyzed by quantitative RT-PCR after Nox-i or DMSO treatment. The level in the DMSO-treated sample was set to 1. The error bars indicate the SEM values.

Table S1: Plasmids used in this study

Plasmid number	Plasmid name	Regulatory sequences	Expressed protein	purpose	ref
65	pCS2transposase	SP6	Tol2 transposase	transgenesis	⁵³
1070	pT22i2Shh24Gal4F	2.4Shha-ABC	Gal4BD-FF	transgenesis	this paper
1190	pT22i5uasHyper7	5xUAS	HyPer7	transgenesis	this paper
1242	pT22i5uasm4C5DaoChe	5xUAS	Igk-mb5-DAO-mCherry	transgenesis	this paper
1243	pT22i5uasm4C5CatChe	5xUAS	Igk-mb5-CAT-mCherry	transgenesis	this paper
1261	pT2i5biGfpF•Shh-SbpChe	5xUAS bidirectional	(i) GFP-Farn (ii) Shh-SBP-mCherry	transgenesis	this paper
237	pCSmChF	SP6	mCherry-Farn	mRNA synthesis	this paper
1313	pcDNA7LGBi	CMV-tetOx2	LgBiT	stable cell line	⁴³
1314	pcDNA2LsiLGBiCD5	CMV-tetOx2	IL2-LgBiT-mb5	stable cell line	this paper
754	pcDNA7Hyper1	CMV-tetOx2	HyPer1	stable cell line	²³
1036	pcDNA2LckHyper	CMV-tetOx2	Lck-HyPer1	stable cell line	this paper
911	pT2iC6mShhYfaChe	sCMV	Shh-YFast-mCherry	transient <i>ex vivo</i> expr	this paper
669	pT2iC6mShhChe	sCMV	Shh-mCherry	transient <i>ex vivo</i> expr	this paper
1330	pKhCTagScLgBiT	T7	CTagSc-LgBiT	bac prod	this paper
832	pStrKdel_ShhsbpChe	CMV	sti-STRP-kdel-ires-Shh-SBP-mCherry	transient <i>ex vivo</i> expr	this paper
1265	pCL9StKDEL	CMV	sti-STRP-kdel	transient <i>ex vivo</i> expr	this paper
1237	pcDNA2SbpShhSbi	CMV-tetOx2	Shh-SBP-HiBiT	transient <i>ex vivo</i> expr	this paper
1311	pcDNA2SilGfpSbpSbi	CMV-tetOx2	IL2-GFP-SBP-HiBiT	transient <i>ex vivo</i> expr	this paper
1312	pcDNA2silSbiChe	CMV-tetOx2	IL2-HiBiT-mCherry	transient <i>ex vivo</i> expr	this paper
966	pcDNA2LckCheDao	CMV-tetOx2	Lck-mCherry-DAO	transient <i>ex vivo</i> expr	this paper
669	pT2iC6mShhChe	sCMV	Shh-mCherry	transient <i>ex vivo</i> expr	this paper

CAT: catalase deprived from its lysosome-targeting signal; CTagSC: His6-bacterial CherryTag-Scission protease site for bacterial expression; DAO: D-Aminoacid oxidase; Farn: C-terminal farnesylation+palmitoylation signal from Ha-Ras; Gal4BD-FF: Gal4 DNA binding domain fused to dimerized minimal activation domain F; HiBiT: small fragment of split nanoluciferase; HyPer: H₂O₂ ratiometric probe; Igk: signal peptide from kappa light chain; IL2: signal peptide from interleukin2; IRES: Internal Ribosome Entry Site; kdel: classical ER retrieval signal; Lck: N-terminal myristoylation+palmitoylation signal from Lck tyrosine kinase; mb5 : minimal transmembrane domain form CD4; LgBiT: large fragment of split nanoluciferase; SBP: streptavidin binding peptide; Shh; Sonic Hedgehog; sti: signal peptide from stromal interaction molecule 1 STIM1; STRP: core streptavidin; YFAST: Yellow fluorogen-activated peptide

Table S2: Stable HeLa cell lines used in this study

Name	Construct used for HeLa Flp-In	Expressed protein	ref
GBi	pcDNA7LGBi	LgBiT	this study
siLGBimb5	pcDNA4siLGBiCD5	IL2-LgBiT-mb5	this study
LckHyPer	pcDNA7LckHyPer	Lck-HyPer1	this study
HyPer	pcDNA7Hyper	HyPer1	²³

Table S3. Sample sizes and number of replicates.

Figure	condition	Sample size	Mean	Std. Deviation	Std. Error of Mean	P-value	Confidence interval
Figure 2							
2C	Shh	4					
	SecGFP	4					
2E	Shh t=5min	2	16	1.697	1.2	T=5min Shh vs SecmCh P>0.9999 t=30min Shh vs SecmCh P<0.0001 t=60min Shh vs SecmCh P<0.0001 t=75min Shh vs SecmCh P=0.0491	
	Shh t=30min	8	100	23.11	8.17		
	Shh t=60min	5	104.2	16.62	7.432		
	Shh t=75min	2	112	5.657	4		
	Secm Ch t=5min	2	11.5	7.778	5.5		
	SecmCh t=30min	7	18.43	16.22	6.129		
	SecmCh t=60min	4	36	7.348	3.674		
	SecmCh t=75min	2	25.5	27.58	19.5		
2G	Ctrl (A)	11	0.1	0.02246	0.006772	A vs B p>0.9999 A vs C p=0.7699 A vs D p=0.0919 A vs E p<0.0001 A vs F p<0.0001	
	1h (B)	10	0.1017	0.03336	0.01055		
	2h (C)	6	0.1387	0.0549	0.02241		
	3h (D)	8	0.1726	0.05061	0.01789		
	4h (E)	7	0.2578	0.07452	0.02816		
	4h30 (F)	8	0.2518	0.09564	0.03381		
	Nox-i	11	0.83	0,08184	0,02467		

Figure 3B							
Shh	Ctrl	14	100	25.46	6.804	0.0007	
	D-Ala	13	53.96	25.69	7.126		
SecGFP	Ctrl	14	100	30.19	8.069	0.9966	
	D-Ala	12	101.7	22.06	6.368		
Figure 3C							
Shh	Ctrl	15	100	16.06	4.147	<0.0001	
	CAT	13	155.5	23.20	6.434		
SecGFP	Ctrl	8	100	9.794	3.463	0.0173	
	CAT	7	75.71	21.90	8.277		
Figure 3E							
Shh	Ctrl	8	100	24.91	8.808	0.0048	
	D-Ala	8	144.6	39.1	13.83		
SecCh	Ctrl	8	7.579	21.88	7.736	0.9380	
	D-Ala	7	12	11.45	4.326		
Figure 3F							
Shh	Ctrl	6	100	19.18	7.832	0.0007	
	CAT	6	52.46	8.507	3.473		
SecCh	Ctrl	5	32.7	23.72	10.61	0.9341	
	CAT	5	28.78	21.23	9.493		
Figure 3H							

	Ctrl t=0 (A)	10	0,1	0.0255	0.008065	A vs B p=0.0091 B vs C p<0.0001	
	Ctrl t=4h (B)	9	0.1395	0.02436	0.008119		
	D-Ala t=4h (C)	11	0.2162	0.03876	0.01169		
Figure 3I							
	Ctrl t=0 (A)	6	0,1	0.03096	0.01264	A vs B p<0.0001 B vs C p=0.0066	
	Ctrl t=4h (B)	6	0.2705	0.07288	0.02975		
	CAT t=4h (C)	6	0.1697	0.02609	0.01065		

Figure 4B							
	26 hpf	8	1,125	0,08582	0,03034	<0,0001	0,95
	28 hpf	8	1,053	0,07437	0,02629	<0,0001	0,95
	30 hpf	13	1,000	0,07127	0,01977		0,95
	33 hpf	5	0,9074	0,07615	0,03406	<0,0001	0,95
	39 hpf	5	0,8569	0,04880	0,02182	0,0005	0,95
	45 hpf	5	0,8545	0,03733	0,01669	0,0027	0,95
Figure 4D							
	25 hpf	12					
	29 hpf	12					
	31 hpf	24					
	35 hpf	12					
	45 hpf	11					

Figure 5C							
	Ctrl ≤9	29	58.3%	10.06			
	Ctrl ≥10	29	41.7%	10.06			
	D-Ala	24	31%	8.59		<0.0001	0.95
	D-Ala	24	69%	8.59		<0.0001	0.95
Figure 5D							
	Ctrl	243	20%	2.57		<0.0001	0.95
	D-Ala	302	24%	2.46			
Figure 5F							
	Ctrl	15	6.2	3.14	0.81		
	Nox-i	22	12.05	5.62	1.2	<0.01	0,95
	HH-i	5	0	0	0	<0.05	0,95

Figure 6A							
mCherry	Exp- (A)	50	100	32.76	4.634	A vs B p=0.5451 C vs D p=0.3243 A vs C p=0.0392 B vs D p<0.0001	
	Exp+ (B)	15	88.43	34.2	8.830		
Shh-mCherry	Exp- (C)	14	133.3	29.17	7.797		
	Exp+ (D)	43	150	50.69	7.716		
Figure 6D							
	Ctrl	82	100	41.8	4.616	<0.0001	
	Shh	104	141.4	42.63	4.181		
Figure 6E							

	Ctrl	40	923.9	806.9	127.6	<0.0001	
	Shh	34	1940	1219	209.1		
Figure 6F							
	Ctrl (A)	113	100	26.1	2.456	A vs B p<0.0001 C vs D p<0.0001 E vs F p=0.8904	
	Shh (B)	128	143.7	48.08	4.250		
	Shh-i (C)	50	95.57	31.02	4.386		
	Shh+Shhi (D)	46	138.9	57.57	8.488		
	NOX-i (E)	58	76.87	29.65	3.894		
	Shh+NOX-i (F)	54	81.45	16.33	2.222		
Figure 6G							
	Ctrl (A)	111	100	29.47	2.798	A vs B p<0.0001 C vs D p=0.3685 E vs F p=0.9802 G vs H p=0.9308	
	Shh (B)	126	180	51.64	4.601		
	Rac1-i (C)	57	106.7	30.53	4.044		
	Shh+Rac1-i (D)	51	119.5	31.6	4.424		
	DOCK-i (E)	33	122.9	43.48	7.57		
	Shh+DOCK-i (F)	52	127.4	44.69	6.198		
	Rac1-i+DOCK-i (G)	25	110.3	28.44	5.688		
	Shh+ Rac1-i+DOCK-i (H)	22	118.7	41.37	8.821		

Figure Sup1A							
	Ctrl	14	1.0	0,2506	0,06472	0,030	0,95
	NOX-i	11	0.83	0,08184	0,02467		

Figure Sup1B							
	Ctrl	298	65.77	2.75	0.1592	<0.0001	0.95
	NOX-i	334	52.69	2.73	0.1495		

Figure Sup2	condition	Sample size	Mean	Std. Deviation	Std. Error of Mean	P-value
	DMSO	3	1.000	0.02160	0.05068	0.9146
	HH-i	3	1.004	0.005068	0.02926	

Table S4. Shh pathway member expression in cell lines**A:** data from The Human Protein Atlas/Cell line database

(https://www.proteinatlas.org/humanproteome/cell+line).

nTPM: number of transcripts per million (mRNA are considered expressed for mTPM>1).

B: this paper, determined by quantitative RT-PCR (see Methods)

nd : non detected, un : untested. Positive value indicates that gene of interest is x fold more expressed than gapdh and negative values x fold less than gapdh

A	HeLa nTPM
<i>shh</i>	0
<i>ihh</i>	0
<i>dhh</i>	0
<i>smo</i>	0
<i>ptch1</i>	3.0
<i>ptch2</i>	0.6
<i>cdon</i>	6.5
<i>elmo2</i>	18.7
<i>elmo3</i>	4.8
<i>dock1</i>	18.8
<i>dock2</i>	3.4
<i>dock3</i>	1.2
<i>dock4</i>	5.8
<i>dock5</i>	40.5

B	HeLa Δ Ct	HEK	Universal probe ID	Left primer	Right primer
<i>gadph</i>			68	ccc cgg ttt cta taa att gag c	ctt ccc cat ggt gtc tga g
<i>hhatt</i>	12.6±2.2	un	22	gct ggg agt cac tgt gga g	gct tgt ggg gag aag tat cg
<i>disp1</i>	7.0±1.4	un	43	tgt gca atg tag ata att cca gga t	gcg atg tag ttt ccc agt gtc
<i>ptch1</i>	3.8±0.2	un	17	tgg gat taa aag cag cga ac	cga ctt act cgt cct cca act t
<i>ptch2</i>	-4.8±1.1	un	17	tgg aac agc tct ggg tag aag	ccc agc ttc tcc ttg gtg ta
<i>scube2</i>	-45±9.3	un	17	agc tgc cat cca cag tac aa	cca ctg atg tgg tgt tgc tc
<i>smo</i>	nd	-3.2	22	gct tcc ggg act atg tgc ta	aaa cat ggc aaa cag gtt ga
<i>boc</i>	3.3±4.6	un	6	cgc caa ccc tct act atg tgg t	aat gcc aga gat ggt cca at

<i>nox1</i>	nd	un	43	ccc agc aga agg ttg tga tt	ttg agg ggc aat taa caa aga
<i>nox2</i>	-5.2±0.5	un	6	aca att gca agt caa cac cct a	ctc agg gtt tca gcc aag g
<i>nox3</i>	nd	un	22	tca agt cca ggc att gtg tt	aac agg ccg tca cag gaa
<i>nox4</i>	nd	un	22	aac caa ggg cca gag tat ca	gct gag gct ctg ctt aga cac