Supplemental Information

The guanine nucleotide exchange factor net1 facilitates the specification of dorsal cell fates in zebrafish embryos by promoting maternal β-catenin activation

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Extended Materials and Methods

Plasmid constructions and RNA interference

Wild-type zebrafish net1 and two net1 mutants L266E and W437L were cloned into pCS2 vectors for eukaryotic expression. To induce the expression of net1 in the cytoplasm, we deleted the its N-terminus containing NLS sequences (ΔN-net1) and added two tandem nuclear export signal (NES) sequences derived from the MAPKK protein [1] to the N terminus of ΔN-net1 (NES-ΔN-net1). Similarly, three tandem NLS sequences derived from the SV40 large T antigen [2] were fused to the N terminus of wild-type net1 (NLS-net1) to facilitate its nuclear expression. Wild-type PAK1, DN-PAK1, CA-PAK1, β-catenin S675D, β-catenin S675A, HDAC1 expression plasmids were kindly gifted from Prof. Wei Wu of Tsinghua University (Beijing, China). For RNA interference in mammalian cells, two human net1 shRNA constructs were generated using a pLKO.1 plasmid, with the following target sequences: 5'-CCCGAGGTGAACAGGATTTAA-3' and 5'-CCAAAGCTCTTCTTGATCAAA-3'. For RNA interference in zebrafish embryos, two siRNAs targeting different regions of zebrafish *net1* mRNA were designed and synthesized by GenePharma. The sequences of siRNAs were as follows: negative control, sense 5'-ACGUGACACGUUCGGAGAATT-3', antisense 5'-UUCUUCGAACGUGUCACGUTT-3'; *net1* siRNA 1, sense 5'-GCUCACGUUUGGUCAAAUATT-3', antisense 5'-UAUUUGACCAAACGUGAGCTT-3'; *net1* siRNA 2, sense 5'-GCAGUGCAAGAGCUUGCUUTT-3', antisense 5'-AAGCAAGCUCUUGCACUGCTT-3'.

Quantitative RT-PCR

Total RNA was extracted with Trizol (Invitrogen) and cDNA was synthesized with high-efficiency reverse transcriptase Revertra Ace (Toyobo). A Biorad CFX96 PCR system was employed to perform qRT-PCR using SYBR® Premix Ex Taq™ dye (Takara).

The primer sequences used to detect the expression of Wnt target genes in HEK293T cells were as follows:

c-Myc	Forward primer	5’-TGCTCCATGAGGAGACA-3’
	Reverse primer	5’-CCTCCAGCAGAAGGTGA-3’
Axin2	Forward primer	5’-AGTGTGAGGTCCACGGAAAC-3’
	Reverse primer	5’-CTTCACACTGCGATGCATTT-3’
LEF1	Forward primer	5’-AACATGGTGGAAAACGAAGC-3’
	Reverse primer	5’-GGGTTGGCAGTGATTGTCTT-3’
GAPDH	Forward primer	5’-GCACCACCAACTGCTTA-3’
	Reverse primer	5’-AGTAGAGGCAGGGATGAT-3’

The primer sequences used to detect the expression of genes related to GTPase-mediated signal transduction in wild-type embryos, *net1* morphants and *net1Δ53* mutants were as follows:

Fgd4	Forward primer	5’-GCGTGCCCAAGTTCAGTCT-3’
	Reverse primer	5’-CGCCTTTATCCAGTCCTCC-3’
plekhg5a	Forward primer	5’-AGACTCACTAAATACCCACTGCTG-3’

	Reverse primer	5’-TTCCTCCCTGTGCCTCATC-3’
arhgef1b	Forward primer	5’-CCTGGAATCGGCTATGACG-3’
	Reverse primer	5’-TGCTCCCTGACACGGTAAT-3’
arhgef28	Forward primer	5’-ATAGCACTCGCAACAAGAACA-3’
	Reverse primer	5’-CAGCCCTTATGGACACTCAC-3’
arhgef4	Forward primer	5’-CCATAGAAGACTGGGAGGGT-3’
	Reverse primer	5’-TTGGTGGTCAAAGAGGAAGA-3’
rapgef3	Forward primer	5’-AGTCAGGCAGTCGGAATGTG-3’
	Reverse primer	5’-TCGTCGTCTTTGGAGTCTTTT-3’
plekhg2	Forward primer	5’-AATAATGGGTGGGTCGCTAA-3’
	Reverse primer	5’-GGCTCAGACGATGGGAAAG-3’
cdc42ep2	Forward primer	5’-AACGACGAAGCCGCAAAG-3’
	Reverse primer	5’-TGTCCAGGTAGCAGGTGAAAT-3’
cdc42ep1a	Forward primer	5’-ACGGTCGGACTCGCCTATA-3’
	Reverse primer	5’-CAGCAGCCCTCTGGAAACT-3’
rab9b	Forward primer	5’-CGCTTCCCCTTTGTAGTGC-3’
	Reverse primer	5’-TAGCCAGAACCTCCCGAAC-3’
rab25a	Forward primer	5’-ACTCGCTCTGTTCAGGTGG-3’
	Reverse primer	5’-TCATAGGTCAGGTGTTTGGTG-3’
rab33a	Forward primer	5’-CGAGGGCGAGAAAATAAAG-3’
	Reverse primer	5’-GACCGTTGCACTCCTGAAT-3’
rab8a	Forward primer	5’-GCAATGGGTATTATGCTGGTT-3’

	Reverse primer	5’-AGCTTCTCGCCTCTGTCTTTA-3’
rab25b	Forward primer	5’-ATCGTAGTGATGCTGGTGGG-3’
	Reverse primer	5’-CGGGTCACTTCTTTGCTGTT-3’
rab11al	Forward primer	5’-ATCGGGGTGGAGTTTGC-3’
	Reverse primer	5’-TCTCATAGGTCAGATGCTTGG-3’
rhoub	Forward primer	5’-AAAATATGTCCCGACAGCG-3’
	Reverse primer	5’-AAAGCACAGCAGGAGAACG-3’
β-actin	Forward primer	5’-ATGGATGATGAAATTGCCGCAC-3’
	Reverse primer	5’-ACCATCACCAGAGTCCATCACG-3’

Generation of net1Δ20 and net1Δ53 mutant lines using Cas9/gRNA system

The humanized Cas9 expression vector was kindly provided by Prof. Jingwei Xiong at College of Life Sciences, Peking University. The gRNA oligonucleotide sequence was: 5’-**TAATACGACTCACTATAG**GGCCAGACCCTCCAGGCCTCATTC*GTTTTAGAGCTAG AAATAGC*-3’, with the T7 site shown in bold, the targeting sequence underlined, and the gRNA scaffold shown in italics. The Cas9 mRNA and gRNA were synthesized *in vitro* as previously described [3]. 150 pg Cas9 mRNA and 20 pg gRNA were co-injected into one cell stage wild-type embryos. Embryos were collected to make genomic DNA for genotyping at 24 hpf. For screening of the F1 fish with mutant alleles, genomic DNA was isolated from the tail of individual fish. The genomic region surrounding gRNA targeted sequences in the *net1* locus was amplified with the forward primer (5’- TTGGCAGTTGTGCAGTTGGTA-3’) and the reverse primer (5’- TTTTCAGTGCTGTCTGGATTCTC-3’). The amplified DNA

fragment was purified for enzymatic digestion with T7 endonuclease I (M0302, NEB) and subjected to Sanger sequencing. Homozygous *net1* mutants were generated by incrossing F1 fish carrying mutated genomic DNA.

Supplemental References

1 Fukuda M, Gotoh I, Gotoh Y, Nishida E. Cytoplasmic localization of mitogen-activated protein kinase kinase directed by its NH2-terminal, leucine-rich short amino acid sequence, which acts as a nuclear export signal. *J Biol Chem* 1996; **271**:20024-20028.

2 Kalderon D, Roberts BL, Richardson WD, Smith AE. A short amino acid sequence able to specify nuclear location. *Cell* 1984; **39**:499-509.

3 Chang N, Sun C, Gao L *et al.* Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. *Cell Res* 2013; **23**:465-472.